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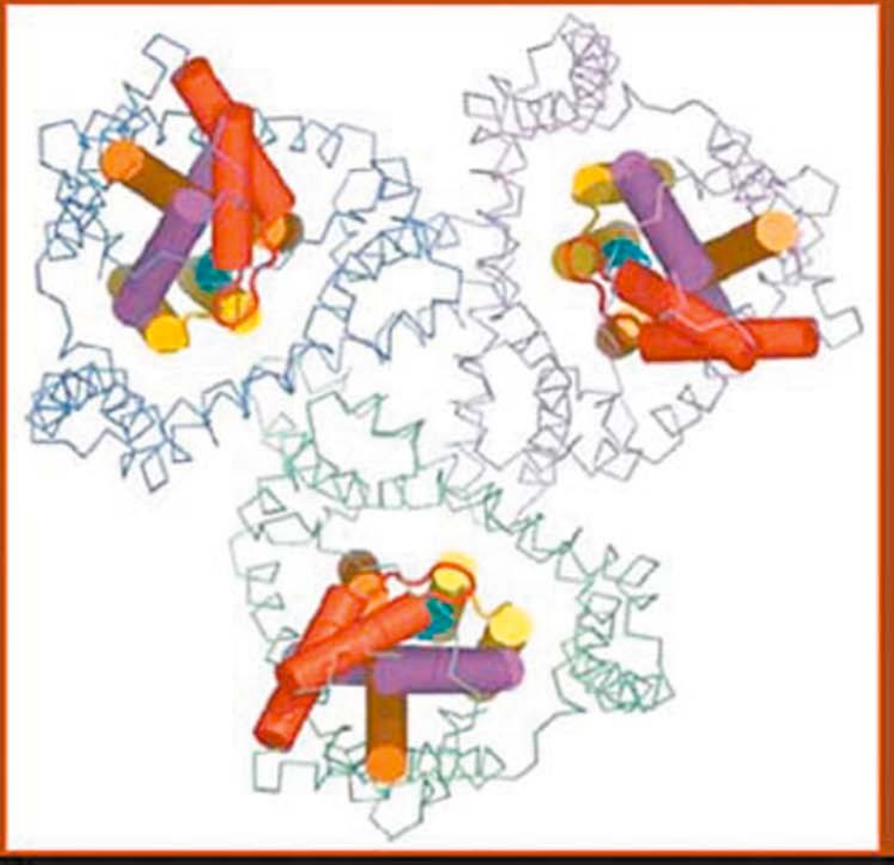

EDITED BY

George Siegel • R. Wayne Albers • Scott Brady • Donald Price

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MOLECULAR, CELLULAR AND MEDICAL ASPECTS

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Basic Neurochemistry

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Acknowledgments and History

We express our debt to all the former authors and editors for their contributions that have made this book so useful over the years. We have carried forward many of these contributions into this seventh edition. In particular, we note with thanks the earlier work of former co-editors, Dr. Bernard Agranoff in the first six editions, Dr. Robert Katzman in the first three editions, Dr. Perry Molinoff in the fourth and fifth editions, and Drs. Stephen Fisher and Michael Uhler in the sixth edition. In revising this book to remain current, all chapters have been updated, some earlier chapters have been dropped and their pertinent content incorporated into other chapters, new chapters and topics amounting to about 25% of the content have been introduced and the book has been expanded as far as practical while keeping to one volume. Illustrations have been redrawn and produced in full color with an eye toward teaching and aesthetic goals. These revisions have involved rotation of authors and inclusion of many new authors. We are indebted to previous authors whose work has been continued and in many cases further developed in this revision. This is actually the way science evolves and is taught. We owe gratitude to the many investigators whose past and current research has made this new edition both necessary and possible. We regret that much important work and citations of historical significance cannot be specifically referenced in a textbook.

We are grateful to Johannes Menzel, Ph.D., of Elsevier, for his guiding the production of this edition and in keeping us all on track to ensure its timely and successful completion. We also thank Jasna Markovac, Sr. Vice President, for her long-standing encouragement and the staff of Elsevier, particularly Karen Dempsey, Kirsten Funk, and art designer Julio Esperas, for their work on this book.

Basic Neurochemistry: Molecular, Cellular and Medical Aspects had its origin in the Conference on Neurochemistry Curriculum initiated and organized by R. Wayne Albers, Robert Katzman and George J. Siegel under the sponsorship of the National Institute for Neurological Diseases and Stroke, June 19 and 20, 1969, Bronx, New York. At this conference, a group of 30 neuroscientists constructed a syllabus outline delineating the scope of a neurochemistry curriculum appropriate for medical, graduate and postgraduate neuroscience students. Out of this outline grew the first edition, edited by Wayne Albers, George Siegel, Robert Katzman and Bernard Agranoff. It was anticipated that the book would evolve with the emergence of the field and would stimulate continuing reappraisal of the scientific and educational aspects of neurochemistry. This has in fact been the case over the years of these seven editions (see Preface).

The original editors elected to assign the copyright and all royalties to the American Society for Neurochemistry (ASN), the royalties to be used for educational purposes, and as may be needed for editorial work and revisions of the book. These funds have been used to subsidize travel of graduate students and postdoctoral fellows to present their research at meetings of the ASN, to send copies of the book to libraries in underdeveloped countries and as prizes to students for their research at the ASN and International Society for Neurochemistry. In addition, these royalties have subsidized the Basic Neurochemistry Lectureships at the annual meetings of the ASN since 1979. Retrospectively, it is of historical interest that the first two Basic Neurochemistry Lectures reflected the meaning of this book in integrating molecular and functional aspects in studying the nervous system.

The first lecture given by Tomas Hokfelt, who pioneered anatomical studies based on amine fluorescence, was entitled, "Neuroanatomy for the Neurochemist" while the second lecture given by Louis Sokoloff, who invented the methodology of functional brain imaging originally based on metabolism of radionuclide labeled-2-deoxyglucose, was entitled "Neurochemistry for the Neuroanatomist". The history and further information concerning the ASN may be found at the website: www.asneurochem.org.

George J. Siegel
R. Wayne Albers
Scott Brady
Donald Price

Preface to the Seventh Edition

This Seventh Edition of *Basic Neurochemistry: Molecular, Cellular and Medical Aspects* is appearing near to the 50th anniversary of the founding of neurochemistry as a discipline. This seems an appropriate time to examine the progress of neurochemistry and of this book. To make this brief, we will look at only two topics, which may be considered "defining" neurochemical subjects: chemical neurotransmission, and learning and memory. Fifty years ago, our understanding of the former was rudimentary and the latter could hardly be discussed in terms of credible biochemical hypotheses, although it was recognized in the preface to the first edition of *Basic Neurochemistry* (*BN1*) in 1972, that a thorough molecular understanding of learning and memory will be an ultimate achievement.

A series of "International Neurochemical Symposia" led to the organization of the International Society for Neurochemistry and subsequently the ASN. The first symposium volume (1954) was titled *Biochemistry of the Developing Nervous System* and the second volume (1956), contains an historically interesting chapter which begins:

> "It is a measure of the changed outlook among neurophysiologists that it has been thought appropriate to include ...[here] ... a discussion on the nature of synaptic transmitter substances other than acetylcholine. ... A few years ago, the whole hypothesis of the chemical mediation of impulse transmission across central synapses was meeting so much opposition that the energies of those who supported it had to be concentrated on the claims of acetylcholine."

This chapter lists tentative evidence for actions at central synapses by histamine, substance P and serotonin; but concludes that

> "pharmacological substances other than acetylcholine have been identified ... but the evidence ... is inadequate to provide solid support for the claims of any of them to be considered as [CNS] transmitter agents."

'Glutamic Acid and Glutamine' was a topic at the same symposium. This chapter's introduction stated that

> "... the high concentration of glutamic acid and glutamine in brain ... has stimulated much research. ... [their] ... metabolism seems quantitatively different in nervous system tissue although up to now no ... unique reactions have been reported."

Sixteen years later, when the first edition of *Basic Neurochemistry* appeared, Sol Snyder's chapter could classify norepinephrine, dopamine and serotonin as

> "putative neurotransmitters in certain neuronal tracts in the brain"

while Eugene Roberts and Richard Hammerschlag state in a chapter on 'Amino Acid Transmitters' that

> "Many lines of evidence ... make it seem probable that GABA is a major inhibitory transmitter in the vertebrate central nervous system".

However, they cautioned that

> "the case for an excitatory role of glutamate ... rests almost entirely on the greater concentrations of glutamate in dorsal over ventral regions of spinal cord and on the excitatory effect of glutamate when applied directly to spinal interneurons and motor neurons."

This seventh edition includes discussions of neurotransmitters ranging from acetylcholine through other amines, amino acids, purines, peptides, steroids and lipids Whereas in most cases their metabolism and receptor interactions are known, much current research involves questions of identification of effector pathways, their regulation and control.

In the *BN1* of 1972, Bernard Agranoff introduces his chapter by warning us of the undeveloped state of the "neurochemistry of learning and memory":

> "There is a common belief among biologists that the detailed mechanisms of behavioral plasticity constitute a major remaining frontier in our understanding of living systems. Certainly it is at this time the most obscure frontier and, accordingly, hypotheses run rampant."

In contrast, Agranoff's chapter in the *BN6* of 1999 is illuminated by studies of genetically engineered "dunce flies" and by fMRI maps of spatial localization made possible by advances in our understanding of the underlying neurochemistry. Most notably, these maps show verbal memory is localized just where Paul Broca claimed it to be in 1865! An important current trend in neurochemistry is the increasing appreciation of functional integration at multiple levels: control of receptor types and subtypes in the plasmalemma, transmission of signals into the cell via networks of molecular interactions that are regulated at both transcriptional and translational levels. The nervous system uniquely employs neurotransmitters and synapses to extend these networks to mediating both efferent and afferent functions. Neuroscientists must increasingly address how systemic brain functions arise from these intricate intracellular and intercellular molecular networks. The 'learning and memory' chapter has progressed from 'dunce flies' to 'smart mice' as Joe Tsien, in *BN7*, outlines experiments that affirm some long held physiologic concepts such as Hebb's rule; and asks further questions about mechanisms of 'remote memory' storage and the possible relation of hippocampal neurogenesis to memory clearance.

These advances reinforce the "unifying objective" of neurochemistry stated in the 1972 preface of *BN1*:

> "Its central, unifying objective is the elucidation of biochemical phenomena that subserve the characteristic activity of the nervous system or are associated with neurological diseases. This objective generates certain subsidiary ... goals ... (1) isolation and identification of components; (2) analysis of their organization ... and (3) a description of the temporal and spatial relations of these components and [of] their interactions to [produce] the activity of the intact organ. A comprehensive description ... should be continuous from the molecular level to the most complex level of integration."

It appears that neurochemistry is now heading more energetically and successfully than ever into the third subsidiary goal.

George J. Siegel
R. Wayne Albers

For the Editors

Pierre Morell, Ph.D. 1941–2003

Pierre with wife, Bonnie

Pierre Morell was an author for at least one of the myelin chapters in all six previous editions of *Basic Neurochemistry*. For editions 4, 5 and 6, I had the privilege and honor of being a co-author with him for both the basic and clinical chapters. He was enthusiastically participating in the early planning for this seventh edition until his untimely death in 2003. The current chapters on the structure and diseases of myelin are still based largely on the strong foundation that he and his co-authors developed in the earlier editions. Pierre's authorship of these chapters over the years is just one example of his numerous contributions to myelin research and neurochemistry in general. Particularly significant were two editions of the book entitled *Myelin*, published in 1977 and 1984 for which he was the sole editor. The latter still serves as a comprehensive reference for basic information and early research on myelin. Pierre was a leader in myelin research throughout his career, with an emphasis on the metabolism and function of myelin lipids and proteins. He frequently investigated abnormalities of myelin caused by malnutrition, toxins and genetic factors to gain important insights into the formation, maintenance and breakdown of myelin sheaths. This prolific research was funded largely by 33 years of continuous support from the National Institutes of Health.

Pierre attended the Bronx High School of Science, Columbia University and The Albert Einstein College of Medicine (AECOM) of Yeshiva University, followed by postdoctoral training at the University of Michigan. He joined the faculty at AECOM in the late 1960s and moved to the University of North Carolina (UNC) at Chapel Hill in 1973, where he was Professor of Biochemistry and Biophysics at the time of his premature death. He was a dedicated and wise mentor to numerous students,

postdoctoral fellows and junior faculty members, many of whom are noteworthy investigators in the myelin field and other areas today. His tireless support of neurochemistry included service in many capacities within the American Society for Neurochemistry, of which he was a founding member, and International Society for Neurochemistry, as well as numerous other professional activities.

In addition to his intellectual and creative talents as a neuroscientist, Pierre was a caring person known for his witty remarks and enthusiastic demeanor in all aspects of life. In recent years, he became an avid scuba diver and teacher of this sport at UNC. His wife Bonnie and two children, David and Sharon, were paramount in his life. Although we in the neurochemistry community will greatly miss this remarkable scientist, colleague and friend, we can celebrate his life and outstanding career.

Richard H. Quarles

PART

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CHAPTER

1

Neurocellular Anatomy

Cedric S. Raine

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UNDERSTANDING NEUROANATOMY IS NECESSARY TO STUDY NEUROCHEMISTRY

Despite the advent of molecular genetics in neurobiology, our understanding of the functional relationships of the components of the CNS remains in its infancy, particularly in the areas of cellular interaction and synaptic modulation. Nevertheless, the fine structural relationships of most elements of nervous system tissue have been described well [1–5]. The excellent neuroanatomical atlases of Peters *et al.* [3] and Palay and Chan-Palay [1] should be consulted for detailed ultrastructural analyses of specific cell types, particularly of neurons with their diverse forms and connections. This chapter provides a concise description of the major cytoarchitectural features of the nervous system, an introduction to the historical literature and a structural roadmap for the neurochemist. Although the fine structure of the organelles of the CNS and PNS is not peculiar to these tissues, the interactions between cell types, such as synaptic contacts between neurons and myelin sheaths around axons, are unique. These specializations and those that allow for the sequestration of the CNS from the outside world, namely, the blood–brain barrier (BBB) and the absence of lymphatics, become major issues in considerations of normal and disease processes in the nervous system. For the sake of simplicity, the present section is subdivided into a section on general organization and a section regarding major cell types.

Diverse cell types are organized into assemblies and patterns such that specialized components are integrated into a physiology of the whole organ. The CNS parenchyma is made up of nerve cells and their afferent and efferent extensions, dendrites and axons, all closely enveloped by glial cells. Coronal section of the cerebral hemispheres of the brain reveals an outer convoluted rim of gray matter overlying the white matter (Fig. 1-1). Gray matter, which also exists as islands within the white matter, contains mainly nerve cell bodies and glia and lacks significant amounts of myelin, the lipid component responsible for the whiteness of white matter. More distally along the neuraxis in the spinal cord, the cerebral situation is reversed: white matter surrounds gray matter, which is arranged in a characteristic H formation (Fig. 1-2).

A highly diagrammatic representation of the major CNS elements is shown in Figure 1-3. The entire CNS is bathed both internally and externally by cerebrospinal fluid (CSF), which circulates throughout the ventricular and leptomeningeal spaces. This fluid, a type of plasma

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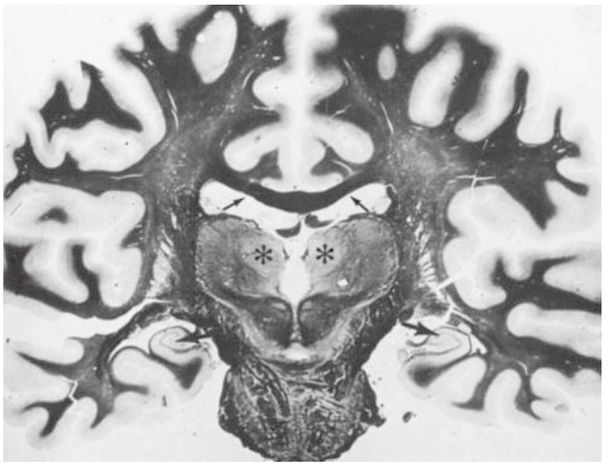

FIGURE 1-1 Coronal section of the human brain at the thalamic level stained by the Heidenhain technique for myelin. Gray matter stains faintly; all myelinated regions are black. The thalamus (*) lies beneath the lateral ventricles and is separated at this level by the beginning of the third ventricle. The roof of the lateral ventricles is formed by the corpus callosum (*small arrows*). Ammon's horns are shown by the *large arrows*. Note the outline of gyri and sulci at the surface of the cerebral hemispheres, sectioned here near the junction of the frontal and parietal cortices.

ultrafiltrate, plays a significant role in protecting the CNS from mechanical trauma, balancing electrolytes and protein and maintaining ventricular pressure (see Ch. 32). The outer surface of the CNS is invested by the triple-membrane system of the meninges. The outermost is the dura mater, derived from the mesoderm, which is tightly adherent to the inner surfaces of the calvaria. The arachnoid membrane is closely applied to the inner surface of the dura mater. The innermost of the meninges, the pia mater, loosely covers the CNS surface. The pia and arachnoid together, derived from the ectoderm, are called the leptomeninges. CSF occupies the subarachnoid space, between the arachnoid and the pia, and the ventricles. The CNS parenchyma is overlaid by a layer of subpial astrocytes, which in turn is covered on its leptomeningeal aspect by a basal lamina (Fig. 1-3). On the inner, or ventricular, surface, the CNS parenchyma is separated from the CSF by a layer of ciliated ependymal cells, which are thought to facilitate the movement of CSF. The production and circulation of CSF are maintained by the choroid plexus, grape-like collections of specialized vascular elements and cells that protrude into the ventricles. Resorption of CSF is effected by vascular structures known as arachnoid villi, located within the leptomeninges over the surface of the brain (see Ch. 32).

Ependymal cells abut layers of astrocytes, which in turn envelop neurons, neurites and vascular components. In addition to neurons and glial cells, such as astrocytes and oligodendrocytes, the normal CNS parenchyma contains blood vessels and microglial cells, the resident macrophages of the CNS.

The PNS and the autonomic nervous system consist of bundles of myelinated and nonmyelinated axons, extending to and from the CNS, which are intimately enveloped by Schwann cells, the PNS counterpart of oligodendrocytes. Nerve bundles are enclosed by the perineurium and the epineurium, which are tough, fibrous, elastic sheaths. Between individual nerve fibers are isolated connective tissue, or endoneurial cells, and blood vessels. The ganglia, such as dorsal root and sympathetic ganglia, are located peripherally to the CNS and are made up of large neurons, usually unipolar or bipolar, surrounded by satellite cells, which are specialized Schwann cells. A dendrite and an axon, both of which can be up to several feet in length, arise from each neuron. Occasionally, within both the CNS and PNS, mast cells can be seen in the perivascular space.

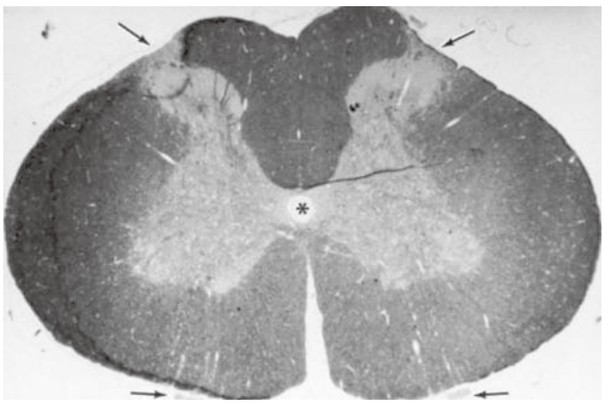

FIGURE 1-2 Transverse section of a rabbit lumbar spinal cord at L-1. Gray matter is seen as a paler-staining area in an H configuration formed by the dorsal and ventral horns with the central canal in the center (*). The dorsal horns would meet the incoming dorsal spine nerve roots at the *upper arrows*. The anterior roots can be seen below (*lower arrows*), opposite the ventral horns, from which they received their fibers. The white matter occupies a major part of the spinal cord and stains darker. Epon section, 1 µm, stained with toluidine blue.

CHARACTERISTICS OF THE NEURON

From a historical standpoint, no other cell type has attracted as much attention or caused as much controversy as the nerve cell. It is impossible in a single chapter to delineate comprehensively the extensive structural, topographical and functional variation achieved by this cell type. Consequently, despite an enormous literature, the neuron still defies precise definition, particularly with regard to function. It is known that the neuronal population usually is established shortly after birth, that mature neurons do not divide and that in humans there is a daily dropout of neurons amounting to approximately 20,000 cells. These facts alone make the neuron unique.

Neurons can be excitatory, inhibitory or modulatory in their effect and motor, sensory or secretory in their function [6]. They can be influenced by a large repertoire of neurotransmitters and hormones (see Ch. 10). This enormous repertoire of functions, associated with different

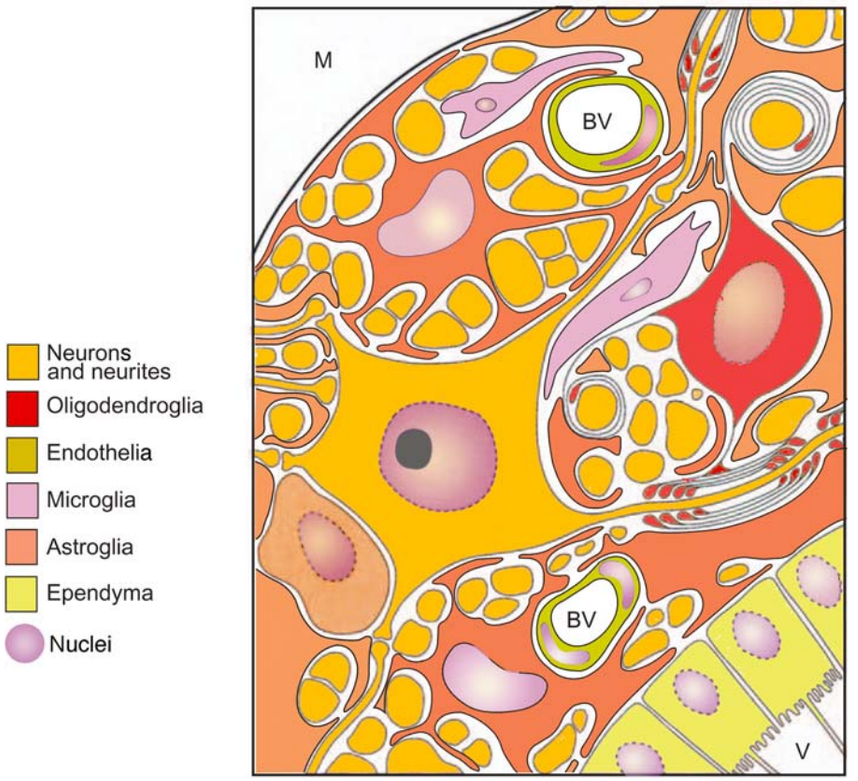

FIGURE 1-3 The major components of the CNS and their interrelationships. Microglia are depicted in light purple. In this simplified schema, the CNS extends from its meningeal surface (*M*) through the basal lamina (*solid black line*) overlying the subpial astrocyte layer of the CNS parenchyma and across the CNS parenchyma proper (containing neurons and glia) and subependymal astrocytes, to ciliated ependymal cells lining the ventricular space (*V*). Note how the astrocyte also invests blood vessels (*BV*), neurons and cell processes. The pia-astroglia (glia limitans) provides the barrier between the exterior (dura and blood vessels) and the CNS parenchyma. One neuron is seen (**center**), with synaptic contacts on its soma and dendrites. Its axon emerges to the right and is myelinated by an oligodendrocyte (**above**). Other axons are shown in transverse section, some of which are myelinated. The oligodendrocyte to the lower left of the neuron is of the nonmyelinating satellite type. The ventricles (*V*) and the subarachnoid space of the meninges (*M*) contain cerebrospinal fluid.

developmental influences on different neurons, is largely reflected in the variation of dendritic and axonal outgrowth. Specialization also occurs at axonal terminals, where a variety of junctional complexes, known as synapses, exist. The subtle synaptic modifications are best visualized ultrastructurally, although immunohistochemical staining also permits distinction among synapses on the basis of transmitter type.

General structural features of neurons are the perikarya, dendrites and axons. The stereotypical image of a neuron is that of a stellate cell body, the perikaryon or soma, with broad dendrites emerging from one pole and a fine axon emerging from the opposite pole. This impression stems from the older work of Purkinje, who first described the nerve cell in 1839, and of Deiters, Ramón y Cajal and Golgi (see [3]) at the end of the 19th century and the early 20th century. However, this picture does not hold true for many neurons. The neuron is the most polymorphic cell in the body and defies formal classification on the basis of shape, location, function, fine structure or transmitter substance. Although early workers described the neuron as a globular mass suspended between nerve fibers, the teased preparations of Deiters and his contemporaries soon proved this not to be the case. Later work, using impregnation staining and culture techniques, elaborated on Deiters's findings. Before the work of Deiters and Ramón y Cajal, both neurons and neuroglia were believed to form syncytia, with no intervening membranes. Today, of course, we are familiar with the specialized membranes and the enormous variety of nerve cell shapes and sizes. They range from the small, globular cerebellar granule cells, with a perikaryal diameter of approximately 6–8 µm, to the pear-shaped Purkinje cells and star-shaped anterior horn cells, both of which may reach diameters of 60–80 µm in humans. Perikaryal size is generally a poor index of total cell volume, however, and it is a general rule in neuroanatomy that neurites occupy a greater percentage of the cell surface area than does the soma. For example, the pyramidal cell of the somatosensory cortex has a cell body that accounts for only 4% of the total surface area, whereas from its dendritic tree the dendritic spines alone claim 43% (Mungai, quoted by Peters *et al.* [3]). Hyden [2] quotes Scholl (1956), who calculated that the

perikaryon of a 'cortical cell' represents 10% of the neuronal surface area. In the feline reticular formation, some giant cells possess ratios between soma and dendrites of about 1:5. A single axon is the usual rule, but some cells, like the Golgi cells of the cerebellum, are endowed with several axons, some of which may show branching.

The extent of the branching displayed by the dendrites is a useful index of their functional importance. Dendritic trees represent the expression of the receptive fields, and large fields can receive inputs from multiple origins. A cell with a less developed dendritic ramification, such as the cerebellar granule cell, has synapses with a more homogeneous population of afferent sources.

The axon emerges from a neuron as a slender thread and frequently does not branch until it nears its target. In contrast to the dendrite and the soma, the axon is myelinated frequently, thus increasing its efficiency as a conducting unit. Myelin, a spirally wrapped membrane (see Ch. 4), is laid down in segments, or internodes, by oligodendrocytes in the CNS and by Schwann cells in the PNS. The naked regions of axon between adjacent myelin internodes are known as nodes of Ranvier (see below).

Neurons contain the same intracellular components as do other cells. No unique cytoplasmic inclusions of the neuron distinguish it from any other cell. Neurons have all the morphological counterparts of other cell types, the structures are similarly distributed and some of the most common, the Golgi apparatus and mitochondria, for example, were described first in neurons (Fig. 1-4).

The nucleus is large and usually spherical, containing a prominent nucleolus. The nucleochromatin is invariably pale, with little dense heterochromatin. In some neurons, such as the cerebellar granule cells, the nucleoplasm may show more differentiation and dense heterochromatin. The nucleolus is vesiculated and clearly delineated from the rest of the nucleoplasm. It usually contains two textures: the pars fibrosa, which are fine bundles of filaments, and the pars granulosa, in which dense granules predominate. An additional juxtaposed structure, found in neurons of the female of some species, is the nucleolar satellite, or sex chromatin, which consists of dense but loosely packed, coiled filaments. The nucleus is enclosed by the nuclear envelope, made up on the cytoplasmic side by the perikaryon inner membrane, which sometimes is seen in continuity with the endoplasmic reticulum (Fig. 1-5), and a more regular membrane on the inner, or nuclear, aspect of the envelope. Between the two is a clear channel of between 20 and 40 nm. Periodically, the inner and outer membranes of the envelope come together to form a single diaphragm, a nuclear pore (Fig. 1-5). In tangential section, nuclear pores are seen as empty vesicular structures, approximately 70 nm in diameter. In some neurons, as in Purkinje cells, that segment of the nuclear envelope which faces the dendritic pole is deeply invaginated.

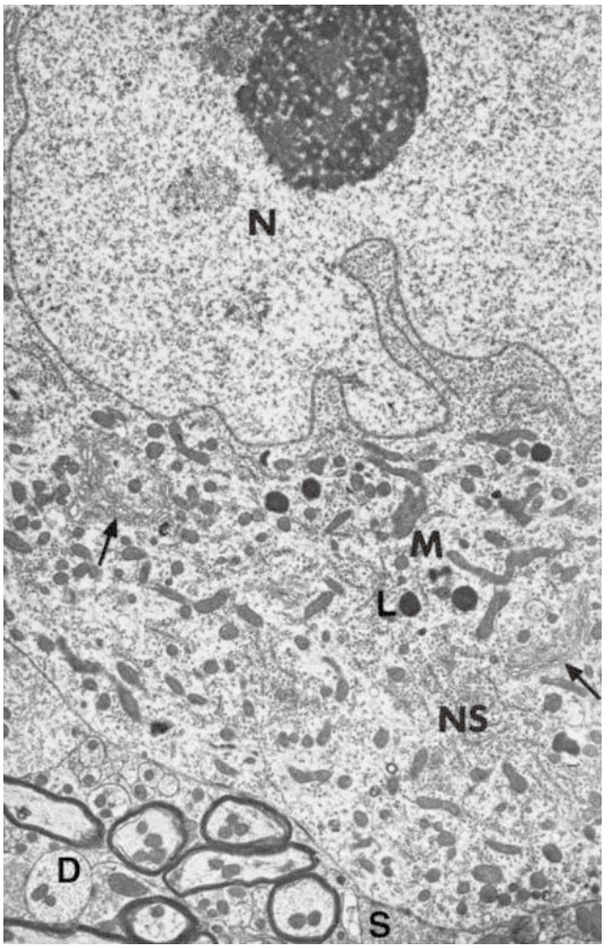

FIGURE 1-4 A motor neuron from the spinal cord of an adult rat shows a nucleus (*N*) containing a nucleolus, clearly divisible into a pars fibrosa and a pars granulosa, and a perikaryon filled with organelles. Among these, Golgi apparatus (*arrows*), Nissl substance (*NS*), mitochondria (*M*) and lysosomes (*L*) can be seen. An axosomatic synapse (*S*) occurs below, and two axodendritic synapses abut a dendrite (*D*). ×8,000.

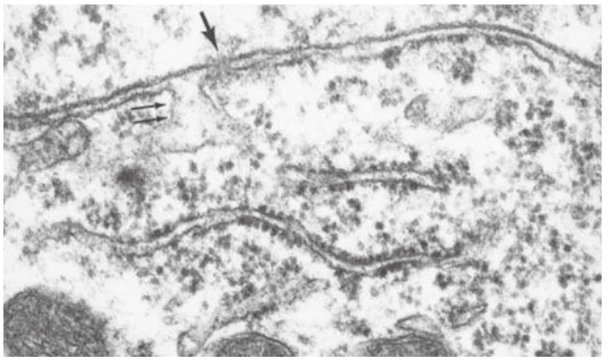

FIGURE 1-5 Detail of the nuclear envelope showing a nuclear pore (*single arrow*) and the outer leaflet connected to the smooth endoplasmic reticulum (ER) (*double arrows*). Two cisternae of the rough ER with associated ribosomes are also present. ×80,000.

The perikaryon, or body of the neuron, is rich in organelles (Fig. 1-4). It often stands out poorly from a homogeneous background neuropil, most of which is composed of nonmyelinated axons and dendrites, synaptic complexes and glial cell processes. Closer inspection shows that, like all cells, the neuron is delineated by a typical triple-layered unit membrane approximately 7.5 nm wide. Among the most prominent features of the perikaryal cytoplasm is a system of membranous cisternae, divisible into rough or granular endoplasmic reticulum (ER), which forms part of the Nissl substance; smooth or agranular ER; subsurface cisternae; and the Golgi apparatus. Although these various components are interconnected structurally, each possesses distinct enzymological properties. Also present within the cytoplasm are abundant lysosomes; lipofuscin granules, which also are termed *aging pigment*; mitochondria; multivesicular bodies; neurotubules; neurofilaments; and ribosomes.

Nissl substance consists of the intracytoplasmic basophilic masses that ramify loosely throughout the cytoplasm and is typical of most neurons (Figs 1-4, 1-5). The distribution of Nissl substance in certain neurons is characteristic and can be used as a criterion for identification. By electron microscopy (EM), this substance is seen to comprise regular arrays or scattered portions of flattened cisternae of the rough ER surrounded by clouds of free polyribosomes. The membranes of the rough ER are studded with rows of ribosomes, which produce the granular appearance of the rough ER. A space of 20–40 nm is maintained within cisternae. Sometimes, cisternal walls meet at fenestrations. Unlike the rough ER of glandular cells or other protein-secreting cells, such as plasma cells, the rough ER of neurons probably produces most of its proteins for use within that neuron, a feature imposed by the extraordinary functional demands placed on the cell. Nissl substance does not penetrate axons but does extend along dendrites.

Smooth endoplasmic reticulum is present in most neurons, although it is sometimes difficult to differentiate it from the rough ER owing to the disorderly arrangement of ribosomes. Ribosomes are not associated with these membranes, and the cisternae usually assume a meandering, branching course throughout the cytoplasm. In some neurons, the smooth ER is quite prominent, for example, in Purkinje cells. Individual cisternae of the smooth ER extend along axons and dendrites (see Chs 8 and 9).

Subsurface cisternae are a system of smooth, membrane-bound, flattened cisternae that can be found in many neurons. These structures, referred to as hypolemmal cisternae by Palay and Chan-Palay [1], abut the plasmalemma of the neuron and constitute a secondary membranous boundary within the cell. The distance between these cisternae and the plasmalemma is usually 10–12 nm and, in some neurons, such as the Purkinje cells, a mitochondrion may be found in close association with the innermost leaflet. Similar cisternae have been described beneath synaptic complexes, but their functional significance is not known. Some authors have suggested that such a system may play a role in the uptake of metabolites. Membrane structures are described in Chapter 2.

The *Golgi apparatus* is a highly specialized form of agranular reticulum and is visualized best using the metal impregnation techniques of Golgi. Ultrastructurally, the Golgi apparatus consists of aggregates of smooth-walled cisternae and a variety of vesicles. It is surrounded by a heterogeneous assemblage of organelles, including mitochondria, lysosomes and multivesicular bodies. In most neurons, the Golgi apparatus encompasses the nucleus and extends into dendrites but is absent from axons. A three-dimensional analysis of the system reveals that the stacks of cisternae are pierced periodically by fenestrations. Tangential sections of these fenestrations show them to be circular profiles. A multitude of vesicles is associated with each segment of the Golgi apparatus, particularly 'coated' vesicles, which proliferate from the lateral margins of flattened cisternae (Fig. 1-6) (see Ch. 9). Such structures have been variously named, but the term alveolate vesicle seems to be generally accepted. Histochemical staining reveals that these bodies are rich in acid hydrolases, and they are believed to represent primary lysosomes [7]. Acid phosphatase is also found elsewhere in the cisternae but in lesser amounts than in alveolate vesicles.

The *lysosome* is the principal organelle responsible for the degradation of cellular waste. It is a common constituent of all cell types of the nervous system and is particularly prominent in neurons, where it can be seen at various stages of development (Fig. 1-4). It ranges in size from 0.1 to 2 μm in diameter. The primary lysosome is elaborated from Golgi saccules as a small, vesicular structure (Fig. 1-6). Its function is to fuse with the membrane of waste-containing vacuoles, termed *phagosomes*, into which

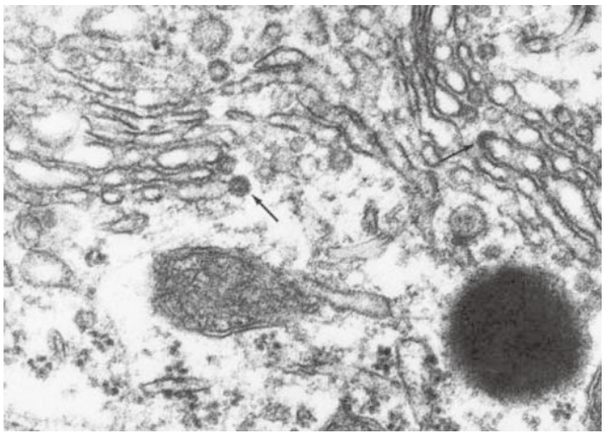

FIGURE 1-6 A portion of a Golgi apparatus. The smooth-membraned cisternae appear beaded. The many circular profiles represent tangentially sectioned fenestrations and alveolate vesicles (primary lysosomes). Two of the latter can be seen budding from Golgi saccules (*arrows*). Mitochondria and a dense body (secondary lysosomes) are also present. ×60,000.

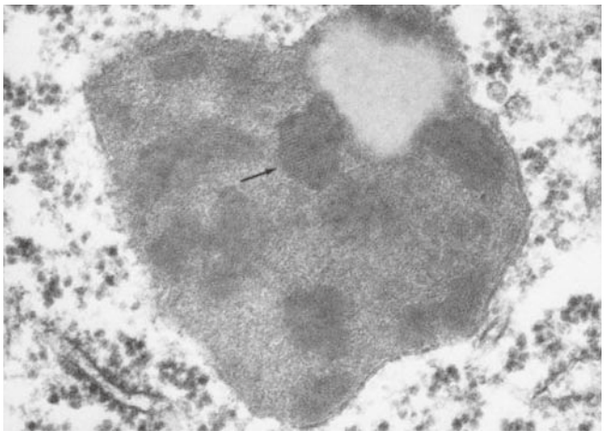

FIGURE 1-7 A lipofuscin granule from a cortical neuron shows membrane-bound lipid (dense) and a soluble component (gray). The denser component is lamellated. The lamellae appear as paracrystalline arrays of tubular profiles when sectioned transversely (*arrow*). The granule is surrounded by a single-unit membrane. Free ribosomes also can be seen. ×96,000.

it releases hydrolytic enzymes (see Ch. 41). The sequestered material is then degraded within the vacuole, and the organelle becomes a secondary lysosome; it is usually electron-dense and large. The matrix of this organelle will give a positive reaction when tested histochemically for acid phosphatase. Residual bodies containing nondegradable material are considered to be tertiary lysosomes, and in the neuron some are represented by lipofuscin granules (Fig. 1-7). These granules contain brown pigment and lamellar stacks of membrane material and are more common in the aged brain [7].

Multivesicular bodies are usually found in association with the Golgi apparatus and are visualized by EM as small, single membrane-bound sacs approximately 0.5 μm in diameter. They contain several minute, spherical profiles, sometimes arranged about the periphery. They are believed to belong to the lysosome series prior to secondary lysosomes because they contain acid hydrolases and apparently are derived from primary lysosomes.

Neurotubules, (neuronal microtubules), have been the subject of intense research [8]. They usually are arranged haphazardly throughout the perikaryon of neurons but are aligned longitudinally in axons and dendrites. Each neurotubule consists of a dense-walled structure enclosing a clear lumen, in the middle of which may be found an electron-dense dot. High-resolution studies indicate that each microtubule wall consists of 13 filamentous subunits arranged helically around a lumen (see also Chs 8 and 28). The diameter of neurotubules varies between 22 and 24 nm. Axonal microtubules display 5 nm filamentous side-arms known to be involved in axoplasmic transport in association with the molecular motor proteins, kinesin and dynein (see below).

Neurofilaments belong to the family of intermediate filaments and usually are found in association with neurotubules. The function of these two organelles has been debated for some time [8,9], and current views of their roles in the maintenance of form and in axoplasmic transport are discussed in Chapters 8 and 28. Neurofilaments have a diameter of approximately 10 nm, are of indeterminate length and frequently occur in bundles. They are constant components of axons but are rarer in dendrites. In the axon, individual filaments possess a minute lumen and interconnect by proteinaceous side-arms, thereby forming a meshwork. Because of these cross-bridges, they do not form tightly-packed bundles in the normal axon, in contrast to filaments within astrocytic processes (see Fig. 1-14), which lack cross-bridges. Neurofilaments within neuronal somata usually do not display cross-bridges and can be found in tight bundles. A form of filamentous structure finer than neurofilaments is seen at the tips of growing neurites, particularly in the growth cones of developing axons. These structures, known as microfilaments, are 5 nm in size and are composed of actin, belonging to a family of molecules (myosins), involved in mechanical stress and organelle transport via nanotubular highways known as 'tunneling nanotubes' [10]. Actin microfilaments facilitate movement and growth since it has been shown that axonal extension can be arrested pharmacologically by treatment with compounds that depolymerize these structures. The biochemistry of neurotubules and neurofilaments is dealt with in more detail in Chapter 8 and in Soifer [8] and Wang *et al.* [9].

Mitochondria are the centers for oxidative phosphorylation and the respiratory centers of all cells. While usually aerobic, some mitochondria (e.g. in some bacteria), are known that function anaerobically. These organelles occur ubiquitously in the neuron and its processes (Figs 1-4, 1-6). Their overall shape may change from one type of neuron to another but their basic morphology is identical to that in other cell types. Mitochondria consist morphologically of double-membraned sacs surrounded by protuberances, or cristae, extending from the inner membrane into the matrix space [7].

The axon becomes physiologically and structurally divisible into the following distinct regions as it egresses: the axon hillock, the initial segment, the axon proper and the axonal termination [3]. The segments differ ultrastructurally in membrane morphology and the content of the rough and smooth ER. The axon hillock may contain fragments of Nissl substance, including abundant ribosomes, which diminish as the hillock continues into the initial segment. Here, the various axoplasmic components begin to align longitudinally. A few ribosomes and the smooth ER persist, and some axoaxonic synapses occur. More interesting, however, is the axolemma of the initial segment, the region for the generation of the action potential, which is underlaid by a dense granular layer similar to that seen at the nodes of Ranvier. Also present in this region are neurotubules, neurofilaments and mitochondria. The arrangement of the neurotubules in the initial segment, unlike their scattered pattern in the distal axon,

is in fascicles; they are interconnected by side-arms [3,10]. Beyond the initial segment, the axon maintains a relatively uniform morphology. It contains the axolemma without any structural modification, except at nodes and the termination, where submembranous densities are seen; microtubules, sometimes cross-linked; neurofilaments, connected by side-arms; mitochondria; and tubulovesicular profiles, probably derived from the smooth ER. Myelinated axons show granular densifications beneath the axolemma at the nodes of Ranvier [6,11], and synaptic complexes may occur in the same regions. In myelinated fibers, there is a concentration of sodium channels at the nodal axon, a feature underlying the rapid, saltatory conduction of such fibers [12] (see Chs 6 and 12). The terminal portion of the axon arborizes and enlarges at its synaptic regions, where it might contain synaptic vesicles beneath the specialized presynaptic junction.

Molecular motors move organelles and particles along axonal microtubules. Molecular motors exist along axonal microtubules and are visualized structurally as 10–15 nm side arms. These structures are known to comprise kinesin and dynein. The kinesins, a large family of >140 proteins, are involved in the transport of a variety of cargo membranous organelles, mRNA, intermediate filaments and signaling molecules. The dyneins form an equally large family and are involved in microtubule bending and flagellar (ciliary) beating. Both kinesin and dynein motors are ATP-dependent and display directionality. Conventional kinesin, a processive dimeric motor, moves material along microtubules in a plus-end direction (anterograde; away from the centrosome), in eight 3 nm center-of-mass steps for each ATP hydrolyzed. The globular heads of kinesin move in a hand-over-hand mechanism, not an inchworm mechanism, as believed previously [13]. Several nonconventional kinesins are known to exist that move materials towards the minus-end of the microtubule, towards the cell body (retrograde). Dyneins generally move cargo retrogradely, towards the minus-end only (see Ch. 28).

The dendrites are the afferent components of neurons and frequently are arranged around the neuronal soma in stellate fashion. In some neurons, they may arise from a single trunk, from which they branch into a dendritic tree. Unlike axons, they generally lack neurofilaments, although they may contain fragments of Nissl substance; however, large branches of dendrites in close proximity to neurons may contain small bundles of neurofilaments. Some difficulty may be encountered in distinguishing small unmyelinated axons or terminal segments of axons from small dendrites. In the absence of synaptic data, they can often be assessed by the content of neurofilaments. The synaptic regions of dendrites occur either along the main stems (Fig. 1-8) or at small protuberances known as dendritic spines or thorns. Axon terminals abut these structures.

The synapse is a specialized junctional complex by which axons and dendrites emerging from different

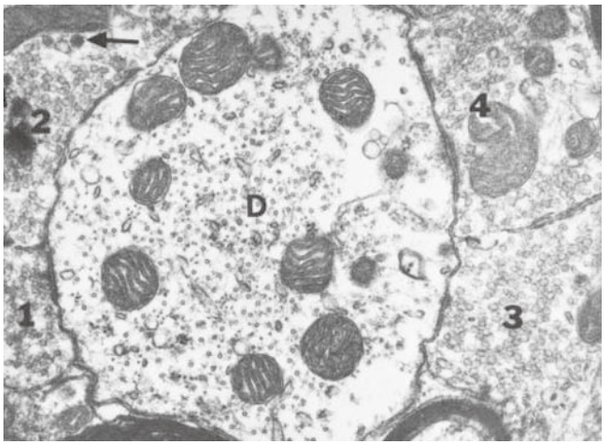

FIGURE 1-8 A dendrite (*D*) emerging from a motor neuron in the anterior horn of a rat spinal cord is contacted by four axonal terminals: terminal *1* contains clear, spherical synaptic vesicles; terminals *2* and *3* contain both clear, spherical and dense-core vesicles (*arrow*); and terminal *4* contains many clear, flattened (inhibitory) synaptic vesicles. Note also the synaptic thickenings and, within the dendrite, the mitochondria, neurofilaments and neurotubules. ×33,000.

neurons intercommunicate [14]. This was proposed first by Sherrington in 1897, who also proposed the term 'synapse'. The existence of synapses was immediately demonstrable by EM and can be recognized today in a dynamic fashion by Nomarski optics (differential interference microscopy), confocal optics, light microscopy and scanning EM. With the development of neurochemical approaches to neurobiology, an understanding of synaptic form and function becomes of fundamental importance. As was noted in the first ultrastructural study on synapses (Palade and Palay in 1954, quoted in [4]), synapses display interface specialization and frequently are polarized or asymmetrical. The asymmetry is due to the unequal distribution of electron-dense material, or thickening, applied to the apposing membranes of the junctional complex and the heavier accumulation of organelles within the presynaptic component. The closely applied membranes constituting the synaptic site are overlaid on the presynaptic and postsynaptic aspects by an electron-dense material similar to that seen in desmosomes and separated by a gap or cleft of 15–20 nm. The presynaptic component usually contains a collection of clear, 40–50 nm synaptic vesicles. These synaptic vesicles are important in packaging, transport and release of neurotransmitters and after their discharge into the synaptic cleft, they are recycled with the axon terminal [6,15]. Also present are small mitochondria approximately 0.2–0.5 μm in diameter (Figs 1-8–1-10). Occasionally, 24 nm microtubules, coated vesicles and cisternae of the smooth ER are found in this region. On the postsynaptic side is a density referred to as the subsynaptic web but, apart from an infrequent, closely applied packet of smooth ER or subsurface cisternae belonging to the hypolemmal system, there are no aggregations of organelles in the dendrite.

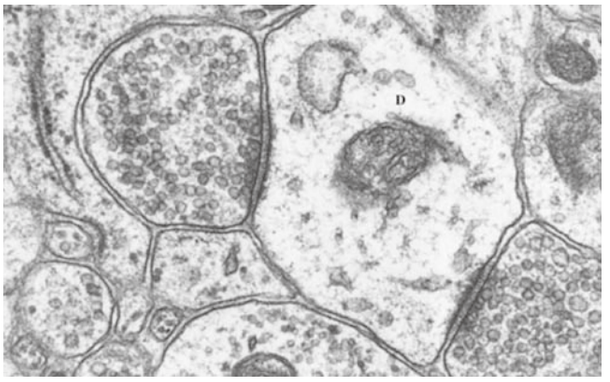

FIGURE 1-9 A dendrite (*D*) is flanked by two axon terminals packed with clear, spherical synaptic vesicles. Details of the synaptic region are clearly shown. ×75,000.

At the neuromuscular junction, the morphological organization is somewhat different. Here, the axon terminal is greatly enlarged and ensheathed by Schwann cells; the postsynaptic or sarcolemmal membrane displays less density and is infolded extensively.

Before elaborating further on synaptic diversity, it might be helpful to outline briefly other ways in which synapses have been classified in the past. Using the light microscope, Ramón y Cajal was able to identify 11 distinct groups of synapses [16]. Today, most neuroanatomists apply a more fundamental classification schema to synapses, depending on the profiles between which the synapse is formed, such as axodendritic, axosomatic, axoaxonic, dendrodendritic, somatosomatic and somatodendritic synapses. Unfortunately, such a list disregards whether the transmission is chemical or electrical and, in the case of chemical synapses, this classification does not address the neurotransmitter involved.

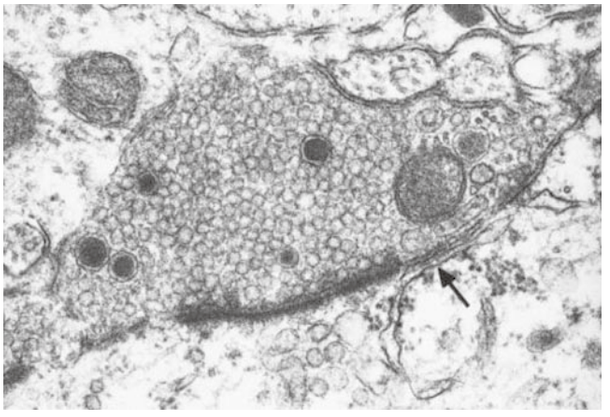

FIGURE 1-10 An axonal terminal at the surface of a neuron from the dorsal horn of a rabbit spinal cord contains both dense-core and clear, spherical synaptic vesicles lying above the membrane thickenings. A subsurface cisterna (*arrow*) is also seen. ×68,000.

In terms of physiological typing, three groups of synapses are recognized: excitatory, inhibitory and modulatory. Some neuroanatomical studies [14] have claimed that excitatory synapses possess spherical synaptic vesicles, whereas inhibitory synapses contain a predominance of flattened vesicles (Fig. 1-8). Other studies [17] have correlated this synaptic vesicular diversity with physiological data. In his study on the cerebellum, Gray [17] showed that neurons with a known predominance of excitatory input on dendrites and an inhibitory input on the cell body possessed two corresponding types of synapse; however, although this interpretation fits well in some loci of the CNS, it does not hold true for all regions. Furthermore, some workers consider that the differences between flat and spherical vesicles may reflect an artifact of aldehyde fixation or a difference in physiological state at the time of sampling. In light of these criticisms, it is clear that confirmation of the correlation between flattened vesicles and inhibitory synapses is required.

Another criterion for the classification of synapses by EM was introduced in 1959 by Gray [17]. Briefly, certain synapses in the cerebral cortex can be grouped into two types, depending on the length of the contact area between synaptic membranes and the amount of postsynaptic thickening. Relationships have been found between type 1 synapses, which have closely apposed membranes over long distances and a large amount of associated postsynaptic thickening, and excitatory axodendritic synapses. Type 2 synapses, which show less close apposition and thickening at the junction, are mainly axosomatic and are believed to be inhibitory. This broad grouping has been confirmed in the cerebral cortex by a number of workers but it does not hold true for all regions of the CNS.

Most of the data from studies on synapses *in situ* or on synaptosomes have been on cholinergic transmission. There is a vast family of chemical synapses that utilize biogenic amines (see Ch. 12) as neurotransmitter substances. Morphologically, catecholaminergic synapses are similar but possess, in addition to clear vesicles, slightly larger dense-core or granular vesicles of variable dimension (Figs 1-8, 1-10). These vesicles were identified first as synaptic vesicles by Grillo and Palay (see Bloom [18]), who segregated classes of granular vesicles based on vesicle and core size, but no relationship was made between granular vesicles and transmitter substances. About the same time, EM autoradiographic techniques were being employed and, using tritiated norepinephrine, Wolfe *et al.* [19] labeled granular vesicles within axonal terminals. Catecholaminergic vesicles generally are classified on a size basis, and not all have dense cores. Another, still unclassified, category of synapses may be the so-called silent synapses observed in CNS tissue both *in vitro* and *in vivo*. These synapses are morphologically identical to functional synapses but are physiologically dormant.

Finally, with regard to synaptic type, there is the well-characterized electrical synapse [20], where current can pass from cell to cell across regions of membrane

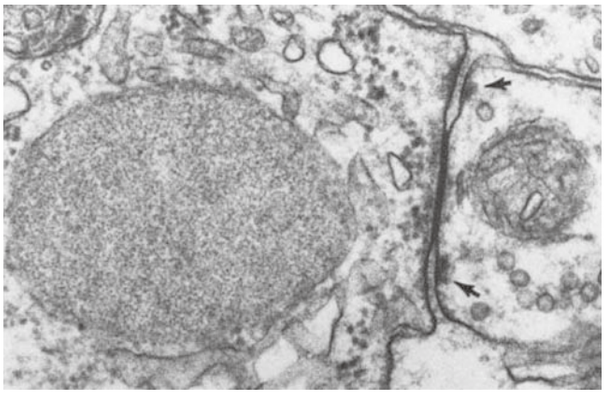

FIGURE 1-11 An electrotonic synapse is seen at the surface of a motor neuron from the spinal cord of a toadfish. Between the neuronal soma (**left**) and the axonal termination (**right**), a gap junction flanked by desmosomes (*arrows*) is visible. (Photograph courtesy of Drs G. D. Pappas and J. S. Keeter.) ×80,000.

apposition that essentially lack the associated collections of organelles present at the chemical synapse. In the electrical synapse (Fig. 1-11), the unit membranes are closely apposed, and indeed, the outer leaflets sometimes fuse to form a pentalaminar structure; however, in most places, a gap of approximately 20 nm exists, producing a so-called gap junction. Not infrequently, such gap junctions are separated by desmosome-like regions [3]. Sometimes, electrical synapses exist at terminals that also display typical chemical synapses; in such cases, the structure is referred to as a mixed synapse. The comparative morphology of electrical and chemical synapses has been reviewed by Pappas and Waxman [20].

Molecular markers can be used to identify neurons. Characterization of the vast array of neuron-specific cytoskeletal elements, such as intermediate filaments, microtubules and their associated proteins [21,22], and the neurotransmitters and their receptors [6,21], has led to the development of correspondingly large numbers of molecular and immunological probes, which now are applied routinely in neuroanatomical analyses. Under normal conditions, the neuron is incapable of participating in T-cell interactions via the expression of major histocompatibility complex (MHC) antigens or the production of soluble mediators, such as cytokines. However, neurons do possess unique proteins, some of which are antigenic, that normally are sequestered by the BBB from the circulating immune system. This theoretically renders the CNS vulnerable to immune-mediated damage should the BBB be breached.

CHARACTERISTICS OF NEUROGLIA

In 1846, Virchow first recognized the existence in the CNS of a fragile, non-nervous, interstitial component made up of stellate or spindle-shaped cells, morphologically distinct from neurons, which he named neuroglia, or 'nerve glue' [3]. It was not until the early part of the 20th century that this interstitial element was classified as consisting of distinct cell types [3,4]. Today, we recognize three broad groups of glial cells: (a) true glial cells or macroglia, such as astrocytes and oligodendrocytes, of ectodermal origin, the stem cell of which is the spongioblast; (b) microglia, of mesodermal origin; and (c) ependymal cells, also of ectodermal origin and sharing the same stem cell as true glia. Microglia invade the CNS at the time of vascularization via the pia mater, the walls of blood vessels and the tela choroidea. Glial cells differ from neurons in that they possess no conventional synaptic contacts and retain the ability to divide throughout life, particularly in response to injury. The rough schema represented in Figure 1-3 demonstrates the interrelationships between glia and other CNS components.

Virtually nothing can enter or leave the central nervous system parenchyma without passing through an astrocytic interphase. The complex packing achieved by the processes and cell bodies of astrocytes underscores their involvement in brain metabolism. Although astrocytes traditionally have been subdivided into protoplasmic and fibrous astrocytes [4], these two forms probably represent the opposite ends of a spectrum of the same cell type. However, Raff *et al.* [23] have suggested that the two groups might derive from different progenitors and that the progenitor of the fibrous astrocyte is the same as that of the oligodendrocyte, although recent evidence supports a lineage for myelinating oligodendrocytes involving motor neurons [24]. The structural components of fibrous and protoplasmic astrocytes are identical; the differences are quantitative. In the early days of EM, differences between the two variants were more apparent owing to imprecise techniques, but, with the development of better procedures, the differences became less apparent.

Protoplasmic astrocytes range in size from 10–40 μm, frequently are located in gray matter in relation to capillaries and have a clearer cytoplasm than do fibrous astrocytes (Fig. 1-12). Within the perikaryon of both types of astrocyte are scattered 9 nm filaments and 24 nm microtubules (Fig. 1-13); glycogen granules; lysosomes and lipofuscin-like bodies; isolated cisternae of the rough ER; a small Golgi apparatus opposite one pole of the nucleus; and small, elongated mitochondria, often extending together with loose bundles of filaments along cell processes. A centriole is not uncommon. Characteristically, the nucleus is ovoid and the nucleochromatin homogeneous, except for a narrow, continuous rim of dense chromatin and one or two poorly defined nucleoli. The fibrous astrocyte occurs in white matter (Fig. 1-13). Its processes are twig-like, being composed of large numbers of 9 nm glial filaments arranged in tight bundles. The filaments within these cell processes can be distinguished from neurofilaments by their close packing and the absence of side-arms (Figs 1-13, 1-14). Desmosomes (mediated by cadherins) and gap junctions (mediated by connexins)

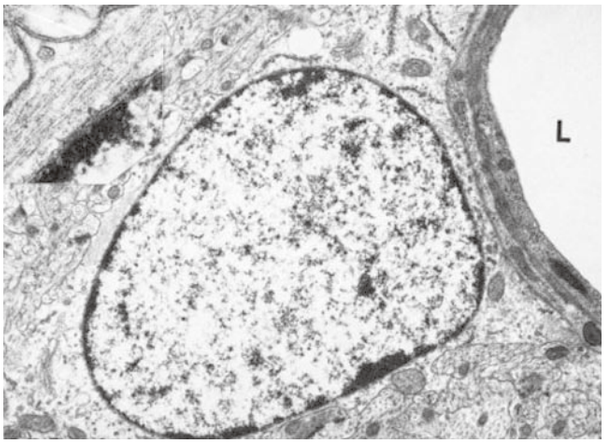

FIGURE 1-12 A protoplasmic astrocyte abuts a blood vessel (lumen at *L*) in rat cerebral cortex. The nucleus shows a rim of denser chromatin, and the cytoplasm contains many organelles, including Golgi and rough endoplasmic reticulum. ×10,000. **Inset (top left):** Detail of perinuclear cytoplasm showing filaments. ×44,000.

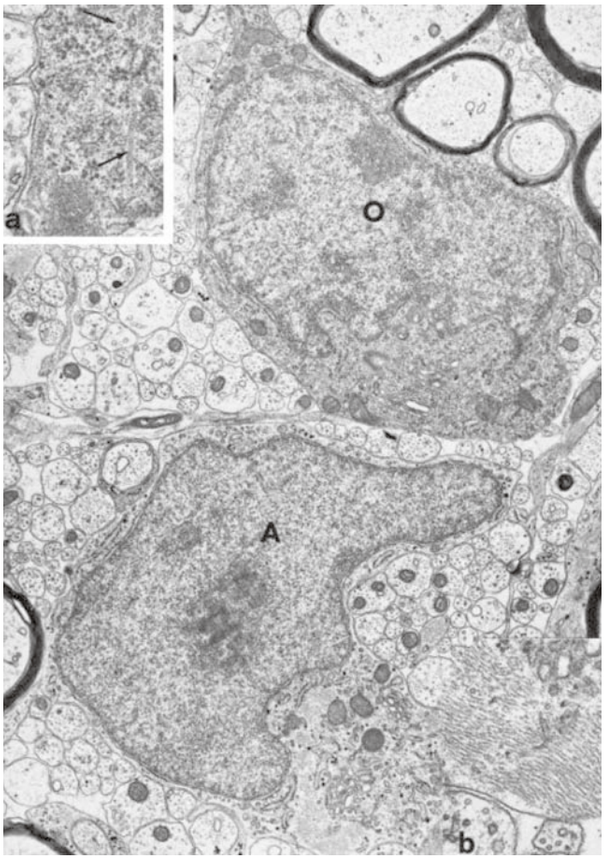

FIGURE 1-13 A section of myelinating white matter from a kitten contains a fibrous astrocyte (*A*) and an oligodendrocyte (*O*). The nucleus of the astrocyte (*A*) has homogeneous chromatin with a denser rim and a central nucleolus. That of the oligodendrocyte (*O*) is denser and more heterogeneous. Note the denser oligodendrocytic cytoplasm and the prominent filaments within the astrocyte. ×15,000. **Inset a:** Detail of the oligodendrocyte, showing microtubules (*arrows*) and absence of filaments. ×45,000. **Inset b:** Detail of astrocytic cytoplasm showing filaments, glycogen, rough endoplasmic reticulum and Golgi apparatus. ×45,000.

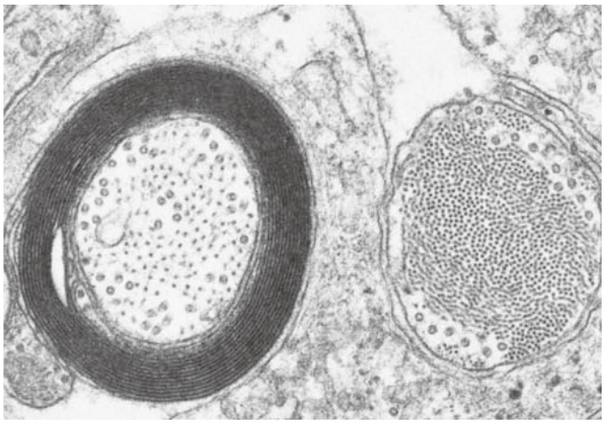

FIGURE 1-14 Transverse sections of a myelinated axon (**left**) and the process of a fibrous astrocyte (**right**) in dog spinal cord. The axon contains scattered neurotubules and loosely packed neurofilaments interconnected by side-arm material. The astrocytic process contains a bundle of closely packed filaments with no cross-bridges, flanked by several microtubules. Sometimes, a lumen can be seen within a filament. ×60,000.

occur between adjacent astrocytic processes. The latter also occur between astrocyte processes and myelin.

In addition to protoplasmic and fibrous forms, regional specialization occurs among astrocytes. The outer membranes of astrocytes located in subpial zones and those facing blood vessels (which are in most cases formed from invaginations of the pial surface as blood vessels penetrated into the CNS from the meningeal surface during development), possess a specialized thickening, sometimes called 'hemidesmosomes'. Desmosomes and gap junctions are very common in these regions between astrocytic processes. In the cerebellar cortex, protoplasmic astrocytes can be segregated into three classes, each ultrastructurally distinct: the Golgi epithelial cell, the lamellar or velate astrocyte and the smooth astrocyte [1].

Astrocyte functions have long been debated. Their major role is related to a connective tissue or skeletal function since they invest, possibly sustain and provide a packing for other CNS components. In the case of astrocytic ensheathment around synaptic complexes and the bodies of some neurons, such as Purkinje cells, it has been speculated that the astrocyte isolates these structures and their neurotransmitters, which may be released into the extracellular space, since some neurotransmitters are capable of precipitating cell damage if extracellular accumulations are not rapidly cleared. In the case of glutamate, an excitatory neurotransmitter, this can lead to glutamate excitotoxicity due to overactivation of ion channels on nerve cells and the excessive entry of calcium, which can trigger apoptosis. Under normal circumstances, the astrocyte is involved in glutamate uptake, whereby it enzymatically detoxifies this transmitter substance, converting it to glutamine, which is then recycled to the neuron (see Chs 5 and 15).

One well-known function of the astrocyte is concerned with repair. Subsequent to trauma, astrocytes invariably proliferate, swell, accumulate glycogen and undergo fibrosis by the accumulation of filaments, expressed neurochemically as an increase in glial fibrillary acidic protein (GFAP). This state of gliosis may be total, in which case all other elements are lost, leaving a glial scar, or it may be a generalized response occurring against a background of regenerated or normal CNS parenchyma. Fibrous astrocytosis can occur in both the gray and white matter, thereby indicating common links between protoplasmic and fibrous astrocytes. With age, both fibrous and protoplasmic astrocytes accumulate filaments. In some diseases, astrocytes become macrophages. It is interesting to note that the astrocyte is probably the most disease-resistant component in the CNS because very few diseases, other than alcoholism, cause depletion of astrocytes.

Another putative role of the astrocyte is its involvement in transport mechanisms (see Ch. 5) and in the BBB system (see Ch. 32). Astrocytes interact with neurons in various metabolic and transport processes. It was believed for some time that transport of water and electrolytes was effected by the astrocyte, a fact never definitively demonstrated and largely inferred from pathological or experimental evidence. It is known, for example, that damage to the brain vasculature, local injury due to heat or cold and inflammatory changes produce focal swelling of astrocytes, presumably owing to disturbances in fluid transport. The astrocytic investment of blood vessels suggests a role in the BBB system, but the studies of Reese and Karnovsky [25] and Brightman [26] indicate that the astrocytic end-feet provide little resistance to the movement of molecules and that blockage of the passage of material into the brain occurs at the endothelial-cell-lining blood vessels (see Ch. 32). CNS endothelial cells display selective transport by transcytosis and the surface of the astrocyte also frequently displays evidence of endocytosis by the presence of clathrin-coated pits, structures known to be involved in a number of secretory and recycling mechanisms. During inflammation, these mechanisms are disrupted and there are alterations in permeability of endothelial tight junctions and formation of edema within and around neighboring astrocytes. Finally, it is believed that astrocytes are responsible for the regulation of local pH levels and local ionic balances.

Molecular markers of astrocytes. Although antigenically distinct from other cell types by virtue of its expressing GFAP [27], there is no documented evidence of astrocytic disease related to an immunological response to GFAP on any astroglial molecule. GFAP remains singularly the most used cytoplasmic marker of astrocytes. Another protein with phenotypic specificity for astrocytes is S100β, a marker that develops earlier than GFAP in astrocytes and can therefore be used to identify less mature cells. A reliable marker for astrocytic membranes remains to be described. Interestingly, there is increasing evidence demonstrating the ability of astrocytes to serve as accessory cells of the immune system in a number of immune-mediated conditions [27,28]. In this regard, astrocytes have been shown *in vitro* to express class II MHC antigens, which are molecules essential for the presentation of antigen to helper/inducer $CD4^+$ T cells, as well as their ability to synthesize a number of cytokines, such as interleukin-1, tumor necrosis factor, interferon γ and several chemokines, e.g. Groα. (see Chs 35 and 39). It appears, therefore, that in circumstances in which the BBB is interrupted, the astrocyte is a facultative phagocyte with the potential to interact with lymphocytes.

Oligodendrocytes are myelin-producing cells in the central nervous system. The ultrastructural studies of Schultz *et al.* (1957) and Farquhar and Hartman (1957) (discussed in [4]) were among the first to contrast the EM features of oligodendrocytes with astrocytes (Fig. 1-12). The study of Mugnaini and Walberg [4] more explicitly laid down the morphological criteria for identifying these cells and, apart from subsequent technical improvements, our EM understanding of these cells has changed little since that time [5,29].

As with astrocytes, oligodendrocytes are highly variable, differing in location, morphology and function, but definable by some morphological criteria. The cell soma ranges from 10 to 20 μm and is roughly globular and more dense than that of an astrocyte. The margin of the cell is irregular compressed against the adjacent neuropil, and may show the presence of coated pits. Few cell processes are seen, in contrast to the astrocyte. Within the cytoplasm, many organelles are found. Parallel cisternae of rough ER and a widely-dispersed Golgi apparatus are common. Free ribosomes occur, scattered amid occasional multivesicular bodies, mitochondria and coated vesicles. Distinguishing the oligodendrocyte from the astrocyte are the apparent absence of glial filaments and the constant presence of 24 nm microtubules (Fig. 1-13). Microtubules are most common at the margins of the cell, in the occasional cell process and in the cytoplasmic loops around myelin sheaths. Lamellar dense bodies, typical of oligodendrocytes, are also present [5]. The nucleus is usually ovoid, but slight lobation is not uncommon. The nucleochromatin stains heavily and contains clumps of denser heterochromatin; the whole structure is sometimes difficult to discern from the background cytoplasm. Desmosomes and gap junctions occur between interfascicular oligodendrocytes [5].

Ultrastructural and labeling studies on the developing nervous system (see Ch. 27) have demonstrated variability in oligodendrocyte morphology and activity. Mori and Leblond (see [5]) separated oligodendrocytes into three groups based on location, stainability and DNA turnover. Their three classes correspond to satellite, intermediate and interfascicular, or myelinating, oligodendrocytes. Satellite oligodendrocytes are small (≈10 μm), restricted to gray matter and closely applied to the surface of neurons.

They are assumed to play a role in the maintenance of the neuron and are potential myelinating cells. Interfascicular oligodendrocytes are large (≈20 μm) during myelination but, in the adult, range from 10 to 15 μm, with the nucleus occupying a large percentage of the cell volume. Intermediate oligodendrocytes are regarded as satellite or potential myelinating forms. The nucleus of these cells is small, the cytoplasm occupying the greater area of the soma.

Myelinating oligodendrocytes have been studied extensively [5,30] (see Ch. 4). Examination of the CNS during myelinogenesis (Fig. 1-15) reveals connections between the cell body and the myelin sheath [31]; however, connections between these elements have never been demonstrated in a normal adult animal, unlike the PNS counterpart, the Schwann cell. In contrast to the Schwann cell (see below), the oligodendrocyte is capable of producing many internodes of myelin simultaneously. It has been estimated that oligodendrocytes in the optic nerve produce between 30 and 50 internodes of myelin [5]. In addition to this heavy structural commitment, the oligodendrocyte possesses a slow mitotic rate and a poor regenerative capacity. Damage to only a few oligodendrocytes, therefore, can be expected to produce an appreciable area of primary demyelination. In most CNS diseases in which myelin is a target, oligodendrocytes are among the most vulnerable elements and the first to degenerate (see Ch. 39).

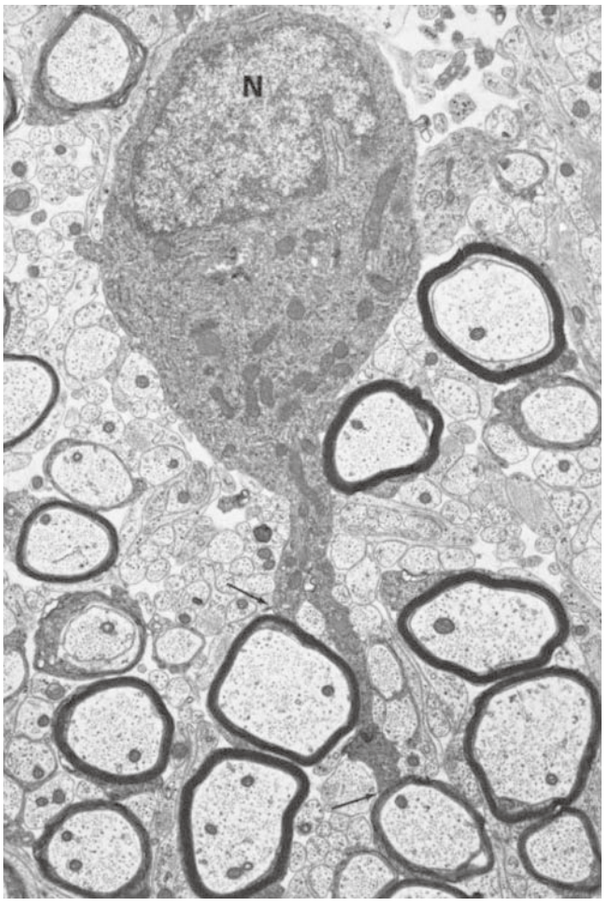

FIGURE 1-15 A myelinating oligodendrocyte, nucleus (*N*), from the spinal cord of a 2-day-old kitten extends cytoplasmic connections to at least two myelin sheaths (*arrows*). Other myelinated and unmyelinated fibers at various stages of development, as well as glial processes, are seen in the surrounding neuropil. ×12,750.

Somewhat analogous to the neuron, the relatively small oligodendrocyte soma produces and supports many more times its own volume of membrane and cytoplasm. For example, consider an average 12 μm oligodendrocyte producing 20 internodes of myelin [5]. Each axon has a diameter of 3 μm and is covered by at least six lamellae of myelin, each lamella representing two fused layers of unit membrane. By statistical analysis, taking into account the length of the myelin internode, which is possibly 500 μm, and the length of the membranes of the cell processes connecting the sheaths to the cell body (≈12 μm), the ratio between the surface area of the cell soma and the myelin it sustains is approximately 1:620. In most cases, however, this ratio is probably in the region of 1:3,000. In rare instances, oligodendrocytes elaborate myelin around structures other than axons in that myelin has been documented around neuronal somata and nonaxonal profiles.

Molecular markers of oligodendrocytes. The oligodendrocyte is potentially highly vulnerable to immune-mediated damage since it shares with the myelin sheath many molecules with known affinities to elicit specific T- and B-cell responses, which lead to its destruction. Chapter 39 describes the immune process in demyelination. Many of these molecules, such as myelin basic protein, proteolipid protein, myelin-associated glycoprotein, myelin/oligodendrocyte protein, galactocerebroside, myelin oligodendrocyte glycoprotein (MOG) and others, have been used to generate specific antibodies, which are routinely applied to anatomical analyses of oligodendrocytes *in vivo* and *in vitro*. However, while the oligodendrocyte expresses no class I or II MHC molecules [32], it has recently been found to demonstrate a wide array of cytokine receptors, both pro-inflammatory and regulatory [33], suggestive of innate and adaptive abilities to participate in immune responses.

The microglial cell plays a role in phagocytosis and inflammatory responses. Of the few remaining types of CNS cell, the most interesting, and probably the most enigmatic, is the microglial cell, a cell of mesodermal origin, located in the normal brain in a resting state (Fig. 1-3) and purported to become a very mobile, active macrophage during disease (see Ch. 35) and the major effector cell in immune-mediated damage in the CNS. Microglia can be stained selectively and demonstrated by light microscopy using Hortega's silver carbonate method, but no comparable technique exists for their

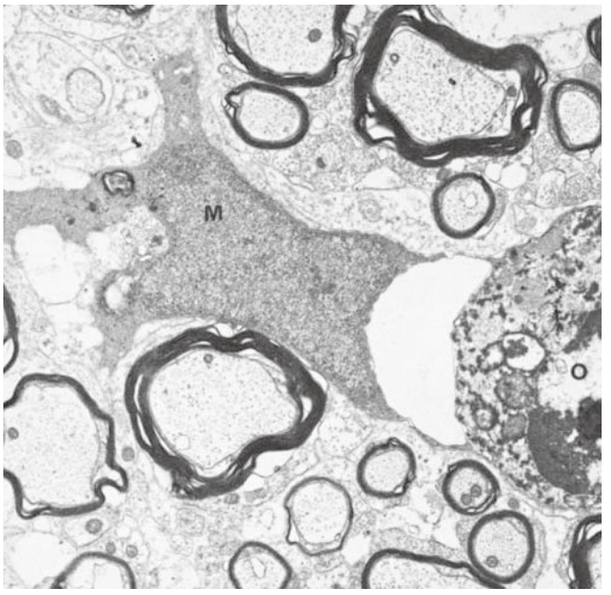

FIGURE 1-16 A microglial cell (*M*) has elaborated two cytoplasmic arms to encompass a degenerating apoptotic oligodendrocyte (*O*) in the spinal cord of a 3-day-old kitten. The microglial cell nucleus is difficult to distinguish from the narrow rim of densely staining cytoplasm, which also contains some membranous debris. ×10,000.

ultrastructural demonstration. A wide array of immunocytochemical markers are available for microglia, including anti-MHC II and anti-CD45 (LCA).The cells have spindle-shaped bodies and a thin rim of densely-staining cytoplasm difficult to distinguish from the nucleus. The nucleochromatin is homogeneously dense and the cytoplasm does not contain an abundance of organelles, although representatives of the usual components can be found. During normal wear and tear, some CNS elements degenerate and microglia phagocytose the debris (Fig. 1-16). Their identification and numbers, as determined by light microscopy, differ from species to species. The CNS of rabbit is richly endowed. In a number of disease instances, such as trauma, microglia are stimulated and migrate to the area of injury, where they phagocytose debris. The relatively brief mention of this cell type in the major EM textbooks [3] and the reported conflicting EM descriptions [34] were indicative of the uncertainty attached to their identification at those timepoints. Pericytes are believed by some to be a resting form of microglial cell. Perivascular macrophages, which are of bone marrow origin and are distinct from parenchymal microglia, have also been described and these cells are known to traffic between the CNS and the lymphoid system.

Molecular markers of microglial cells. In the past 10–15 years there has been a veritable explosion of activity in the field of microglial cell biology with the realization that this cell type is capable of functioning as a highly efficient accessory and effector cell of the immune system. While no particularly microglia-specific molecule has been identified, a number of antibodies raised against monocytic markers and complement receptor molecules stain microglial cells *in situ* and *in vitro*. Microglia express class II MHC upon activation [35–38], frequently in the absence of a T-cell response. This suggests that class II MHC expression may represent a marker of activation or in some way elevate the cells to a state of immunological awareness or competence. Microglia are major producers of a large number of proinflammatory cytokines with known effects upon T cells [33]. Taken in concert, an abundance of evidence for an immunological role for microglia in a wide spectrum of CNS conditions supports the putative monocytic origin of this cell type and bestows upon it the role of the major effector of immune mediated damage in the CNS.

Ependymal cells line the brain ventricles and the spinal cord central canal. Ependymal cells are arranged in single palisade arrays and line the ventricles of the brain and central canal of the spinal cord. They are usually ciliated, their cilia extending into the ventricular cavity. Their fine structure has been elucidated by Brightman and Palay [39]. They possess several features that clearly differentiate them from any other CNS cell. The cilia emerge from the apical pole of the cell, where they are attached to a blepharoplast, the basal body (Fig. 1-17), which is anchored in the cytoplasm by means of ciliary rootlets and a basal foot. The basal foot is the contractile component that determines the direction of the ciliary beat. Like all flagellar structures, the cilium contains the common microtubule arrangement of nine peripheral pairs around a central doublet (Fig. 1-17). In the vicinity of the basal body, the arrangement is one of nine triplets; at the tip of each cilium, the pattern is one of haphazardly organized

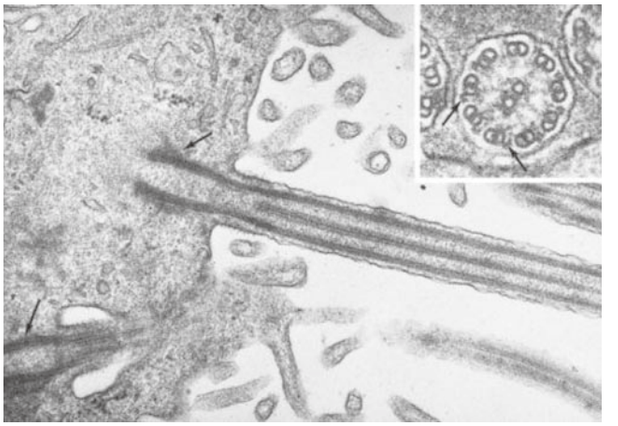

FIGURE 1-17 The surface of an ependymal cell contains basal bodies (*arrows*) connected to the microtubules of cilia, seen here in longitudinal section. Several microvilli are also present. ×37,000. **Inset:** Ependymal cilia in transverse section possess a central doublet of microtubules surrounded by nine pairs, one of each pair having a characteristic hook-like appendage (*arrows*). ×100,000.

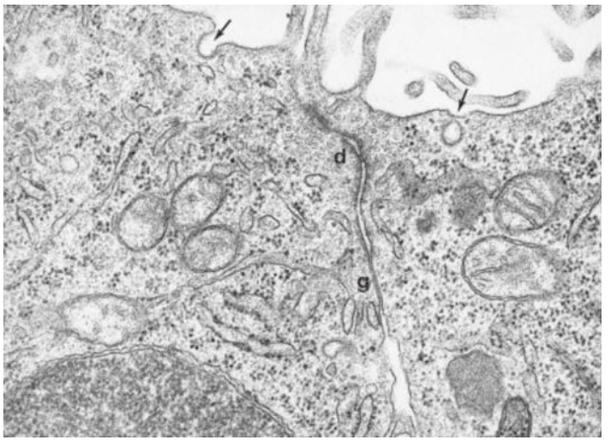

FIGURE 1-18 A typical desmosome (*d*) and gap junction (*g*) between two ependymal cells. Microvilli and coated pits (*arrows*) are seen along the cell surface. ×35,000.

single tubules. Also, extending from the free surface of the cell are numerous microvilli containing actin microfilaments (Fig. 1-17). The cytoplasm stains intensely, having an electron density about equal to that of the oligodendrocyte, whereas the nucleus has a similar density to that of the astrocyte. Microtubules, large whorls of filaments, coated vesicles, rough ER, Golgi-apparatus, lysosomes and abundant small, dense mitochondria are also present in ependymal cells. The base of the cell is composed of involuted processes that interdigitate with the underlying neuropil. The lateral margins of each cell characteristically display long, compound, junctional complexes (Fig. 1-18) made up of desmosomes, termed *zonula adherentes*, and gap junctions [3]. Overlying specialized secretory zones around the ventricles (the subventricular organs) and choroid plexus, the ependymal lining is different and the cells are connected at their apical poles by tight junctions called *zonula occludentes*, the latter expressing the junctional protein occludin. Desmosomes (cadherins) and gap junctions (connexins) are also present at the lateral aspects of the cells [40].

The biochemical properties of these structures are known. Desmosomes display protease sensitivity, divalent cation dependency and osmotic insensitivity; and their membranes are mainly of the smooth type. In direct contrast to desmosomes, the tight junctions as well as gap junctions and synapses display no protease sensitivity, divalent cation dependency or osmotic sensitivity, while their membranes are complex. These facts have been used in the development of techniques to isolate purified preparations of junctional complexes.

The Schwann cell is the myelin-producing cell of the peripheral nervous system. When axons leave the CNS, they lose their neuroglial interrelationships and traverse a short transitional zone where they are invested by an astroglial sheath enclosed in the basal lamina of the glia limitans. The basal lamina then becomes continuous with that of axon-investing Schwann cells, at which point the astroglial covering terminates. Schwann cells, therefore, are the axon-ensheathing cells of the PNS, equivalent functionally to the oligodendrocyte of the CNS (see Ch. 4). Along the myelinated fibers of the PNS, each internode of myelin is elaborated by one Schwann cell and each Schwann cell elaborates one internode [30]. This ratio of one internode of myelin to one Schwann cell is a fundamental distinction between this cell type and its CNS analog, the oligodendrocyte, which is able to proliferate internodes in the ratio of 1:30 or greater. Another distinction is that the Schwann cell body always remains in intimate contact with its myelin internode (Fig. 1-19), whereas the oligodendrocyte extends long, attenuated processes toward its internodes. Periodically, myelin lamellae open up into ridges of Schwann cell cytoplasm, producing bands of cytoplasm around the fiber, Schmidt–Lanterman incisures, reputed to be the stretch points along PNS fibers. These incisures usually are not present in the CNS. The PNS myelin period is 11.9 nm in preserved specimens, which is some 30% less than in the fresh state, in contrast to the 10.6 nm of central myelin. In addition to these structural differences, PNS myelin

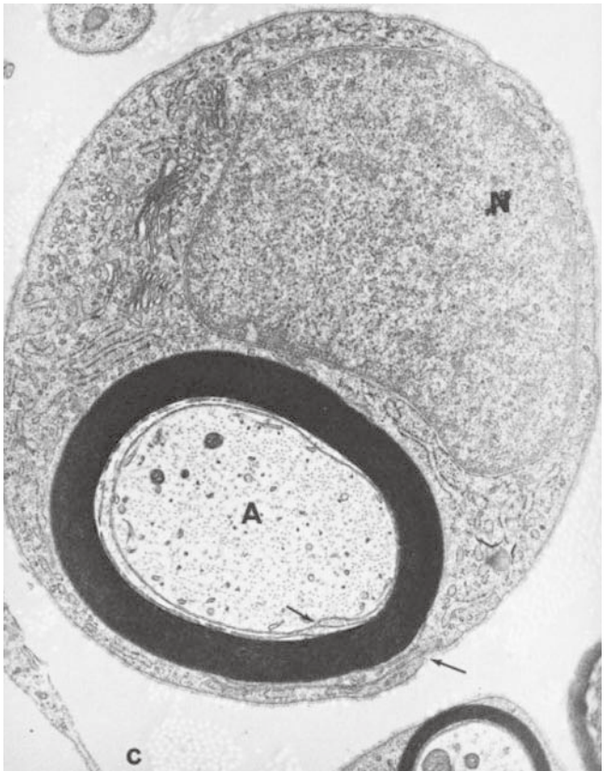

FIGURE 1-19 A myelinated PNS axon (*A*) is surrounded by a Schwann cell nucleus (*N*). Note the fuzzy basal lamina around the cell, the rich cytoplasm, the inner and outer mesaxons (*arrows*), the close proximity of the cell to its myelin sheath and the 1:1 (cell:myelin internode) relationship. A process of an endoneurial cell is seen (**lower left**), and unstained collagen (*c*) lies in the endoneurial space (*white dots*). ×20,000.

differs biochemically and antigenically from that of the CNS (see Ch. 4). Not all PNS fibers are myelinated but, in contrast to nonmyelinated fibers in the CNS, nonmyelinated fibers in the PNS are suspended in groups within the Schwann cell cytoplasm, each axon connected to the extracellular space by a short channel, the mesaxon, formed by the invaginated Schwann cell plasmalemma.

Ultrastructurally, the Schwann cell is unique and distinct from the oligodendrocyte. Each Schwann cell is surrounded by a basal lamina made up of a mucopolysaccharide approximately 20–30 nm thick that does not extend into the mesaxon (Fig. 1-19). The basal laminae of adjacent myelinating Schwann cells at the nodes of Ranvier are continuous, and Schwann cell processes interdigitate so that the PNS myelinated axon is never in direct contact with the extracellular space. These nodal Schwann cell fingers display intimate relationships with the axolemma (Figs 1-20, 1-21), suggesting that the entire nodal complex might serve as an electrogenic pump for the recycling of ions [10]. A similar arrangement between the nodal axon and the fingers of astroglial cells is seen in the CNS. The Schwann cells of nonmyelinated PNS fibers overlap, and there are no nodes of Ranvier.

The cytoplasm of the Schwann cell is rich in organelles. A Golgi apparatus is located near the nucleus, and cisternae of the rough ER occur throughout the cell. Lysosomes, multivesicular bodies, glycogen granules and lipid granules, also termed pi granules, also can be seen. The cell is rich in microtubules and filaments, in contrast to the oligodendrocyte. The plasmalemma frequently shows pinocytic vesicles. Small, round mitochondria are scattered throughout the soma. The nucleus, which stains intensely, is flattened and oriented longitudinally along the nerve fiber. Aggregates of dense heterochromatin are arranged peripherally [3].

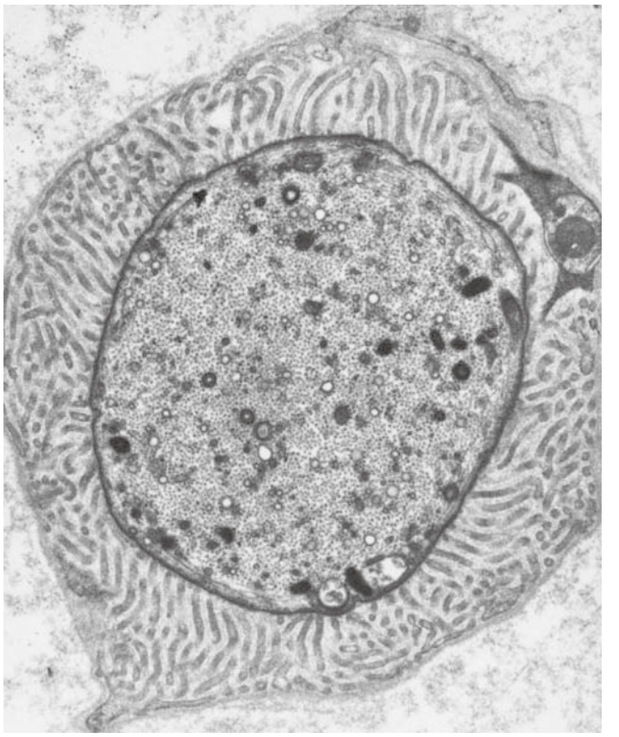

FIGURE 1-21 A transverse section of the node of Ranvier (7–8 μm across) of a large fiber shows a prominent complex of Schwann cell fingers around an axon highlighted by its subaxolemmal densification and closely packed organelles. The Schwann cell fingers arise from an outer collar of flattened cytoplasm and abut the axon at regular intervals of approximately 80 nm. The basal lamina of the nerve fiber encircles the entire complex. The nodal gap substance is granular and sometimes linear. Within the axoplasm, note the transversely sectioned sacs of beaded smooth endoplasmic reticulum (ER); mitochondria; dense lamellar bodies, which appear to maintain a peripheral location; flattened smooth ER sacs; dense-core vesicles; cross-bridged neurofilaments; and microtubules, which in places run parallel to the circumference of the axon (**above left** and **lower right**), perhaps in a spiral fashion. ×16,000.

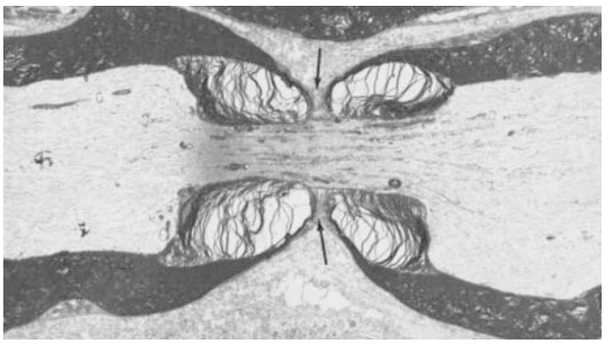

FIGURE 1-20 Low-power electron micrograph of a node of Ranvier in longitudinal section. Note the abrupt decrease in axon diameter and the attendant condensation of axoplasmic constituents in the paranodal and nodal regions of the axon. Paranodal myelin is distorted artifactually, a common phenomenon in large-diameter fibers. The nodal gap substance (*arrows*) contains Schwann cell fingers, the nodal axon is bulbous and lysosomes lie beneath the axolemma within the bulge. Beaded smooth endoplasmic reticulum sacs are also seen. ×5,000.

In sharp contrast to the oligodendrocyte, the Schwann cell responds vigorously to most forms of injury (see Ch. 39). An active phase of mitosis occurs following traumatic insult, and the cells are capable of local migration. Studies on their behavior after primary demyelination have shown that they are able to phagocytose damaged myelin. They possess remarkable reparatory properties and begin to lay down new myelin approximately 1 week after a fiber loses its myelin sheath. Studies on PNS and CNS remyelination [41] have shown that, by 3 months after primary demyelination, PNS fibers are well remyelinated, whereas similarly affected areas in the CNS show relatively little proliferation of new myelin (see Ch. 29). Under circumstances of severe injury, such as transection, axons degenerate and the Schwann cells form tubes, termed *Büngner bands*, containing cell bodies and processes surrounded by a single basal lamina. These structures provide channels along which regenerating axons

might later grow. The presence and integrity of the Schwann cell basal lamina is essential for reinnervation.

The extracellular space between peripheral nerve fibers is occupied by bundles of collagen fibrils, blood vessels and endoneurial cells. Endoneurial cells are elongated, spindle-shaped cells with tenuous processes relatively poor in organelles except for large cisternae of the rough ER. There is some evidence that these cells proliferate collagen fibrils. Sometimes mast cells, the histamine producers of connective tissue, can be seen. Bundles of nerve fibers are arranged in fascicles emarginated by flattened connective tissue cells forming the perineurium, an essential component in the blood–nerve barrier system. Fascicles of nerve fibers are aggregated into nerves and invested by a tough elastic sheath of cells known as the epineurium [42].

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REFERENCES

1. Palay, S. L. and Chan-Palay, V. *Cerebellar Cortex: Cytology and Organization*. New York: Springer, 1974.
2. Hyden, H. The neuron. In J. Brachet and A. E. Mirsky (eds.), *The Cell*. New York: Academic Press, 1960, vol. 5, pp. 215–323.
3. Peters, A., Palay, S. L. and Webster, H. de F. *The Fine Structure of the Nervous System: The Cells and Their Processes*. New York: Oxford University Press, 1991.
4. Mugnaini, E. and Walberg F. Ultrastructure of neuroglia. *Ergeb. Anat. Entwicklungsgesch.* 37: 194–236, 1964.
5. Raine, C. S. Oligodendrocytes and central nervous system myelin. In R. L. Davis and D. M. Robertson (eds.), *Textbook of Neuropathology*, 3rd ed. Baltimore: Williams & Wilkins, 1997, pp. 137–164.
6. Kandel, E. R., Schwarz, J. H. and Jessell, T. M. (eds.), *Principles of Neural Science*. Amsterdam: Elsevier, 1991.
7. Novikoff, A. B. and Holtzman, E. *Cells and Organelles*. New York: Holt, Rinehart & Winston, 1976.
8. Soifer, D. (ed.), Dynamic aspects of microtubule biology. *Ann. N.Y. Acad. Sci.* 466, 1986.
9. Wang, E., Fischman, B., Liem, R. L. and Sun, T.-T. (eds.), Intermediate filaments. *Ann. N.Y. Acad. Sci.* 455, 1985.
10. Rustom, A., Saffrich, R., Markovic, I., Walther, P. and Gerdes, H.-H. Nanotubular highways for intercellular organelle transport. *Science* 303:1007–1010, 2004.
11. Raine, C. S. Differences in the nodes of Ranvier of large and small diameter fibres in the PNS. *J. Neurocytol.* 11: 935–947, 1982.
12. Ritchie, J. M. Physiological basis of conduction in myelinated nerve fibers. In P. Morell (ed.), *Myelin*. New York: Plenum Press, 1984, pp. 117–146.
13. Yildiz, A., Tomishige, M., Vale, R. D. and Selvin, P. R. Kinesin walks hand-over-hand. *Science* 303:676–678, 2004.
14. Peters, A. and Palay, S. L. The morphology of synapses *J. Neurocytol.* 25: 687–700, 1996.
15. Bauerfeind, R., Galli, T. and DeCamilli, P. Molecular mechanisms in synaptic vesicle recycling. *J. Neurocytol.* 25: 701–716, 1996.
16. Bodian, D. Synaptic diversity and characterization by electron microscopy. In G. D. Pappas and D. P. Purpura (eds.), *Structure and Function of Synapses*. New York: Raven Press, 1972, pp. 45–65.
17. Gray, E. G. Electron microscopy of excitatory and inhibitory synapses: a brief review. *Prog. Brain Res.* 31: 141, 1969.
18. Bloom, F. E. Localization of neurotransmitters by electron microscopy. In *Neurotransmitters (Proc. ARNMD)*. Baltimore: Williams & Wilkins, 1972, vol. 50, pp. 25–57.
19. Wolfe, D. E., Potter, L. T., Richardson, K. C. and Axelrod, J. Localizing tritiated norepinephrine in sympathetic axons by electron microscopic autoradiography. *Science* 138: 440–442, 1962.
20. Pappas, G. D. and Waxman, S. Synaptic fine structure: morphological correlates of chemical and electronic transmission. In G. D. Pappas and D. P. Purpura (eds.), *Structure and Function of Synapses*. New York: Raven Press, 1972, pp. 1–43.
21. Liem, R. K. H. Neuronal intermediate filaments. *Curr. Opin. Cell Biol.* 2: 86–90, 1990.
22. Cleveland, D. W. and Hoffman P. N. Neuronal and glial cytoskeletons. *Curr. Opin. Neurobiol.* 1: 346–353, 1991.
23. Raff, M. C., Miller R. H. and Noble M. A. Glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* 303: 390–396, 1983.
24. Rowitch, D. H., Lu, R. Q., Richardson, W. and Kessaris N. An 'oligarchy' rules neural development. *Trends Neurosci.* 25: 417–422, 2002.
25. Reese, T. S. and Karnovsky M. J. Fine structural localization of a blood–brain barrier to exogenous peroxidase. *J. Cell Biol.* 34: 207–217, 1967.
26. Brightman, M. The distribution within the brain of ferritin injected into cerebrospinal fluid compartments. II. Parenchymal distribution. *Am. J. Anat.* 117: 193–220, 1965.
27. Yong, V. W. and Antel J. P. Major histocompatibility complex molecules on glial cells. *Semin. Neurosci.* 4: 231–240, 1992.
28. Benveniste, E. N. Cytokine expression in the nervous system. In R. W. Keane and W. F. Hickey (eds.), *Immunology of the Nervous System*. New York: Oxford University Press, 1997, pp. 419–459.
29. Norton, W. T. (ed.), *Oligodendroglia. Advances in Neurochemistry*, vol. 5. New York: Plenum, 1984.
30. Raine, C. S. Morphology of myelin and myelination. In P. Morell (ed.), *Myelin*, 2nd ed. New York: Plenum, 1984, pp. 1–50.
31. Bunge, R. P. Glial cells and the central myelin sheath. *Physiol. Rev.* 48: 197–248, 1968.

32. Raine, C. S. The Dale E. McFarlin Memorial Lecture: the immunology of the multiple sclerosis lesion. *Ann. Neurol.* 36: 561–572, 1994.
33. Cannella, B. and Raine, C. S. Multiple sclerosis: cytokine receptors on oligodendrocytes predict innate regulation. *Ann. Neurol.* 55: 46–57, 2004.
34. Fujita, S. and Kitamura, T. Origin of brain macrophages and the nature of the microglia. In H. Zimmerman (ed.), *Progress in Neuropathology.* New York: Grune & Stratton, 1976, vol. 2, pp. 1–50.
35. Dickson, D. W., Mattiace, L. A., Kure K., *et al.* Biology of disease. Microglia in human disease, with an emphasis on acquired immune deficiency syndrome. *Lab. Invest.* 64: 135–156, 1991.
36. Matsumoto, Y., Ohmori, K. and Fujiwara, M. Microglial and astroglial reactions to inflammatory lesions of experimental autoimmune encephalomyelitis in the rat central nervous system. *J. Neuroimmunol.* 37: 23–33, 1992.
37. Ling, E. A. and Wong, W. C. The origin and nature of ramified and amoeboid microglia: an historical review and current concepts. *Glia* 7: 84–92, 1993.
38. Perry, V. H. and Gordon, S. Microglia and macrophages. In R. W. Keane and W. F. Hickey (eds.), *Immunology of the Nervous System.* New York: Oxford University Press, 1997, pp. 155–172.
39. Brightman, M. and. Palay, S. L. The fine structure of ependyma in the brain of the rat. *J. Cell Biol.* 19: 415–440, 1963.
40. Milhorat, T. H. (ed.), *Cerebrospinal Fluid and the Brain Edemas.* New York: Neuroscience Society of New York, 1987.
41. Raine, C. S., Wisniewski, H. and Prineas, J. An ultrastructural study of experimental demyelination and remyelination. II. Chronic experimental allergic encephalomyelitis in the peripheral nervous system. *Lab. Invest.* 21: 316–327, 1969.
42. Babel, J., Bischoff, A. and Spoendlin, H. Ultrastructure of the peripheral nervous system and sense organs. In *Atlas of Normal and Pathologic Anatomy.* St Louis: Mosby, 1970, pp. 1–171.

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CHAPTER

2

Cell Membrane Structures and Functions

R. Wayne Albers

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Neurons are specialized to integrate selected extracellular signals, both spatially and temporally. In addition to generating action potentials and, through synaptic activity, signaling other cells, structural modifications are initiated within neurons that may be as transient as ion channel gating or as long lasting as memory. Nearly all of this activity involves cell membranes and many of these membrane functions are discussed in subsequent chapters. This chapter begins with brief discussions of the physical chemistry underlying the lipid and protein components of cell membranes (Figs 2-1–2-4), proceeds to examine some aspects of membrane biochemistry relevant to neurons and their supporting cells, and concludes with discussion of some issues of cell membrane functions that are subjects of current investigations.

To perform its unique functional role each neuron must regulate a host of intracellular activities. that occur in axons and dendrites distant from the cell nucleus. For example, axonal guidance during development, or remodeling of dendritic spines in response to local input, each involves many different complex control systems that are highly localized and largely autonomous. [1].

PHOSPHOLIPID BILAYERS

Cells are bounded by proteins arrayed in lipid bilayers.

The importance of lipids in membrane structure was established early in the 20th century when pioneering biophysicists established positive correlations between cell membrane permeabilities to small non-electrolytes and the oil/water partition coefficients of these molecules. Contemporary measurements of the electrical impedance of cell suspensions suggested that cells are surrounded by a hydrocarbon barrier, which was first estimated to be about 3.3 nm thick. This was originally thought to be a lipid monolayer. Among the pioneering biophysical experiments were those that established that the ratio of the area of a monolayer formed from erythrocyte

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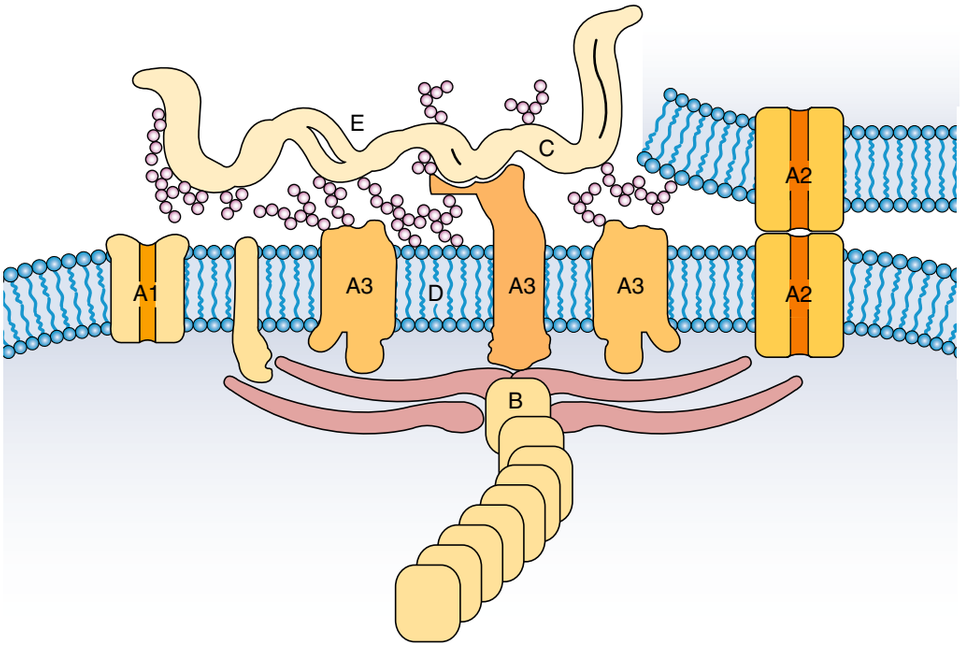

FIGURE 2-1 Overview of plasma membrane structure. Plasma membranes are distinguishable from other cellular membranes by the presence of both glycolipids and glycoproteins on their outer surfaces and the attachment of cytoskeletal proteins to their cytoplasmic surfaces. Interrelations among typical membrane components are depicted. Proteins that are inserted through the lipid bilayer (*A1–A3*), termed 'integral' membrane proteins, are often glycosylated (*lavender circles*), as are some bilayer lipids (*D*) and many components of the extracellular matrix (*E*). Many interactions at the extracellular surface are stabilized by hydrogen bonding among these glycosyl residues. Certain integral membrane proteins can interact by virtue of specific receptor sites with intracellular proteins (*B*), with extracellular components (*C*) and to form specific junctions with other cells (*A2*). A host of integral membrane proteins mediates different signal-transduction and active-transport pathways.

membrane lipids to the surface area of these cells is nearly 2. These and other studies of the physical chemistry of lipids led to the concept of a continuous lipid bilayer as a major component of cell membranes. This concept received support from other approaches, including measurements of X-ray diffraction patterns of intact cell membranes.

Forces acting between lipids and between lipids and proteins are primarily noncovalent, consisting of electrostatic, hydrogen-bonding and van der Waals interactions. These are weak interactions relative to covalent bond formation, but they sum to produce very stable associations. Ionic and polar parts of molecules exposed to water will become hydrated. Substances dissolve in a solvent only if their molecules interact with the solvent more strongly than with each other. In aqueous solution large molecules having two or more domain surfaces of differing polarity will form an internal hydrophobic phase and hydrate the more polar surfaces. Such molecules are termed amphipathic and include most biological lipids and proteins.

Amphipathic molecules can form bilayered lamellar structures spontaneously if they have an appropriate geometry. Most of the major cell membrane lipids have a polar head, most commonly a glycerophosphorylester moiety, and a hydrocarbon tail, usually consisting of two esterified fatty acids. Both domains have similar cross-sectional areas. Consequently, as the head groups interact with each other and with water, and the nonpolar tails aggregate with each other to form an internal phase, the similar cross-sections of the two phases can produce planar bilayers.

Three principal phases with different structures are formed by phospholipids in the presence of water [2] (Fig. 2-2). Although the lamellar, or bilayer, structure is generally found in cell membranes, the hexagonal phases may occur transiently during membrane shape transformations. The importance of molecular geometry for bilayer stability is illustrated by the effects of phospholipase A_2, a component of many venoms, on erythrocytes: this enzyme removes the C-2 fatty acid from phospholipids to produce lysophosphatides. Because of the 'conical' geometry of lysophosphatides, this process ultimately destabilizes bilayers relative to the hexagonal phase structures; this disrupts cell membranes and lyses the cells. Detergents are amphipathic molecules with abilities to transform lipid bilayers into water-soluble micelles. In contrast to the destabilizing effects of lysophosphatides and other detergents, cholesterol stabilizes bilayers by intercalating at the interface between head and tail regions of phospholipid so as to satisfy the bulk requirements for a planar geometry.

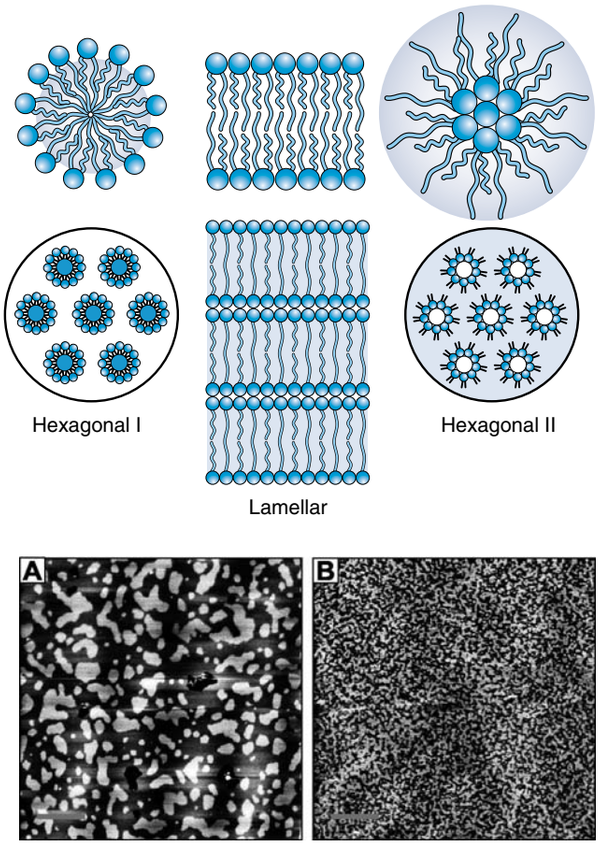

FIGURE 2-2 **Top row:** Complex lipids interact with water and with each other to form different states of aggregation, or 'phases', shown here schematically. *Open circles* or *ellipses* represent the more polar head groups, and *dark lines* and *areas* represent nonpolar hydrocarbon chains. The phase structures are generally classified as illustrated in the **middle row** of the figure. The hexagonal I and lamellar phases can be dispersed in aqueous media to form the micellar structures shown in the **top row.** Hexagonal II phase lipids will form 'reverse micelles' in nonpolar solvents. The stability of lamellar structures relative to hexagonal structures depends upon fatty acid chain length, presence of double bonds, relative sizes of polar head and hydrocarbon tail groups and temperature. **Bottom row:** Atomic Force Microscopic images (6×6 mm, scale bar 1 mm, z-scale of 5 nm) showing (left) domains in bilayers of 1:1 sphingomyelin:dioleylphosphatidylcholine combined with 30% cholesterol and (right) domains of 1:1 dipalmitylphosphatidylcholine:dioleylphosphatidylcholine combined with 30% cholesterol. Lighter areas are higher than darker areas. From [31] with permission.

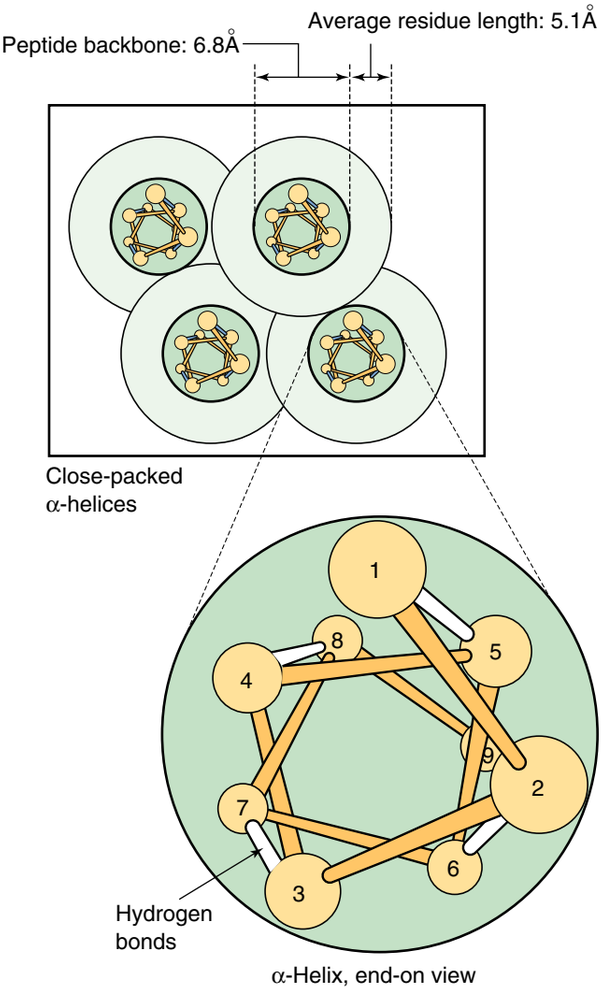

FIGURE 2-3 The transmembrane domains of integral membrane proteins are predominantly α-helices. This structure causes the amino acid side chains to project radially. When several parallel α-helices are closely packed, their side chains may intermesh as shown, or steric constraints may cause the formation of interchain channels. The outwardly directed residues must be predominantly hydrophobic to interact with the fatty acid chains of lipid bilayers. The bilayer is about 3 nm thick. Each peptide residue extends a helix by 1.5 Å. Thus, although local modifications of the bilayer or interactions with other membrane polypeptides may alter this requirement, transmembrane segments usually require about 20 residues to span the bilayer. Integral membrane proteins are characterized by the presence of hydrophobic segments approximating this length.

The multilamellar bilayer structures that form spontaneously on adding water to solid- or liquid-phase phospholipids can be dispersed to form vesicular structures called liposomes. These are often employed in studies of bilayer properties and may be combined with membrane proteins to reconstitute functional membrane systems. A valuable technique for studying the properties of proteins inserted into bilayers employs a single bilayer lamella, also termed a black lipid membrane, formed across a small aperture in a thin partition between two aqueous compartments. Because pristine lipid bilayers have very low ion conductivities, the modifications of ion-conducting properties produced by membrane proteins can be measured with great sensitivity (Ch. 6).

In aqueous systems, membrane lipids may exist in a gel-like solid state or as a two-dimensional liquid. In the case of pure phospholipids, these states interconvert at a well-defined transition temperature, T_c, that increases with alkyl chain length and decreases with introduction of alkyl chain unsaturation. In cell membranes, which have marked heterogeneity in both the polar and nonpolar domains of the bilayer, this state is described as 'liquid disordered'. The presence of sufficient sphingolipids, with

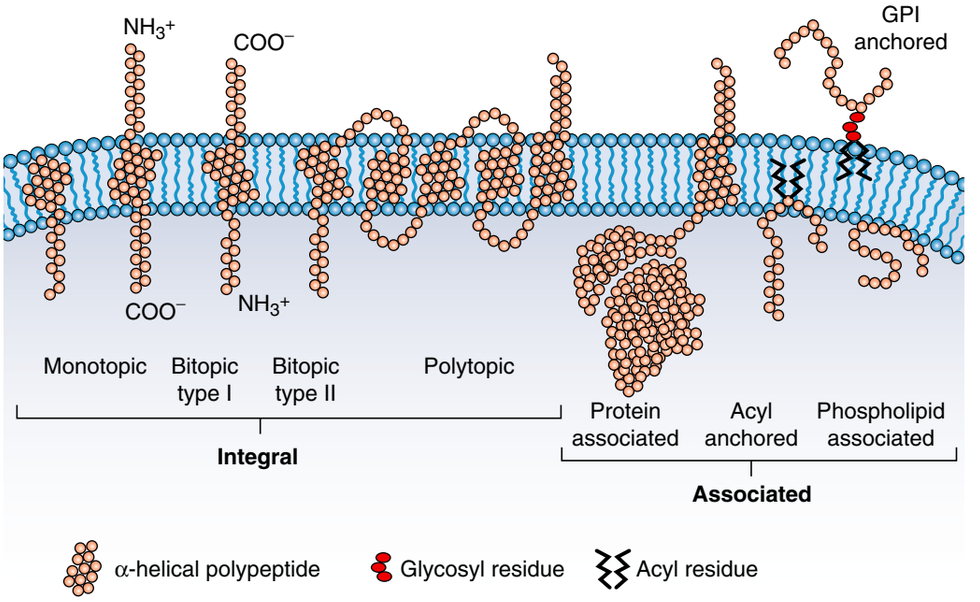

FIGURE 2-4 **Left:** Integral membrane proteins can be classified with respect to the orientation and complexity of their transmembrane segments. **Right:** Proteins may associate with membranes through several types of interactions with the bilayer lipids and by interacting with integral membrane proteins. They can also be 'anchored' by integration into the bilayer of covalently bound lipids. GPI = glycosylphosphatidylinositol.

saturated alkyl chains, and of cholesterol, which has a rigid planar structure, can cause a 'liquid ordered' structure to separate laterally into microdomains that are in phase equilibrium with 'liquid disordered' structures (Fig. 2-2, bottom row). Such microdomains consisting of 'lipid rafts' enriched in cholesterol and sphingomyelin may function, in biomembranes, to concentrate or localize certain membrane proteins, as discussed below.

Alkyl chain heterogeneities cause cell membrane bilayers to remain in the fluid state over a broad temperature range. This permits rapid lateral diffusion of membrane lipids and proteins within the plane of the bilayer. The lateral diffusion rate for an unconstrained phospholipid in a bilayer is of the order of $1\,mm^2\,s^{-1}$; an integral membrane protein such as rhodopsin would diffuse $\approx 40\,nm^2\,s^{-1}$.

MEMBRANE PROTEINS

Membrane integral proteins have transmembrane domains that insert directly into lipid bilayers. Transmembrane domains (TMDs) consist predominantly of nonpolar amino acid residues and may traverse the bilayer once or several times. High-resolution structural information is available for only a few integral membrane proteins, primarily because it is difficult to obtain membrane protein crystals that are adequate for X-ray diffraction measurements.

TMDs usually consist of a helix. The peptide bond is intrinsically polar and can form internal hydrogen bonds between carbonyl oxygens and amide nitrogens, or either of these may be hydrated. Within the lipid bilayer, where water is essentially excluded, peptides usually adopt the α helical configuration that maximizes their internal hydrogen bonding. A length of a helix of 18–21 amino acid residues is sufficient to span the usual width of a lipid bilayer (Fig. 2-3). Because the surface properties of a helix are determined by its side chains, a single helical segment that can insert into or through a bilayer will consist largely of hydrophobic residues.

Integral membrane proteins with one transmembrane domain may have 'soluble' domains at either or both surfaces. An example of a monotopic protein, cytochrome b5 has a single hydrophobic segment that forms a hairpin loop, acting as an anchor to the cytoplasmic surface but probably not totally penetrating the bilayer.

Bitopic proteins with a single transmembrane helix are more common. If oriented with the N-terminus extracytoplasmic, they are classified as type I or, if cytoplasmic, type II (Fig. 2-4). Bitopic membrane proteins are often involved in signal transduction, as exemplified by receptor-activated tyrosine kinases (Ch. 24): agonist occupation of an extracytoplasmic receptor domain can transmit structural changes via a single transmembrane helix to activate the latent kinase activity in a cytoplasmic domain.

Ion channels, transporters and many receptors are polytopic. Polar and helix-destabilizing residues are likely to occur within their transmembrane segments to form the requisite gates, channels or binding domains. Transmembrane helices in polytopic proteins are usually

closely packed. Examples of this are G-protein-coupled receptors (GPCRs; Ch. 19), and the sarcoplasmic Ca^{2+} pump (Ch. 5). Each peptide bond has a significant dipole moment, which is transmitted to the ends of a helix. This circumstance favors close packing of antiparallel helices and is the observed disposition of helices in bacteriorhodopsin [3]. In oligomeric transmembrane proteins, intersubunit packing can encompass extramembranous protein domains and bilayer lipids.

Many transmembrane proteins that mediate intracellular signaling form complexes with both intra- and extracellular proteins. For example, neural cell adhesion molecules (NCAMs) are cell-surface glycoproteins (Ch. 7). The extracellular domains of NCAMs can activate fibroblast growth factor receptors when clustered by reaction with NCAM antibodies [4] or by homotypic binding to domains of adjacent cells (see Fig. 7-2). Activation was found to sequester a complex of NCAM, βI spectrin and $PKC\beta_2$ into rafts, as defined by the operational criteria discussed on p. 28.

Membrane associations can occur by selective protein binding to lipid head groups. One example is spectrin, which binds to phosphatidylinositol-4,5-bisphosphate by means of a pleckstrin-homology (PH) domain [5] (Fig. 2-5). and also to phosphatidyl serine [6] (Fig. 2-6).

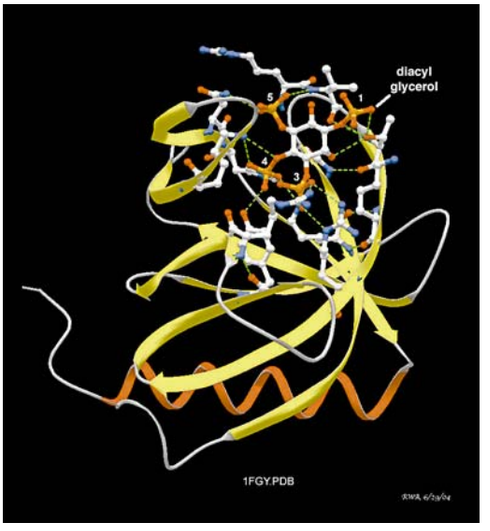

FIGURE 2-5 The pleckstrin homology (PH) domain of GrP1, a GDP–GTP (guanosine 5′ triphosphate) exchange factor for Arf GTPases. Activation of GrP1 occurs when this domain binds to PI(3,4)P2 or PI(3,4,5)P3 produced in the inner leaflet of plasma membranes by a PI3-kinase (Ch. 20). This structure is shown complexed with inositol 1,3,4,5-tetraphosphate as it was crystallized for X-ray diffraction, whereas diacyl glycerol would be esterified to the inositol 1-phosphate in the membrane-bound form. The amino-acid residues (ball and stick models) shown are those that approach the inositol tetraphosphate near enough to form hydrogen bonds (green dashes). Model constructed from Protein Data Base coordinates 1fgy [32] using DeepView 3.7 [33].

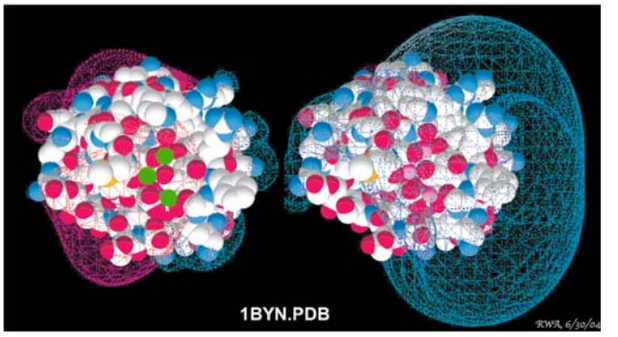

FIGURE 2-6 The C2 Ca^{2+}-dependent lipid-binding domain of synaptotagmin. Although binding calcium to this domain has little effect on its conformation, it produces a markedly increased affinity for negatively charged phospholipids such as phosphatidyl serine. The red and blue grids are isopotential lines over the molecular surface: red = negative, blue = positive potentials. On the left are the isopotential grids for the domain in the absence of calcium (the green circles mark the empty Ca^{2+} binding sites). On the right are isopotential grids after calcium binding (Ca^{2+} atoms = magenta spheres) which intensifies the positive field on the right, which presumably increases its affinity for the negatively charged phospholipids. More sophisticated evaluations of C2 domains from different proteins show that in some cases Ca^{2+} binding drives the binding surface to neutral potential, possibly favoring binding to zwitterionic phospholipids [34]. These models were constructed from Protein Data Base coordinates 1byn [35] using DeepView 3.7 [33].

Ca^{2+} influx initiates protein and membrane associations by several different mechanisms. Allosteric regulation of the hydrophobicity of protein-binding surfaces frequently occurs. One of the best studied examples is the Ca^{2+}-dependent binding of calmodulin to other proteins (Ch. 22). Annexins are a family of proteins that exhibit Ca^{2+}-dependent associations with cell membranes through direct interaction with phospholipids, and conversely, interactions with phospholipids increase their affinities for Ca^{2+} [7].

BIOLOGICAL MEMBRANES

The fluidity of lipid bilayers permits dynamic interactions among membrane proteins. For example, the interactions of a neurotransmitter or hormone with its receptor can dissociate a 'transducer' protein, which in turn will diffuse to interact with other effector proteins (Ch. 19). A given effector protein, such as adenylyl cyclase, may respond differently to different receptors because of mediation by different transducers. These dynamic interactions require rapid protein diffusion within the plane of the membrane bilayer. Receptor occupation can initiate extensive redistribution of membrane proteins, as exemplified by the clustering of membrane antigens consequent to binding bivalent antibodies [8]. In contrast to these examples of lateral mobility, the surface distribution of integral membrane proteins can be fixed by interactions with other proteins. Membranes may also be partitioned into local spatial domains consisting of networks

of cytoskeletal and scaffolding proteins or lipid rafts. This partitioning may restrict the translational motion of enmeshed proteins and yet allow rapid rotational diffusion. Examples of such spatial localization include restriction of Na,K-pumps to the basolateral domains of most epithelial cells, Na^+ channels to nodes of Ranvier and nicotinic acetylcholine receptors to the postsynaptic membranes of neuromuscular junctions.

Because membranes components participate in nearly every cell activity their structures are also dynamic and far from the equilibrium states that are most readily understood in biophysical terms. Newly synthesized bilayer lipids are initially associated with endoplasmic reticulum (Ch.3) whereas phospholipids initially insert into the cytoplasmic leaflet while cholesterol and sphingolipids insert into the luminal endoplasmic reticulum (ER) leaflet. Glycosylation of ceramides occurs as they transit the Golgi compartments, forming cerebrosides and gangliosides in the luminal leaflet. Thus, unlike model systems, the leaflets of ER membranes are asymmetric by virtue of their mode of biosynthesis.

The lipid compositions of plasma membranes, endoplasmic reticulum and Golgi membranes are distinct. All have asymmetric distributions of lipids between cytoplasmic and exocytoplasmic leaflets. Little is known of the intrinsic mechanisms that maintain the distinctions among these interacting membrane classes. As Golgi vesicles fuse into the plasma membrane their luminal surface becomes extracellular at the plasma membrane surface. The aminophospholipids PS and PE remain cytoplasmic but most PC is transported to the exo-leaflet, perhaps by the ATP-dependent ABCA1 transporters that occur in both neurons and glia. Conditions that elevate cell Ca^{2+}, such as anoxia, can activate a Ca^{2+}-dependent 'scramblase' that catalyzes transverse phosphatidylserine movements. Exposure of phosphatidyl-serine extracellularly constitutes a phagocytotic signal to microglia and other phagocytes that express surface receptors for phosphatidylserine [9]. An ATP-dependent aminophospholipid translocase activity in plasma membranes prevents this occurrence in healthy cells.

Cholesterol transport and regulation in the central nervous system is distinct from that of peripheral tissues. Blood-borne cholesterol is excluded from the CNS by the blood–brain barrier. Neurons express a form of cytochrome P-450, 46A, that oxidizes cholesterol to 24(S)-hydroxycholesterol [11] and may oxidize it further to 24,25 and 24,27-dihydroxy products [12]. In other tissues hydroxylation of the alkyl side chain of cholesterol at C22 or C27 is known to produce products that diffuse out of cells into the plasma circulation. Although the rate of cholesterol turnover in mature brain is relatively low, 24-hydroxylation may be a principal efflux path to the liver because it is not further oxidized in the CNS [10].

During brain development *de novo* cholesterol synthesis occurs at high rates at various stages in all brain cell types. In human adults, brain cholesterol constitutes 23% of total body cholesterol, about ten times higher than the average of all tissues. Much of this cholesterol derives from the oligodendrocyte plasma membrane component of myelin; about 80% is associated with myelin in mouse brain and a somewhat larger fraction in human. In contrast to its high content, the metabolic turnover of adult brain cholesterol is relatively low: for humans the turnover rate is estimated to be 0.03% per day for brain compared to 0.7% per day for whole body cholesterol [10].

In adult brain most cholesterol synthesis occurs in astrocytes. Apoprotein E (apoE) is the major apolipoprotein of the CNS and it is secreted by astrocytes. In astrocyte cultures apoE appears in the media as cholesterol-rich particles of a size similar to peripheral HDL (5–12 nm) (Fig. 2-7). The ATP-dependent transporter ABCA1, expressed by both astrocytes and neurons, promotes the formation of the apoE-stabilized high-density lipoprotein (HDL)-sized particles from astrocytic cholesterol.

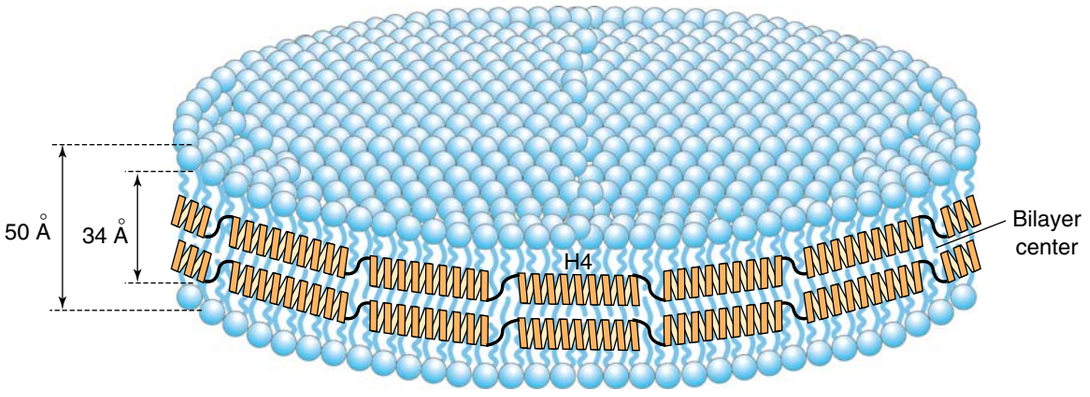

FIGURE 2-7 Putative model of apoE in rHDL. Two molecules of apoE of a total of about four molecules per discoidal particle are depicted to circumscribe the periphery of a bilayer of phospholipids. The helical axes are orientated perpendicular to the phospholipid fatty acyl chains. Adapted from [36] with permission.

Although the extracellular release of cholesterol is sometimes described as a passive 'shedding' process, in astrocytes it seems clear that cholesterol and phospholipid are mobilized from plasma membranes and that their transfer to extracellular apoE to form lipoprotein particles is facilitated by ABCA1 transporters. The mechanism for cholesterol transfer into neurons is less certain. There are seven members of the 'low-density lipoprotein receptor' (LDLR) family and they all are expressed either in developing or adult brain. They are all type I bitopic membrane receptors that uniformly incorporate a characteristic array of domains: they exhibit, extracellularly, a ligand-binding domain and between one and eight epidermal growth factor domains and, intracellularly, one or two NPxY motifs that act both as phosphotyrosine-binding domains and endocytotic signals. Two LDLRs, apoER2 and very-low-density lipoprotein receptor (VLDLR), are apoE receptors expressed on neuron plasma membranes and probably mediate HDL-lipid uptake into neurons. These same receptors interact with signaling ligands and adaptor proteins that mediate neuronal migration during brain development [13]. These signaling pathways are thought to have a role in adult brain, perhaps involving axoplasmic transport of essential components for synaptic remodeling (Chs 9, 53) and subject to damage in Alzheimer's disease (Ch. 47).

Although apoE HDL particles are formed by astrocytes *in vitro,* the brain contents of apoE knockout (–/–) were not found to differ in lipid content in comparison to those obtained from normal animals [14]. A probable explanation is that newly synthesized cholesterol can be transported from astrocyte ER to plasma membrane via an alternative route that employs caveolae to form apoA1-HDL [15].

The astrocytic cholesterol supply to neurons is important for neuronal development and remodeling. This is supported by neuronal cell culture observations [16]. Pure cultures of rat retinal ganglion cells from 8-day-old rats, in the absence of glia or serum, will extend axons and form synapses (autapses) that display low frequency postsynaptic currents. Co-culturing these neurons with glia from 2-day-old rats was observed to stimulate the formation of twice as many synapses and these exhibited about 12 times higher frequencies of postsynaptic activity. Astrocytes and oligodendrocytes, but not microglia, produced similar effects, as did replacement of the glia with glial-conditioned culture medium. ApoE was secreted by glia into their culture medium, but adding recombinant apoE to the retinal ganglion cell cultures did not stimulate the formation of synapses nor did it increase their efficacy. However both of these effects were replicated simply by adding cholesterol to the retinal ganglion cell cultures (Fig. 2-8).

Other investigators have grown similar purified preparations of retinal ganglion cells on channel-inscribed plates that cause the axons to extend linearly to permit measurement of axon elongation rates [17]. These axons will grow across siliconized barriers into side channels, which effectively isolates, externally, the axon membranes from the soma. Glial-conditioned medium was found to stimulate the axon elongation rates about 50% for several days when added to the side channels, but it had no effect if present only in the central compartments containing the neuronal soma. HDL lipoproteins, purified from glial-conditioned medium, stimulated equally well but, unlike the effects on synapses in the previous study, neither pure apoE nor cholesterol could replace the lipoprotein in stimulating axon elongation. The problem of why cholesterol is sufficient to support synapse formation but not axon elongation remains unresolved.

The stimulations of synaptic activity and of axon elongation observed in these experiments were both inhibited by RAP, an inhibitor protein selective for members of the LDLR family. These inhibitions are most readily explained as resulting from blockade of apoE-lipoprotein uptake via neuronal LDL receptors. Developing neurons can synthesize cholesterol but this capacity decreases as neurons mature. Under conditions of rapid extension of neurites, glial support may be necessary to meet the large energy demands of neuronal membrane biosynthesis (Ch. 31) and also to circumvent the limitations of endocytotic vesicle trafficking from neuronal soma to distal axons and dendrites (Ch. 9). Even in adult brain continuous cholesterol synthesis is necessary for the remodeling of synapses that is now recognized to be part of information processing (Ch. 50).

Cholesterol and sphingolipids are synthesized and transported through the ER and Golgi systems [18]. However they are present at much higher levels in the outer leaflet of plasma membranes. Adding cholesterol and sphingomyelin to synthetic lipid bilayer can produce thicker 'liquid ordered' membranes. This has led to proposals that bilayer thickness may be a factor in sorting integral membrane proteins between ER and Golgi. Liquid ordered bilayers have an 'elastic' ability to adjust their thickness as they interact with TMDs. Alkyl chain associations with hydrophobic protein residues are energetically favorable relative to interactions with more polar surfaces. This adjustment has an energetic cost that depends on the extent of bilayer deformation [19]. Because many plasma membrane integral proteins (PMIPs) are polytopic, with TMDs of varying length, the alkyl chain heterogeneity in length and unsaturation of lipid bilayers may be factors in sorting proteins into different membranes.

Mechanisms of selection of integral membrane proteins for transit through or retention by the Golgi system are largely unknown. Shortening the TMD of a plasma membrane protein was found to result in Golgi retention [20] and the converse effect occurs on lengthening the TMD of a normally retained Golgi protein [21]. The lengths of transmembrane domains for Golgi-retained proteins are usually less than for those directed to the plasma membrane.

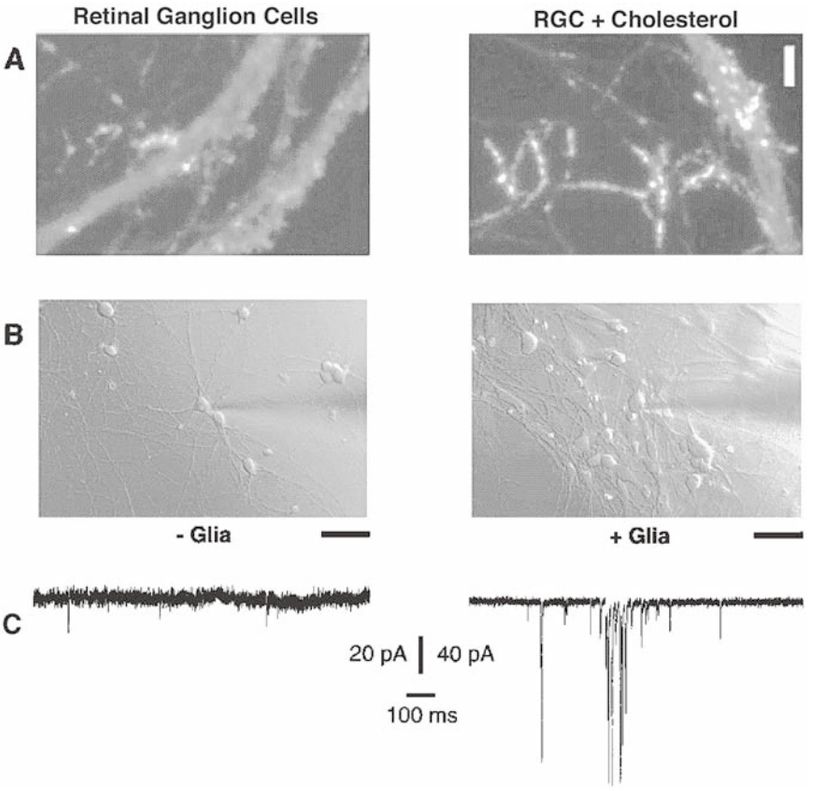

FIGURE 2-8 Cholesterol increases the number and release efficacy of synapses in single retinal ganglion cells. (**A**) Fluorescence micrographs of retinal ganglion cell synapses labeled by a synapsin I–specific antibody. The cells were cultured in the absence (left) or presence (right) of cholesterol. Scale bar = 5 µm. (Reproduced from [37] with permission.) (**B**) Hoffmann-modulation contrast micrographs of retinal ganglion cells cultured for 5 days in defined, serum-free medium in the absence (left) or presence (right) of collicular glia. Scale bar = 50 µm. The density of neurons was similar in both cultures. (**C**) Spontaneous synaptic activity in these cells: whole-cell patch-clamp recordings of spontaneous excitatory postsynaptic currents (EPSCs) at a holding potential of −70 mV. (Reproduced from [38], with permission.)

The structure and roles of membrane microdomains (rafts) in cell membranes are under intensive study but many aspects are still unresolved. Unlike in synthetic bilayers (Fig. 2-2), no way has been found to directly visualize rafts in biomembranes [22]. Many investigators operationally define raft components as those membrane lipids and proteins (a) that remain insoluble after extraction with cold 1% Triton X-100 detergent, (b) that are recovered as a low density band that can be isolated by flotation centrifugation and (c) whose presence in this fraction should be reduced by cholesterol depletion.

Much of the plasma membrane cholesterol is removed by incubating cells with β-methylcyclodextrin for several hours. Cells remain viable after this treatment but the raft fraction is reduced and it is inferred that the depleted proteins are normally associated with cholesterol-dependent lipid rafts. Some, but not all, glycosylphosphatidylinositol (GPI)-anchored proteins are recovered in the fractions defined by this procedure.

A proteomic study of the HeLa cell proteins in raft fractions, identified by these criteria and estimated by quantitative mass spectrometry, has identified 241 'authentic raft proteins' [23]. This analysis found that the 'raft proteins' most enriched relative to total membrane proteins belong to several classes of signaling protein: kinases and phosphatases, heterotrimeric G proteins and small G proteins. Notice that most of the raft proteins identified in this way are not PMIPs but rather they are proteins that associate with the cytoplasmic lipid leaflet or with cytoplasmic domains of PMIPs.

The inability to visualize rafts in biological membranes by direct optical methods suggests that they are very small and/or very transient. This has been investigated by methods such as fluorescence recovery after photolysis (FRAP), which provides information about lateral diffusion, and fluorescence resonant energy transfer (FRET), which can detect 'molecular crowding' when separation distances are less than ≈1 nm. These experiments have generally supported the existence of very small domains of restricted lateral diffusion and of protein clustering in living cell plasma membranes.

GPI-anchored proteins (GP-APs) are synthesized in the cytoplasm and their transport into the ER occurs during the process of acquiring a GPI anchor, which is ultimately sorted into the outer leaflet of plasma membranes [24]. A FRET study of fluorophore-labeled GPI-APs in cultured

cells [25] concluded that most of the GPI-APs existed as monomers in these cells but a significant fraction, 20–40%, exist as very small, dense clusters that may be a signal for endocytosis. They also suggest that the size of these GPI-AP clusters may be controlled by processes that regulate plasma membrane cholesterol.

The membrane-associated small G proteins H-Ras and K-Ras have been studied with respect to their association with cytoplasmic leaflets. These two proteins have nearly identical structures and functions but different membrane anchors, membrane distributions and effector responses. Application of the FRAP method to fluorescent constructs of H-Ras and K-Ras revealed that only H-Ras in its guanosine 5′ diphosphate (GDP)-bound form associates with cholesterol-dependent rafts [26].

In addition to fluorescence methods, another study [27] developed a method to permit electron microscopic localization of Ras anchor domains on cytoplasmic membrane surfaces by immunogold labeling. The particle neighbor distances can be analyzed to obtain information about possible domain structure. Expressing H-Ras and K-Ras in baby hamster kidney cells, a nonrandom particle distribution was obtained from which the estimated mean raft size was 7.5–22 nm and about 35% of the membrane area consists of rafts. The same technique applied to cells that had been incubated with β-cyclodextrin to reduce cholesterol produced completely random distributions of H-Ras. This cholesterol dependence suggests some type of coupling of rafts across the inner and outer membrane leaflets.

Mechanical functions of cells require interactions between integral membrane proteins and the cytoskeleton. These functions include organization of signaling cascades, formation of cell junctions and regulation of cell shape, motility, endo- and exocytosis. Several different families of membrane-associated proteins mediate specific interactions among integral membrane proteins, cytoskeletal proteins and contractile proteins. Many of these linker proteins consist largely of various combinations of conserved protein-association domains, which often occur in multiple variant copies.

In erythrocytes and most other cells, the major structural link of plasma membranes to the cytoskeleton is mediated by interactions between ankyrin and various integral membrane proteins, including Cl^-/HCO_3^- antiporters, sodium ion pumps and voltage-dependent sodium ion channels. Ankyrin also binds to the ≈100 nm, rod-shaped, antiparallel αβ heterodimers of spectrin and thus secures the cytoskeleton to the plasma membrane. Spectrin dimers self-associate to form tetramers and further to form a polygonal network parallel to the plasma membrane (Fig. 2-9D). Neurons contain both spectrin I, also termed erythroid spectrin, and spectrin II, also termed fodrin. Spectrin II is found throughout neurons, including axons, and binds to microtubules, whereas spectrin I occurs only in the soma and dendrites. This spectrin network further binds to actin microfilaments and to numerous other ligands. These associations are probably dynamic. For example, phosphorylation of ankyrin can alter its affinity for spectrin. The functions of the multiple protein-interaction domains of both spectrin and ankyrin have been as yet only partially defined (see Ch. 8).

The spectrin–ankyrin network comprises a general form of membrane-organizing cytoskeleton within which a variety of membrane–cytoskeletal specializations are interspersed. Many of these are concerned with cell-cell or cell-matrix interactions (Ch. 7). The several morphological types of cell–cell junctions are associated with junction-specific structural and linking proteins. For example, tight junctions, also termed zona occludens, are constructed of the integral membrane protein occludin, which binds the linking proteins ZO-1 and ZO-2 [15]. These linking proteins are members of a large family, termed membrane-associated guanylyl kinase homologs (MAGUKs). The general structure of this family has, distributed from the N-terminus to the C-terminus, one or more PDZ-binding domains, a src-homology-3 (SH3) domain (see Ch. 25) and a guanylyl kinase homolog domain. Other members of the PDZ family are expressed in neurons at postsynaptic densities. One of these, PSD-95, contains two N-terminal PDZ domains that can bind to a motif, –E–S/T–D–V–, that occurs in *N*-methyl-D-aspartate (NMDA) receptors. Multimeric clusters of these receptors or channels can be formed through disulfide cross-linking between cysteines of the N-terminal domains of PSD-95 molecules [28]. Different PDZ domains within a single linker protein can display different peptide motif selectivities. Accordingly, it has been suggested that a given linker protein may simultaneously bind to multiple different channels and receptors to produce complex clusters at various postsynaptic sites.

Interaction of rafts with cytoskeleton is suggested by the results of video microscopy. Brightfield video microscopy can record movements of single 40 nm gold particles on the outer surface of cells in culture. Such particles, if coated with an appropriate binding protein can bind to a component of the cell surfaces. In the experiments to be described, gold particles with fluorescein antibody were attached to fluorescein-derivatized dioleyl phosphatidyl ethanolamine under conditions designed to produce beads with predominantly single molecules attached. These were introduced into cells on coverslip cultures, and the movements of these particles were recorded with digital video and analyzed (Fig. 2-10) [29]. Fig. 2-10A shows the video trace of a single particle's movement over 10 seconds, which appears random and unstructured when recorded at 33 ms intervals. The same particle movements, when recorded at 110 μs intervals (300 times faster), reveals a path consisting of random hops from one 'confinement compartment' to another,

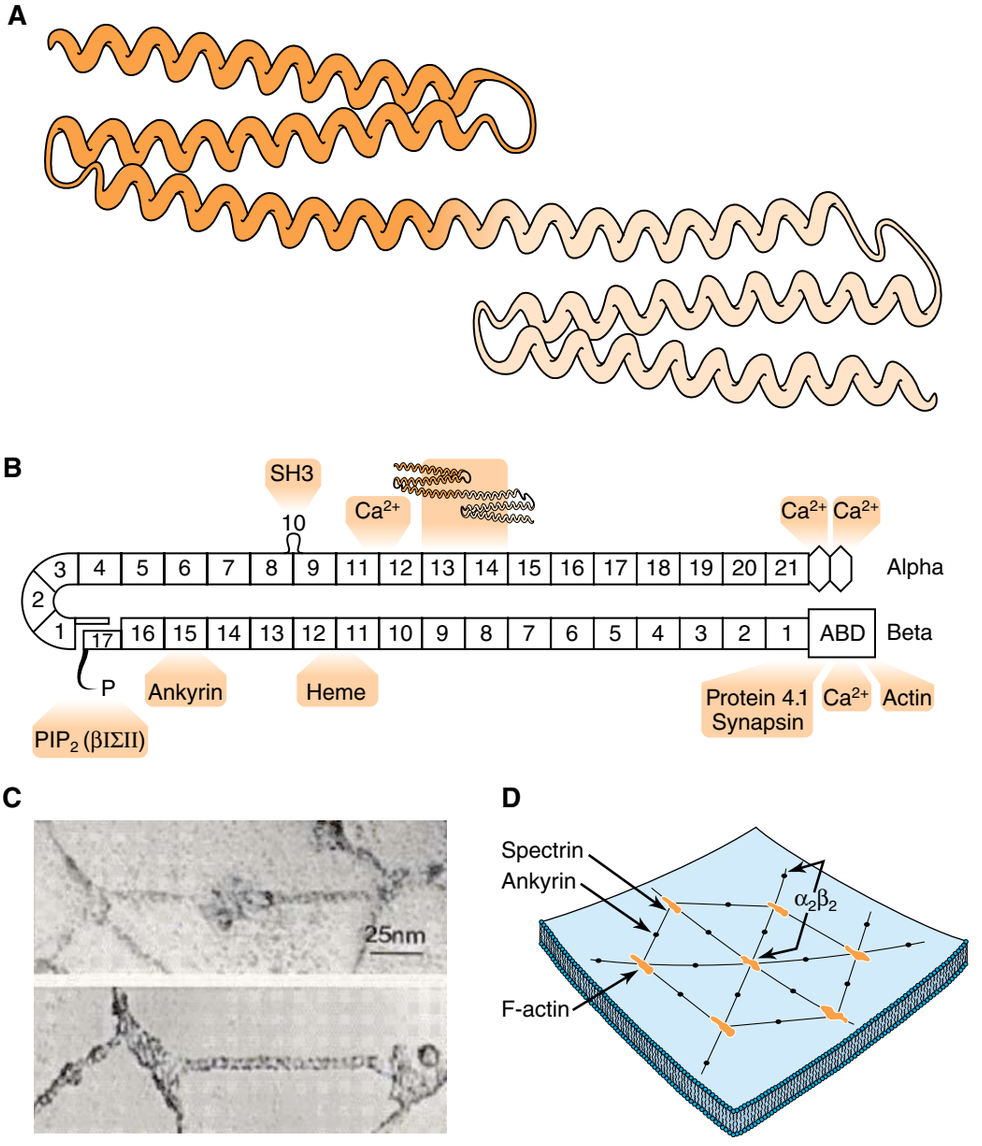

FIGURE 2-9 The ankyrin–spectrin lattice. (**A**) Structural model of a spectrin repeat unit based on the crystal structure of a dimer of the 14th repeat unit of *Drosophila* spectrin. (Adapted from [39], with permission.) (**B**) Cartoon of the domain structure of a spectrin dimer. Many of the repeat units of spectrin constitute binding domains with different specificities. Some of these have been identified and are labeled here. ABD = actin-binding domain; PIP2 = phosphatidylinositol-4,5-bisphosphate domain – occurs only on the βIΣII isoform; SH3 = src homology 3 domain. (Adapted from [40] with permission). (**C**) Electron micrographs of rotary shadowed spectrin tetramers. Note the periodic substructure of spectrin filaments and the putative site of a complex with an ankyrin molecule (top, center). (Courtesy of J. Ursitti.) (**D**) Schematic organization of the spectrin–ankyrin cytoskeleton on the cytoplasmic surface of neurons. $\alpha_2\beta_2$ are the spectrin subunits depicted in **B**. (Redrawn from [41], with permission.)

usually at less than 33 ms intervals and thus only detectable at the higher speeds. Additional experiments were designed to examine the generality of this phenomenon in different cell types and to establish the structure of the confinement compartments. Compartmentation was detected in all of eight mammalian cell types examined with sizes ≈32–230 nm diameter. Prior treatment of the cells with the actin depolymerizing agent cytochalasin on average increased the compartment size approximately twofold. Treatment with an actin stabilizing agent had little effect on the compartment size but increased the median residency time approximately sevenfold. Both the size and the actin dependence suggest that the actin–spectrin network may be the source of the compartments.

Another research group has applied gold particle tracking to measure movements of the μ-opioid GPCR on the surface of GPCR-transfected fibroblasts [30]. They describe the pattern observed in Fig. 2-11 as a 'walking defined diffusion mode'. More than 90% of the observed particles displayed this pattern, which consists of rapid

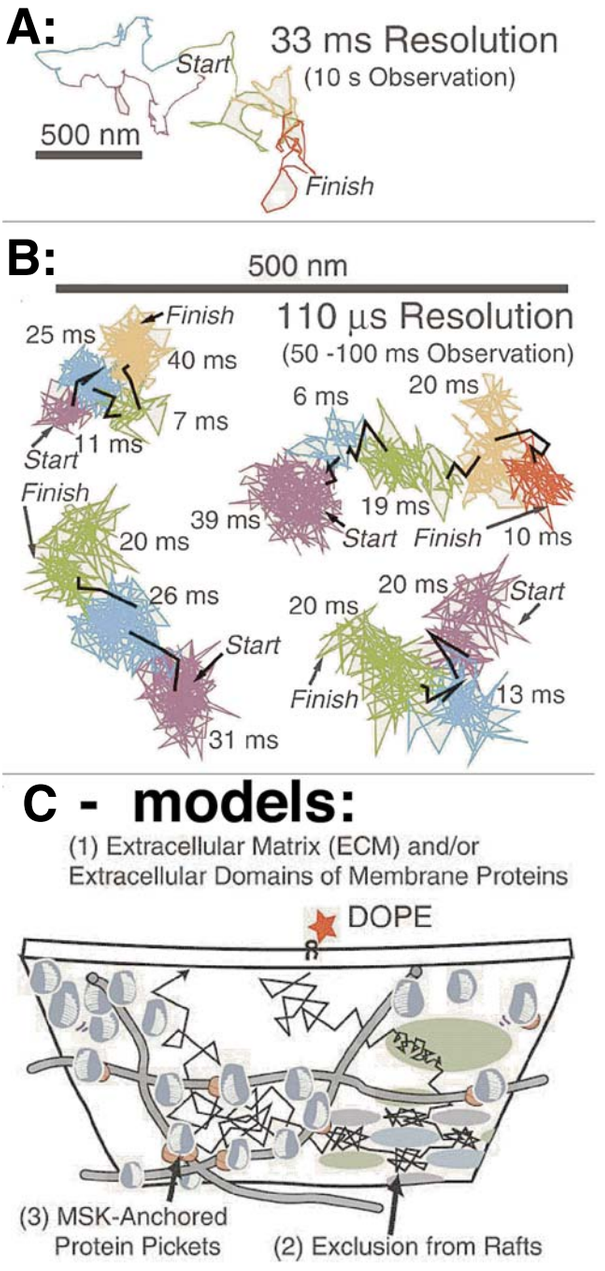

FIGURE 2-10 Tracking a gold particle attached to a single molecule of phosphatidyl ethanolamine. What appears to be simple Brownian diffusion at a time resolution of 33 ms per video frame (**A**) is revealed to actually consist of fast 'hop diffusion' by recording 300 times faster (**B**) at 110 µs per video frame. In (**A**) each color represents 60 frames = 2 seconds. In (**B**) each color indicates an apparent period of confinement within a compartment and black indicates intercompartmental hops. The residency time for each compartment is indicated. The hypothetical explanations are illustrated in part (**C**) and discussed in the text. Adapted from [29].

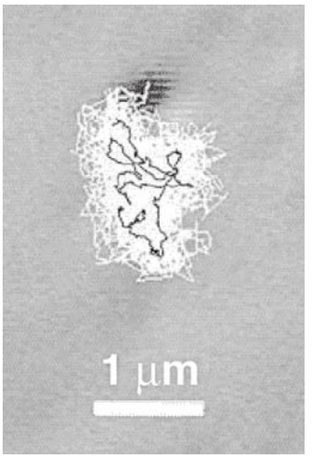

FIGURE 2-11 Video-enhanced Differential Interference Contrast (DIC) images of gold-labeled µ-opioid GPCR on the surface of a GPCR-transfected fibroblast. The white trace is the trajectory of one particle over 2 minutes at 25 frames/s. The black trace is the mean square displacement of the particle as a function of time. Reproduced from Figure 1 of [30], with permission.

diffusion within a 'domain area' (with a mean size of about 150 nm) combined with a much slower (≈10-fold) drift of the whole domain.

These authors interpret their data as the natural result of restrictions imposed on the free diffusion of the labeled receptor by encounters with other transmembrane proteins in the bilayer. However they consider that their data are incompatible with the hop and skip model based on spectrin mesh confinement.

REFERENCES

1. Fivaz, M. and Meyer, T. Specific localization and timing in neuronal signal transduction mediated by protein-lipid interactions. *Neuron* 40: 319–330, 2003.
2. Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed. New York: Wiley Interscience, 1980.
3. Kimura, Y., Vassylyev, D. G., Miyazawa, A. *et al.* Surface of bacteriorhodopsin revealed by high-resolution electron crystallography. *Nature* 389: 206–211, 1997.
4. Leshchynska, I., Sytnyk, V., Morrow, J. S. and Schachner, M. Neural cell adhesion molecule (NCAM) association with PKCβ2 via βI spectrin is implicated in NCAM-mediated neurite outgrowth. *J. Cell Biol.* 161: 625–639, 2003.
5. Wang, D. and Shaw, G. The association of the C-terminal region of βIΣII spectrin to brain membranes is mediated by a PH domain, does not require membrane proteins, and coincides with an inositol-1,4,5 triphosphate binding site. *Biochem. Biophys. Res. Comm.* 217: 608–615, 1995.
6. An, X. and Guo, X. Phosphatidylserine binding sites in erythroid spectrin: location and implications for membrane stability. *Biochemistry* 43: 310–315, 2004.
7. Mollenhauer, H. Annexins: what are they good for? *Cell Mol. Life Sci.* 53: 506–507, 1997.

8. Poo, M. Mobility and localization of proteins in excitable membranes. *Ann. Rev. Neurosci.* 8: 369–406, 1985.
9. DeSimone, R., Ajmone-Cat, M. A. and Minghetti, L. Atypical anti-inflammatory activation of microglia induced by apoptotic neurons. *Mol. Neurobiol.* 29: 197–212, 2004.
10. Dietschy, J. M. and Turley, S. D. Thematic review series: brain lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J. Lipid Res.* 45: 1375–1397, 2004.
11. Lund, E. G., Xie, C., Kotti, T., Turley, S. D., Dietschy, J. M. and Russell, D. W. Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. *J. Biol. Chem.* 278: 22980–22988, 2003.
12. Mast, N., Norcross, R., Andersson, U. *et al.* Broad substrate specificity of human cytochrome P450 46A1 which initiates cholesterol degradation in the brain. *Biochemistry* 42: 14284–14292, 2003.
13. Beffert, U., Stolt, P. C. and Herz, J. Functions of lipoprotein receptors in neurons. *J. Lipid Res.* 45: 403–409, 2004.
14. Han, X., Cheng, H., Fryer, J. D., Fagan, A. M. and Holtzman, D. M. Novel role for apolipoprotein E in the central nervous system: modulation of sulfatide content. *J. Biol. Chem.* 278: 8043–8051, 2003.
15. Ito, J., Nagayasu, Y., Kato, K., Sato, R. and Yokoyama, S. Apolipoprotein A-I induces translocation of cholesterol, phospholipid, and caveolin-1 to cytosol in rat astrocytes. *J. Biol. Chem.* 277: 7929–7935, 2002.
16. Goritz, C., Mauch, D. H., Nagler, K. and Pfrieger, F. W. Role of glia-derived cholesterol in synaptogenesis: new revelations in the synapse–glia affair. *J. Physiol. Paris* 96: 257–263, 2002.
17. Hayashi, H., Campenot, R. B., Vance, D. E. and Vance, J. E. Glial lipoproteins stimulate axon growth of central nervous system neurons in compartmented cultures. *J. Biol. Chem.* 279: 14009–14015, 2004.
18. Bjorkhem, I. and Meaney, S. Brain cholesterol: long secret life behind a barrier. *Arterioscler. Thromb. Vasc. Biol.* 24: 806–815, 2004.
19. Lundbaek, J. A., Andersen, O. S., Werge, T. and Nielsen, C. Cholesterol-induced protein sorting: an analysis of energetic feasibility. *Biophys. J.* 84: 2080–2089, 2003.
20. Sivasubramanian, N. and Nayak, D. P. Mutational analysis of the signal-anchor domain of influenza virus neuraminidase. *Proc. Natl. Acad. Sci. U.S.A.* 84: 1–5, 1987.
21. Munro, S. Lipid rafts: elusive or illusive? *Cell* 115: 377–388, 2003.
22. Kusumi, A., Koyama-Honda, I. and Suzuki, K. Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic* 5: 213–230, 2004.
23. Foster, L. J., de Hoog, C. L. and Mann, M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. U.S.A.* 100: 5813–5818, 2003.
24. Murakami, Y., Siripanyapinyo, U., Hong, Y. *et al.* PIG-W is critical for inositol acylation but not for flipping of glycosylphosphatidylinositol-anchor. *Mol. Biol. Cell* 14: 4285–4295, 2003.
25. Sharma, P., Varma, R., Sarasij, R. C., Gousset, K., Krishnamoorthy, G. and Rao M, Mayor S. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* 116: 577–589, 2004.
26. Niv, H., Gutman, O., Kloog, Y. and Henis, Y. I. Activated K-Ras and H-Ras display different interactions with saturable non-raft sites at the surface of live cells. *J. Cell Biol.* 157: 865–872, 2002.
27. Parton, R. G. and Hancock, J. F. Lipid rafts and plasma membrane microorganization: insights from Ras. *Trends Cell Biol.* 14: 141–147, 2004.
28. Hueh, Y., Kim, E. and Sheng, M. Disulfide-linked head-to-head multimerization in the mechanism of ion channel clustering by PSD-95. *Neuron* 18: 803–814, 1997.
29. Murase, K., Fujiwara, T., Umemura, Y. *et al.* Ultrafine membrane compartments for molecular diffusion as revealed by single molecule techniques. *Biophys. J.* 86: 4075–4093, 2004.
30. Daumas, F., Destainville, N., Millot, C., Lopez, A., Dean, D. and Salome, L. Confined diffusion without fences of a G-protein-coupled receptor as revealed by single particle tracking. *Biophys. J.* 84: 356–366, 2003.
31. Van Duyl, B. Y., Ganchev, D., Chupin, V., de Kruijff, B. and Killian, J. A. Sphingomyelin is much more effective than saturated phosphatidylcholine in excluding unsaturated phosphatidylcholine from domains formed with cholesterol. *FEBS Lett.* 547: 101–106, 2003.
32. Lietzke, S. E., Bose, S., Cronin, T. *et al.* Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains. *Mol. Cell* 6: 385–394, 2000.
33. Guex, N., Diemand, A., Peitsch, M. C. and Schwede, T. *Deep View Swiss Pdb Viewer.* Basel: Swiss Institute of Bioinformatics, 2001. Available on line at: www.expasy.org/spdbv/.
34. Murray, D. and Honig, B. Electrostatic control of the membrane targeting of C2 domains. *Mol. Cell* 9: 145–154, 2002.
35. Shao, X., Fernandez, I., Sudhof, T. C. and Rizo, J. Solution structures of the Ca^{2+}-free and Ca^{2+}-bound C2A domain of synaptotagmin I: does Ca^{2+} induce a conformational change? *Biochemistry* 37: 16106–16115, 1998.
36. Narayanaswami, V., Maiorano, J. N., Dhanasekaran, P. *et al.* Helix orientation of the functional domains in apolipoprotein e in discoidal high density lipoprotein particles. *J. Biol. Chem.* 279: 14273–14279, 2004.
37. Mauch, D. H., Nagler, K., Schumacher, S. *et al.* CNS synaptogenesis promoted by glia-derived cholesterol. *Science* 294: 1354–1357, 2001.
38. Pfrieger, F. W. and Barres, B. A. Synaptic efficacy enhanced by glial cells in vitro. *Science* 277: 1684–1687, 1997.
39. Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C. and Branton, D. Crystal structure of the repetitive segments of spectrin. *Science* 262: 2027–2030, 1993.
40. Ursitti, J. A., Kotula, L., DeSilva, T. M., Curtis, P. J. and Speicher, D. W. Mapping the human erythrocyte β-spectrin dimer initiation site using recombinant peptides and correlation of its phasing with the α-actinin dimer site. *J. Biol. Chem.* 271: 6636–6644, 1996.
41. Goodman, S. R., Zimmer, W. E., Clark, M. B., Zagon, I. S., Barker, J. E. and Bloom, M. L. Brain spectrin: of mice and men. *Brain Res. Bull.* 36: 593–606, 1995.

CHAPTER

3

Lipids

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Lipids have critical roles in nervous system structure and function. Synaptic complexes and myelin are characterized by unique lipid compositions that contribute to the specialized properties of these nervous system structures. Multiple signaling pathways involving lipid intermediates regulate cell differentiation and synaptic transmission. Lipid modification of proteins is a key mechanism for modulating the activity of trophic factors and receptors. Since lipids constitute about one-half of brain tissue dry weight, it is not surprising that lipid biochemistry and neurochemistry have evolved together. Like other tissues, the brain contains phospholipids, sterols and sphingolipids. Many complex lipids, including gangliosides, cerebrosides, sulfatides and phosphoinositides, were first discovered in brain, where they are highly enriched compared to other tissues. Phospholipids account for the high total phosphorus content of brain, which led to an alchemical mystique in the 19th century that associated phosphorescence with thought and to the apocryphal claim that fish are good 'brain food' since fish, too, are rich in phosphorus.

PROPERTIES OF BRAIN LIPIDS

Lipids have multiple functions in brain. They have two principal functions in the body: as repositories of chemical energy in storage fat, primarily triglycerides, and as structural components of cell membranes. The brain contains virtually no triglyceride, so it is in their role as membrane components that brain lipids initially commanded the attention of neurochemists. Later, some biomessenger functions of nonmembrane lipids, such as steroid hormones and eicosanoids, became evident. Some membrane lipids, such as inositides and phosphatidylcholine, which were previously believed to have only a structural role, also have important functions in signal transduction across biological membranes. Cholesterol and sphingolipids play a central role in formation of lipid rafts, which function in protein trafficking and signaling at the cell

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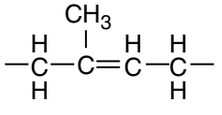
surface (see Ch. 2). In addition, lipids covalently coupled to proteins play a major role in anchoring marker proteins within biomembranes (see below). These discoveries established that lipids participate in both the function and the structure of neural membranes.

Membrane lipids are amphiphilic molecules. All membrane lipids have a small polar, or hydrophilic, and a large nonpolar, or hydrophobic, component. The hydrophilic regions of lipid molecules associate with water and water-soluble ionic compounds by hydrogen and electrostatic bonding. The hydrophobic regions cannot form such bonds and therefore associate with each other outside the aqueous phase. Depending on the relative dominance of the hydrophobic and hydrophilic regions of a given lipid molecule, these amphiphiles will form either aggregates, also termed micelles, or bilayers. Lipid molecules containing comparatively large polar groups, such as lysolipids, gangliosides and natural or synthetic detergents, which are fairly soluble in water, tend to form micelles once the solubility limit, or critical micellar concentration, is reached. Most membrane lipids tend to associate in a hydrophobic 'tail-to-tail' fashion to form bilayers (see Ch. 2).

The hydrophobic components of many lipids consist of either isoprenoids or fatty acids and their derivatives. Lipids were originally defined operationally, on the basis of their extractability from tissues with organic solvents such as a chloroform/methanol mixture, but this is no longer the sole criterion. For example, the protein component of myelin proteolipid is extractable into lipid solvents but, nevertheless, is not considered to be a lipid since its structure is that of a highly hydrophobic polypeptide. In fact, many integral membrane proteins contain 'hydrophobic' membrane-spanning regions (see Ch. 2). Conversely, gangliosides are considered to be lipids on the basis of their structure, even though they are water-soluble. It is apparent, then, that lipids are defined not only by their physical properties but also on the basis of their chemical structure. Chemically, lipids can be defined as compounds containing long-chain fatty acids and their derivatives or linked isoprenoid units. Fatty acids in lipids are either esterified to the trihydroxy alcohol glycerol or are present as amides of sphingosine, a long-chain dihydroxyamine. The isoprenoids are made up of branched-chain units and include sterols, primarily cholesterol.

Isoprenoids have the unit structure of a five-carbon branched chain. Isoprenoid units have the formula C_5H_8 and the structure:



The most abundant of these in brain is cholesterol. Unlike other tissues, normal adult brain contains virtually no cholesterol esters. Desmosterol, the immediate biosynthetic precursor of cholesterol, is found in developing brain and in some brain tumors but not in normal adult brain. Other isoprenoid substances present in brain are the dolichols, very long (up to C_{100}) branched-chain alcohols that are cofactors for glycoprotein biosynthesis; squalene, which is the linear C_{30} precursor of all steroids; and the carotenoids, including retinal and retinoic acid. Some isoprene units, such as farnesyl (C_{15}) and geranylgeranyl (C_{20}), have been shown to be covalently linked via thioether bonds to membrane proteins (see below for structures of some of these compounds and for the numbering system for cholesterol).

Brain fatty acids are long-chain carboxylic acids that may contain one or more double bonds. The brain contains a variety of straight-chain monocarboxylic acids, usually with an even number of carbon atoms ranging from C_{12} to C_{26}. The hydrocarbon chain may be saturated or may contain one or more double bonds, all in *cis* (Z) configuration. When multiple double bonds are present, they are nonconjugated and almost always three carbons apart. The unsaturated fatty acids are classified by the location of the double bond most distal from the carboxyl end. The most prevalent series, n being the number of carbon atoms in the fatty acid, are n-3 (n minus 3), n-6 and n-9. Thus, linoleic acid, which has 18 carbons (Fig. 3-1), is a member of the n-6 family because the double bond most distal from the carboxyl end is at 18-6, or the C-12 position. Since the next double bond is separated by three carbon atoms, it is between C_9 and C_{10}. A similar, widely used but nonstandard nomenclature employs the omega (ω) designation, indicating the position of the first double bond counting from the methyl (ω-carbon) end. These nomenclature conventions are convenient from both the biochemical and the nutritional points of view since fatty acids are elongated or degraded *in vivo* by two carbon units from the carboxyl end and animals need certain polyunsaturated fatty acids, termed essential fatty acids, in their diet, as discussed below and in Chapter 33. The complete shorthand notation for fatty acids consists of the number of carbon atoms followed by the number of double bonds and the position of the first double bond. Linoleic acid is thus 18:2 (n-6) or, alternatively, 18:2ω6. The brain contains some unusual fatty acids, such as very long (20–26 carbons), odd-numbered and 2-hydroxy fatty acids, prevalent in the cerebrosides. A list of major brain fatty acids with their common names and structures is given in Figure 3-1.

COMPLEX LIPIDS

Glycerolipids are derivatives of glycerol and fatty acids. Most brain glycerolipids are derivatives of phosphatidic acid (PtdOH), which is diacylated *sn*-glycerol-3-phosphate. The notation *sn* refers to stereochemical

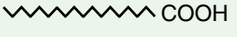
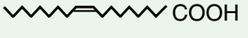
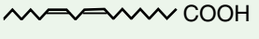
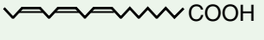
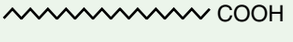
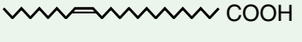
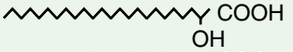
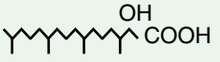
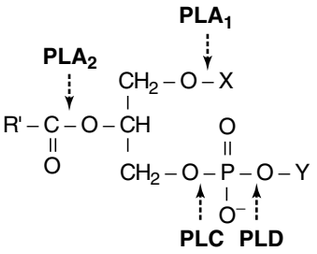
Structure	Chemical name	Trivial name	Abv.
	Dodecanoic acid	Lauric acid	12:0
	Tetradecanoic acid	Myristic acid	14:0
	Hexadecanoic acid	Palmitic acid	16:0
	Octadecanoic acid	Stearic acid	18:0
	9-Octadeceanoic acid	Oleic acid	18:1(n-9)
	9,12-Octadecadienoic acid	Linoleic acid	18:2(n-6)
	9,12,15-Octadecatrienoic acid	Linolenic acid	18:3(n-3)
	5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4(n-6)
	5,8,11,14,17-Eicosapentenoic acid	EPA	20:5(n-3)
	4,7,10,13,16,19-Docosahexenoic acid		22:6(n-3)
	Tetracosanoic acid	Lignoceric acid	24:0
	15-Tetracoseanoic acid	Nervonic acid	24:1(n-9)
	2-Hydroxytetracosanoic acid	Cerebronic acid	24h:0
	3,7,11,15-Tetramethylhexadecanoic acid	Phytanic acid	

FIGURE 3-1 Structures of some fatty acids of neurochemical interest (see also Fig. 3-7 and text). The 'n minus' nomenclature for the position of the double bond(s) is given here. Note that the position of the double bond from the carboxyl end can be indicated by the symbol Δ, so that linoleic acid may be also be designated as $18{:}2^{\Delta 9,12}$. The linolenic acid shown is the α isomer.

numbering, with the secondary hydroxyl group of glycerol at C-2 shown on the left, that is, the l-configuration of Fischer's projection, and the phosphate at C-3. This special nomenclature is employed because, unlike the trioses or other carbohydrates, glycerol does not have a reporter carbonyl group to assign an absolute D- or L-configuration. As shown in Figure 3-2, the hydroxyl groups on C-1 and C-2 of glycerolipids are esterified with fatty acids. The substituent at *sn*-1 is usually saturated, whereas that at *sn*-2 is unsaturated. In addition, there are lipid species in which *sn*-1 is ether-linked either to an aliphatic alcohol, termed an alkyl, or to an α,β-unsaturated alcohol, alk-1-enyl. The latter lipids are referred to as plasmalogens (Fig. 3-2). While diacylglycerophospholipids are saponifiable, that is, they contain alkali-labile ester bonds, and are acid-stable, the alkenyl ethers are alkali-stable and acid-labile. Alkyl ethers are stable to both acids and bases. A useful general term that includes all of these various aliphatic substituents, acyl, alkenyl and alkyl, is 'radyl,' for example, 1,2-diradyl-*sn*-glycerol-3-phosphorylethanolamine is a term that includes phosphatidylethanolamine (PtdEtn) as well as its plasmalogen analogs.

If positions 1 and 2 are acylated and the *sn*-3 hydroxyl group is free, the lipid is 1,2-diacyl-*sn*-glycerol (DAG). The DAGs play both a biosynthetic (see later) and a cellular regulating role in that they activate protein kinase C (PKC) (see Chs 20 and 23). In addition, DAGs can be fusogenic and have been proposed to play a role in altering cell morphology, for example, in fusion of synaptic vesicles (see Ch. 9). Other non-phosphorus-containing glycerides of interest are DAG-galactoside and its sulfate. These minor glycolipids are found primarily in white matter and appear to be analogous to their sphingosine-containing counterparts, the cerebrosides, described below.

Glycerophospholipid classes are defined on the basis of the substituent base at *sn*-3 of the diacylglycerophosphoryl (phosphatidyl) function (Fig. 3-2). The bases are short-chain, polar alcohols phosphodiester-linked to PtdOH. The amount and distribution of these lipids vary with brain regions and with age [1, 2]. In quantitatively decreasing order in adult human brain, they are PtdEtn, including plasmalogens; phosphatidylcholine (PtdCho, 'lecithin'); and phosphatidylserine (PtdSer). The phosphoinositides include phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns-4-P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2); they are quantitatively minor phospholipids but play an important role in signal transduction. They are also abbreviated as PI, PIP and PIP_2 respectively, and are discussed in more detail, as are the phosphatidylinositide-3-phosphate (PI3P) family of inositides, in Chapter 20. The phosphatidylglycerols in brain, as in other tissues, are present in mitochondrial membranes. Of these, cardiolipin (bisphosphatidylglycerol) is the most prevalent.



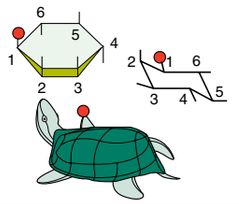
Y	Lipid	Abv.
H	Phosphatidate	PtdOH
$CH_2-CH_2-\overset{+}{N}H_3$	Phosphatidylethanolamine	PtdEtn
$CH_2-CH_2-\overset{+}{N}(CH_3)_3$	Phosphatidylcholine	PtdCho
$CH_2(NH_3^+)-CH(COO^-)-H$	Phosphatidylserine	PtdSer
	Phosphatidylinositol	PtdIns
$CH_2-CH(OH)-CH_2HO$	Phosphatidylglycerol	PtdGro
Phosphatidylglycerol	Cardiolipin	PtdGroPtd

FIGURE 3-2 The structure of phosphoglycerides. In most lipids, X is acyl, that is, R–(C=O). In alkyl ethers, present mainly in brain ethanolamine phosphoglycerides (2–3%), X is a long-chain hydrocarbon (C_{16}, C_{18}). For plasmalogens, which constitute about 60% of adult human brain PtdEtn, X is 1-alk-1′enyl (i.e. –CH=CH–R). *Arrows* indicate sites of enzymatic hydrolysis of the phosphoglycerides. *PLA_1*, phospholipase A_1; *PLA_2*, phospholipase A_2; *PLC*, phospholipase C; *PLD*, phospholipase D. Note that *myo*-inositol is written in the D-configuration, where the 1′ position is linked to the PtdOH moiety. For polyphosphoinositides, additional phosphate groups are present in the 3, 4 or 5 positions. See Chapter 21 for further detail regarding the stereochemistry of inositol and the use of the turtle representation.

Each phospholipid class in a given tissue has a characteristic fatty acid composition. Though the same fatty acid may be present in a number of lipids, the quantitative fatty acid composition is different for each class of lipids and remains fairly constant during the growth and development of the brain. A typical distribution profile of the major fatty acids in rat brain phospholipids is given in Table 3.1. Not only do the phosphoglycerides differ in the structure of the polar head groups, or phospholipid classes, but within each class there are a variety of combinations of pairs of fatty acids, giving rise to molecular species that differ in the nature and positional distribution of fatty acids esterified to the glycerol backbone. For example, the 1-stearoyl, 2-arachidonyl (18:0-20:4) species is predominant in inositides, while 22:6 acids are enriched in PtdEtn and PtdSer [3]. The fatty acid substituents for a given phospholipid class isolated from white and gray matter may differ dramatically. Thus, white matter PtdEtn

TABLE 3-1 Distribution profile of the major individual molecular species in the diacylglycerol moieties of rat brain phosphoglycerides*

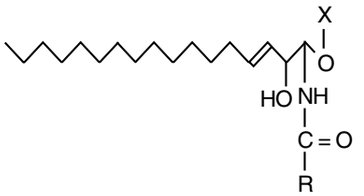
Fatty acid						
C-1	**C-2**	**PtdIns (mol %)**	**PtdIns4,5P_2 (mol %)**	**PtdCho (mol %)**	**PtdEtn (mol %)**	**PtdSer (mol %)**
16:0	22:6	1.4	0.1	3.3	4.8	0.8
16:0	20:4	7.8	9.5	4.4	2.3	0.6
18:1	20:3	4.1	1.1	Tr	Tr	Tr
18:0	22:6	Tr	1.0	2.5	17.6	42.4
14:0	16:0	0.6	0.4	3.1	1.5	0.8
18:0	22:5	1.0	0.7	0.4	0.2	5.3
18:0	20:4	49.5	66.1	3.8	22.5	3.8
18:1	18:1	1.7	2.1	3.4	11.1	7.0
16:0	18:1	12.7	6.5	36.2	15.8	9.1
16:0	16:0	6.9	1.4	19.2	0.7	Tr
18:0	18:1	7.0	4.6	14.1	14.8	23.7

*Adapted from [3]. PtdIns, phosphatidylinositol; PtdIns(4,5)P_2, phosphatidylinositol-4,5-*bis*phosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

contains 42% 18:1 and 3% 22:6, while gray matter PtdEtn contains only 12% 18:1 and 24% 22:6 [4]. As noted below, brain lipids contain some unusually long and polyunsaturated fatty acids from both the ω3 and ω6 families of essential fatty acids, which cannot be biosynthesized in the animal body *de novo* (see also Ch. 33). This implies the existence of a mechanism for transporting essential fatty acids across the blood–brain barrier. There is considerable interest in the role of the polyunsaturated fatty acids and their metabolites in brain after breakdown of their parent phospholipids in conditions such as ischemia and anoxia (see Chs 31 and 32).

In sphingolipids, the long-chain aminodiol sphingosine serves as the lipid backbone. Sphingosine resembles a monoradyl glycerol but has asymmetric carbons at both C-2 and C-3. The chiral configuration is like that of the tetrose D-erythrose. That is, the amino group at C-2 and hydroxyl group at C-3 are in *cis* configuration (2S, 3R). Unlike unsaturated fatty acids, the double bond between C-4 and C-5 in sphingosine is in the *trans* (E) configuration. In the IUPAC-IUB nomenclature, the saturated analog of sphingosine, dihydrosphingosine or D-erythro-2-amino-1,3-octadecanediol, is termed sphinganine and sphingosine is (E-4) sphingenine. While in most sphingolipids the sphingosine is 18 carbons long, in brain gangliosides there is a significant representation of the C_{20} homolog.

The amino group of sphingosine is acylated with long-chain fatty acids and the *N*-acylated product is termed a ceramide (Fig. 3-3). C-1 of ceramide is linked to different head groups to form various membrane lipids. For example, sphingomyelin is the phosphodiester of ceramide and choline. The fatty acids in sphingomyelin have a bimodal distribution: in white matter they are mostly 24 carbons long (lignoceric and nervonic) while in gray matter stearic acid (18:0) predominates (see Table 3.1). Most of the glycolipids in brain consist of ceramide glycosidically linked at C-1 with different mono- or polysaccharides. The major glycolipid of mammalian brain is galactocerebroside, in which galactose is β-glycosidically linked to ceramide; it constitutes about 16% of total adult human brain lipid. Sulfatide is galactocerebroside esterified to sulfate at the 3 position of galactose and constitutes about 6% of brain lipid. Cerebrosides are present mainly in brain white matter, especially in myelin, and generally contain very-long-chain normal (lignoceric and nervonic), α-hydroxy (cerebronic) and odd-numbered fatty acids, such as 23:0 and 23h:0. Myelin is a specialized plasma membrane that surrounds nerve processes and is elaborated by oligodendroglial cells in the CNS and by Schwann cells in the peripheral nervous system (see Chs 1 and 4). A number of neurological disorders appear to involve selectively myelin (Ch. 38). Brain also contains many other glycolipids that are polysaccharide derivatives of glucocerebroside (Cer-Glc). Many monosaccharides, such as galactose (Gal), glucose (Glc), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose and others, are present in various linkages in these carbohydrate head groups. One important carbohydrate is sialic acid, or *N*-acetyl (or *N*-glycolyl)-neuraminic acid (NANA), an *N*-acylated, nine-carbon amino sugar (Fig. 3-4B)



X	Lipid	Abv.
H	Ceramide	Cer
O^- $P-O-CH_2CH_2\overset{+}{N}(CH_3)_3$ O	Sphingomyelin	CerPCho
CH_2OH HO O OH OH	Galactocerebroside	CerGal
CH_2OH O OH OH OH	Glucocerebroside	CerGlc
Lactose (Glc-Gal)	Lactosylceramide	CerLac

FIGURE 3-3 Structure of some simple sphingolipids. X may be a complex polysaccharide either containing sialic acid (gangliosides) or not (globosides). See also Figures 3-4 and 3-9 for the nomenclature and structure of some of the complex brain sphingolipids.

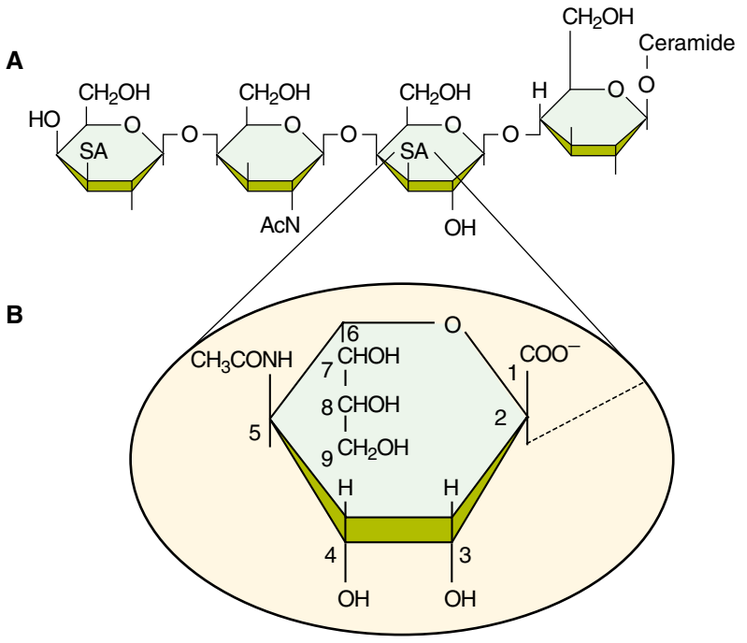


FIGURE 3-4 (**A**) The structure of a major brain ganglioside, which is termed GD1a according to the nomenclature of Svennerholm. G denotes ganglioside, D indicates disialo, 1 refers to the tetrasaccharide (Gal-GalNac-GalGlc-) backbone and a distinguishes positional isomers in terms of the location of the sialic acid residues (see also Fig. 3-9). In IUPAC-IUB nomenclature, this ganglioside is termed IV^{3}NeuAc,II^{3}NeuAc-Gg$_4$Cer, where the roman numerals indicate the sugar moiety (from ceramide) to which the sialic acids (NeuAC) are attached and the arabic numeral superscript denotes the position in the sugar moiety where NeuAC are attached; Gg refers to the ganglio (Gal-GalNAc-Gal-Glc) series and the subscript 4 to the four-carbohydrate backbone for the 'ganglio' series. (**B**) The structure of sialic acid, also called *N*-acetyl neuraminic acid (NeuAc or NANA). Human brain gangliosides are all *N*-acetyl derivatives; however, some other mammalian, such as bovine, brain may contain the *N*-glycolyl derivatives. The metabolic biosynthetic precursor for sialylation of glycoconjugates is CMP-sialic acid, forming the phosphodiester of the 5′OH of cytidine and the 2-position of neuraminic acid.

containing a free carboxyl group. NANA is enzymatically formed by condensation of *N*-acetyl (or *N*-glycolyl)-mannosamine with phosphoenolpyruvate. The sialic acid-containing glycolipids contain a free carboxylic group and are termed gangliosides. Many gangliosides have been identified in neural and other tissues, and their classification and nomenclature are somewhat complex. Svennerholm classified the gangliosides according to the number of sialic acid residues present in the molecule and its relative migration rate on thin-layer chromatograms (Fig. 3-5). IUPAC-IUB has proposed a different systematic nomenclature for both gangliosides and neutral glycolipids, or globosides. The structure and nomenclature of a major brain ganglioside are given in Figure 3-4A (see below for other gangliosides).

ANALYSIS OF BRAIN LIPIDS

Chromatographic methods are employed to analyze and classify brain lipids. The lipids from brain are generally extracted with a mixture of chloroform and methanol using variations of a method originally described by Folch *et al.* In most procedures, the tissue or homogenate is treated with 19 volumes of a 2:1 (v/v) mixture of chloroform methanol. A single liquid phase is formed, leaving behind a residue of macromolecular material, primarily protein, with lesser amounts of DNA, RNA and polysaccharides. The subsequent addition of a small amount of water to the $CHCl_3$-methanol extract leads to separation into chloroform-rich and aqueous methanol phases; the lower chloroform phase contains the lipids, whereas low-molecular-weight metabolites and polar lipids, such as gangliosides, are in the upper phase. If the lower phase is evaporated to dryness and taken back up in a lipid solvent such as chloroform, proteolipid protein remains undissolved and can be removed at this point. Gangliosides can be extracted from the aqueous phase by repartitioning into an apolar solvent. Acidic phospholipids such as the polyphosphoinositides are poorly extracted at neutral pH, so it is necessary to acidify the initial chloroform methanol mixture for their recovery [6]. Unfortunately, the acidity leads to cleavage of plasmalogens, primarily alkenyl-acyl PtdEtn. There is thus no single procedure that results in quantitative recovery of all brain lipids. Lipid classes are separated from a lipid extract by thin-layer chromatography (TLC), ion-exchange chromatography or high-performance liquid chromatography (HPLC) using silicic acid as the stationary phase. For analysis of individual fatty acids in a given lipid class,

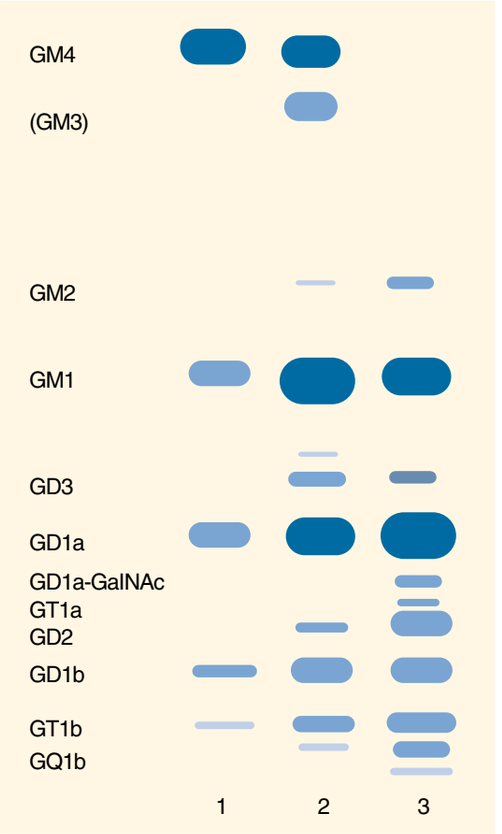


FIGURE 3-5 Diagrammatic representation of thin-layer chromatograms of gangliosides from normal human white matter (*lane 2*) and gray matter (*lane 3*). *Lane 1* contains a mixture of isolated standards. Each lane contains about 7 µg sialic acid. Merck precoated HPTLC plates (silica gel 60, 200 µm thick) were used. The plate was developed with chloroform–methanol–water, 60:40:9 (containing 0.02% $CaCl_2 \cdot 2H_2O$). The bands were visualized with resorcinol–hydrochloric acid reagent. See legend to Figures 3-4 and 3-9 for nomenclature. (Courtesy of R. K. Yu, see also Yu and Ando [5].)

methyl esters can be prepared directly by alkaline methanolysis of extracted lipid bands scraped from TLC plates following visualization, usually with a fluorescent spray. The amide-bound fatty acids of the sphingolipids require more vigorous conditions of methanolysis, such as treatment with hot HCl methanol. The methyl esters are then separated by gas–liquid chromatography (GLC). The molecular species can also be separated by reverse-phase HPLC. For this purpose, a reporter group, such as the ultraviolet-absorbing benzoyl group, can be attached either directly to the lipids in the carbohydrate portion of glycolipids or to the DAG backbone of lipid after hydrolysis of the polar head group [3]. In this method, separation of the derivatized DAGs is achieved on the basis of their differences in hydrophobicity. Gangliosides are separated from each other by HPTLC, as shown in Figure 3-5.

The lipid composition of mammalian brain analyzed by these methods is found to change with age and is different from one region to another [1]. A typical lipid composition of gray and white matter in adult human brain is given in Table 3-2.

Recently, a quantitative electrospray ionization/mass spectrometry method (ESI/MS) has been developed to analyze the molecular profile, or 'lipidome' of different lipid classes in very small samples. In this method, total lipid extracts from tissues or cultured cells can be directly analyzed. By manipulating the ionization method, the mass spectrographs of polar or even non-polar lipids can be obtained [8]. This method and the use of lipid arrays allow precise and quantitative identification of the lipid profile of a given tissue, and map functional changes that occur.

BRAIN LIPID BIOSYNTHESIS

Acetyl coenzyme A is the precursor of both cholesterol and fatty acids. The hydrophobic chains of lipids, that is, fatty acids and isoprenoids, are biosynthesized from the same two-carbon donor, acetyl coenzyme-A (acetyl-CoA), with differences in condensation leading to different products. In cholesterol biosynthesis, two acetyl-CoAs are condensed to form acetoacetyl-CoA, which can be further condensed with a third acetyl-CoA to form a C_6 branched-chain dicarboxylic acyl-CoA, termed β-hydroxy-β-methyl-glutaryl (HMG)-CoA. HMGCoA is reduced by $2NADPH^+$ to form mevalonic acid, and this reduction is catalyzed by the enzyme HMGCoA reductase, the principal regulatory enzyme for the biosynthesis of isoprenoids [9]. Mevalonic acid undergoes pyrophosphorylation by two consecutive reactions with ATP, and the product is decarboxylated to form isopentenyl pyrophosphate. This C_5H_8 isoprene unit is the building block of all isoprenoids. Two isoprene units, isopentenyl pyrophosphate and dimethyl allyl pyrophosphate, condense to form geranyl pyrophosphate (C_{10}), which then condenses with another C_5 unit to form farnesyl pyrophosphate (C_{15}), the precursor of many different isoprenoids, such as dolichol, a very-long-chain (up to C_{100}) alcohol; a redox coenzyme, ubiquinone; and cholesterol. Polyisoprenyl pyrophosphates also alkylate some proteins via a thioether bond, which bonds them to biomembranes (see below). During cholesterol biosynthesis, two farnesyl pyrophosphate molecules reductively condense in a head-to-head manner to form squalene, a C_{30} hydrocarbon. Squalene is oxidatively cyclized to form lanosterol, a C_{30} hydroxysteroid. After three demethylations, lanosterol is converted to cholesterol (C_{27}). An outline of the pathway of biosynthesis of cholesterol is shown in Figure 3-6. Once formed, brain cholesterol turns over very slowly, and there is both metabolic and analytic evidence to indicate an accretion of brain cholesterol with age.

Fatty acids are biosynthesized via elongation of C_2 units. Here, acetyl-CoA is carboxylated by bicarbonate to form

TABLE 3-2 Lipid composition of normal adult human brain*

	Gray matter (%)			*White matter (%)*		
Constituent	**Fresh wt**	**Dry wt**	**Lipid**	**Fresh wt**	**Dry wt**	**Lipid**
Water	81.9	–	–	71.6	–	–
Chloroform–methanol – insoluble residue	9.5	52.6	–	8.7	30.6	–
Proteolipid protein	0.5	2.7	–	2.4	8.4	–
Total lipid	5.9	32.7	100	15.6	54.9	100
Upper-phase solids	2.2	12.1	–	1.7	6.0	–
Cholesterol	1.3	7.2	22.0	4.3	15.1	27.5
Phospholipid, total	4.1	22.7	69.5	7.2	25.2	45.9
PtdEtn	1.7	9.2	27.1	3.7	13.2	23.9
PtdCho	1.9	10.7	30.1	2.4	8.4	15.0
Sphingomyelin	0.4	2.3	6.9	1.2	4.2	7.7
Phosphoinositides	0.16	0.9	2.7	0.14	0.5	0.9
PtdSer	0.5	2.8	8.7	1.2	4.3	7.9
Galactocerebroside	0.3	1.8	5.4	3.1	10.9	19.8
Galactocerebroside sulfate	0.1	0.6	1.7	0.9	3.0	5.4
Ganglioside, total†	0.3	1.7	–	0.05	0.18	–

*Modified from Suzuki [7].

†Phospholipid fractions include plasmalogen, assuming that all plasmalogen is present as PtdEtn. Ratios of PtdEtn to PtdCho are 4:1 in white matter and 1:1 in gray matter. In intact brain (based on analysis of rapidly microwaved rat brain), phosphoinositides are present in both white and gray matter in the ratio of 5:0.3:1 for phosphatidylinositol (PtdIns) phosphatidylinositol-4-phosphate (PtdIns4P_2), phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P_2). Gangliosides are calculated on the basis of total sialic acid, assuming that sialic acid constitutes 30% of the weight of a typical ganglioside; GD_{1a} is the major ganglioside of both gray and white matter. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine.

malonyl-CoA, which then condenses with an acyl-CoA to form a β-ketoacyl-CoA and CO_2. This release of CO_2 (HCO_3^-) drives the reaction forward and elongates the chain by acetyl units. The ketone group is then enzymatically reduced, dehydrated and hydrogenated, resulting in an acyl-CoA that is two carbons longer than the parent acyl-CoA. NADPH acts as the reducing agent for the reduction of both the ketone group and the double bond. All four reactions, condensation, reduction, dehydration and hydrogenation, are carried out by fatty acid synthase, a large, multifunctional, dimeric enzyme. This cycle is repeated until the proper chain length (>C_{12}) is attained, after which the fatty acid is hydrolyzed from its thioester link with the enzyme. Preformed or exogenous fatty acids are extended by a similar mechanism and catalyzed by enzyme(s) present in the endoplasmic reticulum [10]. There is also a minor mitochondrial chain-elongation system in which acetyl-CoA rather than malonyl-CoA is utilized to lengthen the fatty acid chain. Fatty acids are converted to unsaturated fatty acids mainly in the endoplasmic reticulum. Fatty acyl-CoA desaturases, of which Δ^9-desaturases are most active, remove two hydrogens from the CH_2 CH_2 groups of long-chain intermediates, such as octadecanoyl-CoA, by oxidizing them with molecular oxygen. In brain, this enzyme is responsible for the conversion of stearic acid (18:0) to oleic acid (18:1ω9) and palmitic acid (16:0) to palmitoleic acid (16:1ω7) (see Fig. 3-1). The electrons are transferred via cytochrome b_5 which in turn is reduced to NADH via cytochrome b_5 reductase. In brain, polyunsaturated fatty acids, such as arachidonic (20:4ω6) and docosahexenoic acids (22:6ω3), are major phospholipid components. They are formed by chain elongation and desaturation of shorter-chain fatty acids (Fig. 3-7). In animals, additional double bonds can be introduced only between an existing double bond and the fatty acid carboxyl group. For example, stearic acid (18:0) is converted to oleic acid (18:1ω9) in brain but cannot be further converted to linoleic acid (18:2ω6). This means that the fatty acids of the ω3 and ω6 series can be obtained only via dietary sources, mainly from plants. They are termed 'essential fatty acids' and have important physiological roles. If the ω3 and ω6 precursors are not available in the diet, then ω9 fatty acids are further chain-elongated and desaturated to form abnormal fatty acids, as a compensatory response of the brain. One of these is 20:3ω9 (Fig. 3-7), termed 'Mead acid' after its discovery by James Mead in the tissues of animals fed a fat-free diet over extended periods. Mead acid substitutes for arachidonic acid and, like arachidonic acid in normal animals, is enriched in the inositides of essential fatty acid-deficient animals.

Fatty acids are degraded by two-carbon units in a reverse manner analogous to their biosynthesis. The acyl-CoAs are first dehydrogenated to α,β-unsaturated acyl-CoA, and then hydrated to β-hydroxyacyl-CoA, followed by oxidation to β-ketoacyl-CoA. The C–C bond between C-2 and C-3 of the latter compound is broken by a free CoA molecule via thiolysis to form an acyl-CoA that is two carbons shorter and acetyl-CoA. Unlike fatty acid biosynthesis, each step of the β oxidation of fatty acids is

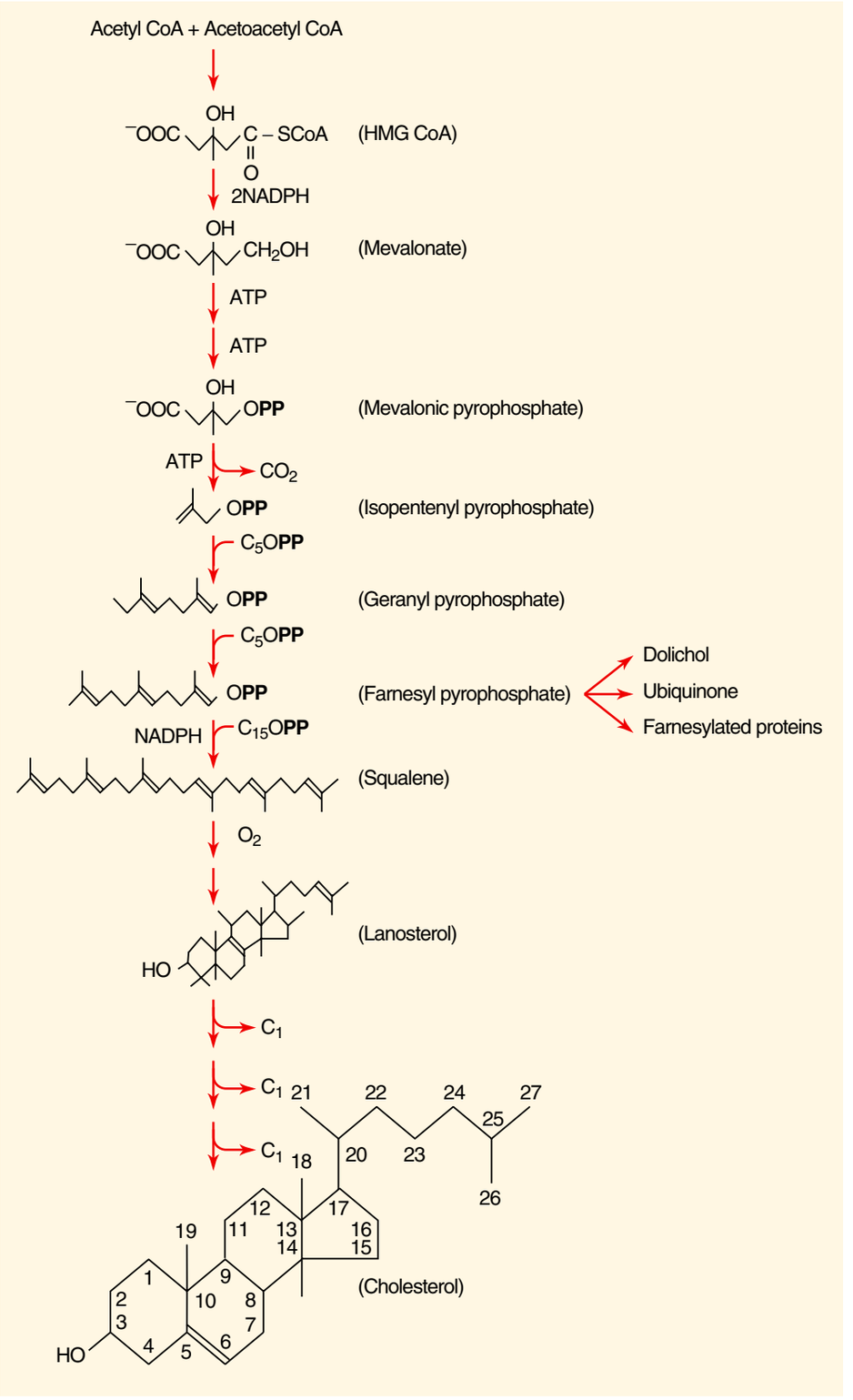


FIGURE 3-6 Pathways of biosynthesis of isoprenoids.

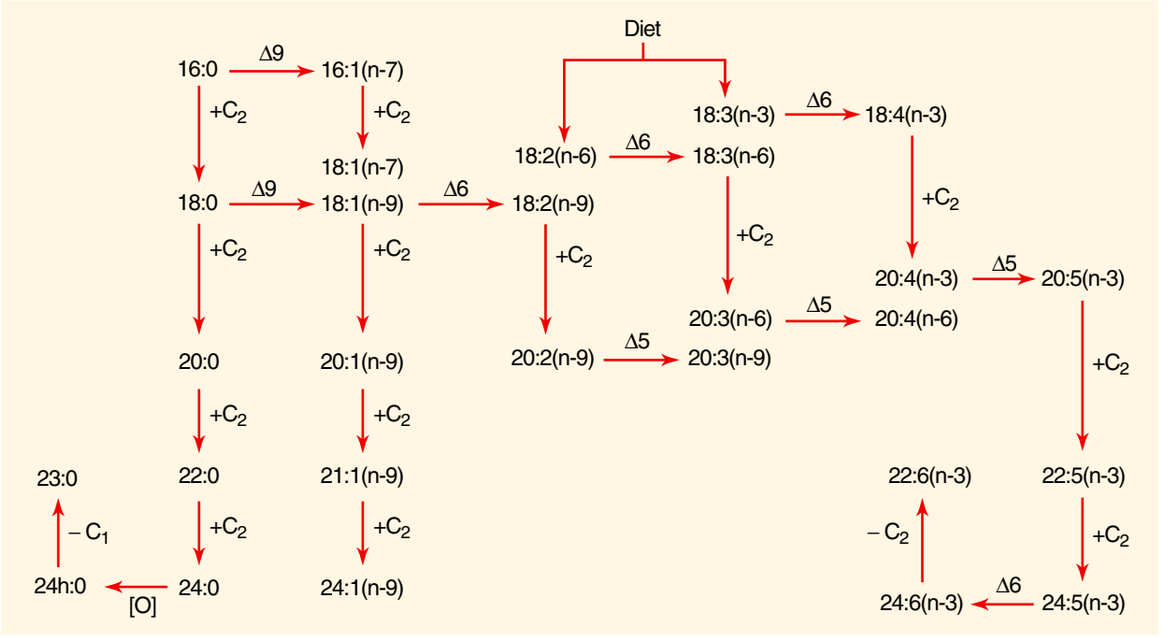


FIGURE 3-7 Pathways for the interconversion of brain fatty acids. Palmitic acid (16:0) is the main end product of brain fatty acid synthesis. It may then be elongated, desaturated, and/or β-oxidized to form different long chain fatty acids. The monoenes (18:1 Δ^7, 18:1 Δ^9, 24:1 Δ^{15}) are the main unsaturated fatty acids formed de novo by Δ^9 desaturation and chain elongation. As shown, the very long chain fatty acids are α-oxidized to form α-hydroxy and odd numbered fatty acids. The polyunsaturated fatty acids are formed mainly from exogenous dietary fatty acids, such as linoleic (18:2, n–6) and α-linoleic (18:2, n–3) acids by chain elongation and desaturation at Δ^5 and Δ^6, as shown. A Δ^4 desaturase has also been proposed, but its existence has been questioned. Instead, it has been shown that unsaturation at the Δ^4 position is effected by 'retroconversion' i.e. Δ^6 unsaturation in the endoplasmic reticulum, followed by one cycle of β-oxidation (–C2) in peroxisomes [11]. This is illustrated in the biosynthesis of DHA (22:6, n–3) above. In severe essential fatty acid deficiency, the abnormal polyenes, such as 20:3, n–9 are also synthesized *de novo* to substitute for the normal polyunsaturated acids.

catalyzed by a distinct enzyme. These are present both in mitochondria and in peroxisomes. Though the biochemical steps are similar in the two cellular compartments, there are some differences between peroxisomal and mitochondrial β-oxidation pathways. In mitochondria, the first dehydrogenation is carried out by an FAD-containing enzyme, which is coupled to oxidative phosphorylation, thus generating ATP. In peroxisomes, however, this dehydrogenation is carried out by a flavin-containing oxidase, which reacts directly with molecular oxygen to form H_2O_2, which is further decomposed by peroxisomal catalase to H_2O and O_2, thus wasting the chemical energy. Two separate mitochondrial enzymes, enoyl-CoA hydratase and β-hydroxy acyl-CoA dehydrogenase, catalyze the next two reaction steps, while in peroxisomes both the reactions are catalyzed by a single multifunctional enzyme protein. The peroxisomal β-oxidation pathway is probably responsible for the oxidation of very-long-chain fatty acids (>C_{22}), which are enriched in brain. Evidence for this is provided by a number of genetic diseases involving peroxisomal disorders, such as Zellweger's cerebrohepatorenal syndrome and adrenoleukodystrophy, in which there is an accumulation of such very-long-chain fatty acids [12], especially in neural tissues (see Ch. 41).

In addition to the classical β oxidation of fatty acids, known to occur in all tissues, significant α oxidation, especially of the fatty acids of galactocerebroside, occurs in brain. In this reaction, carbon 2, termed the α carbon, of a long-chain fatty acid is hydroxylated, then oxidized and decarboxylated to form a fatty acid one carbon shorter than the parent fatty acid. This quantitatively minor α-hydroxylation pathway may explain the origins of both the comparatively large amounts of odd carbon fatty acids and of 2-hydroxy fatty acids in brain galactocerebrosides. Another α-oxidation pathway normally present in liver and other tissues is defective in the genetic disorder Refsum's disease. This results in the failure to metabolize the dietary branched-chain fatty acid phytanic acid (see Fig. 3-1), which can be initially metabolized only by ω oxidation in these patients [13]. In Refsum's disease, this branched-chain fatty acid accumulates in nervous tissues, resulting in severe neuropathy (see Ch. 41)

Phosphatidic acid is the precursor of all glycerolipids. *sn*-Glycerol-3-phosphate (G-3-P), formed from the reduction of dihydroxyacetone phosphate (DHAP) by NADH catalyzed by glycerophosphate dehydrogenase, is consecutively acylated with two acyl-CoAs to form PtdOH. Alternatively, DHAP may be first acylated then reduced by NADPH to lysophosphatidate, or 1-acyl-GP, which is further acylated to form PtdOH. Acyl DHAP is also the precursor of ether lipids. The ether bond is formed in a

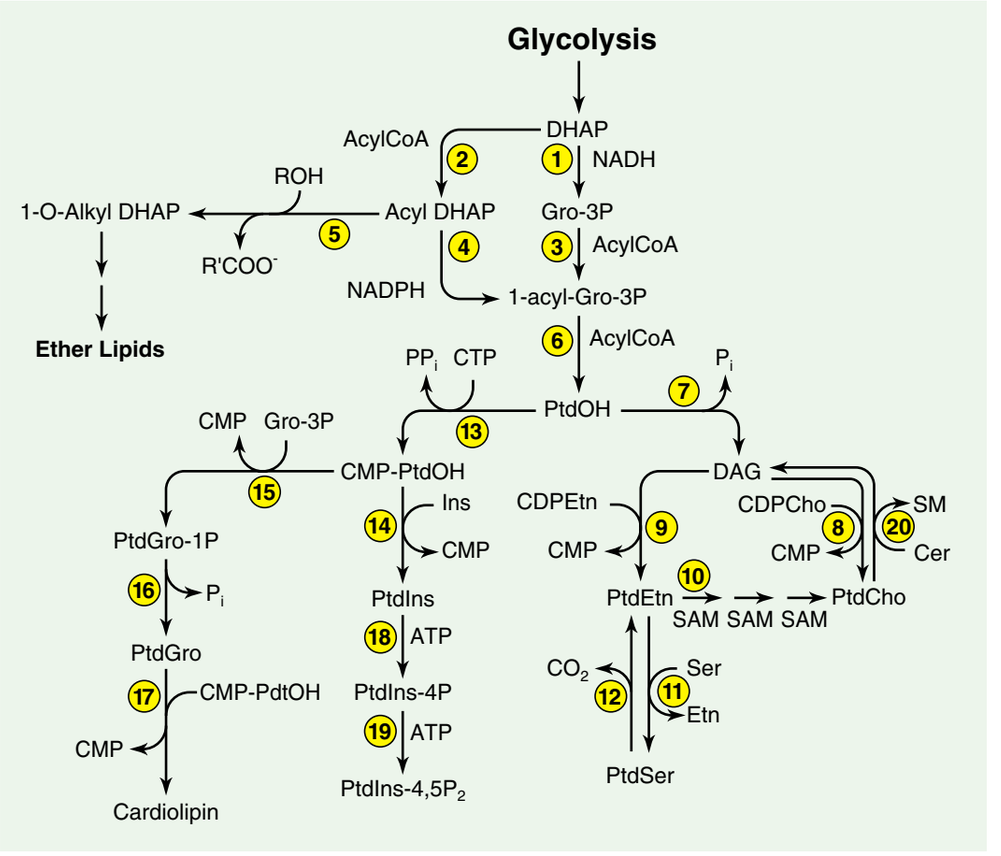


FIGURE 3-8 Schematic representation of glycerophospholipid biosynthesis. Note that dihydroxyacetone phosphate (*DHAP*) may be reduced to glycerophosphate or may be first acylated and then serve as a precursor of ether lipid. The alkyl analog of phosphatidic acid (i.e. 1-*O*-alkyl,2-acyl-*sn*-glycerol-3-P) is converted to the alkyl analog of phosphatidylethanolamine (*PtdEtn*) by the same diacylglycerol (*DAG*) pathway as shown for the diacyl lipids, and the alkyl analog of PtdEtn is dehydrogenated to form the 1-alk-1′enyl analog of PtdEtn, plasmalogen (not shown). As mentioned in the text, phosphatidic acid (*PtdOH*) is converted to DAG, which is converted to the major brain lipids phosphatidylcholine (*PtdCho*) and PtdEtn. The acidic lipids are formed via the conversion of PtdOH to CDP-DAG (CMP-PtdOH). PtdCho and PtdEtn are interconverted either via methylation or via base-exchange reactions to phosphatidylserine (*PtdSer*). Not only PtdEtn (as shown) but also PtdCho is converted to PtdSer by base-exchange reaction. Exchange of the head group of PtdCho with ceramide to form sphingomyelin is also shown. The enzymes catalyzing lipid biosynthesis are as follows: *1*, glycerophosphate dehydrogenase; *2*, dihydroxyacetone phosphate acyltransferase; *3*, *sn*-glycerol-3-phosphate acyltransferase; *4*, acyl/alkyl dihydroxyacetone phosphate reductase; *5*, alkyl dihydroxyacetone phosphate synthase; *6*, 1-acyl glycerol-3-phosphate acyltransferase; *7*, phosphatidate phosphohydrolase; *8*, diacylglycerol cholinephosphotransferase; *9*, diacylglycerol ethanolaminephosphotransferase; *10*, phosphatidylethanolamine *N*-methyl transferase and phosphatidyl-*N*-methylethanolamine *N*-methyl transferase; *11*, phosphatidylethanolamine:serine transferase; *12*, phosphatidylserine decarboxylase; *13*, phosphatidate cytidyltransferase; *14*, phosphatidylinositol synthase; *15*, CDP-DAG:glycerol-3-phosphate phosphatidyltransferase; *16*, phosphatidylglycerol phosphatase; *17*, cardiolipin synthase; *18*, phosphatidylinositol-4-kinase; *19*, phosphatidylinositol-4-phosphate 5-kinase; *20*, phosphatidylcholine:ceramide cholinephosphotransferase.

reaction where the acyl group of acyl DHAP is substituted by a long-chain alcohol to form 1-*O*-alkyl DHAP, which is then reduced and converted to 1-alkyl,2-acyl-*sn*-G-3-P, and which is in turn converted to the alkyl ether analog of PtdEtn, the precursor of Ptd-Etn plasmalogen (Fig. 3-8).

Phosphatidate may be hydrolyzed to 1,2-diacyl-*sn*-glycerol (DAG), which is the precursor of the zwitterionic membrane lipids PtdCh, PtdEtn and PtdSer. PtdCho is formed by the transfer of the phosphocholine group from CDP-choline to DAG, and PtdEtn is formed by a corresponding transfer of the head group from CDP-ethanolamine. The enzymes catalyzing the synthesis of CDP-choline and CDP-ethanolamine regulate the overall biosynthesis of PtdCho and PtdEtn. In a minor alternative pathway, Ptd-Etn is converted to PtdCho by sequential methylations, the methyl donor being *S*-adenosylmethionine. In animals, there is no direct pathway for the formation of PtdSer. PtdSer is formed in brain by a base-exchange reaction between Ptd-Etn, or PtdCho, and serine. PtdSer is in turn decarboxylated in mitochondria to form PtdEtn [14].

The acidic phospholipids are synthesized by a completely different pathway, in which the phosphate group in PtdOH is retained in the product. In this scheme, PtdOH is converted to the liponucleotide CMP-PtdOH (CDP-DAG) (Fig. 3-8). CDP-DAG reacts with inositol to form PI

or with *sn*-glycerol-3-phosphate (Gro3-P) to form phosphatidyl glycerophosphate, which is then converted to cardiolipin (bisphosphatidylglycerol), a mitochondria-specific phospholipid. PI is phosphorylated in the inositol moiety to form PIP, which is an intermediate in a pathway that mediates signal transduction across membranes (see Ch. 20). The pathways from brain phospholipid biosynthesis, including the enzymes that catalyze each step, are summarized in Figure 3-8.

The newly biosynthesized phosphoglycerides undergo deacylation to the corresponding lysolipids, which can be further degraded or reconverted to the parent lipids by reacylation, often with a different fatty acyl substitute. The reacylation of lysolipids occurs by transferring acyl groups from acyl-CoAs or from other phospholipids either by CoA-dependent or CoA-independent acyltransferase. The acyltransferase(s) catalyzing the reacylation reactions is very specific toward the acyl donor and lysolipid substrates. It is thought that the specific distribution of fatty acids in each individual class of membrane phosphoglycerides is regulated by these 'deacylation–reacylation' mechanisms. Thus, the initial fatty acid composition of a biosynthesized lipid may not reflect its ultimate composition.

Most of the enzymes catalyzing the biosynthesis of glycerolipids are bound to endoplasmic reticular membranes, although those catalyzing the biosynthesis of cardiolipin are mitochondrial. The acyl-DHAP pathway enzymes, obligatory for the synthesis of ether lipids, are in peroxisomes [15], a finding that explains the deficiency of ether lipids in patients suffering from genetic peroxisomal disorders, as noted above.

The phosphoglycerides are hydrolyzed by specific phospholipases, as indicated in Figure 3-2. The acyl groups at C-1 and C-2 are hydrolyzed by phospholipases A_1 and A_2 (PLA_1, PLA_2), respectively. The presence of PLA_1 in brain is inferential. The head groups are hydrolyzed by class-specific phospholipases. Thus, PtdCho and PI are cleaved by different phospholipases. The bond between DAG and phosphate is hydrolyzed by PLC, whereas that between the phosphate and the polar alcohol is hydrolyzed by phospholipase D. These enzymes can be important not only for the catabolism of these lipids but also for the generation of biological signal-transduction-messenger lipid products, such as DAG or arachidonic acid (see Ch. 20). Many of these enzymes are regulated, indirectly or directly, by cell-surface receptors. The brain also contains specific hydrolases, plasmalogenase and lysoplasmalogenase, which catalyze the hydrolysis of the alkenyl ether bond to form long-chain aldehydes and lysolipids or glycerophosphorylethanolamine, respectively.

Sphingolipids are biosynthesized by adding head groups to the ceramide moiety. Sphinganine, also termed dihydrosphingosine, is biosynthesized by a decarboxylating condensation of serine with palmitoyl-CoA to form a keto intermediate, which is then reduced by NADPH (Fig. 3-9). Sphinganine is acylated, then dehydrogenated to form ceramide. Free sphingosine, also termed sphingenine, 'salvaged' from sphingolipid breakdown can be enzymatically acylated with acyl-CoA to form ceramide.

Ceramide is the precursor of all sphingolipids; sphingomyelin is formed by a reaction that transfers the head group of PtdCho to ceramide to form sphingomyelin and DAG (Figs 3-8, 3-9), while sphingosine-containing glycolipids are formed from consecutive glycosylation of ceramide by various nucleotide carbohydrate derivatives. For example, galactocerebroside is formed by glycosylation of ceramide with UDPGal, whereas glucocerebroside is formed by glycosylation of ceramide with UDPGlc [16]. The latter, Cer-Glc, is the precursor of neutral glycolipids, also termed globosides, and acidic glycolipids, also termed gangliosides. The CMP derivative of the *N*-acetyl (or *N*-glycolyl) neuraminic acid NANA, or NeuAc, is the donor of this moiety to form gangliosides. Some of the reactions forming these complex glycolipids are shown in Figure 3-9. The specificity of these membrane-bound glycosyl transferases toward the lipid substrate and to the water-soluble nucleotide derivatives determine the structures of the products.

These same glycolipids are broken down by specific hydrolases present in lysosomes and stimulated by non-catalytic lysosomal proteins. A congenital deficiency of either one of the hydrolases or in the helper proteins results in the accumulation of lipid intermediates in lysosomes, leading to a lysosomal storage disease. For example, in Gaucher's disease, Cer-Glc accumulates because of a defect in its hydrolysis, whereas in Tay–Sachs disease, the GM2-ganglioside concentration is increased because of a deficiency in the hydrolase releasing *N*-acetylgalactosamine (see Ch. 41).

GENES FOR LIPID-SYNTHESIZING ENZYMES

Because of interest in these genetic diseases, genes coding for a number of these hydrolytic enzymes have been identified and cloned. Progress has also been made in the elucidation of genes coding for biosynthetic enzymes, including several transferases in the pathway for ganglioside formation and the UDP-galactosyltransferase that leads to cerebroside formation (see Ch. 4). In recent years, dozens of genes coding for enzymes catalyzing synthesis of cholesterol, phospholipids, galactolipids and gangliosides have been cloned. In pathways for phospholipid synthesis, the DNA coding for the key enzyme CDP-DAG synthase has been cloned from a human cell line [17].

Mutant or knockout mice defective in specific enzymes involved in lipid synthesis have provided powerful tools for genetic analysis of lipid function in the nervous system. For example, disruption of the genes for ceramide galactosyl transferase or galactosyl ceramide sulfotransferase,

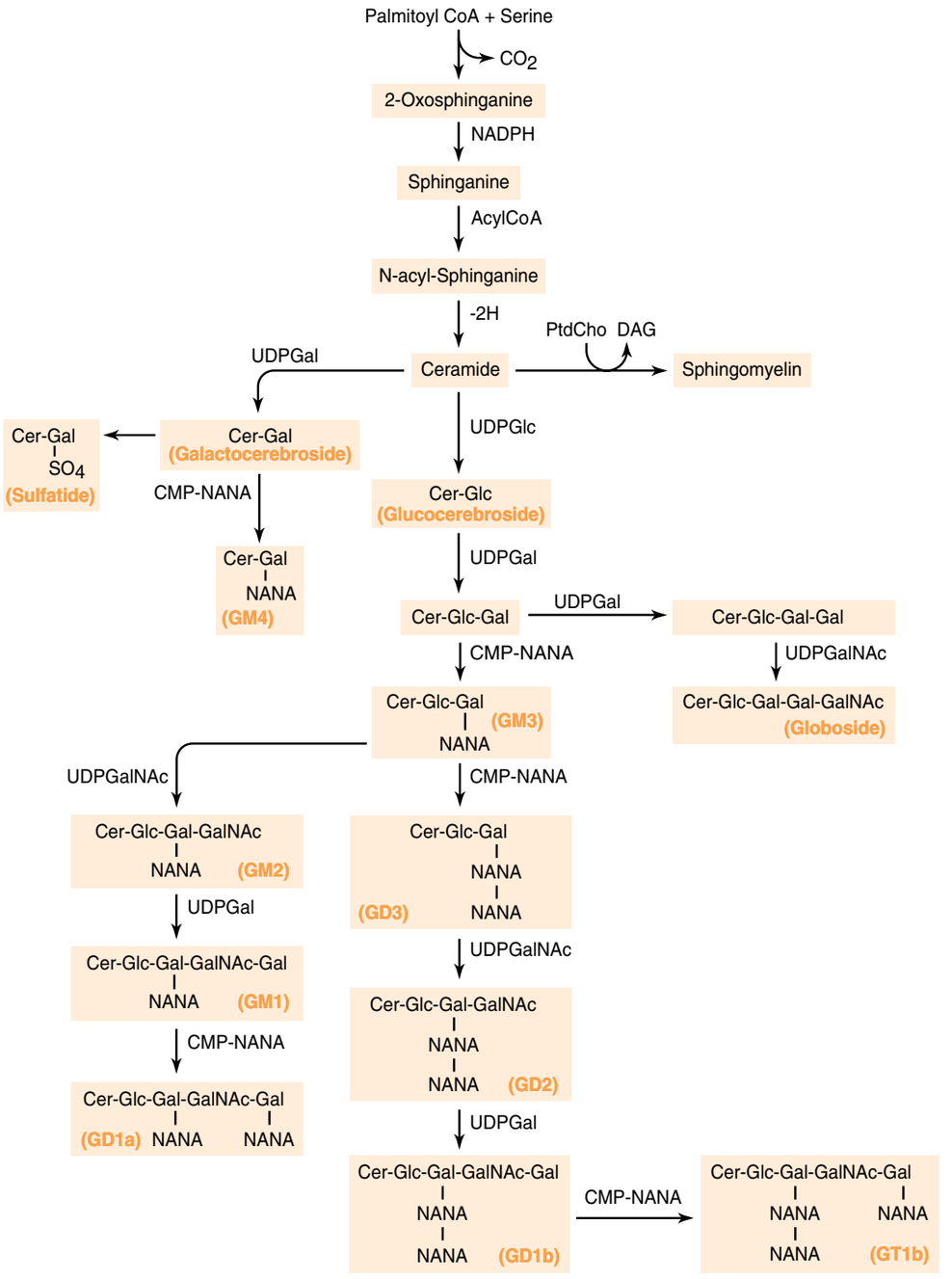


FIGURE 3-9 Pathways for biosynthesis of sphingolipids. Ceramide (*Cer*) is the precursor of all sphingolipids. Ceramide is converted to cerebroside (*Cer-Gal*), the main brain glycolipid, which is further converted to cerebroside sulfate (sulfatide) as shown. Cer-Gal is also converted to ganglioside (*GM4*), which is present in brain myelin. Most other gangliosides originate from Cer-Glc, and the main pathways for formation of these lipids are shown. The abbreviations using Svennerholms's nomenclature are shown in parentheses. (See Figs 3-4, 3-5.) The first letter, G, is for ganglioside. The second letter, M, D, T or Q, represents the number of sialic acid residues. Isomeric configurations of NANAs are distinguished by a and b. The main gangliosides of adult human brain are GM1, GD1a, GD1b and GT1b.

enzymes synthesizing galactocerebroside and sulfatide, both major sphingolipid components of myelin, gave unexpected results. While myelination and compaction of myelin was not affected initially, the mice displayed abnormal paranodal junctions and later disruption of myelin stability. Comparison of the two kinds of knockout showed that sulfatide plays a critical role in the proper localization and maintenance of Na^+ channels at the paranode [18] (see Chapter 4). Negative phenotypes can be informative as well. The hypothesis that complex gangliosides play a role in synaptic transmission was examined by deleting GM2/GD2 synthase, an enzyme

that catalyzes an early step in ganglioside biosynthesis, then testing neurotransmitter release at the neuromuscular junction [19]. Transmitter release was not altered under normal conditions, indicating a redundancy in complex ganglioside function at the synapse.

LIPIDS IN THE CELLULAR MILIEU

Lipids are transported between membranes. As indicated above, lipids are often biosynthesized in one intracellular membrane and must be transported to other intracellular compartments for membrane biogenesis. Because lipids are insoluble in water, special mechanisms must exist for the inter- and intracellular transport of membrane lipids. Vesicular trafficking, cytoplasmic transfer-exchange proteins and direct transfer across membrane contacts can transport lipids from one membrane to another. The best understood of such mechanisms is vesicular transport, wherein the lipid molecules are sorted into membrane vesicles that bud out from the donor membrane and travel to and then fuse with the recipient membrane. The well characterized transport of plasma cholesterol into cells via receptor-mediated endocytosis is a useful model of this type of lipid transport. [9, 20]. A brain specific transporter for cholesterol has been identified (see Chapter 5). It is believed that transport of cholesterol from the endoplasmic reticulum to other membranes and of glycolipids from the Golgi bodies to the plasma membrane is mediated by similar mechanisms. The transport of phosphoglycerides is less clearly understood. Recent evidence suggests that net phospholipid movement between subcellular membranes may occur via specialized zones of apposition, as characterized for transfer of PtdSer between mitochondria and the endoplasmic reticulum [21].

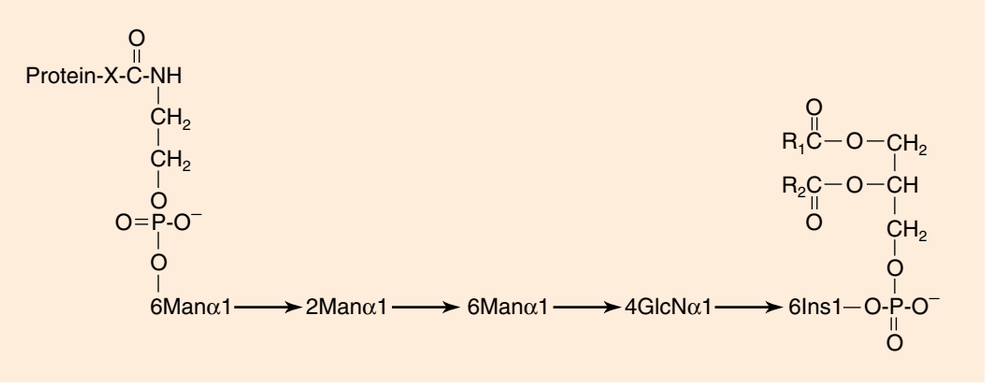
Membrane lipids may be asymmetrically oriented. In the 'fluid-mosaic' model of biomembranes, the lipids form a bimolecular leaflet in which proteins are embedded (see Ch. 2). This model, with some modifications, is useful in explaining a number of membrane phenomena, but it does not take into account the complex arrangement and function of various polar head groups and different fatty acids present in biomembrane lipids. In some biomembranes, such as those of red blood cells, the choline-containing phospholipids PtdCho and sphingomyelin are known to be enriched in the outer leaflet, while the amino lipids PtdEtn and PtdSer are concentrated in the inner leaflet of the plasma membrane. This arrangement probably also exists in the plasma membrane of most other cells. Studies with the serine-binding protein annexin V indicate that PtdSer appears in the plasma membrane outer leaflet in apoptosis [22]. The glycolipids, especially the gangliosides, are enriched in the extracellular side of the plasma membrane, where they may function in intercellular communication and act as receptors for certain ligands; for example, GM1 acts as a receptor for cholera toxin and GD1b for tetanus toxin. It is not clear how this asymmetric distribution of lipids in biomembranes originates. Lipids can move freely within the same plane of the bilayer but their movement from one leaflet of the bilayer to another is thermodynamically restricted. It is postulated that membranes contain proteins that catalyze a 'flip-flop' transbilayer movement of lipids. Specificity of such 'flippase', 'floppase' and 'scramblase' activities may be responsible for the asymmetric distribution of lipids between the inner and outer leaflets of the membrane bilayer. In support of this hypothesis, it has been shown that plasma membrane contains an ATP-dependent phospholipid translocase that selectively catalyzes the transport of the aminophospholipids PtdSer and PtdEtn but not of PtdCho from the outer to the inner lipid layer of the membrane [23]. A glucosyl ceramide translocase, required for transport of glucosyl ceramide from the cytoplasmic surface to the luminal surface of the Golgi membrane for synthesis of neutral glycosphingolipids, has been identified as multiple drug resistance protein 1 [24] (see Chs 2 and 5).

Some proteins are bound to membranes by covalently linked lipids. In recent years, a number of membrane-bound proteins have been shown to be covalently linked with various lipids, which anchor the protein to the lipid bilayer. PI-anchored proteins constitute a major family of membrane-tethered proteins (Box 3-1). In myelin proteolipid protein, fatty acids (16:0, 18:0, 18:1) are attached to the cysteine moieties in the protein as thioesters. A number of cellular proteins are also acylated with myristic acid (14:0) to the free amino group of *N*-terminal amino acids. A class of proteins, including Ras, a proto-oncogene product, has been shown to form covalent links with farnesyl (C_{15}) or C_{20} isoprenes via a thioether linkage to cysteine [25]. The lipid anchor in Ras, which occupies a central position in intracellular signal transduction, is required for its activity. The Hedgehog family of proteins contains palmitic acid linked to the amino terminal domain and cholesterol covalently linked to the carboxy terminal signaling domain [26], making them the only sterolated proteins identified to date. These proteins are of critical importance in patterning during development and in tumorigenesis (see Chapter 29). Lipidated Hedgehog proteins are secreted from cells via the membrane transporter Dispatched, and then transduce signals through two membrane bound receptors, Patched and Smoothened.

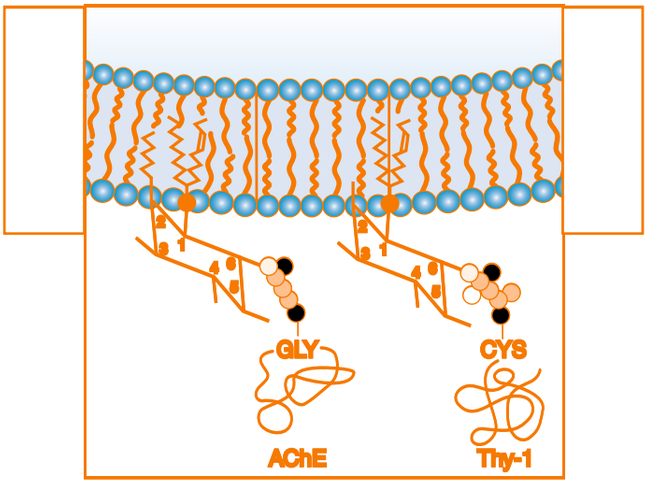
Lipids have multiple roles in cells. Recent discoveries show that the same lipid may have both structural and regulatory roles in the cell. For example, while arachidonic acid (20:4ω6) is a major constituent of brain inositides and PtdEtn, the free acid is also a precursor of a number of important biomessengers, the eicosanoids, such as prostaglandins, prostacyclins, leukotrienes and thromboxanes

BOX 3-1
Glycosylphosphatidylinositol-Anchored Proteins

A bacterial phosphatidylinositol specific phospholipase C (PI-PLC) had been available for many years before it was demonstrated to strip a number of membrane-bound proteins from eukaryotic cell surfaces [1]. Such proteins are anchored by a PI moiety in which the 6 position of inositol is glycosidically linked to glucosamine, which in turn is bonded to a polymannan backbone (Fig. 3-10). The polysaccharide chain is joined to the carboxyl terminal of the anchored protein via amide linkage to ethanolamine phosphate. The presence of a free NH_2 group in the glucosamine residue makes the structure labile to nitrous acid. Bacterial PI-PLC hydrolyzes the bond between DAG and phosphatidylinositols, releasing the water-soluble protein polysaccharide-inositol phosphate moiety. These proteins are tethered by glycosylphosphatidylinositol (GPI) anchors. They occur widely in nature, from trypanosome cell-surface antigens to placental alkaline phosphatase. In yeast and protozoa, cell-surface proteins appear to be ceramide- or GPI-anchored. The concept of a hydrophilic cell-surface protein tethered by a membrane lipid may be misleading since some GPI-anchored proteins appear to fulfill roles generally served by membrane-spanning proteins in higher animal cells, such as signal-transducing receptor proteins, ion channels and transporters. GPI-anchored proteins of neurobiological interest include rat Thy-1 antigen, neural cell adhesion molecules (NCAMs), Nogo receptor and prion protein [2] (see Chs 30 and 48). The prion GPI-anchor contains sialic acid and is found in lipid rafts. If normal prion protein is modified so that it cannot form a GPI anchor on the cell membrane, the abnormal



A



B

FIGURE 3-10 Structure of phosphatidylinositol anchors. (**A**) The backbone structure of a glycosylphosphatidylinositol (GPI) anchor. Additional phosphoethanolamine or galactose may be attached to one of the mannose moieties. (**B**) The structure of two mammalian GPI anchors, Thy-1 antigen and erythrocyte acetylcholinesterase (*AChE*). AChE has an additional fatty acid moiety (16:0) attached to the 2 position of inositol. Phosphorylethanolamine, *black circles*; mannose, *orange circles*; galactose, *white circles*; GlcN, *pale orange circles*. See Chapter 21 for other inositol lipids.

6. Hajra, A. K., Fisher, S. K. and Agranoff, B. W. Isolation, separation and analysis of phosphoinositides from biological sources. In A. A. Boulton, G. B. Baker and L. A. Horrocks (eds), *Neuromethods (Neurochemistry). Lipids and Related Compounds*, vol. 8. Clifton, NJ: Humana Press, 1987.
7. Suzuki, K. Chemistry and metabolism of brain lipids. In G. J. Siegel, R. W. Albers, B. W. Agranoff and R. Katzman (eds). *Basic Neurochemistry*, 3rd edn. Boston: Little, Brown & Co., 1981, pp. 355–370.
8. Han, X. and Gross, R.W. Global analysis of cellular lipidomes directly from crude biological samples by ESI mass spectrometry: a bridge to lipidomics. *J. Lipid Res.* 44: 1071–1079, 2003.
9. Brown, M. S. and Goldstein, J. L. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34–47, 1986.
10. Cinti, D. L., Cook, L., Nagi, M. N. and Suneja, K. J. The fatty acid chain elongation system of mammalian endoplasmic reticulum. *Prog. Lipid Res.* 31: 1–51, 1992.
11. Sprecher, H. Metabolism of highly unsaturated n–3 and n–6 fatty acids. *Biochim. Biophys. Acta* 1486: 219–231, 2000.
12. Moser, H. W., Smith, K.D., Watkins, PA., Powers, J. and Moser, A. B. X- linked adrenoleukodystrophy. In C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle, C. Barton, K. Kinzler and B. Vogelstein (eds), *The Metabolic and Molecular Basis of Inherited Disease*, 8th edn. New York: McGraw-Hill, 2001, pp. 3257–3301.
13. Wanders, R. J., Jakobs, C. and Skjeldal, O. H. Refsum disease. In C. R. Scriver, A. L. Beaudet, W. S. Sly *et al.* (eds), *The Metabolic and Molecular Basis of Inherited Disease*, 8th edn. New York: McGraw-Hill, 2001, pp. 3303–3321.
14. Kennedy, E. P. The biosynthesis of phospholipids. In J. A. F. Op den Kamp, B. Roelofsen and K. W. A. Wirtz (eds), *Lipids and Membranes: Past, Present and Future.* Amsterdam: Elsevier, 1986, pp. 171–206.
15. Hajra, A. K. Glycerolipid biosynthesis in peroxisomes (microbodies). *Prog. Lipid Res.* 34: 343–364, 1995.
16. Radin, N. S. Biosynthesis of the sphingoid bases: a provocation. *J. Lipid Res.* 25: 1536–1560, 1984.
17. Heacock, A.M., Uhler, M. D. and Agranoff, B. W. Cloning of CDP-diacylglycerol synthase from a human neuronal line. *J. Neurochem.* 67: 2200–2203, 1996.
18. Ishibashi, T., Dupree, J. L., Ikenaka, K. *et al.* A myelin galactolipid, sulfatide, is essential for maintenance of channels on myelinated axons but not essential for initial cluster formation. *J. Neurosci.* 22: 6507–6514, 2002.
19. Bullens, R. W., O'Hanlon, G. M., Wagner, E. *et al.* Complex gangliosides at the neuromuscular junction are membrane receptors for autoantibodies and botulinum neurotoxin but redundant for normal synaptic function. *J. Neurosci.* 22: 6876–6884, 2002.
20. Maxfield, F. R. and Wustner, D. Intracellular cholesterol transport. *J. Clin Invest.* 110: 891–898, 2002.
21. Voelker, D. R. New perspectives on the regulation of intermembrane glycerophospholipid traffic. *J. Lipid Res.* 44: 441–449, 2003.
22. Williamson, P., Schlegel, R. A. Transbilayer phospholipid movement and the clearance of apoptotic cells. *Biochim. Biophys. Acta* 1585: 53–63, 2002.
23. Daleke, D. L. and Lyles, J. E. Identification and purification of aminophospholipid flippases. *Biochim. Biophys. Acta* 1486: 108–112, 2000.
24. De Rosa, M. F., Sillence, D.,Ackerley, C. and Lingwood, C. Role of multiple drug resistance protein 1 in neutral but not acidic glycosphingolipid biosynthesis. *J. Biol. Chem.* 279: 7867–7876, 2003.
25. Glomset, J. A., Gebb, M. H. and Farnsworth, C. C. Prenyl proteins in eukaryotic cells: a new type of membrane anchor. *Trends Biochem. Sci.* 15: 139–142, 1990.
26. Bijlsma, M., Spek, C. and Pepplenbosch, M. Hedgehog: an unusual signal transducer. *Bioessays* 26: 387–394, 2004.
27. Sugira, T. and Waku, K. Cannabinoid receptors and their endogenous ligands. *J. Biochem.* 132: 7–12, 2002.
28. Mendelson, W. B. and Basile, A. S. The hypnotic actions of the fatty acid amide, oleamide. *Neuropsychopharmacology* 25(5 Suppl.): S36–S39, 2001
29. Satoh, S., Matsumura, H., Suzuki, F. and Hayaishi, O. Promotion of sleep mediated by the A2α-adenosine receptor and possible involvement of this receptor in the sleep induced by prostaglandin D_2 in rats. *Proc. Natl. Acad. Sci. U.S.A.* 93: 5980–5984, 1996.
30. Bazan, N. G. Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *J. Lipid Res.* 44: 2221–2233, 2003.
31. Buccliero, R. and Futerman, H. The roles of ceramide and complex sphingolipids in neuronal cell function. *Pharm. Res.* 47: 409–419, 2003.
32. Gulbins, E. Regulation of death receptor signaling and apoptosis by ceramide. *Pharmacol. Res.* 47: 393–399, 2003.

GENERAL REFERENCES

Prescott, M. P. (ed.) A thematic series on phospholipases. *Journal of Biological Chemistry 1997 Minireview Compendium.* Bethesda, MD: American Society for Biochemistry and Molecular Biology, 1997.

Vance, D. E. and Vance, J. (eds) *Biochemistry of Lipids, Lipoproteins and Membranes. New Comprehensive Biochemistry,* vol. 36. Amsterdam: Elsevier Science, 2002.

BOX 3-1—cont'd
Glycosylphosphatidylinositol-Anchored Proteins

misfolded prion protein can no longer induce the disease scrapie [3]. Interest in GPI-anchored protein complexes also stems from indications that they can act as signals for protein sorting and localization [4], and may be linked to trophic proteins that mediate chemical affinity gradients during development. The inositol glycan complex of GPI may also have messenger-like properties in growth factor actions. A defect in GPI-anchor formation gives rise to the blood disorder paroxysmal nocturnal hemoglobinuria. For some GPI anchors, the PI moiety may contain alkyl ethers at C-1 of the glycerol, generally not found in free PI, and the 2-position of inositol can be esterified with a fatty acid, which renders the molecule resistant to PLC action. The lipid polysaccharide backbone is biosynthesized by sequential addition of the carbohydrate moiety to PtdIns, which initially reacts with UDPGlcNAc to form PI-6–1Glc NAc, and is then deacetylated. D-mannose moieties are next transferred from dolichol-P-mannose to make the polysaccharide backbone, which then undergoes phosphodiesteratic linkage with phosphoethanolamine derived from phosphatidylethanolamine [5, 6]. The GPI is anchored via amide linkage of the ethanolamine amino group and the C-terminal amino acid of a protein, and the complex is then transported to the outer surface of the plasma membrane. While the sequence of carboxy-terminal amino acids that signal GPI linkage is complex, it has been possible to splice nucleotide sequences coding for such sequences to mRNAs and to transfect cells which then produce novel cell-surface proteins. Proteins released by phospholipase have been shown to be reincorporated and GPI-anchored, even into other cell types. This has led to the concept of 'painting' cell surfaces for potential therapeutic uses, such as prevention of transplant rejection [7].

1. Low, M. G. The glycosyl-phosphatidyl anchor of membrane proteins. *Biochim. Biophys. Acta.* 988: 4217–454, 1989.
2. Prado, M. A., Alves-Silva, J., Magalhaes, A. L. *et al.* PrPc on the road: trafficking of the cellular prion protein. *J. Neurochem.* 88: 769–781, 2004.
3. Aguzzi, A. Prion toxicity: all sail and no anchor. *Science* 308: 1420–1421, 2005.
4. Mayor, S. and Riezman, H. Sorting GPI-anchored proteins. *Nat. Rev. Mol. Cell. Biol.* 5: 110–120, 2004.
5. Udenfriend, S. and Kodukula, K. How glycophosphatidylinositol-anchored membrane proteins are made. *Annu. Rev. Biochem.* 64: 563–591, 1995.
6. Hwa, K. Y. GPI-linked glycoconjugates: structure, biosynthesis and function. *Adv. Exp. Med. Biol.* 491: 207–214, 2001.
7. Medof, M. E., Nagargian, S. and Tykocinski, M. L. Cell-surface engineering with GPI-anchored proteins. *FASEB J.* 10: 574–586, 1996.

(see Ch. 33). Arachidonic acid itself acts as a biomessenger by activating certain isoforms of PKC. It may also be found in a derivatized form. It has been identified in amide linkage with ethanolamine as anandamide or esterified to the 2-position of glycerol, and both have been proposed as possible endogenous ligands for brain cannabinoid receptors [27]. Oleic acid amide has been reported to be an endogenous sleep-promoting factor [28], as has the prostanoid prostaglandin D_2 [29].

DAG is an important precursor for lipid biosynthesis in the endoplasmic reticulum, but in the plasma membrane it acts as a second messenger, activating PKC. Major structural lipids, such as PI and PtdCho, are also intimately involved in the signal-transduction process (see Ch. 23). The ether lipid 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, termed platelet activating factor and commonly referred to as PAF, has potent biomessenger activity in aggregating platelets, releasing eicosanoids and modulating glutamate release and plasticity at the synapse [30] (see Ch. 33). Lysophosphatidic acids have been identified as extracellular ligands acting via several G-protien-coupled receptors. Sphingolipids, including ceramide, sphingosine and sphingosine-1-phosphate, have been implicated in cell regulatory processes, such as cell-cycle arrest, apoptosis and stress-activated protein kinase actions [31, 32]. For example, tumor necrosis factor, the cytokine interleukin-1β and nerve growth factor act through their receptors to induce sphingomyelin hydrolysis to ceramide, which then activates a number of downstream activities, including protein kinases and phosphatases, triggering cell-cycle arrest, proliferation, differentiation or cell death (see also Chs 33 and 35).

REFERENCES

1. Sastry, P. S. Lipids of nervous tissue: Composition and metabolism. *Prog. Lipid Res.* 24: 69–176, 1985.
2. Wells, M. A. and Dittmer., J. C. A comprehensive study of the postnatal changes in the concentration of the lipids of developing rat brain. *Biochemistry* 10: 3169–3175, 1967.
3. Lee, C. and Hajra, A.K. Molecular species of diacylglycerols and phosphoglycerides and the postmortem changes in the molecular species of diacylglycerols in rat brain. *J. Neurochem.* 56: 370–379,1991.
4. O'Brien, J. S. and Sampson, E. L. Lipid composition of the normal human brain: gray matter, white matter, and myelin. *J. Lipid Res.* 6: 537–544. 1965.
5. Yu, R. K. and Ando, S. Structures of some new complex gangliosides. In L. Svennerholm, P. Mandel, H. Dreyfus and P.-F. Urban (eds), *Structure and Function of Gangliosides.* New York: Plenum Press, 1980, pp. 33–45.

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CHAPTER

4

Myelin Formation, Structure and Biochemistry

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The morphological distinction between white matter and gray matter is one that is useful for the neurochemist. White matter, so called for its glistening white appearance, is composed of myelinated axons, glial cells and blood vessels. Gray matter contains, in addition, the nerve cell bodies with their extensive dendritic arborizations. The predominant element of white matter is the myelin sheath, which comprises about 50% of the total dry weight and is responsible for the gross chemical differences between white and gray matter.

THE MYELIN SHEATH

The myelin sheath is a greatly extended and modified plasma membrane, which is wrapped around the nerve axon in a spiral fashion. A comprehensive review of the older literature on the structure, biochemistry and other aspects of myelin is available in a book published 20 years ago [1], whereas newer developments in the myelin field are covered in detail in a recent two-volume set [2]. The myelin membranes originate from, and are part of, Schwann cells in the PNS and oligodendrocytes in the CNS (see Ch. 1). Each myelin-generating cell furnishes myelin for only one segment of any given axon. The periodic interruptions where short portions of the axon are left uncovered by myelin are the *nodes of Ranvier*, and they are critical to the functioning of the axon and the myelin. The segments of myelinated axons between nodes are called internodes.

Myelin facilitates conduction. Myelin is an electrical insulator, although its function of facilitating conduction in axons has no exact analogy in electrical circuitry [3]. In unmyelinated fibers, impulse conduction is propagated by local circuits of ion current that flow into the active region of the axonal membrane, through the axon, and

†Pierre Morell tragically passed away early in the preparation of this chapter. A remembrance of his life and work appears on p. xxiii

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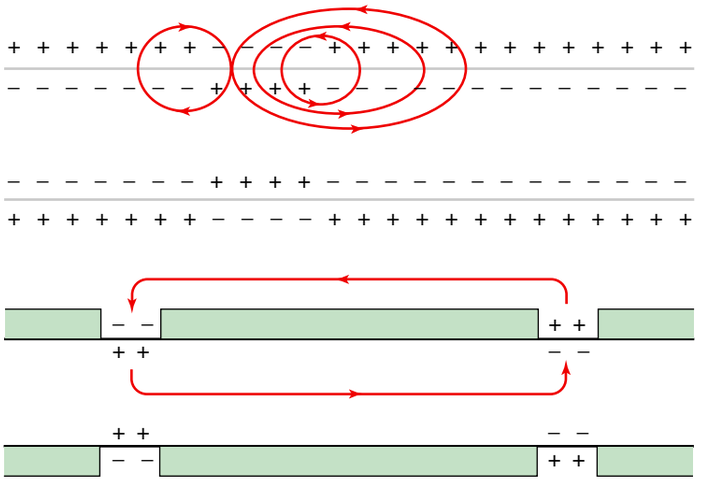

FIGURE 4-1 Impulse conduction in unmyelinated (*top*) and myelinated (*bottom*) fibers. The arrows show the flow of action currents in local circuits into the active region of the membrane. In unmyelinated fibers the circuits flow through the adjacent piece of membrane but in myelinated fibers the circuit flow jumps to the next node.

out through adjacent sections of the membrane (Fig. 4-1). These local circuits depolarize the adjacent piece of membrane in a continuous sequential fashion. In myelinated axons, the excitable axonal membrane is exposed to the extracellular space only at the nodes of Ranvier; this is the location of sodium channels. When the membrane at the node is excited, the local circuit generated cannot flow through the high-resistance sheath and therefore flows out through and depolarizes the membrane at the next node, which might be 1 mm or farther away (Fig. 4-1). The low capacitance of the sheath means that little energy is required to depolarize the remaining membrane between the nodes, which results in an increased speed of local circuit spreading. Active excitation of the axonal membrane jumps from node to node; this form of impulse propagation is called *saltatory conduction* (Latin *saltare*, 'to jump'). Such movement of the wave of depolarization is much more rapid than is the case in unmyelinated fibers. Furthermore, because only the nodes of Ranvier are excited during conduction in myelinated fibers, sodium flux into the nerve is much less than in unmyelinated fibers, where the entire membrane is involved.

Comparison of two different nerve fibers which both conduct at 25 m/s at 20°C demonstrates the advantage of myelination. The 500 µm diameter unmyelinated giant axon of the squid requires 5,000 times as much energy and occupies about 1,500 times as much space as a 12 µm diameter myelinated nerve in a frog. Conduction velocity in myelinated fibers is proportional to the diameter, while in unmyelinated fibers it is proportional to the square root of the diameter. Thus, differences in energy and space requirements between the two types of fiber are exaggerated at higher conduction velocities. If nerves were not myelinated and equivalent conduction velocities were maintained, the human spinal cord would need to be as large as a good-sized tree trunk. Myelin, then, facilitates conduction while conserving space and energy [3].

Myelin has a characteristic ultrastructure. Myelin, as well as many of its morphological features, such as nodes of Ranvier and Schmidt–Lantermann clefts, can be seen readily in the light microscope (Fig. 4-2). Further insight comes from biophysical studies of structures with parallel axons, sciatic nerve as representative of the PNS and optic nerve or tract as representative of the CNS. Myelin, when examined by polarized light, exhibits both a lipid-dependent and a protein-dependent birefringence. Low-angle X-ray diffraction studies of myelin provide electron density plots of the repeating unit that show three peaks (each corresponding to protein plus lipid polar groups) and two troughs (lipid hydrocarbon chains). The repeat distance varies somewhat depending on the species and whether the sample is from CNS or PNS. Thus, the results from these two techniques are consistent with a protein–lipid–protein–lipid–protein structure, in which

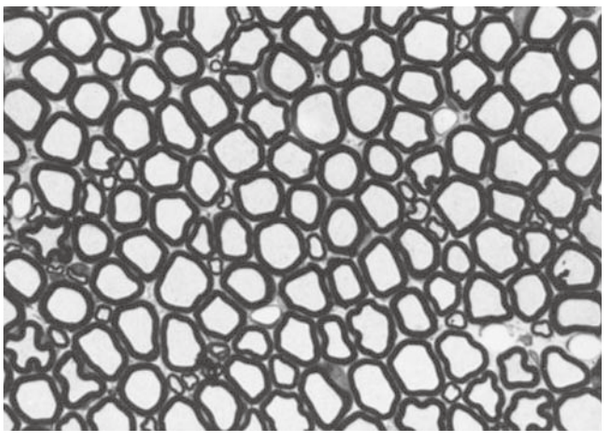

FIGURE 4-2 Light micrograph of a 1 µm Epon section of rabbit peripheral nerve (anterior root), stained with toluidine blue. The myelin sheath appears as a thick black ring around the pale axon. (Courtesy of Dr Cedric Raine.)

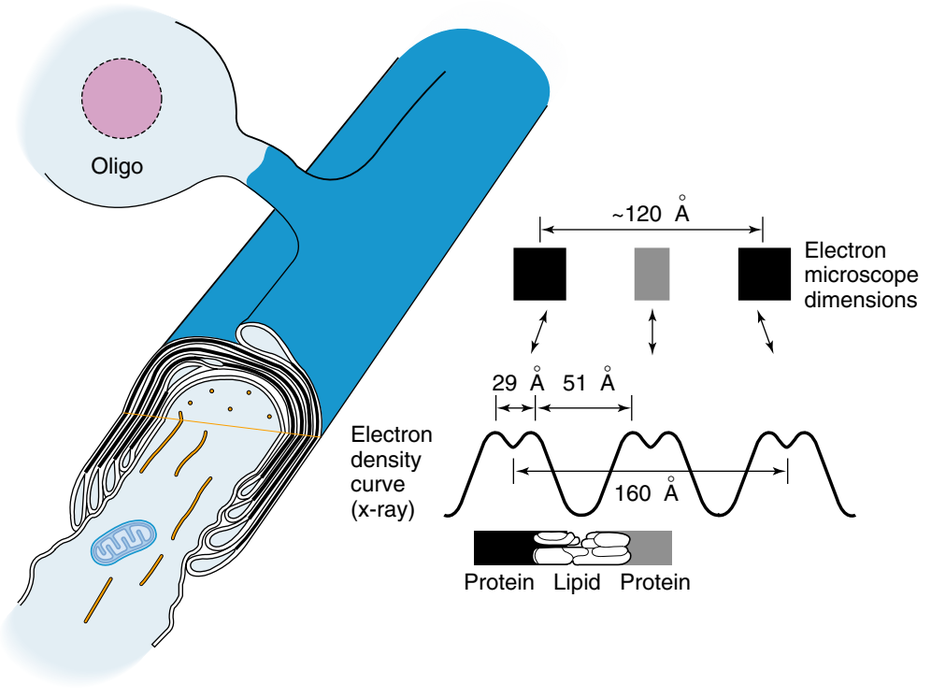

FIGURE 4-3 A composite diagram summarizing some of the ultrastructural data on CNS myelin. At the top an oligodendroglial cell is shown connected to the sheath by a process. The cutaway view of the myelin and axon illustrates the relationship of these two structures at the nodal and paranodal regions. (Only a few myelin layers have been drawn for the sake of clarity.) At the internodal region, the cross-section reveals the inner and outer mesaxons and their relationship to the inner cytoplasmic wedges and the outer loop of cytoplasm. Note that, in contrast to PNS myelin, there is no full ring of cytoplasm surrounding the outside of the sheath. The lower part of the figure shows roughly the dimensions and appearance of one myelin repeating unit as seen with fixed and embedded preparations in the electron microscope. This is contrasted with the dimensions of the electron density curve of CNS myelin obtained by X-ray diffraction studies in fresh nerve. The components responsible for the peaks and troughs of the curve are sketched below. (Adapted with permission from Norton, W. T. The myelin sheath. In E. S. Goldensohn and S. H. Appel (eds), *Scientific Approaches to Clinical Neurology*. Philadelphia: Lea & Febiger, 1977, pp. 259–298.)

the lipid portion is a bimolecular leaflet and adjacent protein layers are different in some way. Figure 4-3 shows data for mammalian optic nerve with a repeat distance of 80 Å. This spacing can accommodate one bimolecular layer of lipid (about 50 Å) and two protein layers (about 15 Å each). The main repeating unit of two such fused unit membranes is twice this, or 160 Å. (See Kirschner and Blaurock [4] for discussion and references.) Although it is useful to think of myelin in terms of alternating protein and lipid layers, this concept has been modified to be compatible with the 'fluid mosaic' model of membrane structure that includes intrinsic transmembrane proteins as well as extrinsic proteins.

Information concerning myelin structure is also available from electron microscope studies, which visualize myelin as a series of alternating dark and less dark lines (protein layers) separated by unstained zones (the lipid hydrocarbon chains) (Figs 4-4 to 4-7). There is asymmetry in the staining of the protein layers. The less dark, or intraperiod, line represents the closely apposed outer protein

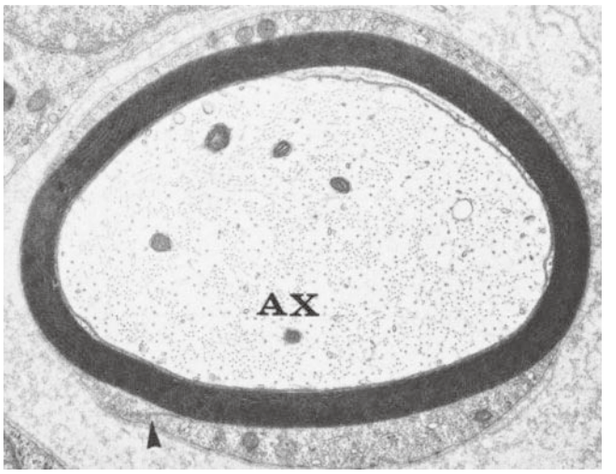

FIGURE 4-4 Electron micrograph of a single peripheral nerve fiber from rabbit. Note that the myelin sheath has a lamellated structure and is surrounded by Schwann cell cytoplasm. The outer mesaxon (*arrowhead*) can be seen in lower left. AX, axon. (Courtesy of Dr Cedric Raine.)

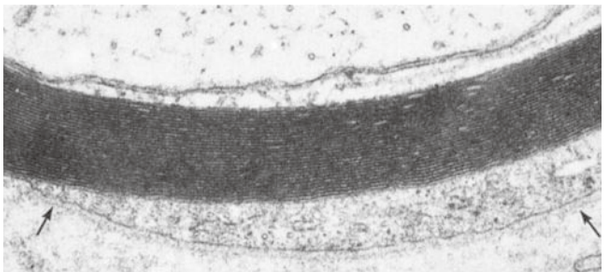

FIGURE 4-5 Higher magnification of Figure 4-4 to show the Schwann cell cytoplasm covered by basal lamina (*arrows*).

layers of the original cell membrane; the membranes are not actually fused, as they can be resolved as a double line at high resolution (Figs 4-6, 4-7). The dark, or major period, line is the fused, inner protein layers of the cell membrane. The repeat distances observed by electron microscopy are less than those calculated from the low-angle X-ray diffraction data, a consequence of the considerable shrinkage that takes place after fixation and dehydration. However, the difference in periodicity between the PNS myelin and CNS myelin is maintained; peripheral myelin has an average repeat distance of 119 Å and the central myelin of 107 Å.

Nodes of Ranvier. Two adjacent segments of myelin on one axon are separated by a node of Ranvier. In this region the axon is not covered by myelin. At the paranodal region and the Schmidt–Lantermann clefts (see below), the cytoplasmic surfaces of myelin are not compacted and Schwann or glial cell cytoplasm is included within the sheath. To visualize these structures, one may refer to Figures 4-8 and 4-9, which show that if myelin were unrolled from the axon it would be a flat, spade-shaped sheet surrounded by a tube of cytoplasm. Thus, as shown in electron micrographs of longitudinal sections of axon paranodal regions, the major dense line formed by apposition of the cytoplasmic faces opens up at the edges of the sheet, enclosing cytoplasm within a loop (Figs 4-3, 4-9).

FIGURE 4-6 Magnification of the myelin sheath of Figure 4-4. Note that the intraperiod line (*arrows*) at this high resolution is a double structure. (Courtesy of Dr Cedric Raine.)

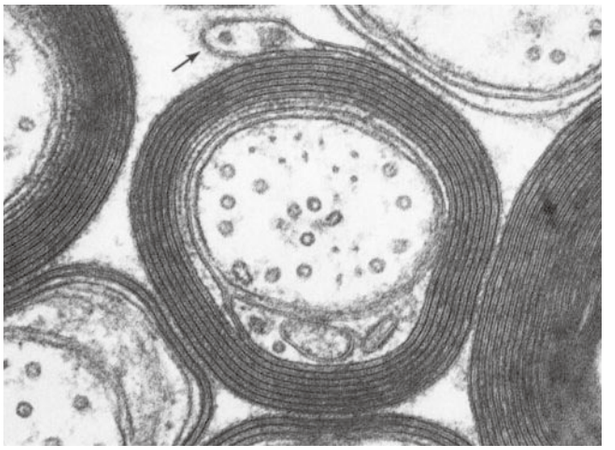

FIGURE 4-7 A typical CNS myelinated fiber from the spinal cord of an adult dog. Contrast this figure with the PNS fiber in Figure 4-4. The course of the flattened oligodendrocytic process, beginning at the outer tongue (*arrow*), can be traced. Note that the fiber lacks investing cell cytoplasm and a basal lamina–as is the case in the PNS. The major dense line and the paler, double intraperiod line of the myelin sheath can be discerned. The axon contains microtubules and neurofilaments.

These loop-shaped terminations of the sheath at the node are called *lateral loops*. The loops form membrane complexes with the axolemma called *transverse bands*, whereas myelin in the internodal region is separated from the axon by an extracellular gap of *periaxonal space*. The transverse bands are helical structures that seal the myelin to the axolemma but provide, by spaces between them, a tortuous path from the extracellular space to the periaxonal space.

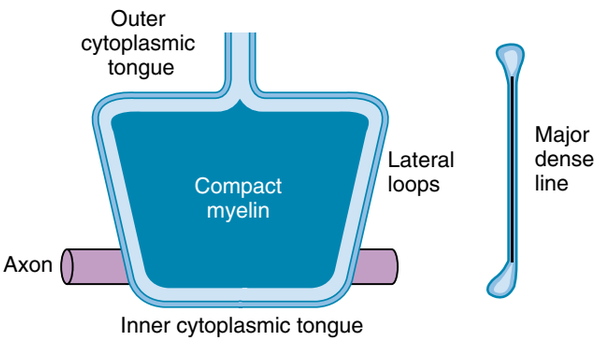

FIGURE 4-8 A diagram showing the appearance of CNS myelin if it were unrolled from the axon. One can visualize this structure arising from Figure 4-3 if the glial cell process were pulled straight up and the myelin layers separated at the intermediate period line. The whole myelin internode forms a spade-shaped sheet surrounded by a continuous tube of oligodendroglial cell cytoplasm. This diagram shows that the lateral loops and inner and outer cytoplasmic tongues are parts of the same cytoplasmic tube. The drawing on the right shows the appearance of this sheet if it were sectioned along the vertical line, indicating that the compact myelin region is formed of two unit membranes fused at the cytoplasmic surfaces. The drawing is not necessarily to scale. (Adapted from Hirano, A. and Dembitzer, H. M. A structural analysis of the myelin sheath in the central nervous system. *J. Cell Biol.* 34: 555–567, 1967.)

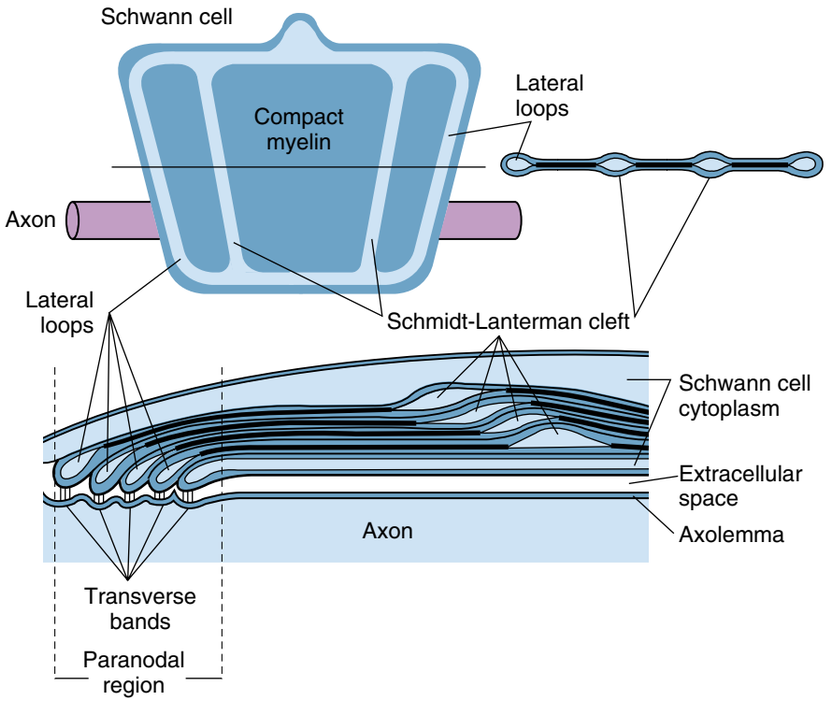

FIGURE 4-9 A diagram similar to Figure 4-8 but showing one Schwann cell and its myelin sheath unrolled from a peripheral axon (**top left**). The sheet of PNS myelin is, like CNS myelin, surrounded by a tube of cytoplasm and has additional tubes of cytoplasm, which make up the Schmidt–Lantermann clefts, running through the internodal regions. The horizontal section (top right) shows that these additional tubes of cytoplasm arise from regions where the cytoplasmic membrane surfaces have not fused. The diagram at the bottom is an enlarged view of a portion of the top left diagram, with the Schwann cell and its membrane wrapped around the axon. The tube forming the lateral loops seals to the axolemma at the paranodal region, and the cytoplasmic tubes in the internodal region form the Schmidt–Lantermann clefts. These drawings are not to scale. (Adapted from Hirano, A. and Dembitzer, H. M. A structural analysis of the myelin sheath in the central nervous system. *J. Cell Biol.* 34: 555–567, 1967.)

Schmidt–Lantermann clefts are structures where the cytoplasmic surfaces of the myelin sheath have not compacted to form the major dense line and therefore contain Schwann or glial cell cytoplasm (Fig. 4-9). They are common in peripheral myelin but rare in the CNS. These inclusions of cytoplasm are present in each layer of myelin. The clefts can be visualized in the unrolled myelin sheet as tubes of cytoplasm similar to the tubes making up the lateral loops but in the middle regions of the sheet, rather than at the edges (Fig. 4-9).

Myelin is an extension of a glial plasma membrane. Myelination in the PNS is preceded by invasion of the nerve bundle by Schwann cells, rapid multiplication of these cells and segregation of the individual axons by Schwann cell processes. Smaller axons (≤1 μm), which will remain unmyelinated, are segregated; several may be surrounded by one Schwann cell, each within its own pocket, similarly to the single axon shown in Figure 4-10A. Large axons (≥1 μm) destined for myelination are enclosed singly, one cell per axon per internode. These cells line up along the axons with intervals between them; the intervals become the nodes of Ranvier.

Before myelination the axon lies in an invagination of the Schwann cell (Fig. 4-10A). The plasmalemma of the cell then surrounds the axon and joins to form a double membrane structure that communicates with the cell surface. This structure, called the *mesaxon*, then elongates around the axon in a spiral fashion (Fig. 4-10). Thus, formation of myelin topologically resembles rolling up a sleeping bag: the mesaxon winds about the axon, and the cytoplasmic surfaces condense into a compact myelin sheath and form the major dense line. The two external surfaces form the myelin intraperiod line.

In the CNS, myelin is formed by oligodendrocytes. This has many similarities but also points of difference with respect to myelination in the PNS. CNS nerve fibers are not separated by connective tissue nor are they surrounded by cell cytoplasm, and specific glial nuclei are not obviously associated with particular myelinated fibers. CNS myelin is a spiral structure similar to PNS myelin: it has an inner mesaxon and an outer mesaxon that ends in a loop, or tongue, of glial cytoplasm (Fig. 4-3). Unlike peripheral nerve, where the sheath is surrounded by Schwann cell cytoplasm on the inside and outside (Fig. 4-10), the cytoplasmic tongue in the CNS is restricted to a small

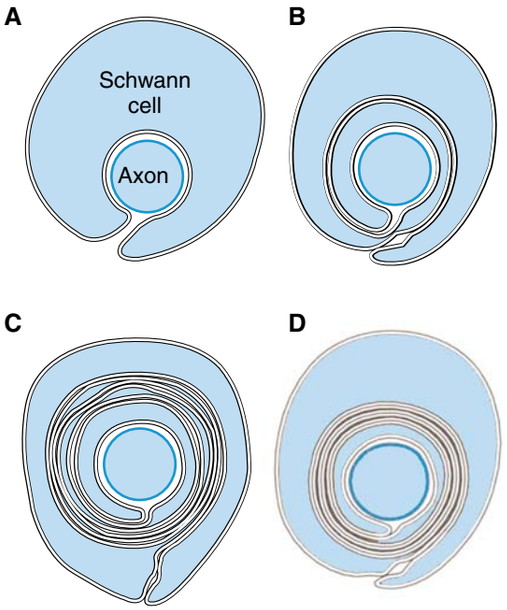

FIGURE 4-10 Myelin formation in the peripheral nervous system. (**A**) The Schwann cell has surrounded the axon but the external surfaces of the plasma membrane have not yet fused in the mesaxon. (**B**) The mesaxon has fused into a five-layered structure and spiraled once around the axon. (**C**) A few layers of myelin have formed but are not completely compacted. Note the cytoplasm trapped in zones where the cytoplasmic membrane surfaces have not yet fused. (**D**) Compact myelin showing only a few layers for the sake of clarity. Note that Schwann cell cytoplasm forms a ring both inside and outside of the sheath. (Adapted with permission from Norton, W. T. The myelin sheath. In E. S. Goldensohn and S. H. Appel (eds), *Scientific Approaches to Clinical Neurology*. Philadelphia: Lea & Febiger, 1977, pp. 259–298.)

portion of the sheath (Figs 4-3, 4-8). This glial tongue is continuous with the plasma membrane of the oligodendroglial cell through slender processes. One oligodendrocyte can myelinate as many as 40 or more separate axons.

Myelin deposition in the PNS may result in a single axon having up to 100 myelin layers, and it does not appear that myelin is laid down by a simple rotation of the Schwann cell around the axon. In the CNS, such rotation is precluded by the fact that one glial cell can myelinate several axons. During myelination, there are increases in the length of the internode, the diameter of the axon and the number of myelin layers. Myelin is therefore expanding in all planes at once. Any mechanism to account for this growth must assume that the membrane system is able to expand and contract, and that layers slip over each other.

Myelin affects axonal structure. The presence of a myelin sheath affects the structure of the axon that it surrounds [5], presumably optimizing its properties for transmission of action potentials by saltatory conduction. Generally, one of the effects of myelin is to increase axonal diameter by inducing biochemical changes in components of the axonal cytoskeleton such as neurofilaments (see Ch. 8). The effects of myelin on axonal structure imply that there are signaling mechanisms from myelin or myelin-forming glia to axons. A common theme, emerging from recent research on transgenic mice deficient for some of the myelin proteins described later in this chapter, is that, in addition to their roles in the structure of the myelin sheaths, several of them are necessary for the normal formation, maintenance and survival of the axons that are ensheathed.

CHARACTERISTIC COMPOSITION OF MYELIN

The composition of myelin is well characterized because it can be isolated in high yield and purity by subcellular fractionation. If CNS tissue is homogenized in media of low ionic stren gth, myelin peels off the axons and reforms in vesicles of the size range of nuclei and mitochondria. Because of their high lipid content, these myelin vesicles have the lowest intrinsic density of any membrane fraction of the nervous system. Procedures for isolation of myelin take advantage of both of these properties – large vesicle size and low density [1]. Peripheral nerve myelin can be isolated by similar techniques, but especially vigorous homogenization conditions are required because of the large amounts of connective tissue and, sometimes, adipose tissue present in the nerve. The slightly lower density of PNS myelin requires some adjustment of gradient composition to prevent loss of myelin.

Myelin *in situ* has a water content of about 40%. The dry mass of both CNS and PNS myelin is characterized by a high proportion of lipid (70–85%) and, consequently, a low proportion of protein (15–30%). By comparison, most biological membranes have a higher ratio of proteins to lipids. The currently accepted view of membrane structure is that of a lipid bilayer with integral membrane proteins embedded in the bilayer and other extrinsic proteins attached to one surface or the other by weaker linkages. Proteins and lipids are asymmetrically distributed in this bilayer, with only partial asymmetry of the lipids. The proposed molecular architecture of the layered membranes of compact myelin fits such a concept (Fig. 4-11). Models of compact myelin are based on data from electron microscopy, immunostaining, X-ray diffraction, surface probes studies, structural abnormalities in mutant mice, correlations between structure and composition in various species, and predictions of protein structure from sequencing information [4].

Central nervous system myelin is enriched in certain lipids. Table 4-1 lists the composition of bovine, rat, and human myelin compared to bovine and human white matter, human gray matter, and rat whole brain [1] (see Ch. 3). While there are no absolutely 'myelin-specific' lipids, cerebroside (galactosyl ceramide) is the most typical of myelin. With the exception of early development,

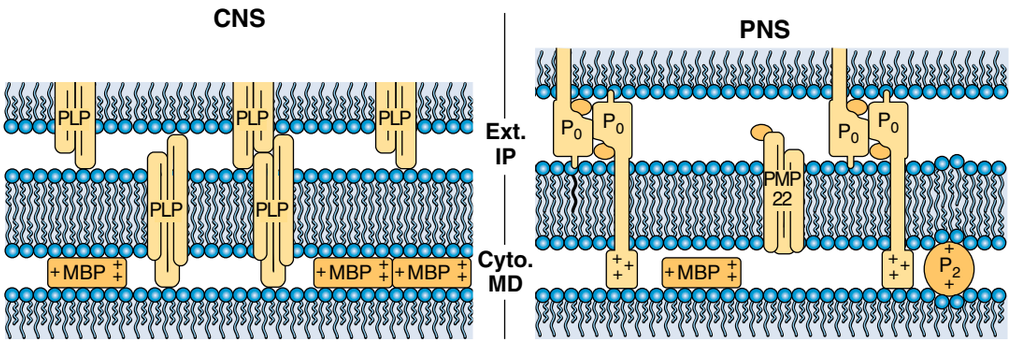

FIGURE 4-11 Diagrammatic representation of current concepts of the molecular organization of compact CNS and PNS myelin. The apposition of the extracellular (*Ext.*) surfaces of the oligodendrocyte or Schwann cell membranes to form the intraperiod (*IP*) line are shown in the upper part of the figure. The apposition of the cytoplasmic (*Cyto.*) surfaces of the membranes of the myelin-forming cells to form the major dense (*MD*) line are shown in the lower part of the figure. The width of the lipid bilayers and the spacing of the intraperiod and major dense lines in this figure are proportional to those determined by X-ray diffraction [4]. See the text for a detailed description of this model. The dark orange structures on P_0 and PMP represent the single oligosaccharide moieties on each protein. The blip at the apex of P_0 represents the tryptophan residue, which X-ray analysis suggests may interact with the apposing bilayer, but the expected tetramerization of P_0 is not shown for diagrammatic simplification. Although PLP molecules may exhibit homophilic interactions as suggested at one position in the figure, there is no strong experimental evidence to support this as in the case of P_0. These diagrams do not include CNP, MAG and other quantitatively minor proteins of isolated myelin, because they probably do not play a major structural role in most of the compact myelin. In fact, many of them are localized selectively in regions of myelin sheaths distinct from the compact myelin.

the concentration of cerebroside in brain is directly proportional to the amount of myelin present. As much as one-fifth of the total galactolipid in myelin is sulfatide, in which the 3-hydroxyl moiety on the galactose of cerebroside is sulfated. Presumably, the glycolipids in myelin, as in other membranes, are preferentially localized on the extracellular membrane face at the intraperiod line. Because of the specificity and quantitative significance of galactocerebroside in oligodendrocytes and myelin, it had long been thought that it would be essential for the formation and maintenance of myelin, but in fact it is not. A UDP-galactose:ceramide galactosyltransferase-null mouse was generated, which eliminates the obligate terminal step in cerebroside biosynthesis and thereby additionally sulfatide formation [6]. Thus, these mice synthesize no cerebroside or sulfatide. Surprisingly, the myelin formed by these mice is relatively normal, although there are subtle structural alterations in the myelin sheaths and neurological abnormalities, both of which become progressively more severe with age. Particularly severe defects occur in

TABLE 4-1 Composition of central nervous system myelin and brain

	Myelin			*White matter*			
Substance*	**Human**	**Bovine**	**Rat**	**Human**	**Bovine**	**Gray matter (human)**	**Whole brain (rat)**
Protein	30.0	24.7	29.5	39.0	39.5	55.3	56.9
Lipid	70.0	75.3	70.5	54.9	55.0	32.7	37.0
Cholesterol	27.7	28.1	27.3	27.5	23.6	22.0	23.0
Cerebroside	22.7	24.0	23.7	19.8	22.5	5.4	14.6
Sulfatide	3.8	3.6	7.1	5.4	5.0	1.7	4.8
Total galactolipid	27.5	29.3	31.5	26.4	28.6	7.3	21.3
Ethanolamine phosphatides	15.6	17.4	16.7	14.9	13.6	22.7	19.8
Lecithin	11.2	10.9	11.3	12.8	12.9	26.7	22.0
Sphingomyelin	7.9	7.1	3.2	7.7	6.7	6.9	3.8
Phosphatidylserine	4.8	6.5	7.0	7.9	11.4	8.7	7.2
Phosphatidylinositol	0.6	0.8	1.2	0.9	0.9	2.7	2.4
Plasmalogens†	12.3	14.1	14.1	11.2	12.2	8.8	11.6
Total phospholipid	43.1	43.0	44.0	45.9	46.3	69.5	57.6

*Protein and lipid figures in percentage dry weight; all others in percentage total lipid weight.
†Plasmalogens are primarily ethanolamine phosphatides.

the CNS paranodal loops, where glia–axon tight junctions are located. Abnormalities in the PNS of these knockout mice are much less severe. Mice lacking the sulfotransferase that converts cerebroside to sulfatide exhibited similar paranodal disorganization in the CNS, indicating that sulfatide is important for establishing the normal oligodendroglial–axon interactions in the paranodal region [6, 7]. The lack of sulfatide also results in abnormal distribution of Na^+ and K^+ channels in the paranodal and nodal regions of myelinated axons. In addition to their role in myelin itself, experiments with cultured oligodendrocytes have demonstrated that both galactocerebroside and sulfatide also have important functions in the differentiation of oligodendrocytes, with sulfatide being particularly important [7].

In addition to cerebroside/sulfatide, the major lipids of myelin are cholesterol and phospholipids [1]. On a molar basis, CNS myelin preparations contain cholesterol, phospholipid and galactolipid in a ratio varying between 4:3:2 and 4:2:2. Thus, myelin contains substantially more molecules of cholesterol than any other single lipid, although on the basis of weight the content of galactolipids is comparable and total phospholipids are most abundant (Table 4-1). A characteristic phospholipid, and the single most prominent one, is ethanolamine-containing plasmalogen (glycerophospholipid containing an alkenyl ether bond– see Ch. 3). Lecithin is also a major myelin constituent, and sphingomyelin is a relatively minor one. Cholesterol is enriched on the extracellular face of the myelin membrane, whereas ethanolamine plasmalogen is asymmetrically localized to the cytoplasmic half of the bilayer. Not only is the lipid class composition of myelin highly characteristic of this membrane, the fatty acid composition of many of the individual lipids is distinctive.

The data in Table 4-1 indicate that myelin accounts for much of the total lipid of white matter, and that the lipid composition of gray matter is quite different from that of myelin. The composition of brain myelin from all mammalian species studied is very much the same. There are, however, some species differences; for example, myelin of rat has less sphingomyelin than does that of bovine or human (Table 4-1). Although not shown in the table, there are also regional variations; for example, myelin isolated from the spinal cord has a higher lipid-to-protein ratio than brain myelin from the same species.

In addition to the lipids of CNS myelin listed in Table 4-1, there are some other minor lipids, including polyphosphoinositides (see Ch. 3),which account for between 5% and 8% of the total myelin phosphorus; some fatty acid esters of galactocerebroside; and two galactosyldiglycerides [1]. Myelin from mammals also contains 0.1–0.3% ganglioside (complex sialic acid-containing glycosphingolipids). The major ganglioside in CNS myelin is a monosialoganglioside (GM1) and there are very low amounts of the polysialogangliosides characteristic of neuronal membranes. Myelin from certain species (including human) contains an additional novel ganglioside as a major component: sialosylgalactosylceramide (GM4).

Peripheral and central nervous system myelin lipids are qualitatively similar. However, there are quantitative differences. PNS myelin has less cerebroside and sulfatide and considerably more sphingomyelin than CNS myelin. Of interest is the presence of the LM1 ganglioside, sialosyl-lactoneotetraosylceramide, as a characteristic component of myelin in the PNS of some species. These differences in lipid composition between CNS and PNS myelin are not, however, as dramatic as the differences in protein composition discussed below.

Central nervous system myelin contains some unique proteins. The protein composition of CNS myelin is simpler than that of other brain membranes, with the myelin basic protein (MBP) and proteolipid protein (PLP) making up 60–80% of the total in most species. Many other proteins and glycoproteins are present to a lesser extent. With the exception of MBP, myelin proteins are neither easily extractable nor soluble in aqueous media. However, like other membrane proteins, they may be solubilized in sodium dodecylsulfate solutions and, in this condition, can be separated readily by electrophoresis in polyacrylamide gels. This technique separates proteins primarily according to their molecular weight (a common notation is M_r for relative molecular mass, and another is to state molecular weight in kilodaltons, kDa). The presence of bound carbohydrates or unusual structural features distort somewhat the relationship between electrophoretic migration and molecular weight, so that terminology for location of a protein in such a gel is taken to mean 'apparent' molecular weight. The protein composition of human and rat brain myelin are illustrated in Figure 4-12, B and D, respectively. The quantitative predominance of two proteins in human CNS myelin is clear, i.e. MBP and PLP. These two proteins are major constituents of all mammalian CNS myelin membranes and similar proteins are present in myelin membranes of many lower species. The overall orientation of these two proteins in compact CNS myelin is depicted in Fig. 4-11.

Proteolipid protein. Myelin PLP, also known as the Folch–Lees protein [8, 9], has the unusual physical property of solubility in organic solvents. The molecular mass of PLP is about 30,000, although it migrates anomalously on sodium dodecyl sulfate (SDS) gels and gives a lower apparent molecular mass. The amino acid sequence, strongly conserved during evolution, contains four membrane spanning domains, and PLP is described as one of the tetraspan proteins. Both the N- and C-termini are on the cytoplasmic side, as shown in Fig. 4-11. An important role for PLP in stabilizing the intraperiod line of CNS myelin has generally been assumed, based largely on the fact that the extracellular loops of this protein are present at this location. Furthermore, the CNS intraperiod line is

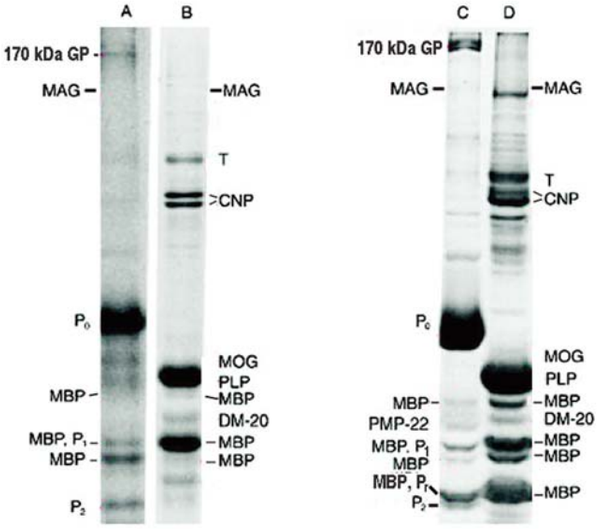

FIGURE 4-12 Polyacrylamide gel electrophoresis of myelin proteins in the presence of sodium dodecyl sulfate (SDS). The proteins of human PNS myelin (**A**), human CNS myelin (**B**), rat PNS myelin (**C**) and rat CNS myelin (**D**) were solubilized with the detergent SDS, electrophoresed and stained with Coomassie brilliant blue. The electrophoretic system separates proteins primarily according to their molecular size with the smallest proteins migrating the farthest toward the bottom of the gel. Abbreviations for the proteins are the same as in the text or defined below. The three MBP bands in lanes **A** and **B** are the 17.2, 18.5, and 21.5 kDa isoforms generated by alternative splicing of the mRNA in humans, and the four MBP bands in lanes **C** and **D** are the 14.0, 17.0, 18.5, and 21.5 kDa isoforms generated in rats (see Fig. 4-13). The 18.5 kDa MBP and the 14 kDa MBP are also called P_1 and P_2, respectively, in the terminology for the PNS. The 26 kDa MOG is probably the faint band just above PLP that is most apparent in lane **D**. CNP migrates as a tight doublet, and the lower and upper bands are sometimes referred to as CNP1 and CNP2, respectively. Note that the location shown for MAG (which stains too faintly to be seen well on the gels) is just above a discrete Coomassie-blue-stained band in lane **D**, which is probably the 96 kDa subunit of Na^+, K^+-ATPase. T, tubulin. 170 kDa GP, 170 kDa glycoprotein.

abnormally condensed both in the PLP knockout mice and in spontaneously occurring PLP mutants [10] (Table 4-2), confirming a structural role for PLP in determining the membrane spacing at the intraperiod line. PLP has an alternatively spliced isoform, DM20 (M_r = 20,000), which is present in CNS myelin at lower concentration than PLP (Fig. 4-12). DM20 has similar physical properties to PLP and is identical in sequence, except for a deletion of 35 amino acids in the intracellular domain [8, 9]. PLP/DM20 contains about 4–6 mol of fatty acids (primarily palmitate, oleate or stearate) per mole of protein in ester linkage at several cysteines. There is rapid turnover of the fatty acids independent of the peptide backbone.

The PLP gene is expressed very early in development, and in fact DM20 mRNA appears earlier than PLP during development, even before myelin formation in embryos and in premyelinating oligodendrocytes [9]. It is thought that it might have a role in oligodendrocyte migration or differentiation in addition to a structural role in myelin. The *PLP/DM20* gene may have evolved from an ancestral gene encoding a pore-forming polypeptide, lending support to the hypothesis that myelin may be involved in ion movement. Although PLP and DM20 serve important functions, they are not essential. Contrary to the general expectation that PLP would be needed for formation of compact, multilamellar myelin, a knockout mouse for *PLP/DM20* is initially relatively normal with respect to myelin formation (except for the difference in the intraperiod line spacing), life span and motor performance [9]. This suggests that other proteins or lipids of myelin may contribute to adherence of the extracellular faces of the bilayers at the intraperiod line. On the other hand, myelin in the PLP-null mutant is extra sensitive to osmotic shock during fixation, suggesting that PLP does enhance the stability of myelin, possibly by forming a 'zipper-like'

TABLE 4-2 Some spontaneously occurring animal mutants affecting myelin

Names of mutants	Inheritance*	Affected gene	Comments	References
Jimpy mouse, *rumpshaker* mouse, myelin-deficient (md) rat, shaking dog	X-linked	Proteolipid protein (PLP)	Variable degrees of oligodendrocyte death and CNS myelin deficiency; decreased spacing at intraperiod line of compact CNS myelin; see text	1, 9, 10, 43, 44
Shiverer mouse, myelin-deficient mouse	AR	Myelin basic protein (MBP)	Deletion or inversion of several MBP exons; very little functional MBP expressed; severe CNS hypomyelination and failure of compaction of major dense line; see text	1, 10, 43
Trembler mouse (PMP-22)	AD	Peripheral myelin protein-22 (PMP-22)	Hypomyelination specific for the PNS; caused by point mutations in transmembrane domains; see text	1, 45
Quaking mouse	AR	QKI family of proteins (QKI5, QKI6, QKI7 expressed in oligodendrocytes)	Hypomyelination more severe in CNS than PNS; abnormal expression of RNA-binding proteins likely to interfere with normal splicing or transport of mRNAs for myelin proteins; see text	1, 46–48
Taiep rat (acronym: **t**rembling, **a**taxia, **i**mmobility, **e**pilepsy, **p**aralysis)	AR	Unknown	Impaired myelin formation followed by demyelination in the CNS; accumulation of microtubules in oligodendrocytes interferes with transport of myelin proteins or mRNAs; see text	49

*AD, autosomal dominant; AR, autosomal recessive; CNS, central nervous system; PNS, peripheral nervous system.

structure after it is compacted. Furthermore, in older *PLP/DM20* knockout mice, there is significant axonal degeneration, suggesting that while myelin can form in the absence of PLP/DM20, CNS myelin devoid of PLP/DM20 cannot sustain normal axonal function. Despite the apparent similarity of the PLP and DM20, DM20 cannot replace PLP in transgenic mice [11] – the same long-term axonal degeneration occurs in mice expressing exclusively DM20 protein. This may be because PLP uniquely interacts both with inositol hexakisphosphate [12], a molecule involved in vesicle transport, and with integrins, modulating interaction with the extracellular matrix [13]. Thus, PLP has selective and apparently important functions in the CNS relative to DM20. While the loss of PLP/DM20 has clear neuropathological consequences in older animals, the loss of these proteins is significantly less serious than expression of mutated or excess PLP/DM20. Both human patients (see Ch. 38) and genetically engineered or naturally occurring animal mutants (Table 4-2) with defects in the PLP gene exhibit hypomyelination and often early death. This may result from production of either abnormal protein that cannot fold correctly or simply increased amounts of normal PLP [9], which induce an unfolded protein response and are toxic to oligodendrocytes.

While PLP/DM20 expression is highest in oligodendrocytes in the CNS, PLP/DM20 mRNA is also expressed in myelinating Schwann cells in the PNS [9], where small amounts of protein are synthesized although not incorporated into myelin in appreciable amounts. It is also expressed in nonmyelinating Schwann cells of the PNS. The levels of PLP and DM20 mRNA are differentially regulated in myelinating and nonmyelinating Schwann cells, with DM20 mRNA being expressed more in nonmyelinating Schwann cells and PLP mRNA being expressed more in myelinating Schwann cells. In addition to expression in the CNS and PNS, low levels of DM20 expression have been found in thymus and heart [10], again suggesting that this protein has unique functions unrelated to formation and maintenance of compact myelin. Furthermore, a novel alternatively spliced form of the protein that is soluble has recently been identified in neurons and oligodendrocytes [10]. This protein may have yet other functions.

Myelin basic proteins. The MBP of myelin has long been of interest because it was the initial myelin antigen, which, when injected into an animal, elicited a cellular immune response that produced the CNS autoimmune disease called *experimental allergic encephalomyelitis* (EAE, see Ch. 38). MBP can be extracted from myelin as well as from white matter with either dilute acid or salt solutions; once extracted, it is very soluble in water. The MBP genes from a number of species are highly conserved, and as with the PLP gene, the MBP gene is alternatively spliced [10, 14, 15]. The classical MBP gene has seven exons, with the full length MBP (21,500 M_r) containing all seven exons, although this protein is one of the minor MBP proteins in myelin. Exons 2, 5B and 6 are present or absent in four other MBP proteins found in myelin. The most abundant MBP in human myelin contains exons 1B, 3, 4, 6 and 7 (18.5 kDa MBP), whereas in rodent myelin both the 18.5 kDa MBP and a 14 kDa MBP containing exons 1B, 3, 4, 5 and 7 are the most abundant. Two different minor MBPs of approximately 17 kDa exist, which are encoded by exons 1B, 2, 3, 4, 5B and 7 or 1B, 3, 4, 6 and 7 respectively. A diagrammatic representation of some of these alternative splicing schemes is presented in Figure 4-13. The ratio of the MBPs changes with development, with more 14 kDa MBP found in mature rodent tissue. In immature oligodendrocytes, the MBP mRNA is localized in the cell body. However, as the cell matures, the MBP mRNA is localized in the myelin processes, far from the cell body, presumably because newly translated MBP associates rapidly with membranes at its site of synthesis [16].

The MBPs are extrinsic proteins localized exclusively at the cytoplasmic surface in the major dense line (Fig. 4-11), a conclusion based on their amino acid sequence, inaccessibility to surface probes and direct localization at the electron microscope level by immunocytochemistry. There is evidence to suggest that MBP forms dimers, and it is believed to be the principal protein stabilizing the major dense line of CNS myelin, possibly by interacting with negatively charged lipids. A severe hypomyelination and failure of compaction of the major dense line in MBP deficient *shiverer* mutants supports this hypothesis (Table 4-2).

The MBPs are highly unfolded in solution, with essentially no tertiary structure. They show microheterogeneity upon electrophoresis in alkaline conditions. This is due to a combination of phosphorylation, loss of the C-terminal arginine, and deamidation. There is also heterogeneity in the degree of methylation of an arginine at residue 106. The rapid turnover of the phosphate groups present on many of the MBP molecules [17] suggests that this post-translational modification might influence the close apposition of the cytoplasmic faces of the membrane (whether phosphorylation modifies this process in a dynamic manner is a topic of speculation). The physiological significance of the heterogeneity of MBPs, which results from alternative splicing and from unique post-translational modifications, is an open question.

Intriguingly, the classical MBP gene is actually part of a larger gene, *golli* (**g**ene of the **ol**igodendrocyte **li**neage), which is more than 100 kb in length [14]. This gene has three transcription start sites, two of which are used to transcribe the MBP mRNAs, while the most 5′ transcription start site generates *golli* mRNAs (Fig. 4-13). Transcripts from this upstream promoter are expressed more ubiquitously than MBP mRNAs. Thus, they are expressed in neurons and oligodendrocytes in the nervous system and in T cells in the immune system. Most interestingly from an evolutionary perspective, the *golli* proteins contain a 133 amino acid domain that contains both unique *golli* sequences and classic MBP sequences. The *golli* proteins are expressed during embryonic development and in

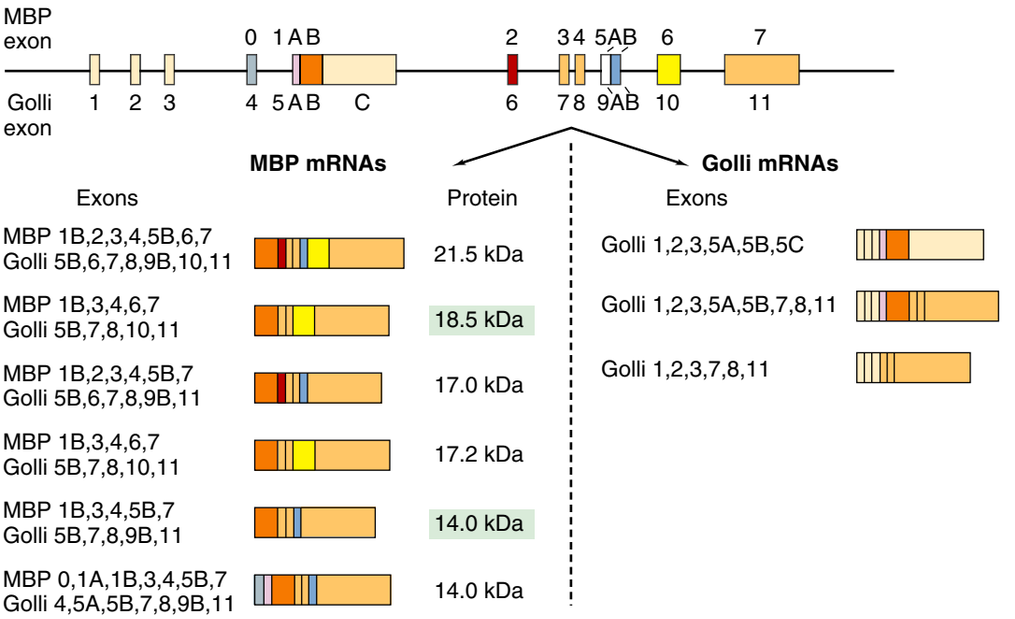

FIGURE 4-13 The amino acid sequences corresponding to the various mouse MBPs are encoded in a gene containing at least 11 exons (separated by introns – DNA regions whose base sequence does not code directly for proteins). This gene is depicted here but the sizes of the exons or introns are not accurately represented. The exons are depicted in boxes with the original *MBP* exon numbering above, and the *golli*/*MBP* exon numbering below. Some of the introns are over 100,000 bases in length and could not be shown accurately here. This gene can be spliced into two sets of mRNAs: the MBP mRNAs and the *golli* mRNAs. The *MBP* exons can be spliced to give an mRNA containing the original seven *MBP* exons, which are exons 5B, 6, 7, 8, 9B, 10 and 11 of the *golli/MBP* gene; this mRNA encodes the 21.5 kDa MBP. Alternative MBP mRNA splicings result primarily in mRNA species with deletions of *MBP* exons 2 (red) and/or 6 (yellow) (*golli* exons 6 and/or 10), which encode the other MBPs, although in humans, elimination of *MBP* exons 2 and 5B (blue) (*golli* exons 6 and 9B) can generate a 17.2 kDa MBP. A unique *MBP* mRNA (M41) encoding a 14 kDa MBP (**bottom**) was identified in which a novel *MBP* transcription site was used (exon 0/4, gray), and *MBP* exons 1A and 1B (*golli* exons 5A and 5B). Additionally, a unique *MBP* sequence upstream of the classical *MBP* exon 5 was identified (exon 5A/9A, white), which may be spliced into some *MBP* mRNAs, although the full sequence of these mRNAs has not been determined [15]. The exons forming the various *MBP* mRNA species and proteins are indicated. There are three well-characterized *golli* mRNAs (BG21, J37 and TP8 [14]), which are transcribed from *golli* exon 1, and which may or may not contain exons from the *MBP* exons 3, 4, 7. (Adapted from a figure published in reference [14].)

postnatal tissue, and the proteins are found in multiple subcellular localizations, including nuclei, cytoplasm and cellular processes. Their function is not yet understood, although there is the suggestion that they may be involved in process extension in neural cells [10, 14].

2′:3′-Cyclic nucleotide 3′-phosphodiesterase: In addition to PLP and MBP, there are many higher-molecular-weight proteins present in myelin (Fig. 4-12). These vary in amount depending on species (rodents generally have more than larger mammals) and age (immature myelin has more). A doublet with $M_r \approx 46$ kDa and 48 kDa is present in CNS myelin, which comprises several percent of total myelin protein and has the enzyme activity, 2′:3′-cyclic nucleotide 3′-phosphodiesterase (CNP) [18]. Although there are low levels of CNP associated with other cell types, it is greatly enriched in CNS myelin and oligodendrocytes, for which it is a commonly used biochemical marker. It is expressed at a much lower concentration in Schwann cells at the onset of myelination and does not increase during development with the accumulation of myelin as in the CNS. The enzyme is extremely active with the substrate 2′, 3′-cAMP, as well as cGMP, cCMP and cUMP analogs, which are all hydrolyzed to the corresponding 2′-isomer. This may be a nonphysiological activity, because only the 3′:5′ cyclic nucleotides have been shown to have biological activity. Nevertheless, evolutionary conservation of the catalytic site indicates that its amino acid sequence probably has an important function, although the precise role of CNP has remained elusive over the many years since it was discovered. Two CNP polypeptides are generated by alternative splicing of the mRNA, with the larger polypeptide having an extra 20 amino acids at the N-terminus. Immunocytochemistry demonstrates that CNP is not a major component of compact myelin, but is concentrated in specific regions of the myelin sheaths associated with cytoplasm, such as the oligodendroglial processes, inner and outer tongue processes, and lateral loops. The protein is in the cytoplasm but much of it associates with membranes, because both isoforms are isoprenylated at the C-terminus and acylated. Some clues about its function have come from reports that it binds to cytoskeletal elements such as F-actin and tubulin and that overexpression in cultured non-neural cells promotes outgrowth of processes. Such findings suggest that its function may be in regulating cytoskeletal dynamics to promote process outgrowth and

differentiation in oligodendrocytes. Furthermore, aberrant myelination occurring *in vivo* in transgenic mice overexpressing CNP similarly suggests that it could be an early regulator of cellular events that culminate in CNS myelination. However, it is also important to note that the amino acid sequence of CNP puts it in a superfamily of RNA-processing enzymes whose physiological roles are unclear, so the relevance of this to oligodendrocytes and myelination is also unclear. An interesting possibility combining some of the above information is that CNP could be involved in some specialized aspects of RNA transport and/or processing in oligodendrocytes. Yet most puzzling of all is the phenotype displayed by the recently generated CNP-null mice, which appear to myelinate entirely normally but as adults exhibit axonal swelling, neurodegeneration and premature death. It has been speculated that CNP is a multifunctional protein with an initial role in oligodendroglial differentiation that can be compensated for by another protein, and a second function essential for the normal interaction of oligodendrocytes with axons leading to axonal degeneration in its absence [18]. Clearly, more research is needed to fully understand the functions of this intriguing myelin/oligodendrocyte-related protein.

Myelin-associated glycoprotein and other glycoproteins of CNS myelin. The myelin-associated glycoprotein (MAG) is a quantitatively minor, 100 kDa glycoprotein in purified CNS and PNS myelin [19, 20] that electrophoreses at the position shown in Figure 4-12. However, because of its small amount (<1% of total protein) and weak staining by Coomassie blue, it does not correspond to one of the discrete protein bands visible in the figure. MAG has a single transmembrane domain that separates a heavily glycosylated extracellular part of the molecule, composed of five Ig-like domains and eight or nine sites for N-linked glycosylation, from an intracellular carboxy-terminal domain. Its overall structure is similar to that of neural-cell adhesion molecule (N-CAM). MAG in rodents occurs in two developmentally regulated isoforms, which differ in their cytoplasmic domains and are generated by alternative splicing of its mRNA. The isoform with a longer C-terminal tail (L-MAG) predominates early in development during active myelination of the CNS, whereas the isoform with a shorter cytoplasmic tail (S-MAG) increases during development to become prominent in adult rodents.

MAG is not present in compact, multilamellar myelin but is located in the periaxonal glial membranes of myelin sheaths. This location next to the axon and its membership in the Ig superfamily (see Ch. 7) suggest that it functions in adhesion and signaling between myelin-forming cells and the axolemma. Indeed, substantial evidence has now accumulated that MAG is involved in signaling in both directions between glia and axons, although its most important functions appear to be different in the CNS and the PNS. MAG is in the 'siglec' [**s**ialic acid –binding **i**mmuno**g**lobulin-like **lec**tins] subgroup of the Ig superfamily and binds to glycoproteins and gangliosides with terminal α2–3 linked sialic acid moieties. Thus, some of the axolemmal binding partner(s) for MAG are likely to be sialoglycoconjugates. A relationship of MAG to other adhesion proteins also is demonstrated by the presence in most species of a sulfate-containing epitope in its oligosaccharide moieties that reacts with the HNK-1 monoclonal antibody. The carbohydrate HNK-1 epitope is expressed on many neural adhesion proteins, including N-CAM and MAG, and has been shown to function in cell–cell interactions.

MAG had long been thought to function in important signaling mechanisms from axons to oligodendrocytes during myelination. However, it is now known that MAG is not essential for myelin formation because MAG-null mice myelinate relatively normally. Nevertheless, in the CNS, these knockouts exhibit a significant delay of myelination, periaxonal and paranodal structural abnormalities, redundant myelin loops and supernumerary myelin sheaths. In addition, there is degeneration of periaxonal oligodendroglial processes in aging MAG-null mice, suggesting the occurrence of a 'dying-back oligodendrogliopathy'. Therefore, the absence of MAG causes oligodendrocytes to form myelin less efficiently during development and become dystrophic with aging. Furthermore, although the neurological deficit in MAG-null mice is mild, double knockouts in which the absence of MAG is combined with the genetic ablation of other proteins result in more severe CNS phenotypes than either knockout alone. These *in vivo* findings suggest that MAG-mediated signaling from axons to oligodendrocytes is needed for efficient myelination and maintenance of healthy mature oligodendroglia. As with other proteins in the Ig superfamily, it is likely that the interaction of MAG with its ligand(s) on the axolemma mediates cell–cell signaling by mechanisms involving phosphorylation. The cytoplasmic domains of MAG are phosphorylated on serine and theonine residues by protein kinase C, and L-MAG is also phosphorylated on tyrosine-620. Furthermore, the cytoplasmic domain of L-MAG has been shown to interact with fyn tyrosine kinase, phospholipase Cγ and other oligodendroglial proteins. The L-MAG isoform appears to be particularly important for CNS myelination, because genetically engineered mice lacking only the L-isoform exhibit the same CNS abnormalities as total knockouts but not the PNS pathology of total knockouts described below.

There are a large number of other glycoproteins associated with white matter and myelin, and a few in addition to MAG that have been cloned and characterized. One of these is a minor 26 kDa protein called the myelin-oligodendrocyte glycoprotein (MOG) [21]. MOG is also a transmembrane glycoprotein, contains a single Ig-like domain and one site for N-linked glycosylation, and expresses the adhesion-related HNK-1 epitope. Unlike MAG, which is sequestered at the interior of CNS myelin

sheaths, MOG is localized on the outside surface of myelin sheaths and oligodendrocytes, apparently directed by a basolateral membrane targeting signal in its cytoplasmic domain. Consistent with its surface localization, MOG has been implicated as a target antigen in autoimmune aspects of demyelinating diseases of the CNS and is a leading candidate to be an important antigen in multiple sclerosis (see Ch. 38). Its surface location also suggests that it may function in signal transduction, transmitting information from the extracellular matrix or adjacent myelin sheaths to oligodendrocytes. This role is further suggested by changes in cultured oligodendrocytes when MOG is cross-linked on the cell surface with anti-MOG antibodies [7]. However, its physiological function remains obscure, because the recent generation of MOG-null mice yielded an apparently normal phenotype.

Another glycoprotein with a similar name to MOG is the oligodendrocyte-myelin glycoprotein (OMgp) [19, 22]. It was first characterized as a 120 kDa phosphatidylinositol-linked glycoprotein in human white matter and subsequently cloned. It is not a member of the Ig superfamily but is characterized by a cysteine-rich motif at the N-terminus, a series of tandem leucine-rich repeats and the HNK-1 epitope. These properties suggest that it may function in cell–cell interactions. However, unlike MAG and MOG, it is not specific to myelin-forming cells and is also expressed in neurons. It has attracted substantial interest in recent years because it is one of the myelin-associated inhibitors of axonal regeneration (see below), but its function with regard to myelination is unclear at this time.

Peripheral nervous system myelin also contains unique proteins.

P_0 glycoprotein. Gel electrophoretic analysis (Fig. 4-12A, C) shows that a single 30 kDa protein, P_0, accounts for more than half of the PNS myelin protein. P_0 is a type 1 membrane glycoprotein containing about 220 amino acids after removal of its signal sequence. Rat P_0 contains a single extracellular Ig-like domain of 124 amino acids, a hydrophobic transmembrane domain of 26 amino acids and an intracellular domain of 69 amino acids [19, 23]. The amino-terminal extracellular domain has a single site for N-linked glycosylation, and the glycans at that site are very heterogeneous, with many containing sialic acid and sulfate. In addition to glycosylation, other posttranslational modifications of P_0 include phosphorylation and acylation.

The principal difference in the overall protein composition of PNS and CNS myelin is that P_0 replaces PLP as the major protein, although myelin-forming Schwann cells do express very low levels of PLP. It is interesting to note that PLP and P_0 proteins, which are so different in sequence, post-translational modifications and membrane topology, may have similar roles in the formation of structures as closely related as myelin of the CNS and PNS respectively. Expression of P_0 in transfected cells results in cell–cell interactions that are due to homophilic binding of its extracellular domains, suggesting that P_0 stabilizes the intraperiod line of PNS myelin by similar homophilic binding (Fig. 4-11). The relatively large, glycosylated, extracellular Ig-like domain of P_0 probably accounts for the greater separation of extracellular surfaces in PNS myelin relative to CNS myelin where closer apposition of these surfaces is possible in the presence of the smaller extracellular domains of PLP. Evidence reviewed by Kirschner *et al.* [23] suggests that homophilic interactions between P_0 molecules involve both protein–protein and protein–carbohydrate interactions. Furthermore, investigation of the crystal structure of the P_0 extracellular domain suggests that P_0 molecules cluster on each membrane surface as tetramers. The crystal structure also suggested that a tryptophan residue at the apex of the extracellular domain could interact directly with the lipid bilayer of the apposing membrane. P_0 protein also has a relatively large positively charged domain on the cytoplasmic side of the membrane that contributes significantly to stabilization of the major dense line in the PNS. The complete knockout of P_0 has profound consequences for myelin structure, in contradistinction to the previously noted, relatively benign CNS consequence of deletion of the PLP gene. P_0-null mice exhibit abnormal motor coordination, tremors, occasional convulsions and a severe hypomyelination with thin, noncompacted myelin sheaths.

Expression of the correct amount of P_0 is apparently essential for normal myelin formation and maintenance. Young mice heterozygous for the P_0 -null mutation appear normal but develop progressive demyelination with age, which resembles chronic inflammatory demyelinating neuropathy and may involve inflammatory mechanisms. Furthermore, transgenic mice overexpressing P_0 exhibit a dose-dependent dysmyelinating neuropathy ranging from transient hypomyelination to severe arrest of myelination and impaired sorting of axons. The critical dosage of P_0 required for normal myelin formation is similar to observations with other myelin proteins and may reflect the necessity for appropriate amounts of myelin proteins to form stoichiometric complexes in compact myelin. However, in the case of P_0, the pathology that occurs with overexpression may also reflect a mistargeting of the protein and an interesting misuse of its obligate homophilic adhesive properties. Some of the extra P_0 is inappropriately located in normally dynamic mesaxonal membranes, causing them to adhere like compact myelin and halting myelination. It is clear that control of P_0 expression is complex, involving interactions with the axon and basal lamina, rate of cell division, inhibitory and stimulatory growth factors, cAMP levels and transcription factors. It also should be noted that low basal levels of P_0 are expressed in Schwann cells and neural crest cells early in embryonic development well before myelination, which suggests that P_0 could have other functions, potentially involving Schwann-cell–axon interactions and signal transduction. The cytoplasmic domain of P_0 is phosphorylated on serine and tyrosine residues and this might be indicative

of signaling mechanisms within Schwann cells during early development as well as later during myelination [17].

Peripheral myelin protein-22. In addition to the major P_0 glycoprotein, compact PNS myelin contains a 22 kDa protein called peripheral myelin protein-22 (PMP-22) that accounts for less than 5% of the total protein (Fig. 4-12C) [19, 24]. Similarly to P_0, PMP-22 has a single site for N-linked glycosylation. However, unlike P_0, which is nerve-specific, PMP-22 is expressed in many other tissues. It has four hydrophobic potential transmembrane domains and is a tetraspan protein like the major PLP of CNS myelin, but there is no sequence homology to PLP. It is in a highly homologous family of small hydrophobic tetraspan proteins that also include epithelial membrane proteins (EMP-1, -2 and –3). It is referred to as a 'growth arrest protein' because its cDNA was first cloned from nondividing fibroblasts, and the synthesis of PMP-22 and other myelin proteins ceases when Schwann cells begin to proliferate following nerve transection. Although the tetraspan PMP-22 is localized primarily in compact PNS myelin, as shown in Fig. 4-11, it is not known if its extracellular or cytoplasmic domains play an important structural role for myelin. The relatively small amount of PMP-22 and the fact that it is present in the plasma membranes of both myelinating and nonmyelinating Schwann cells suggest that it may have a dynamic function in myelin assembly or maintenance rather than a major structural role. Its tetraspan structure, similar to that of PLP, suggests that one of its roles might be similar to one of the functions of PLP in CNS myelin. PMP-22 has been shown to form complexes with P_0, and this interaction with P_0 may be relevant to its function. Also, as is the case with P_0 and PLP, any significant deviation in gene dosage for PMP-22, or disruption caused by point mutations, has severe functional consequences. Mutations of the PMP-22 gene cause the dysmyelinating phenotypes in *trembler* mice (Table 4-2) and some neuropathies in humans (see Ch. 38). In addition, the association of PMP-22 with growth arrest in Schwann cells and other cell types suggests that it may have an unknown role in regulation of growth or differentiation.

P_2 protein. PNS myelin contains a positively charged protein different from MBP that is referred to as P_2 (M_r ≃15,000). It is unrelated in sequence to MBP and is a member of a family of cytoplasmic fatty acid binding proteins (FABP) that are present in a variety of cell types [25]. The amount of P_2 protein is variable among species, accounting for about 15% of total protein in bovine PNS myelin, 5% in humans and less than 1% in rodents. P_2 protein is generally considered a PNS myelin protein but it is expressed in small amounts in CNS myelin sheaths of some species. P_2 is an antigen for experimental allergic neuritis, the PNS counterpart of EAE (see Chs 36 and 38). P_2 appears to be present in the major dense line of myelin sheaths, where it may play a structural role similar to MBP (Fig. 4-11). Interestingly, the larger amounts of P_2 protein that are in myelin of some species correlate with increased widths of the major dense lines as determined by X-ray diffraction, and there appears to be substantially more P_2 in large sheaths than small ones [4]. The large variation in the amount and distribution of the protein from species to species and sheath to sheath raises so far unanswered questions about its function. Its similarities to cytoplasmic proteins in other cells, whose functions appear to involve solubilization and transport of fatty acids and retinoids, suggest that it might function similarly in myelin assembly or turnover, but there is currently no direct experimental evidence to support this hypothesis.

Some classically defined myelin proteins are common to both peripheral and central myelin.

Myelin basic protein. In PNS myelin, MBP varies from approximately 5% to 18% of total protein, in contrast to the CNS, where it is close to 30% [1]. In rodents, the same four 21, 18.5, 17 and 14 kDa MBPs found in the CNS are present in the PNS. In adult rodents, the 14 kDa MBP is the most prominent component and is termed P_r in the PNS nomenclature. The 18.5 kDa component is present and is often referred to as the P_1 protein in the nomenclature of peripheral myelin proteins. Another species-specific variation in human PNS is that the major basic protein is not the 18.5 kDa isoform that is most prominent in the CNS but rather a form of about 17 kDa. It appears that MBP does not play as critical a role in myelin structure in the PNS as it does in the CNS. For example, the *shiverer* mutant mouse, which expresses no MBP (Table 4-2), has a greatly reduced amount of CNS myelin, with no compaction of the major dense line. By contrast, *shiverer* PNS has essentially normal myelin, both in amount and structure, despite the absence of MBP. This CNS/PNS difference in the role of MBP is probably because the cytoplasmic domain of P_0 has an important role in stabilizing the major dense line of PNS myelin. Animals doubly deficient for P_0 and MBP have a more severe defect in compaction of the PNS major dense line than P_0-null mice, which indicates that both proteins contribute to compaction of the cytoplasmic surfaces in PNS myelin [23].

Myelin-associated glycoprotein. As in the CNS, MAG is present in the periaxonal membranes of myelin-forming Schwann cells, but it is also present in the Schwann cell membranes of the Schmidt–Lanterman incisures, paranodal loops and the outer mesaxon [19, 20]. Therefore, in addition to a role in Schwann-cell–axon interactions, MAG may also function in interactions between adjacent Schwann cell membranes at these other locations in the PNS. Both isoforms of MAG are present in the rodent PNS, although S-MAG is the predominant isoform at all ages. PNS myelination in MAG-null mice is initially more normal than CNS myelination. However, as the mice age they develop a peripheral neuropathy characterized by degeneration of myelinated axons, which is the most

severe phenotypic abnormality displayed by the knockout mice. The pathology is associated with decreased axonal caliber, increased neurofilament density, reduced expression and phosphorylation of neurofilaments and eventually axonal degeneration. These findings demonstrate an essential role for MAG in signaling from Schwann cells to axons that is necessary for the maintenance of normal myelinated axons in the PNS. Thus, MAG is another example of a myelin-related glial protein whose absence has profound effects on the ensheathed axon.

In this regard, it is noteworthy that MAG is one of several neural proteins (also including Nogo and OMgp) that inhibit neurite outgrowth in tissue culture and axonal regeneration *in vivo* (see Ch. 30). This inhibitory activity has been studied intensively in recent years, since it is extremely important for understanding factors that prevent axonal regeneration following neural injury [26]. This area of research has led to remarkable progress in identifying neuronal MAG receptors, and to the identification of a MAG-mediated signaling mechanism that affects neurons and also could be important for the normal maintenance of myelinated axons. Thus, a physiologically important signal promoting the stability of mature myelinated axons could be interpreted inappropriately by a plastic developing neurite *in vitro* or a regenerating neurite *in vivo*, thereby inhibiting its growth. The MAG receptor on neurites that transmits this inhibitory signal appears to be a complex localized in raft-like signaling domains, which consists of gangliosides, the glycosylphosphatidylinositol-anchored Nogo receptor and the p75 neurotrophin receptor. This neuronal receptor complex involved in MAG's effects on neurite outgrowth is also likely to function within myelinated axons to promote axonal stability, but this remains to be established.

It is noteworthy that the axonal degeneration that occurs in the PNS of MAG-null mice is not observed in the CNS, possibly because other CNS myelin proteins enhance axonal stability. These could include PLP and/or CNP, both of which are needed for axonal stability in the CNS where they are present in much higher concentration. In summary, it appears that the most important function of MAG in the PNS is transmitting a signal from Schwann cells to axons that is needed for the stability of myelinated axons, whereas its principal function in the CNS is to transmit a signal in the reverse direction that promotes efficient myelination and oligodendrocyte vitality.

Myelin sheaths contain other proteins, some of which have only recently been established as myelin-related. The proteins described above represent most of the well-established myelin proteins that are myelin-specific or have been studied primarily in the context of myelin and demyelinating diseases. However, myelin sheaths contain numerous other proteins in smaller amounts that are also in many other cells and/or have only been identified relatively recently. Some of these are in compact myelin but others are enriched in specialized structures within myelin sheaths that are distinct from compact myelin. Some of these proteins, which may be among the many minor bands seen on myelin protein gels (Fig. 4-12), are described here briefly.

Tetraspan proteins. Intriguingly, there are numerous tetraspan proteins (containing four transmembrane spanning domains) in myelin and related glial membranes [27], including PLP/DM20, PMP-22, myelin and lymphocyte protein (MAL/MVP17/VIP17) and plasmolipin in compact myelin; and oligodendrocyte-specific protein (OSP)/claudin-11, CD9 and connexins in the specialized associated structures of the myelin sheaths, such as the tight junctions or the paranodal loops. The presence or absence of these proteins can be essential to the specialized structure and function of myelin. The paranodal loops, which form the tight junctions between glial processes and axons in the paranodal regions of the sheaths (Figs 4-3, 4-9), are crucial for normal firing of myelinated axons. Rapid saltatory conduction of nerve impulses in myelinated fibers is thus dependent on the structural integrity of nodes of Ranvier and of the tight junctions at this location, which prevent ion leaking into the internodes.

Other than PLP/DM20 and PMP22, one of the earliest myelin tetraspan proteins characterized was the 17 kDa **m**yelin **a**nd **l**ymphocyte tetraspan protein (MAL). This protein was initially identified in compact myelin as MVP17, a novel myelin membrane protein [28], which was quickly demonstrated to be identical to MAL and VIP17. MAL (MVP17/VIP17) is part of the apical sorting machinery in non-neural polarized cells and it has been proposed to be involved in protein sorting in myelin membrane domains. It associates with glycosphingolipid-enriched protein/lipid rafts and may function in their sorting and transport to myelin [29, 30]. MAL has been established to be part of an extended gene family, which includes plasmolipin, another myelin tetraspan protein [31]. Plasmolipin is also associated with glycosphingolipid-enriched membrane domains from myelin. Thus, this family of proteins, two of which are found in compact myelin, may be involved in sorting of proteins or in signal transduction through lipid rafts in myelin.

Many of the other myelin tetraspan proteins are localized in specialized myelin structures. Claudins are a family of tight junction proteins found in many tissues, which form barriers to the diffusion of solutes between adjacent cells. Tight junctions in the paranodal regions of the PNS that act as barriers for diffusion of small ions involve the tetraspan claudin-5 [32]. OSP is found in the radial component of myelin; it was initially identified by differential screening as a novel tetraspan protein found in oligodendrocytes but was quickly established to be also a member of the claudin family, i.e., claudin-11 [33]. The radial component is a specialized ultrastructural feature in CNS myelin but not PNS myelin, which appears as lines of tight junctions with reduced spacing between extracellular leaflets. These lines of tight junctions extend in spiraled

fashion across the whole thickness of CNS myelin sheaths from one paranodal region to the other. The CNS myelin tight junctions between adjacent layers of spiraled membranes probably contribute stability and, most importantly, act as a barrier to the diffusion of ions that is essential for the normal electrophysiological function of myelinated axons. The principal protein component of these tight junctions is OSP/claudin-11 [33] and, in OSP/claudin-11 null mice, these tight junctions are missing from CNS myelin. Thus, OSP/claudin-11 is essential for formation of the radial component in CNS myelin. In addition to its role in formation of tight junctions, OSP/claudin-11 is also involved in oligodendrocyte migration, possibly through its interactions with OSP/claudin-11 associated protein (OAP)-1 and β_1 integrin [33].

CD9 is a well-characterized hematopoietic tetraspan protein that has been shown to be present in CNS and PNS myelin, although it is present at higher levels in PNS myelin. In other cells, it is involved in integrin signaling and cell adhesion and motility. It is expressed at late stages of myelination and in the CNS is primarily found in paranodal junctions [34]. While compact CNS myelin is apparently normal in CD9-null animals, the paranodal loops are often disconnected from axonal membranes, and the transverse bands of the paranodal loops are lost. In the PNS, in addition to altered paranodes, hypermyelination occurs. Thus, this tetraspan protein appears to act primarily at paranodes, where it is crucial for normal paranodal junctions.

Another type of membrane contact in the paranodal regions is gap junctions, which provide a radial pathway for diffusion of small molecules across the lateral loops. In particular the tetraspan proteins connexin-32 and connexin-29 are found in myelin [35], predominantly in noncompact domains of myelin, including paranodes and Schmidt–Lanterman incisures. While gap junctions typically form between adjacent cells, in myelin they form between adjacent layers of membrane. In Schwann cells, functional gap junctions provide a radial pathway of interconnection throughout the myelin. It has been proposed that this radial pathway through the myelin mediates spatial buffering of extracellular potassium during action potential activity as well as communication from the adaxonal domain of myelin to the cell body. Similar radial pathways containing gap junctions probably also exist in CNS myelin. Connexin-32 mutations are associated with the peripheral neuropathy Charcot–Marie–Tooth disease (see Ch. 38) but, interestingly, have little CNS pathology.

Paranodal proteins other than tetraspan proteins. The overall structure of the axonal membrane at the nodes of Ranvier themselves is essential for normal axonal firing, and this appears to be regulated in *trans* by the proteins and lipids in the paranodal myelin membranes. The axonal and glial membranes in this region of the fiber demonstrate an exquisite division into highly specialized domains, whose biochemical structures are currently a very active subject of research. The correct positioning of sodium channels exclusively at the nodes and not in the internodes is necessary for generating action potentials. Similarly, the potassium channels are localized very specifically to the juxtaparanodal axonal membrane and, as noted above, cerebroside- and sulfatide-null mice have disorganized paranodes and consequently altered nodal membranes, for example altered sodium channel organization. A variety of membrane proteins, including several members of the immunoglobulin superfamily, are selectively localized in these nodal and paranodal domains and must play important roles in the formation and stabilization of these complex structures. For example, an important *trans* interaction at the paranode occurs between neurofascin-155 on the glial membrane and contactin/Caspr (contactin-associated protein) multimers on the axonal membrane to form septate-like junctions. A detailed description of the proteins and lipids in these structures is beyond the scope of this chapter, and the reader is referred to excellent recent reviews of this area that are available elsewhere [32, 36].

Enzymes associated with myelin. Several decades ago it was generally believed that myelin was an inert membrane that did not carry out any biochemical functions. More recently, however, a large number of enzymes have been discovered in myelin [37]. These findings imply that myelin is metabolically active in synthesis, processing and metabolic turnover of some of its own components. Additionally, it may play an active role in ion transport with respect not only to maintenance of its own structure but also to participation in ion buffering near the axon.

A few enzymes, such as the previously mentioned CNP, are believed to be fairly specific for myelin/oligodendrocytes. There is much more in the CNS than in peripheral nerve, suggesting some function more specialized to the CNS. In addition, a unique pH 7.2 cholesterol ester hydrolase is also enriched in myelin. On the other hand, there are many enzymes that are not myelin-specific but appear to be intrinsic to myelin and not contaminants. These include cAMP-stimulated kinase, calcium/calmodulin-dependent kinase, protein kinase C, a neutral protease activity and phosphoprotein phosphatases. The protein kinase C and phosphatase activities are presumed to be responsible for the rapid turnover of MBP phosphate groups, and the PLP acylation enzyme activity is also intrinsic to myelin.

Myelin enzymes involved in structural lipid metabolism consist of a number of steroid-modifying enzymes and cholesterol-esterifying enzymes, UDP-galactose:ceramide galactosyl-transferase and many enzymes of glycerophospholipid metabolism, including all the enzymes necessary for phosphatidyl ethanolamine synthesis from diradyl-*sn*-glycerol and ethanolamine. It is likely that phosphatidylcholine can also be synthesized within myelin. Perhaps even more elemental building blocks can be assembled

into lipids by myelin enzymes, since acyl-coenzyme A (CoA) synthetase is present in myelin, suggesting the capacity to integrate free fatty acids into myelin lipids. The extent of the contribution of these enzymes in myelin (relative to enzymes within the oligodendroglial perikaryon) to metabolism of myelin lipids is not known.

Other enzymes present in myelin include those involved in phosphoinositide metabolism; phosphatidylinositol kinase, diphosphoinositide kinase, the corresponding phosphatases and diglyceride kinases. These are of interest because of the high concentration of polyphosphoinositides of myelin and the rapid turnover of their phosphate groups. This area of research has expanded towards characterization of signal transduction system(s), with evidence of G proteins and phospholipases C and D in myelin.

Certain enzymes shown to be present in myelin could be involved in ion transport. Carbonic anhydrase has generally been considered a soluble enzyme and a glial marker but myelin accounts for a large part of the membrane-bound form in brain. This enzyme may play a role in removal of carbonic acid from metabolically active axons. The enzymes 5′-nucleotidase and Na^+, K^+-ATPase have long been considered specific markers for plasma membranes and are found in myelin at low levels. The 5′-nucleotidase activity may be related to a transport mechanism for adenosine, and Na^+, K^+-ATPase could well be involved in transport of monovalent cations. The presence of these enzymes suggests that myelin may have an active role in ion transport in and out of the axon. In connection with this hypothesis, it is of interest that the PLP gene family may have evolved from a pore-forming polypeptide [9].

Neurotransmitter receptors associated with myelin. Neurotransmitter receptors have been identified on oligodendrocytes and oligodendrocyte progenitor cells as well as in compact myelin [37, 38]. Their functional role has not been established but some intriguing hypotheses have been put forth. A wide variety of these receptors has been found, including muscarinic acetylcholine (mACh) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, *N*-methyl-D-aspartate (NMDA) receptors and kainate receptors. It has been proposed that muscarinic receptors may be involved in phosphatidylinositol signaling in myelin or oligodendrocytes. There is recent evidence that muscarinic receptor activation alters integrin function in oligodendrocytes by modulating binding to extracellular matrix molecules [13]. With regard to adult pathologies, there is an increasing literature indicating that AMPA receptors may mediate glutamate cytotoxicity in oligodendrocyte progenitor cells.

Other myelin-related proteins. Another protein in compact CNS myelin is the myelin-associated oligodendrocytic basic protein, which is localized in the major dense line in several 8–12 kDa isoforms and appears to function in controlling axonal diameter and the arrangement of the radial component [39]. PNS myelin sheaths have long been known to contain a 170 kDa glycoprotein (see earlier editions of this chapter) that accounts for about 5% of the total myelin protein and may be related to the recently characterized L-periaxin-dystrophin-related protein 2-dystroglycan (PDG) complex associated with the Schwann plasma membrane [36, 40]. This PDG complex is essential for stable axon–glia interactions and mutations of the periaxin gene lead to profound disruptions of axonal ensheathment and segmental demyelination.

Small amounts of proteins characteristic of cells and membranes in general can also be found in myelin. There is evidence that tubulin is an authentic myelin-related component (**Fig. 4-12B, D,** CNS myelin). The 48 kDa myelin/oligodendrocyte-specific protein (MOSP) is a component found only in CNS myelin and oligodendroglial membranes, which appears to associate with tubulin [41].

DEVELOPMENTAL AND METABOLIC ASPECTS OF MYELIN

The developmental progress of myelination varies between regions and species. As the nervous system matures, portions of the PNS myelinate first, then the spinal cord, and the brain last [1]. In all parts of the nervous system there are many small axons that are never myelinated. It is generally true that large neural pathways become myelinated before they are completely functional. A relevant observation is that rats and other nest-building animals are quite helpless at birth and myelinate predominantly postnatally. By contrast, grazing animals such as horses, cows and sheep have considerably more CNS myelin at birth and a correspondingly higher level of complex activity immediately postnatally.

Synthesis of myelin components is very rapid during deposition of myelin. Nervous system development is marked by several overlapping periods, each defined by a major event in brain growth and structural maturation [1]. In the rat, whose CNS undergoes considerable development postnatally, the maximal rate of cellular proliferation (much of this involving oligodendroglial precursor cells) occurs at 10 days. The rat brain begins to form myelin postnatally at about 10–12 days. The maximal rate of accumulation of myelin in the rat occurs at about 20 days of age, although accumulation continues at a decreasing rate throughout adulthood. A remarkable amount of membrane biogenesis occurs in oligodendrocytes during the period of maximum myelination. Myelin accumulates in a 20-day-old rat brain at a rate of about 3.5 mg/day. Rough calculations based on the number of oligodendrocytes and the amount of myelin deposited indicate that on average the amount of myelin membrane made by each cell per day is more than three times the weight of its own perikaryon. This very rapid myelin synthesis early in development has been demonstrated biochemically by the

very rapid incorporation of radioactive precursors into myelin and substantial increases of enzymes involved in synthesizing myelin components.

Sorting and transport of lipids and proteins takes place during myelin assembly. After myelin components have been synthesized they must be assembled to form the membranes making up myelin sheaths [16]. The biogenesis of these sheaths is an extraordinary process of membrane formation and modeling. In the CNS, this requires the spiraling of numerous oligodendroglial processes around axons and their tight layering to form compact myelin. Furthermore, there is additional modeling of specialized membrane domains with different composition at the inside and outside of the sheaths and in the paranodal glia–axon junctions. The two major proteins of compact CNS myelin, PLP and MBP, enter the myelin by different routes. PLP is synthesized on membrane-bound polysomes in the perikaryon and transported in membranous vesicles to the myelin being formed at the end of the oligodendroglial processes. By contrast, MBP is synthesized on free polysomes, which are actually located in very close proximity to the newly forming myelin at the end of oligodendroglial processes [16]. Its mRNA is transported from the perikaryon to the vicinity of myelin formation in ribonucleoprotein granules by a microtubule-based translocation system. These differences in the route of entry are reflected in different kinetics of incorporation of proteins into myelin membranes in experiments involving labeling with radioactive amino acids after intracranial injection or incubation of brain slices. Radioactive MBP is synthesized and integrated into myelin very rapidly with a lag time of only a few minutes, whereas substantial amounts of radioactive PLP do not appear in myelin until after about 45 minutes [1]. Other proteins that are selectively localized in specialized regions of the myelin sheath such as the inner and outer surfaces (e.g. MAG and MOG, respectively) or in the paranodal regions (e.g. neurofascin-155) must be sorted and transported by different mechanisms involving specific sorting signals. It is likely that the sorting mechanisms are related to the apical and basolateral targeting that occurs in simple polarized epithelial cells. Using the model system of transfected Madin–Darby canine kidney (MDCK) epithelial cells, it was shown that MOG sorts exclusively to MDCK basolateral membranes, whereas PLP sorts exclusively to MDCK apical membrane [42]. However, sorting in myelin-forming cells probably also involves more complicated mechanisms because of the complex variety of membrane domains in myelin sheaths. Furthermore, some of the lipids and proteins in myelin forming cells are associated with raft-like domains, which are enriched in cholesterol, glycosphingolipids and glycosylphosphatidylinositol-linked proteins [7]. These rafts are likely to play an important role in the trafficking of membrane components and signal transduction mechanisms involved in the assembly of myelin sheaths. Much research designed to elucidate these phenomena and other aspects of myelin assembly is ongoing. However, a more detailed description of this research is beyond the scope of this chapter, and the reader is referred to more comprehensive references [7, 16].

The composition of myelin changes during development. The composition of myelin isolated from immature, rapidly developing brain is different from that of the adult [1]. As rat brain matures, the content of galactolipids, MBP and PLP in purified myelin increases, whereas phosphatidylcholine and high-molecular-weight proteins decrease. These studies on the composition of myelin from immature brains are consistent with the concept that myelin first laid down by oligodendrocytes may represent a transitional form with properties intermediate between those of mature compact myelin and the oligodendroglial plasma membrane. However, interpretation of biochemical studies on purified myelin is complicated by the fact that myelin fractions isolated by conventional procedures can be separated into subfractions of different densities. The lighter fractions are enriched in multilamellar myelin, whereas the denser fractions contain a large proportion of single membrane vesicles that morphologically resemble microsomes or plasma membrane fragments. Generally speaking, as one goes from light myelin subfractions to heavier ones, the lipid:protein ratio and the amount of MBP decrease, the amount of PLP decreases or remains relatively constant, and the amounts of MAG, CNP and other high-molecular-weight proteins increase. The interpretation of these findings is that the light subfractions contain primarily compact myelin while the heavier fractions are enriched in other oligodendroglial-derived membranes from the cell processes, the inner and outer surfaces of the sheaths, and the paranodal loops. Therefore, the differing lipid and protein composition of isolated immature myelin may reflect either transitional forms of developing myelin or a greater content of associated oligodendroglial membranes relative to compact myelin recovered from the thinner immature myelin sheaths, or a combination of these factors. Nevertheless, metabolic studies with radioactive precursors lend support to the view that the heavier fractions isolated from developing brain represent at least in part transitional membranes in the process of conversion to compact myelin. For example, PLP appears first in the heavier fractions and later in lighter fractions in a manner that suggests a precursor–product relationship.

Spontaneous mutations in experimental animals provide insights about the structure and assembly of myelin. The myelin mutants often have names relating to their characteristic tremor due to the myelin deficit, e.g. *shiverer*, *jimpy*, *quaking* and *trembler* mice (Table 4-2). Although some of the mutants have been studied for many years [1], it is only recently that recombinant DNA techniques have led to identification of the primary genetic defects in most of them. Some of them are good

models for inherited human diseases affecting myelin, as described in Ch. 38. Furthermore, the naturally occurring mouse mutants, which may express abnormal myelin proteins, sometimes have phenotypes that differ from null mutants produced experimentally by homologous recombination and are thereby instructive with regard to the mechanisms of myelin formation and loss.

Mutation of either of the major structural proteins of CNS myelin can lead to a severe hypomyelination; MBP in *shiverer* or myelin-deficient (mld) mice and PLP in *jimpy* mice and several other animal mutants [10, 43, 44] (Table 4-2). Ultrastructural abnormalities in the small amount of myelin that is formed by these mutants have been informative with regard to the structural roles of these proteins in compact CNS myelin. Thus the cytoplasmic surfaces of the spiraled oligodendrocyte membranes do not compact to form a major dense line in the absence of MBP in *shiverer* mutants, and the intraperiod line is more condensed than normal in *jimpy* mice, which express very little PLP. The ultrastructure of PNS myelin is normal in both of these mutants since PLP is virtually absent from peripheral myelin and P_0 appears to be capable of stabilizing both the intraperiod and major dense lines of myelin of the PNS in the absence of MBP. The *shiverer* phenotype can be corrected by introducing normal MBP into transgenic mutant mice, resulting in almost complete correction of the shivering, early death and failure of CNS myelin compaction.

The severity of the myelin deficiency in PLP mutants varies. Most PLP mutations are quite severe, but the PLP mutation in *rumpshaker* mice causes a relatively mild hindlimb shaking phenotype. By contrast, there is severe reduction and abnormal structure of CNS myelin in *jimpy* mice, along with a profound loss of oligodendrocytes. Furthermore, attempts to correct the *jimpy* phenotype by introducing the normal gene have not been successful. Thus the *jimpy* PLP mutation appears to be dominant, causing a misfolded protein response that leads eventually to oligodendrocyte death with morphological features of apoptosis. These oligodendroglial abnormalities appear to be the primary cause of the severe hypomyelination in *jimpy* mice rather than the absence of PLP in the myelin, since, as noted above, the PLP-null mouse has relatively normal myelin. The PLP mutants demonstrate the fact that protein mutations may be far more severe than simple loss of the protein. It must be noted that mutation of the minor isoforms of these proteins expressed in glia, neurons and other cell types may also impact these phenotypes [10].

Two allelic *trembler* mutations, which affect only the PNS, result from different point mutations in transmembrane domains of peripheral myelin protein-22 (PMP-22) [1, 45] (Table 4-2). The *trembler* phenotypes are characterized by hypomyelination, continued Schwann cell proliferation and partial paralysis of the limbs. These murine mutants are animal models for some of the inherited human neuropathies caused by abnormalities of the PMP-22 gene (Ch. 38). Because the function of PMP-22 is uncertain, the mechanisms by which the point mutations in this protein cause the *trembler* phenotype are unclear. However, as with the PLP mutations, the pathology may result from an unfolded protein response to abnormal protein that is retained in the endoplasmic reticulum.

A number of spontaneous animal mutants are not direct mutations of compact myelin protein genes but rather appear to cause defects in cellular processes needed for myelin assembly or maintenance. One example is the *quaking* mouse, with hypomyelination in both the CNS and PNS but a greater deficiency of CNS myelin [1, 46] (Table 4-2). Unlike the *shiverer* and *jimpy* mice, which form almost no myelin and die early, *quaking* mice generate more myelin and live to adulthood. However, the myelin sheaths are thin and poorly compacted, especially in the CNS. The myelin isolated from the CNS of this mutant has all the known myelin proteins but its overall composition resembles that of very immature normal mice. Although the *quaking* mouse was one of the first dysmyelinating mutants to be described and has been extensively studied, the genetic lesion was identified only recently. The *quaking* gene encodes a family of RNA-binding proteins generated by mRNA splicing. These QKI proteins appear to regulate the alternative splicing and/or transport of oligodendroglial mRNAs for myelin proteins such as MBP and MAG [46–48].

The more recently identified 'taiep' rat mutant (Table 4-2) has impaired accumulation of CNS myelin for up to 2 months followed by a period of demyelination [49]. Adult taiep rats have only 10–25% of the normal amount of CNS myelin. The primary genetic lesion has not yet been identified but the mutant oligodendrocytes exhibit an abnormal accumulation of microtubules during development, suggesting that the mutation may involve a microtubule-associated protein. Biochemical and immunocytochemical studies indicate that excessive microtubule accumulation interferes with transport of myelin proteins and/or their mRNAs, eventually leading to a failure of myelin maintenance [49].

Myelin components exhibit great heterogeneity of metabolic turnover. One of the novel characteristics of myelin demonstrated in early biochemical studies was that its overall rate of metabolic turnover is substantially slower than that of other neural membranes [1]. A standard type of experiment was to evaluate lipid or protein turnover by injecting rat brains with a radioactive metabolic precursor and then follow loss of radioactivity from individual components as a function of time. Structural lipid components of myelin, notably cholesterol, cerebroside and sulfatide, as well as proteins of compact myelin, are relatively stable, with half-lives of the order of many months. One complication in interpreting these studies is that the metabolic turnover of individual myelin components is multiphasic – consisting of an initial rapid loss of radioactivity followed by a much longer slower loss.

For example, initially MBP and PLP exhibit half-lives of 2–3 weeks, but later their half-lives are too long to be calculated accurately. A possible interpretation of these data is that some of the newly formed myelin remains in outer layers or near cytoplasmic pockets (incisures and lateral loops) where it is accessible for catabolism – thus accounting for the rapidly turning-over pool. The more stable metabolic pool would consist of deeper layers of myelin less accessible for metabolic turnover.

By contrast to the relatively slow rate of overall metabolism, the presence of signal transduction systems in myelin sheaths [7, 16] indicates that some aspects of myelin metabolism probably involve rapid events with half-lives on the order of minutes. For example, the monoesterified phosphate groups of polyphosphatidylinositol (those at positions 4 and 5) are labeled very quickly even in mature animals, and this is presumably related to the function of phosphoinositides in signal transduction (see Ch. 20). Additionally, the phosphate groups on MBP turn over rapidly [17]. Although the representation of myelin structure in **Figure 4-11** is static, studies that demonstrate relatively rapid metabolism of certain myelin components suggest that there may be some dynamic aspect of myelin structure, such as occasional separation of the cytoplasmic faces of the membranes. The dynamic nature of myelin sheaths probably contributes to the maintenance and functional state of axons. Clearly recent studies on the pathology of multiple sclerosis demonstrate the crucial nature of myelin both for nerve conduction and axonal survival (see Ch. 38). A more complete understanding of the formation and maintenance of myelin sheaths awaits conceptual and analytical advances.

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REFERENCES

1. Morell, P. *Myelin*. Plenum Press, New York, 1984.
2. Lazzarini, R. A. *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004.
3. Waxman, S. G. and Bangalore, L. Electrophysiological consequences of myelination. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 117–141.
4. Kirschner, D. A. and Blaurock, A. E. Organization, phylogenetic variations and dynamic transitions of myelin. In R. E. Martenson (ed.), *Myelin: biology and chemistry*. Boca Raton, FL: CRC Press, 1992, pp. 3–78.
5. Trapp, B. D. and Kidd, G. J. Structure of the myelinated axon. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 3–27.
6. Marcus, J. and Popko, B. Galactolipids are molecular determinants of myelin development and axo-glial organization. *Biochim. Biophys. Acta* 1573, 406–413, 2002.
7. Taylor, C. M., Marta, C. B., Bansal, R. *et al.* The Transport, Assembly and Function of Myelin Lipids. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 57–88.
8. Greer, J. M. and Lees, M. B. Myelin proteolipid protein–the first 50 years. *Int. J. Biochem. Cell. Biol.* 34, 211–215, 2002.
9. Hudson, L. D. Proteolipid protein gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 401–420.
10. Campagnoni, A. T. and Skoff, R. P. The pathobiology of myelin mutants reveal novel biological functions of the MBP and PLP genes. *Brain Pathol.* 11, 74–91, 2001.
11. Stecca, B., Southwood, C. M., Gragerov, A. *et al.* The evolution of lipophilin genes from invertebrates to tetrapods: DM-20 cannot replace proteolipid protein in CNS myelin. *J. Neurosci.* 20, 4002–4010, 2000.
12. Yamaguchi, Y., Ikenaka, K., Niinobe, M. *et al.* Myelin proteolipid protein (PLP), but not DM-20, is an inositol hexakisphosphate-binding protein. *J. Biol. Chem.* 271, 27838–27846, 1996.
13. Gudz, T. I., Schneider, T. E., Haas, T. A. *et al.* Myelin proteolipid protein forms a complex with integrins and may participate in integrin receptor signaling in oligodendrocytes. *J. Neurosci.* 22, 7398–7407, 2002.
14. Campagnoni, A. T. and Campagnoni, C. W. Myelin basic protein gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 387–400.
15. Aruga, J., Okano, H. and Mikoshiba, K. Identification of the new isoforms of mouse myelin basic protein: the existence of exon 5a. *J. Neurochem.* 56, 1222–1226, 1991.
16. Trapp, B. D., Pfeiffer, S. E., Anitei, M. *et al.* Cell biology of myelin assembly. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 29–55.
17. Eichberg, J. and Iyer, S. Minireview: Phosphorylation of myelin proteins: Recent Advances. *Neurochem. Res.* 21, 527–535, 1996.
18. Braun, P. E., Lee, J. and Gravel, M. 2′,3′-cyclic nucleotide 3′-phosphodiesterase: Structure, biology and function. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 499–522.
19. Quarles, R. H. Myelin sheaths: glycoproteins involved in their formation, maintenance and degeneration. *Cell. Mol. Life Sci.* 59, 1851–1871, 2002.
20. Georgiou, J., Tropak, M. P. and Roder, J. C. Myelin-associated glycoprotein gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 421–467.
21. Pham-Dinh, D., Dautigny, A. and Linington, C. Myelin oligodendrocyte glycoprotein gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 469–497.
22. Vourc'h, P. and Andres, C. Oligodendrocyte myelin glycoprotein (OMgp): evolution, structure and function. *Brain Res. Rev.* 45, 115–124, 2004.
23. Kirschner, D. A., Wrabetz, L. and Feltri, M. L. The P_0 gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 523–545.

24. Suter, U. PMP-22 gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 547–564.
25. Martenson, R. and Uyemura, K. Myelin P2, a neuritogenic member of the family of cytoplasmic lipid binding proteins. In R. E. Martenson (ed.), *Myelin: biology and chemistry*. Boca Raton, FL: CRC Press, 1992, pp. 509–530.
26. Filbin, M. T. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat. Rev. Neurosci.* 4, 703–713, 2003.
27. Bronstein, J. M. Function of tetraspan proteins in the myelin sheath. *Curr. Opin. Neurobiol.* 10, 552–557, 2000.
28. Kim, T., Fiedler, K., Madison, D. L. *et al.* Cloning and characterization of MVP17: a developmentally regulated myelin protein in oligodendrocytes. *J. Neurosci. Res.* 42, 413–422, 1995.
29. Frank, M. MAL, a proteolipid in glycosphingolipid enriched domains: functional implications in myelin and beyond. *Prog. Neurobiol.* 60, 531–544, 2000.
30. Erne, B., Sansano, S., Frank, M. *et al.* Rafts in adult peripheral nerve myelin contain major structural myelin proteins and myelin and lymphocyte protein (MAL) and CD59 as specific markers. *J. Neurochem.* 82, 550–562, 2002.
31. Magyar, J. P., Ebensperger, C., Schaeren-Wiemers, N., *et al.* Myelin and lymphocyte protein (MAL/MVP17/VIP17) and plasmolipin are members of an extended gene family. *Gene* 189, 269–275, 1997.
32. Scherer, S. S., Arroyo, E. J. and Peles, E. Functional organization of the nodes of Ranvier. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 89–116.
33. Gow, A. The claudin 11 gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 565–568.
34. Ishibashi, T., Ding, L., Ikenaka, K. *et al.* Tetraspanin protein CD9 is a novel paranodal component regulating paranodal junctional formation. *J. Neurosci.* 24, 96–102, 2004.
35. Scherer, S. S. and Paul, D. L. The connexin 32 and connexin 29 genes. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 599–608.
36. Scherer, S. S. and Arroyo, E. J. Recent progress on the molecular organization of myelinated axons. *J. Peripher. Nerv. Syst.* 7, 1–12, 2002.
37. Ledeen, R. W. Enzymes and receptors of myelin. In R. E. Martenson (ed.), *Myelin: biology and chemistry*. Boca Raton, FL: CRC Press, 1992, pp. 531–570.
38. Belachew, S., Rogister, B., Rigo, J. M. *et al.* Neurotransmitter-mediated regulation of CNS myelination: a review. *Acta Neurolog. Belg.* 99, 21–31, 1999.
39. Yoshikawa, H. Myelin-associated oligodendrocytic basic protein modulates the arrangement of radial growth of the axon and the radial component of myelin. *Med. Electron Microsc.* 34, 160–164, 2001.
40. Sherman, D. L. and Brophy, P. J. The periaxin gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 633–642.
41. Dyer, C. A. and Matthieu, J. M. Antibodies to myelin/oligodendrocyte-specific protein and myelin/oligodendrocyte glycoprotein signal distinct changes in the organization of cultured oligodendroglial membrane sheets. *J. Neurochem.* 62, 777–787, 1994.
42. Kroepfl, J. F. and Gardinier, M. V. Mutually exclusive apicobasolateral sorting of two oligodendroglial membrane proteins, proteolipid protein and myelin/oligodendrocyte glycoprotein, in Madin–Darby canine kidney cells. *J. Neurosci. Res.*, 66, 1140–1148, 2001.
43. Baumann, N. and Pham-Dinh, D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol. Rev.* 81, 871–927, 2001.
44. Nave, K. A. and Griffiths, I. R. Models of Pelizeaus–Merzbacher disease. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 1125–1142.
45. Wrabetz, L., Feltri, M. L. and Suter, U. Models of Charcot–Marie–Tooth disease. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 1143–1168.
46. Hardy, R. J. The QKI gene. In R. A. Lazzarini (ed.), *Myelin biology and disorders*. San Diego, CA: Elsevier Academic Press, 2004, 654–659.
47. Larocque, D., Pilotte, J., Chen, T. *et al.* Nuclear retention of MBP mRNAs in the quaking viable mice. *Neuron* 36, 815–829, 2002.
48. Wu, J. I., Reed, R. B., Grabowski, P. J. *et al.* Function of quaking in myelination: regulation of alternative splicing. *Proc. Natl Acad. Sci. U.S.A.* 99, 4233–4238, 2002.
49. Song, J., Carson, J. H., Barbarese, E. *et al.* RNA transport in oligodendrocytes from the taiep mutant rat. *Mol. Cell. Neurosci.* 24, 926–938, 2003.

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CHAPTER

5

Membrane Transport

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INTRODUCTION

Primary active cation transporters energize many of the most basic neural functions. They transform free energy from ATP hydrolysis into electrochemical energy that is stored in the transmembrane concentration gradients of Na^+, K^+, Ca^{2+} and protons. These energy stores are employed via membrane channel proteins for signaling, and also by *secondary transporters* that selectively concentrate many other ions and molecules. Secondary transporters subserve many different neural functions such as packaging neurotransmitters into vesicles and terminating signals at synapses. Also classified as transporters are *facilitator*

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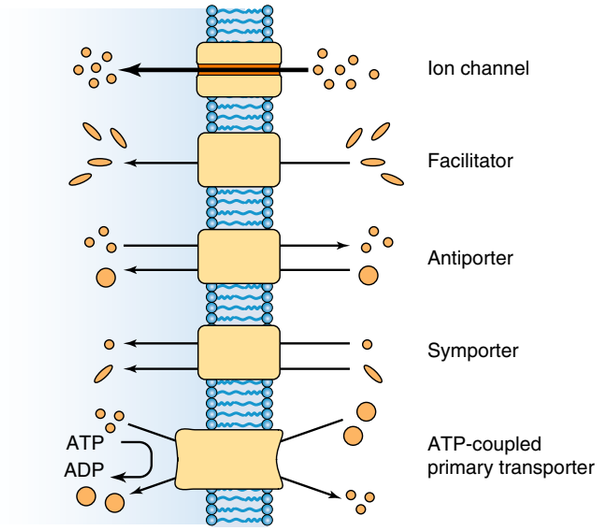

FIGURE 5-1 Types of membrane transport proteins. Ion channels provide gated diffusion paths across cell membranes that are regulated by membrane voltage, interactions with ligands, and/or by phosphorylation (see Ch. 6). Facilitators or uncoupled transporters provide highly selective pathways, e.g. for D-glucose or water, but are not coupled to energy sources and therefore cannot concentrate their substrates. Transporters that are coupled to energy sources can alter the steady-state distribution of their substrate ions and/or molecules. Secondary transporters derive energy from existing ion gradients to transport a second ion or molecule in a direction that is either the same as (symport) or opposite to (antiport) that of the energizing ion. Primary transporters couple a chemical reaction to protein conformational transitions, which supply energy to generate concentration gradients of one or more substrates across cell membranes. For identifying and classifying transporters and channels, see online databases [41].

proteins; they enable specific molecules to diffuse across membranes, often under regulatory control, but are incapable of 'uphill' transport (Fig. 5-1).

Relative to ion channels which, when open, allow diffusion of thousands of ions per channel per millisecond (Ch. 6), transporters typically require milliseconds to move 1–3 ions or molecules per reaction cycle in the uphill direction. Thus transporters are usually expressed at much higher densities in membranes than are ion channels.

PRIMARY ION TRANSPORTERS

The primary transporters discussed in this chapter belong to three distinct genomic superfamilies that differ markedly in structure and reaction mechanism. These P-, V_0V_1- and ABC-superfamily members all catalyze reactions with ATP that drive conformational cycles to move substrates across membranes and 'uphill' to higher concentrations.

P-type transporters share the same general reaction mechanism. Most pumps belonging to the P-type transporter superfamily have evolved to create cation gradients. In the case of the P-type Na^+,K^+ pumps, the Na^+ and K^+ concentration gradients generated across all eukaryotic plasma membranes constitute a large potential energy store that is employed for electrical signaling and for driving secondary transporters. Ca^{2+} gradients are also generated by P-type transporters, but these gradients are employed primarily for intracellular signaling (Ch. 22). The structures and reaction mechanisms of the Na^+,K^+ and Ca^{2+} pumps are similar and have provided insights into the functioning of the whole class of P-type transporters.

Because of the distinct roles of the two different substrate cations in its reaction mechanism, the Na^+,K^+ pump most conveniently illustrates the mechanism of P-type pumps. The Na^+,K^+ pumps are driven by a cycle of conformational transitions that is driven by phosphorylation of their catalytic sites, activated by cytoplasmic Na^+, and hydrolysis of the same phosphorylated sites, activated by extracellular K^+ (Fig. 5-2).

The cycle is initiated by ATP binding to the catalytic site and cytoplasmic Na^+ binding to all three ionophoric sites. The catalytic sites contain an aspartyl residue, which becomes phosphorylated only when all three Na^+ binding sites are occupied by sodium ions. Phosphorylation of the aspartyl is readily reversible when the pump molecule is in the E1 conformation; that is, the energy states of the E1 aspartylphosphate bond and of the ATP phosphate bond are similar. This is demonstrated by the ability of the phosphorylated enzyme to rephosphorylate ADP to ATP under conditions that inhibit step 3 in Figure 5-2. However, the active pump formation of the aspartylphosphate bond initiates a rapid transition to the E2-P conformation, in which the ionophoric sites have changed their selectivity, causing the three Na^+ to be discharged extracellularly in exchange for binding two K^+. This initiates hydrolysis of the aspartylphosphate bond in the E2 state which, in concert with ATP binding, causes E2 to revert to E1. In consequence of this the ionophoric sites discharge the two K^+ into the cytoplasm. ATP binding initiates the next transport cycle. This transport reaction can occur at a rate of ≈10,000 cycles per minute.

As noted above, a Na^+,K^+ pump exchanges three Na^+ for two K^+ per transport cycle. This produces a net outward flow of positive charge, which can generate an electrogenic potential. The extent of membrane hyperpolarization by the pump can be evaluated by measuring the reduction in membrane potential produced by selective Na^+,K^+ pump inhibitors such as ouabain. The electrogenic potential is usually small, 10 mV or less, because opposing ion currents flow through channels or secondary transporters in the contiguous membrane. However, in some neurons and muscle cells, sodium pump hyperpolarization can shorten the duration of an action potential and contribute to negative afterpotentials. In heart muscle, hyperpolarization due to Na^+ pumping occurs after sustained increases in firing rate and may be a factor in cardiac arrhythmias. [1].

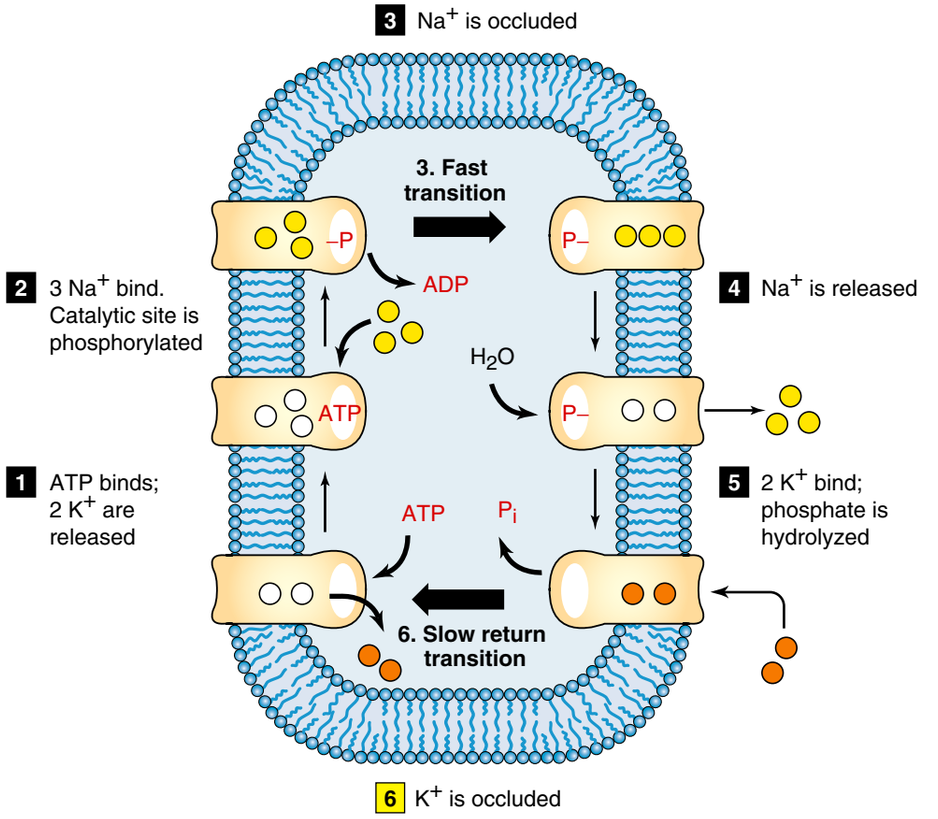

FIGURE 5-2 The mechanism of the ATP-dependent sodium pump. The sequence of reaction steps is indicated by the *large arrows*. On the left side, pump molecules are in the E1 conformation, which has high affinity for Na^+ and ATP and low affinity for K^+. Ionophoric sites are accessible only from the cytoplasmic side. *Step 1.* K^+ is discharged as metabolic energy is added to the system by ATP binding. *Step 2.* Three Na^+ bind and the enzyme is reversibly phosphorylated. *Step 3.* The conformational transition from E1~P to E2–P, shown at the top, is the 'power stroke' of the pump during which the ionophoric sites, with their three bound Na^+, become accessible to the extracellular side and decrease their affinity for Na^+. Part of the free energy of the enzyme acylphosphate has been dissipated in this process. *Step 4.* Three Na^+ dissociate from E2-P. *Step 5.* Two K^+ bind and more free energy is dissipated as the enzyme acylphosphate is hydrolyzed. At this point the two K^+ become tightly bound ('occluded') and in *step 6* E2 reverts to E1, carrying the K^+ to the cytoplasmic side. Each step of this cycle is experimentally reversible.

A major fraction of cerebral energy production is consumed by the Na^+,K^+ pump. Most of this is required to compensate for extrusion of intracellular Na^+ that enters through channels during electrical activity and through Na^+-dependent secondary transporters during neurotransmitter recovery. Assuming typical values for Na^+ and K^+ concentration gradients, that is, $[Na^+]_e/[Na^+]_i \approx 12$ and $[K^+]_i/[K^+]_e \approx 50$, then ΔG is about 3.8 kcal (16 kJ) per mole of Na^+ exchanged for K^+. Hydrolysis of a high-energy phosphate bond of ATP may yield as much as 12≈kcal/mol under physiological conditions, thus permitting the exchange of about three equivalents of cation for each mole of ATP hydrolyzed. From this stoichiometry, it appears that the chemical free energy of ATP hydrolysis must be utilized with about 95% efficiency ($3 \times 3.8/12$) to operate the sodium pump under physiological conditions. Other processes utilizing energy stores are listed in Table 31-1.

Na,K-ATPase is a heterodimer consisting of a catalytic α-subunit and an accessory β-subunit. Four isoforms of the α-subunit and three of the β-subunit are expressed in mammals. Three of the α-subunit isoforms are expressed in brain and will be discussed in a later section.

The β-subunits (≈40 kDa) are monotopic glycoproteins and exhibit some characteristics of cell adhesion molecules. At least one of the three β-subunit isoforms must be coexpressed to translocate an α-subunit from the endoplasmic reticulum (ER) to the plasmalemma. The three β-subunit isoforms have about 45% sequence identity. The β1 is most generally expressed [2]. The β2 was initially described as an 'adhesion molecule on glia' (AMOG) because it is transiently expressed on the surface of cerebellar Bergmann glia during the differentiation of granule cells. As the brain matures, it becomes widely expressed on astrocytes and disappears from most neurons. The particular β-subunit isoform paired with an α subunit

has little effect on pump parameters [3]. However, the different β isoforms may influence the ultimate cellular and subcellular localizations of the Na^+ pumps [4].

There are also Na^+ pump γ-subunits, a family of ≈15 kDa proteolipids that can associate with sodium pump α-subunits. However they are not necessary for pump activity nor are they expressed in all cells. The γ-subunits are classed as members of the FXYD gene family and several are expressed in brain [5]. The kidney expresses almost exclusively Na^+,K^+ pumps with α_1-subunits throughout the functionally diverse segments of the nephron, but different γ-subunits are expressed in different segments. From this it is inferred that different γ-subunits optimize the sodium pump for operation under the varying ionic environments [6].

In the central nervous system, Na,K-ATPase is most highly expressed in regions where high levels of Na^+/K^+ exchange are expected. These include axonal terminals, nodes of Ranvier, dendritic processes and neuronal soma, but not in myelin wrappings. In the spinal cord, neuronal α1 expression is restricted to a set of laterally situated anterior horn cells and to intermediolateral thoracic cord cells. Different dorsal root ganglion cells express α3 alone or together with α1 but do not express α1 alone. In retinal pigment epithelium and choroid plexus ependyma, the Na^+ pump is most concentrated on apical (luminal) surfaces (Fig. 5-3). With these exceptions, epithelial cells adapted for secretion or reabsorption express the Na^+ pump exclusively on the basolateral or abluminal surfaces.

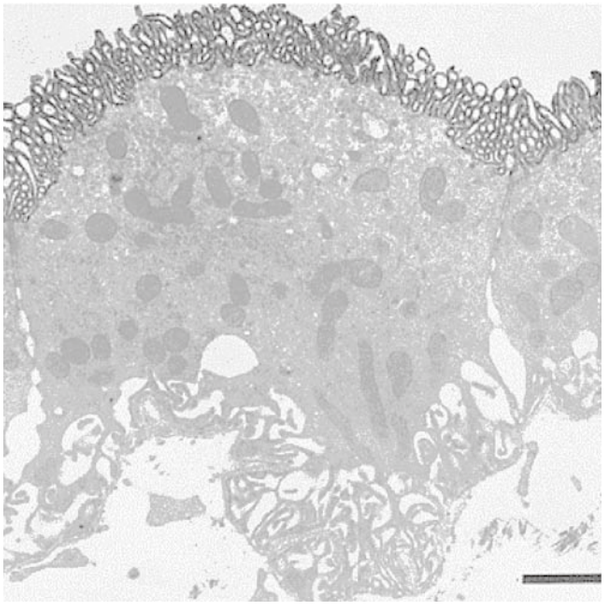

FIGURE 5-3 Immunocytochemical localization of the Na,K pump in choroid plexus. Choroid plexus contains epithelial cells with intensely stained microvillar and intermicrovillar plasma membranes. The basal and lateral plasma membrane surfaces are not stained. Bar = 2 μm. (With permission from reference [81].)

Na,K-ATPase expression patterns change with development, aging and dementia. The Na,K-ATPase activity, relative to total protein, increases about tenfold just prior to the stage of rapid myelination in rats. This occurs 2–12 days postnatally and corresponds to the time of glial proliferation, elaboration of neuronal and glial processes and increasing neuronal excitability. Neurons may play a role in upregulating glial expression of the α2 isoform during peak periods of myelination and the effect is probably dependent on contact [7]. Developmental changes in Na,K-ATPase in brain appears to depend on thyroidal influences. Glial cell cultures from 1-day old rat brain express progressively increasing amounts of α1, α2, β1 and β2 isoforms as a function of increasing triiodothyronine exposure [8].

In cerebral cortex of human (Fig. 5-4) and rat, α3 mRNA is found clustered over pyramidal and other neuronal soma while α1 mRNA is distributed diffusely through the neuropil. In non-demented, aged humans the expression of α1 mRNA does not significantly change, although there are significant but small reductions in α3 mRNA of neuronal perikarya. In contrast, in dementing Alzheimer's disease (Ch. 47), the contents of α3 mRNA in neuronal perikarya and neuropil are markedly diminished early in the neurodegenerative process, prior to neuronal dystrophic changes (Fig. 5-4).

The α-subunit isoforms are products of separate genes that differ in their 5′-flanking regulatory sequences. These may enhance or obstruct the assembly of the preinitiation complex of RNA polymerase and relevant transcription factors [9]. Physiological isoform expression varies with cell type and maturity. In rat heart, the predominant form shifts from neonatal α3 to adult α2.

The α3 isoform is expressed mainly in adult neurons and neonatal cardiomyocytes. The α3 gene exhibits three positively regulating *cis* elements that bind NP-Y, Sp1 and Sp2 [10]. Its neuron specificity appears to be related to a neural-restrictive silencer element and a positively acting *cis* element [11].

The 'basal promoter,' required for constitutive expression of the α1 gene, is a positive regulatory element termed ARE [12] containing binding sites for several transcription factors, including the cAMP-responsive element (CRE). CRE occurs within promoters of many cAMP-inducible genes and can interact with CRE-binding (CREB) and CRE–modulatory (CREM) proteins and with activating transcription factor (ATF)-1. These are a subgroup within the leucine zipper family defined by their amino acid-sequence similarity and ability to dimerize with each other. The CRE site is subject to regulation by several pathways, some involving cAMP, Ca^{2+} and transforming growth factor (TGF)β. Phosphorylation of ATF-1 and CREB by PKA or PKC enhances their binding to the

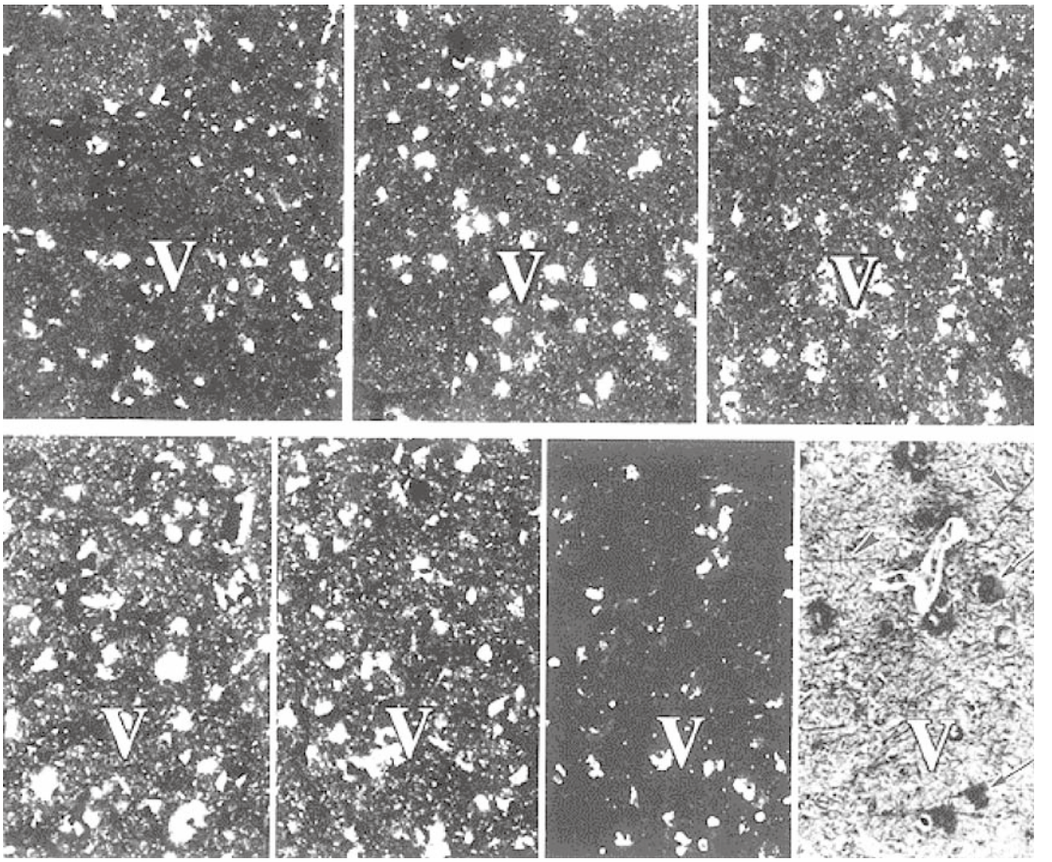

FIGURE 5-4 In situ hybridization detection of α1- and α3-mRNAs of Na,K-ATPase in human superior frontal cortex (layers IV, V). **Top row, α1.** Left to right: 39-year-old normal, 78-year-old normal, 78-year-old Alzheimer's disease (AD) subject. Note the mildly increased hybridization signal for α1-mRNA in the normal age-matched and AD brains compared to the young control brain. **Bottom row, α3.** Sections adjacent to those in the top row. *Far right:* Bielschowsky stain of adjacent AD cortex. Note the small decrease in hybridization signal in the aged normal relative to the young control and the large decrease in signal in AD relative to young and age-matched control brains. The Bielschowsky silver stain shows dark-appearing amyloid plaques in the AD brain. (With permission from reference [82].)

ATF/CRE site and may be required for transcription of the ATPase α1 gene [13].

Na,K-ATPase is subject to hormonal regulation. In the rat hippocampus, the dentate granular cells express the α1 and α3 isoforms and, in adrenalectomized rats, aldosterone can selectively regulate α3 in these neurons. In other cortical neurons that express both mRNAs, however, aldosterone has no effect on either [14], and, in kidney, aldosterone regulates the level of expression of α1. Isoform regulation by aldosterone is evidently determined by local factors such as cell type and location. These positive or negative local factors in gene regulation have been termed the 'cell context'. The cAMP or CREB protein system also interacts with the mineralocorticoid receptor system to modify α-subunit transcription [15]. In rat renal cortex, aldosterone increases α-1 subunit mRNA and this was shown to be directly due to aldosterone rather than to increased Na^+ flux [16]. Aldosterone also upregulates α3 and β1 isoform mRNAs in brains of adrenalectomized rats [17].

Na^+ can also regulate Na,K-pump expression. Alterations in the local ionic environment, through changes in cytoplasmic $Ca^{2+}$and Na^+, can affect expression of the Na,K pump via early-response genes [18].

Na,K-pump activity can be controlled by rapid endocytic removal and insertion in response to GPCR-regulated phosphorylation of α-subunits. For example, renal proximal tubule epithelia secrete dopamine, which acts on autocrine-D1-like receptors to remove Na,K pumps from their basolateral plasma membrane (Fig. 5-5). Pump endocytosis is initiated by phosphorylation at ser-18 of its α1 subunit by ζ-PKC, which permits interaction of PI3K-IA with the α-subunit and AP-2, thus inducing clathrin binding (see Ch. 9). Upregulation of Na,K pumps by recruitment requires α1 phosphorylation at both ser-11 and ser-18, which can be via β-PKC in response to serotonin- or angiotensin-activated GPCRs that leads to AP-1 binding. Another activation path for Na,K-pump

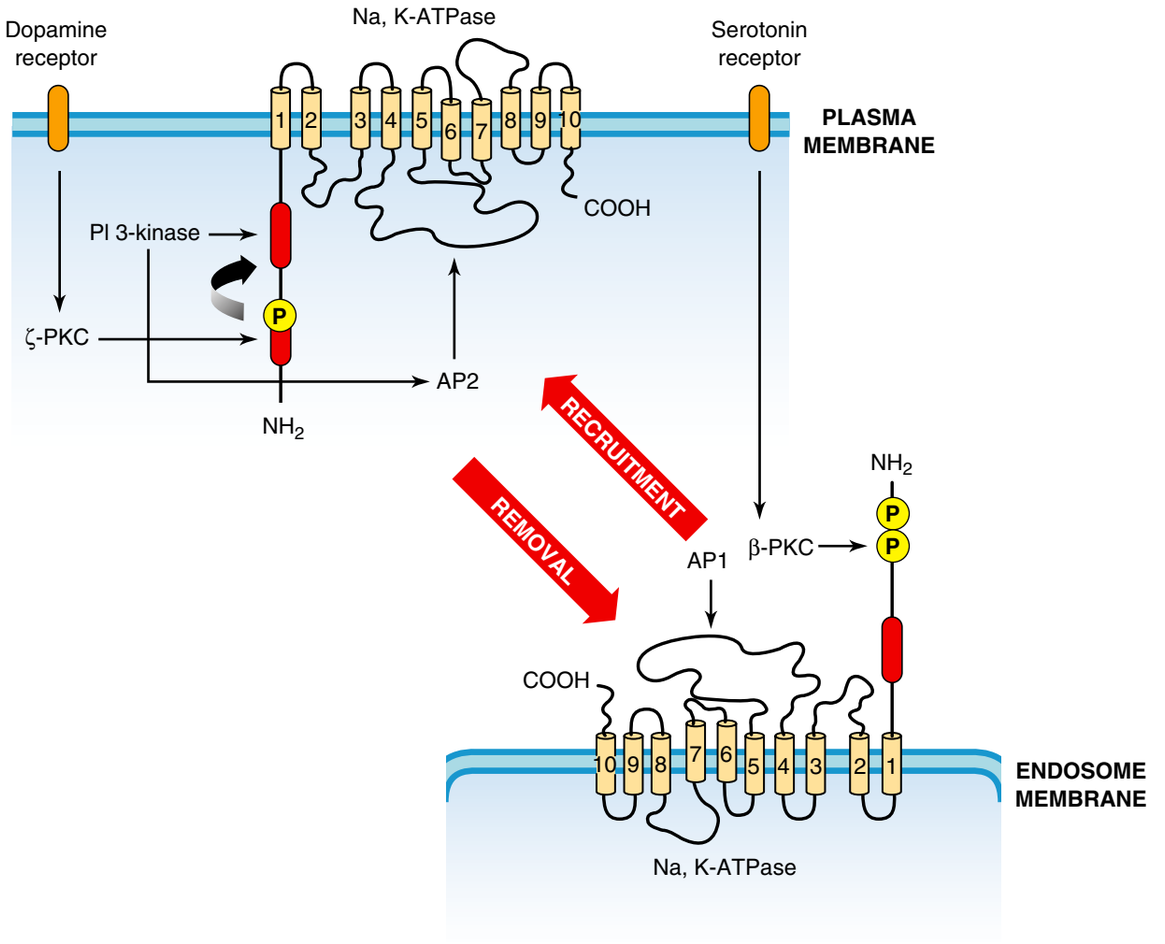

FIGURE 5-5 In kidney epithelial cells, which express only the α1 isoform, down-regulation of Na,K pumps can be initiated by dopamine via GPCR activation of endocytosis and this is controlled by phosphorylation of a single Ser-residue within the N-termini of the Na,K-ATPase α1-subunits to be internalized. Mutation analysis suggests that PI3K-IA activation ensues from its binding to a proline-rich domain present in the Na,K-ATPase α1-subunit, but which only becomes accessible to the kinase after ζ-PKC-dependent Ser-phosphorylation. PI3K-IA activation recruits the AP-2 adaptors that transfer plasma membrane vesicles into early endosomes. Thus, the Na,K-ATPase serves as its own scaffold, organizing the receptor signals that ultimately down-regulate its activity. Serotonin, acting through GPCR, can activate β-PKC phosphorylation of two serines within the N-termini of the endocytosed Na,K-ATPase α1-subunits. This evidently recruits AP-1 adaptors that initiate re-incorporation of Na,K pumps into plasma membrane. However, in the case of cultured striatal spiny neurons, which express all three Na,K-pump isoforms, regulation is more complex: although dopamine produces endocytosis-based down-regulation, this can be prevented by glutamate and glutamate can produce net pump activation by recruiting the α2 isoform [20]. (Adapted with permission from Fig. 4 of reference [83]).

recruitment to plasma membranes appears to involve α1 subunit phosphorylation on tyr-527 [19].

Endocytosis and recruitment of Na,K pumps occurs in primary isolates of striatal neurons. However in these neurons only α2 subunits were removed in response to dopamine whereas α1 subunits were selectively recruited in response to glutamate [20].

The cellular and subcellular distributions of α-subunit isoforms provide clues to their different physiological functions. The four isoforms exhibit about 85% sequence identity. The most substantial differences occur in their N-terminal regions and in an 11-residue sequence of the large cytoplasmic loop. When measured in cell cultures, the isoforms differ in their apparent affinities for intracellular Na^+ ($\alpha1 < \alpha2 < \alpha3$) [21] and extracellular K^+ ($\alpha3 < \alpha2 = \alpha1$) [22]. In adult tissues, α1 is the major isoform in most cells and functions as the 'housekeeping' or 'bulk Na extrusion' isoform. The α2, α3 and α4 isoforms are subject to unique forms of regulation and subcellular localization. In adult mammalian brains α1 is expressed in both neurons and glia, α2 is expressed in glia and some neurons, and α3 is expressed in most neurons.

In tissue culture, astrocytes, hippocampal neurons and arterial myocytes all express α1 diffusely in their plasmalemma. In contrast, immunocytochemically stained α2 in astroglia and α3 in myocytes and neurons display reticular patterns and colocalize with the Na^+/Ca^{2+} exchanger (NCX) (Fig. 5-6). These plasmalemma reticular patterns coincide with the patterns of staining for junctional complexes on sarcoplasmic reticulum and, in astroglia and neurons, junctional complexes on endoplasmic reticulum [23].

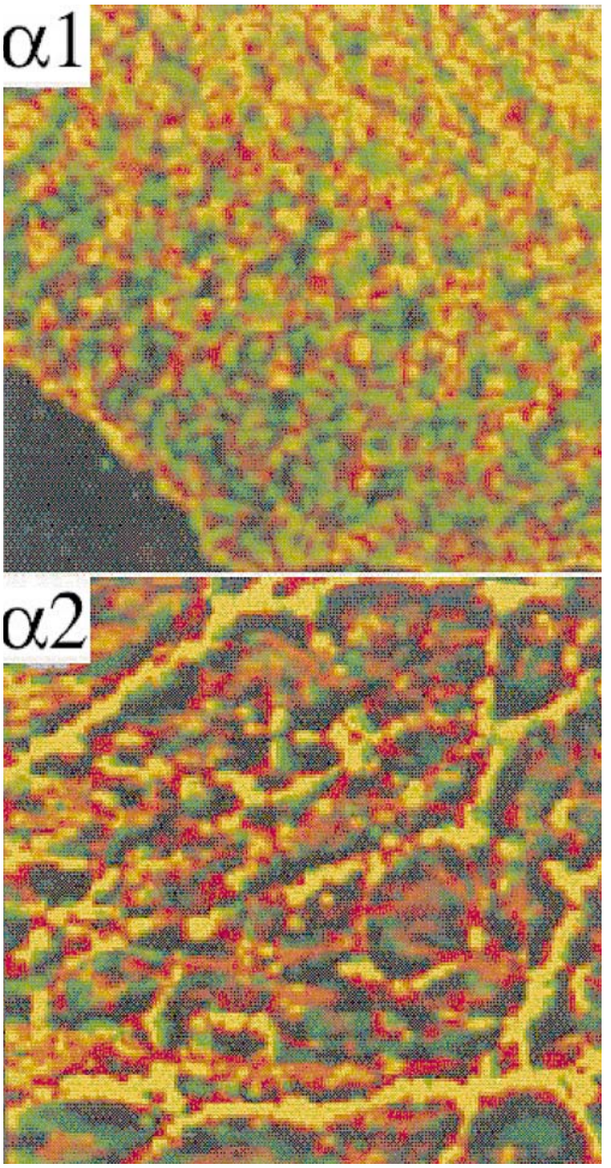

FIGURE 5-6 Localization of Na^+ pump α1 and α2 isoforms in primary cultured astrocytes. Cells were incubated with monoclonal antibodies specific for α1 or α2. The original fluorescent images were filtered by 'nearest neighbor' deblurring and the restored images compare portions of two astrocytes labeled with these antibodies. Both cells were later treated with a fluorescent dye, DiOC, to stain ER and mitochondria. When the respective antibody-labeled images (green) and DiOC-stained images (red) are superimposed, a yellow reticular pattern is observed where the ER and Na,K pumps coincide. (Adapted with permission from Fig. 4 of reference [23]).

The Ca^{2+} pumps of smooth reticulum and ER normally reduce cytoplasmic $[Ca^{2+}]$ to <1 μM. However, rapid restoration of such low $[Ca^{2+}]$ subsequent to plasma membrane depolarizations requires coordinate activity of the plasmalemma Na^+/Ca^{2+} exchanger (see discussion below). Because the energy source for driving this exchanger is the plasmalemma Na^+ gradient, inhibition of the Na,K-ATPase by a cardiac glycoside or an endogenous regulatory molecule [24] will increase cytosolic $[Na^+]$ and, secondarily, increase cytosolic $[Ca^{2+}]$, which then increases heart muscle contractility. This is the presumed cardiotonic mechanism of digitalis and related cardiosteroids.

Genetic evidence supports the importance of coordinated expression and distributions of α2 or α3 Na^+/K^+ pump isoforms with the Na^+/Ca^{2+} exchanger (NCX) and Ca^{2+} pumps to function in excitable and contractile cells: deletion of one copy of the α2 Na^+/K^+ pump gene in mice leads to *increased* contractile force in cardiac and skeletal muscle while deletion of one copy of the α1 gene leads to *reduction* of contractile force [25]. In rat optic nerve astrocytes, deletion of the α2 gene or ouabain treatment of cells expressing α2 leads to increased 'capacitative calcium entrance' responses, which reflect a decreased ability to rapidly remove cytosolic Ca^{2+} [26].

Na,K pumps may function as signal transducers. As discussed above, cardioactive steroids have long been employed to increase cardiac force. This occurs because they inhibit Na^+/K^+ pumps, thus increasing cytoplasmic $[Na^+]$, slowing Na^+/Ca^{2+} exchanger activity and increasing cytosolic $[Ca^{2+}]$. That sodium pumps have an evolutionarily conserved and highly selective binding site for cardioactive steroids has prompted a search for a possible endogenous 'ouabain-like substance' or OLS. Such substances have, in fact, been identified in brain, adrenal cortex, plasma and urine, although at very low concentrations. Elevated levels of OLS have been associated, experimentally and clinically, with salt retention, hypertension and cardiac hypertrophy. Their chemical structures appear very similar to ouabain [27].

Recent work has linked cardioactive steroid binding to the sodium pump, not only with pump inhibition but also with activation of the extracellular-receptor-activated protein kinase (ERK) signaling cascade. After exposure of contracting rat hearts to a positive inotropic dose of ouabain, the content of phosphorylated/activated ERK1/2 increases two- to threefold whereas Src and the α2 isoform of Na,K-ATPase increase by 50–60% in isolated caveolae. Isolated caveolin-enriched membrane fractions from rat cardiac myocytes contain nearly all of the cardiac-specific caveolin-3, 20–30% of the total sodium pumps and they are enriched in the signaling proteins, Src, epidermal growth factor receptor (EGFR) and ERK1/2 [28]. Such observations suggest that OLS may act on signaling systems involving some of these proteins.

Ca^{2+} PUMPS

ATP-dependent Ca^{2+} pumps and Na^+,Ca^{2+} antiporters act in concert to maintain a low concentration of free cytosolic Ca^{2+}. The concentration of cytosolic free calcium ion, $[Ca^{2+}]_i$, in unstimulated cells is between 10^{-8} and 10^{-7} mol/l, which is more than 10,000-fold lower than extracellular free Ca^{2+}. Most intracellular Ca^{2+} is stored in

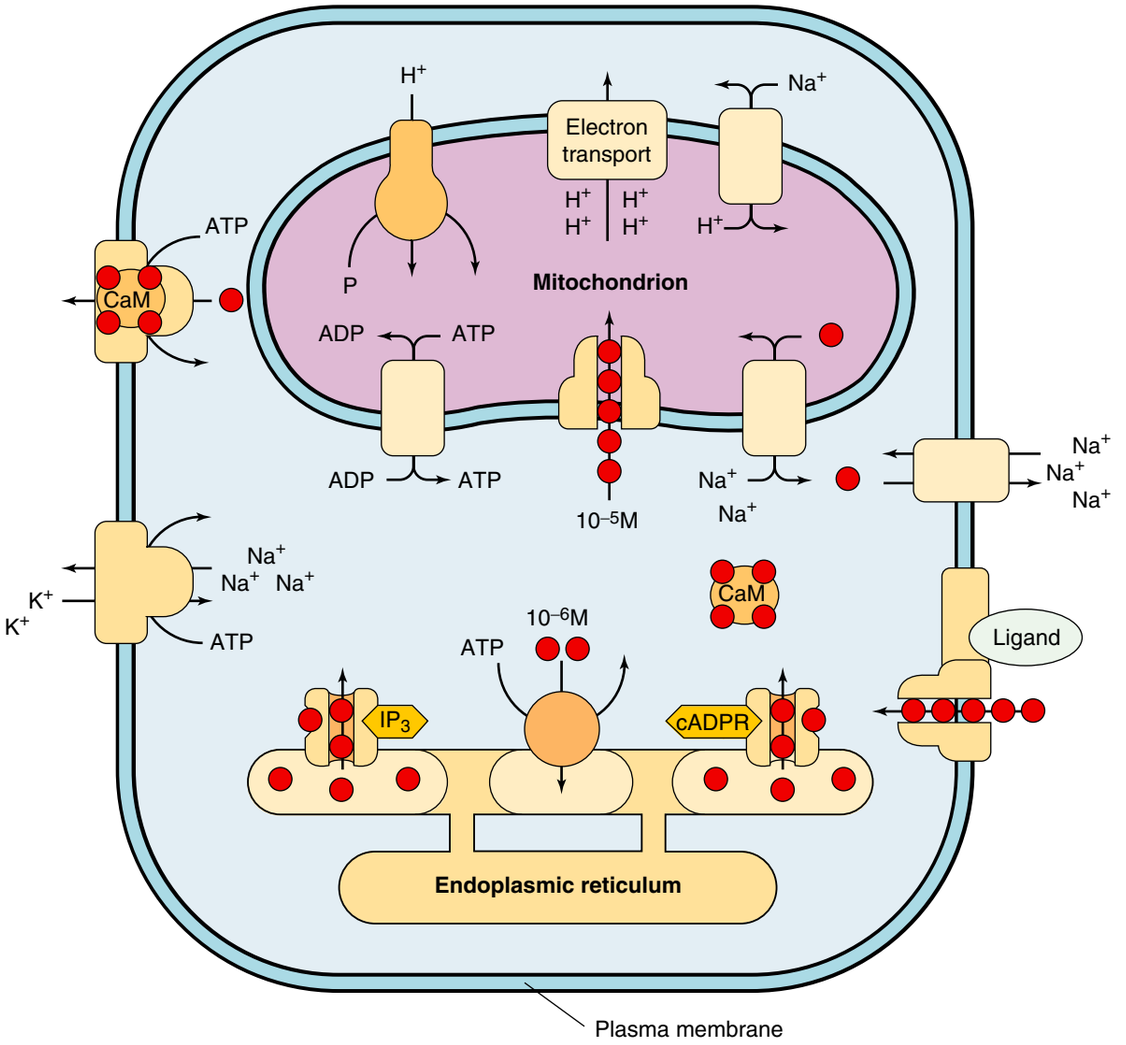

FIGURE 5-7 Calcium homeostasis. In the figure, calcium ions are represented by filled red circles. Ca^{2+} enters cells through a variety of ligand- and voltage-regulated channels, but basal cytoplasmic free Ca^{2+} is maintained at less than micromolar levels. Cytoplasmic Ca^{2+} is regulated coordinately by a Na^+/Ca^{2+} antiporter in plasma membranes and by P-type Ca-ATPases in plasma membranes and endoplasmic reticulum. The driving force for Na^+/Ca^{2+} antiporter exchange is the inwardly directed Na^+ gradient, which is maintained by α2 or α3 Na,K-ATPases. Mitochondria can participate transiently in Ca^{2+} homeostasis if the capacities of these other systems are exceeded. ER stores of Ca^{2+} can be released by second messengers, such as IP_3 or Ca^{2+} itself, in response to various receptor systems.

the endoplasmic reticulum. Because cytoplasmic $[Ca^{2+}]$ has many different intracellular signaling functions, its regulation is complex. and its impairment can be catastrophic (Fig. 5-7).

The primary plasma-membrane Ca^{2+} transporter (PMCA) is a P-type pump with high affinity for Ca^{2+} (K_m = 100–200 nmol/l) but relatively low transport capacity [19]. The stoichiometry of PMCA is one Ca^{2+} transported for each ATP hydrolyzed. These pumps probably do not carry out bulk movements of Ca^{2+} but are most effective in maintaining very low concentrations of cytosolic Ca^{2+} in resting cells. A distinguishing characteristic of the PMCAs is that, in addition to binding Ca^{2+} as a substrate, they are further activated by binding Ca^{2+}/calmodulin. The effect of calmodulin binding is to increase the affinity of the substrate Ca^{2+} site by 20- to 30-fold. This highly cooperative activation mechanism makes the PMCAs very sensitive to small changes in $[Ca^{2+}]_i$. A group of at least five PMCAs forms a multigene family. Three isoforms, PMCA1–3, occur in brain and each has a distinct distribution [20].

The smooth endoplasmic reticulum calcium pumps (SERCA) found in brain were first identified in sarcoplasmic reticulum. The three isoforms of SERCA are products of separate genes: SERCA-1 is expressed in fast-twitch skeletal muscle; SERCA-2a in cardiac/slow-twitch muscle; SERCA-2b, an alternatively spliced form, is expressed in smooth muscle and non-muscle tissues; SERCA-3 is

expressed in endothelial, epithelial, and lymphocytic cells and platelets. SERCA-2b is the major form expressed in brain, predominantly in neurons.

The uniquely high resolution structural data available for the SERCA1a Ca^{2+} pump illuminates the structure of all P-type transporters. Unlike the Na,K pump, the catalytic subunit of the SERCA Ca^{2+} pumps is active and does not require association with another subunit. However, the cardiac isoform, SERCA-2a, associates with a small membrane protein, phospholamban, that can regulate heartbeat strength and rate. The Ca^{2+}-pump reaction mechanism is essentially the same as that illustrated in Figure 5-2 for Na,K pumps except that two Ca^{2+} are exchanged in each cycle, probably for four protons. The primary sequences for the α-subunits of the Na,K- and H,K-ATPases can be superimposed on these Ca^{2+}-pump structures with only minor adjustments necessary to allow for small deletions and insertions.

Notice, in Figure 5-8, that the substrate cations are bound to ionophoric sites consisting of side chains contributed by four adjacent transmembrane helices, and

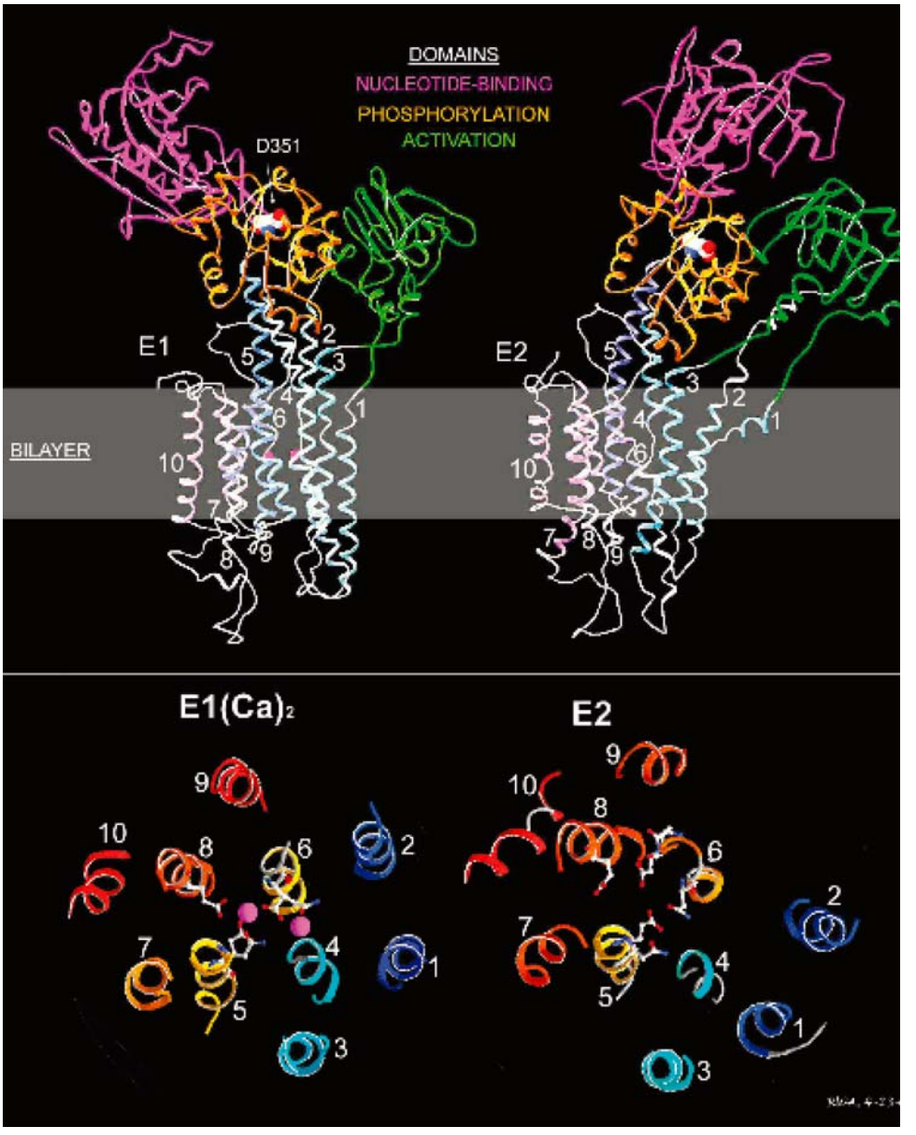

FIGURE 5-8 Structures of the SERCA1a calcium pump: $E1(Ca)_2$ was obtained from crystals formed in the presence of Ca^{2+}; E2 was obtained from crystals formed in the absence of Ca^{2+} and in the presence of the selective inhibitor, thapsigargin [84]. The two conformations are viewed in a plane perpendicular to (*top*) and parallel within (*bottom*) the lipid bilayer. The ten transmembrane segments are numbered in their order from N- to C-terminal, and colored sequentially from blue to red. The magenta spheres represent the two Ca^{2+}-binding sites in the E1 structures and, in the lower figures, the side chains that interact with Ca^{2+} are shown in stick form for both conformations. In the top figures, the N, A, and P cytoplasmic domains are colored magenta, orange and green. The aspartyl residue, D351, that is phosphorylated and dephosphorylated in each pump cycle, is shown as a space-filling model. (Models constructed from Protein Data Base coordinates 1eul and 1iwo using DeepView 3.7.) [85].

that the E2 configurations of these helices are rotated relative to the E1 configurations. Examination of the paths of these helices through the bilayer does not reveal any obvious 'channel' through the membrane. This is consistent with studies of the reaction mechanism, which have shown that, at successive stages of the cycle, the cations either have access from only one side of the membrane or are 'occluded' within the pump molecule.

The P pumps have three clearly demarcated cytoplasmic domains, which in Figure 5-8 are colored magenta (N or nucleotide-binding), orange (P or phosphoryl), and green (A or activator). Notice that transmembrane helix 5, which contributes to the ionophoric domain, extends as a helix well into the cytoplasmic P domain, transforming into a β-sheet structure just at its boundary with the 'signature sequence', ICSDKTGTL. This is conserved in all P pumps and includes the aspartyl residue (space-filled residue in Fig. 5-8), which reacts with ATP and water within each catalytic cycle.

The sodium and calcium pumps can be isolated to near purity and still exhibit most of the biochemical properties of the 'native' pump. Some kinetic properties of these pumps in 'native membranes' are altered or disappear as membrane preparations are purified. For example, when measured in intact membranes, the time-dependencies of phosphorylation and dephosphorylation of the pump catalytic sites exhibit biphasic fast to slow rate transition; this characteristic progressively disappears as the membranes are treated with mild detergents. One suggested explanation is that, as the pumps begin to cycle, the catalytic subunits associate into higher oligomers that may permit more efficient transfer of the energy from ATP into the ion transport process [29, 30]. Some structural evidence indicates that Na,K pumps exist in cell membranes as multimers of $(\alpha\beta)_2$ [31].

P-type copper transporters are important for neural function. Wilson's and Menke's diseases have major neurological components (Ch. 45). The Wilson's disease gene codes for a transporter, expressed chiefly in liver, that probably functions in Cu^{2+} excretion. The Menke's disease gene codes for a closely related transporter that regulates intestinal Cu^{2+} absorption [32].

V_0V_1-PROTON PUMPS

The V-ATPase pumps protons into Golgi-derived organelles. Its specialized neuronal function is to generate the electrochemical gradient that energizes the H^+-antiporters that load neurotransmitters into presynaptic vesicles (Ch. 10). The V_0V_1 structure of these pumps (Fig. 5-9) is similar to that of the F_0F_1-ATP synthases and they pump protons via a similar rotor and stator mechanism. Unlike F_0F_1, which animals express only in mitochondria, the particular Golgi organelle that V_0V_1 targets depends on the isoform of subunit a that is expressed [33].

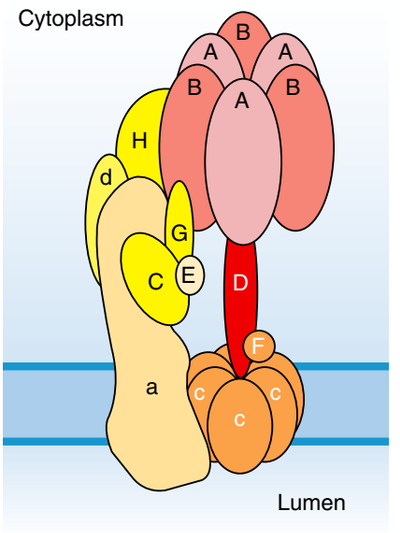

FIGURE 5-9 Structural organization of the vesicular proton pump, V-ATPase: V-ATPase is composed of numerous subunits organized as illustrated by color coding: the cytoplasmic domain, V1, contains eight different subunits (A–H), with three copies of the ATP binding subunits A and B; the membrane domain, termed V_0, is a hexameric ring composed of proteolipid subunits (5 c and 1 c″) and single copies of subunits a and d. Functionally, V-ATPase consists of a rotor (*white lettering*) and a stator domain (*black lettering*). The rotor consists of the hexameric proteolipid c ring and a stalk made of subunits D and F; the stator consists of the remaining subunits, which are fixed to the membrane via subunit a. Expression of different subunit a isoforms targets the V-ATPase to different membranes. ATP binding to and hydrolysis by the three subunits A presumably act on subunit D to induce rotation via asymmetric conformational transitions as has been described for the F_0F_1 ATP synthase. Rotation of the hexameric c ring translocates protons from the cytoplasmic interface of the proteolipid hexamer into the lumen of synaptic or Golgi-derived vesicles. The V_1 domain reversibly dissociates, in physiological conditions, to regulate V-ATPase activity. (With permission from reference [86].)

ATP-BINDING CASSETTES

The ABC transporters are products of one of the largest gene superfamilies. Each consists of two cytoplasmic nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). The NBDs are highly conserved across the ABC family and contain motifs typical of ATP-binding sites, whereas the TMD structures vary, probably because they are adapted to the wide variety of substrates. In eukaryotes the C-terminal of each NBD is linked to a TMD. In some cases the functional unit is $(NBD\text{-}TMD)_2$ and, in others, the first TMD is covalently linked to the second NBD.

The three-dimensional structures of several ABC transporters from prokaryotes have been determined.

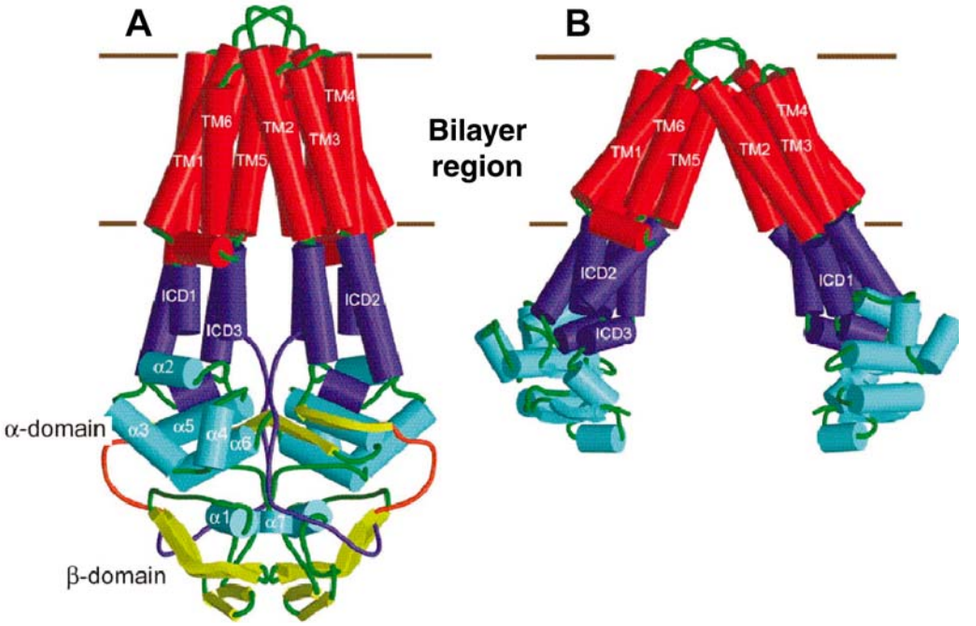

FIGURE 5-10 (**A**) Structure of a multidrug resistance ABC transporter, VC-MsbA, expressed by *Vibrio cholerae*. View looking into the chamber opening (side view). The transmembrane domain (TMD), intracellular domain (ICD) and nucleotide-binding domain (NBD) are colored red, dark blue and pale blue respectively. The transmembrane and NBD α-helices are indicated and the connecting loops are shown in green. The loop connecting the α- and β-domains of the NBD is shown in orange. The ATP-binding β-sheets are highlighted in yellow. Brown lines indicate the approximate location of the cell membrane. (**B**) A corresponding view of a very similar ABC transporter expressed by *Escherichia coli*, Eco-MsbA. Crystals of these two protein were obtained that exhibit closed and open conformations, respectively. (With permission from Fig. 2 of reference [87].)

Figure 5-10 compares NBD structures from nearly identical transporters from *Vibrio cholerae* and *Escherichia coli* that crystallized with their TMDs in two 'closed' and 'open' conformations respectively. Hypothetically, substrate would be acquired and transported as a result of energizing a conformational cycle by coupling it to ATP hydrolysis.

In the closed conformation (Fig. 5-10A) the two NBD domains bind to each other and, as shown in Figure 5-11, nucleotide binding occurs in pockets formed between these two domains. The stoichiometry and nucleotide selectivity of these binding pockets have been determined for an ABC transporter that functions to export peptides from yeast mitochondria [34]. The results of this study has led to the proposed reaction cycle diagrammed and described in Figure 5-12.

ABCA1 functions to translocate cholesterol and phospholipids outward across the plasma membrane after their delivery to the inner plasma membrane leaflet via vesicular pathways [35]. This occurs in astrocytes and developing neurons. Astrocytes also secrete apoE. Extracellular apoE binds and interacts with ABCA1 to promote cholesterol and PC efflux from cultured astrocytes through a mechanism that results in apoE-stabilized

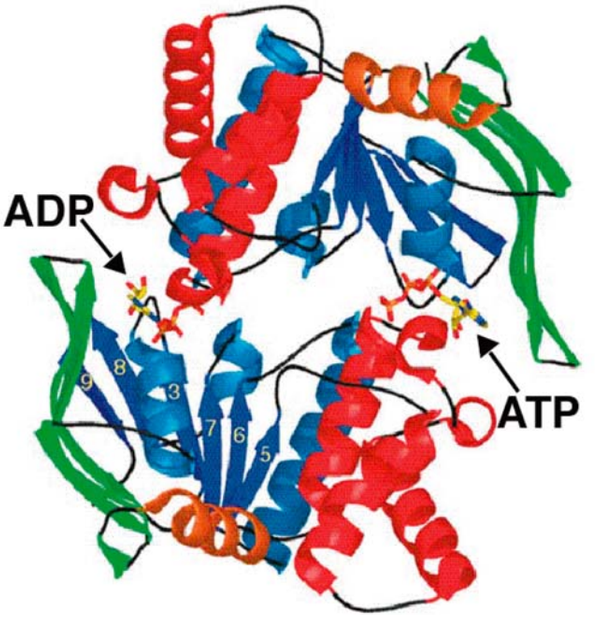

FIGURE 5-11 Ribbon diagram of an NBD dimer (PDB 1f30). β strands are depicted as arrows and α helices as coiled ribbons. The two nucleotides, shown as stick models, bind to form part of the interface that stabilizes the dimeric interaction. (With permission from Fig. 5 of reference [88].)

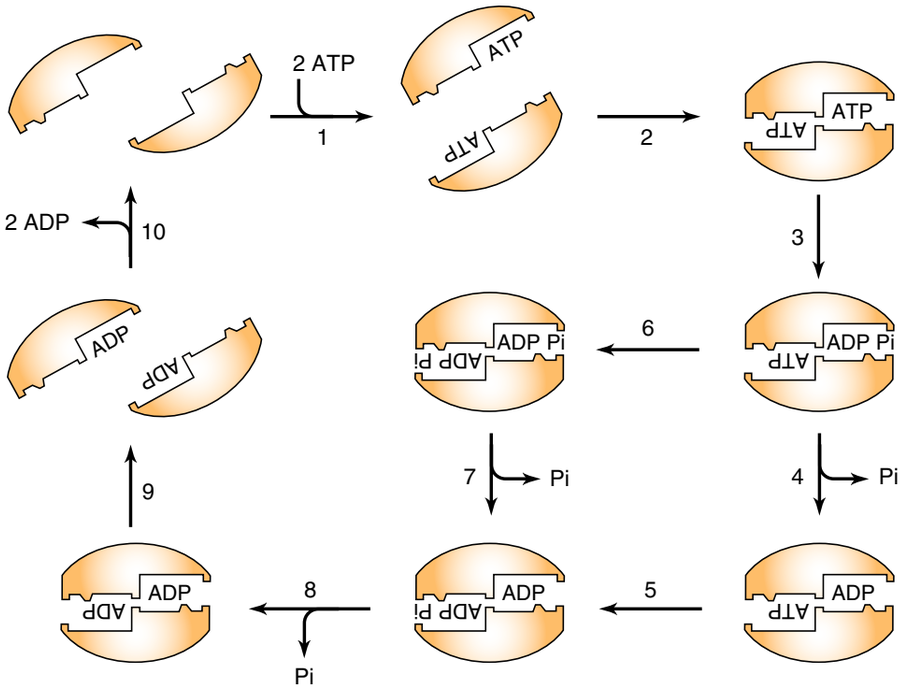

FIGURE 5-12 A processive clamp model for the ATPase cycle of the ABC transporter Mdl1p. ATP binding (*step 1*) on NBD domains of both monomers induces formation of the dimer (*step 2*). After ATP hydrolysis by the first NBD (*step 3*), either the P_i is released first (*step 4*), followed by hydrolysis of the second ATP (*step 5*) and release of the second P_i (*step 8*), or the second ATP is hydrolyzed first (*step 6*) and then both phosphates are set free (*steps 7* and *8*). After both ATPs are hydrolyzed to ADP and both phosphates are released, the dimeric complex dissociates (*step 9*) and ADP (*step 10*) is released. The hydrolysis cycle can then start again with ATP binding. (With permission from Fig. 7 of reference [34].)

HDL-like particles (see Fig. 2–7). In the absence of apolipoprotein, ABCA1 is rapidly proteolyzed by calpain with a $T_{1/2}$ of only about 20 minutes in some cells. However, apolipoproteins protect ABC1A from calpain and increase its level of expression [36]. Thus the interaction of apolipoprotein with ABCA1 is part of the regulatory process of cholesterol efflux. A closely related transporter, ABCA2, is expressed in oligodendrocytes and is markedly upregulated during myelinization [37].

Another ABC subfamily, the multidrug-resistance proteins (MDR), can 'flip' amphipathic molecules, including membrane phospholipids and sphingolipids, from the inner to the outer leaflet of plasma membranes [38]. One member, MDR3, can selectively transport phosphatidylcholine [39]. MDR1or 'P-glycoprotein' is the classic MDR, consisting of two cassettes within a single peptide chain. By pumping the drugs out of the cells its elevated expression during chemotherapy can decrease the chemosensitivity of cancer cells. P-glycoprotein is expressed at high concentrations in the luminal membranes of brain capillaries [40] and probably accounts for many drug-exclusion functions of the blood–brain barrier. Adrenoleukodystrophy and the Zellweger's syndrome (Ch. 41) result from defects in two different genes that specify 'single cassette' proteins targeted to peroxisomes.

SECONDARY ACTIVE TRANSPORT

Secondary transporters employ energy stored in ion gradients to transport other ions and molecules. Many of these are symporters or antiporters linked to Na^+ or proton gradients (Fig. 5-1). However the situation is often more complex, involving more than one ion gradient. A more systematic nomenclature for secondary transporters has recently been adopted. It is based on genomic analyses of the evolutionary relationships among these transporters which are members of a large 'solute carrier family'(SLC) [41]. Examples are discussed in the following sections.

The recovery of neurotransmitters from synaptic clefts and their storage in cytoplasmic vesicles is accomplished by the tandem actions of the secondary transporters in plasma and vesicular membranes. Sodium-dependent symporters mediate neurotransmitter reuptake from synaptic clefts into neurons and glia, whereas proton-dependent antiporters concentrate neurotransmitters from neuronal cytoplasm into synaptic vesicles (Fig. 5-13).

While the extent of the demands on cerebral energy metabolism of neurotransmitter reuptake is still somewhat controversial, it contributes substantially to the

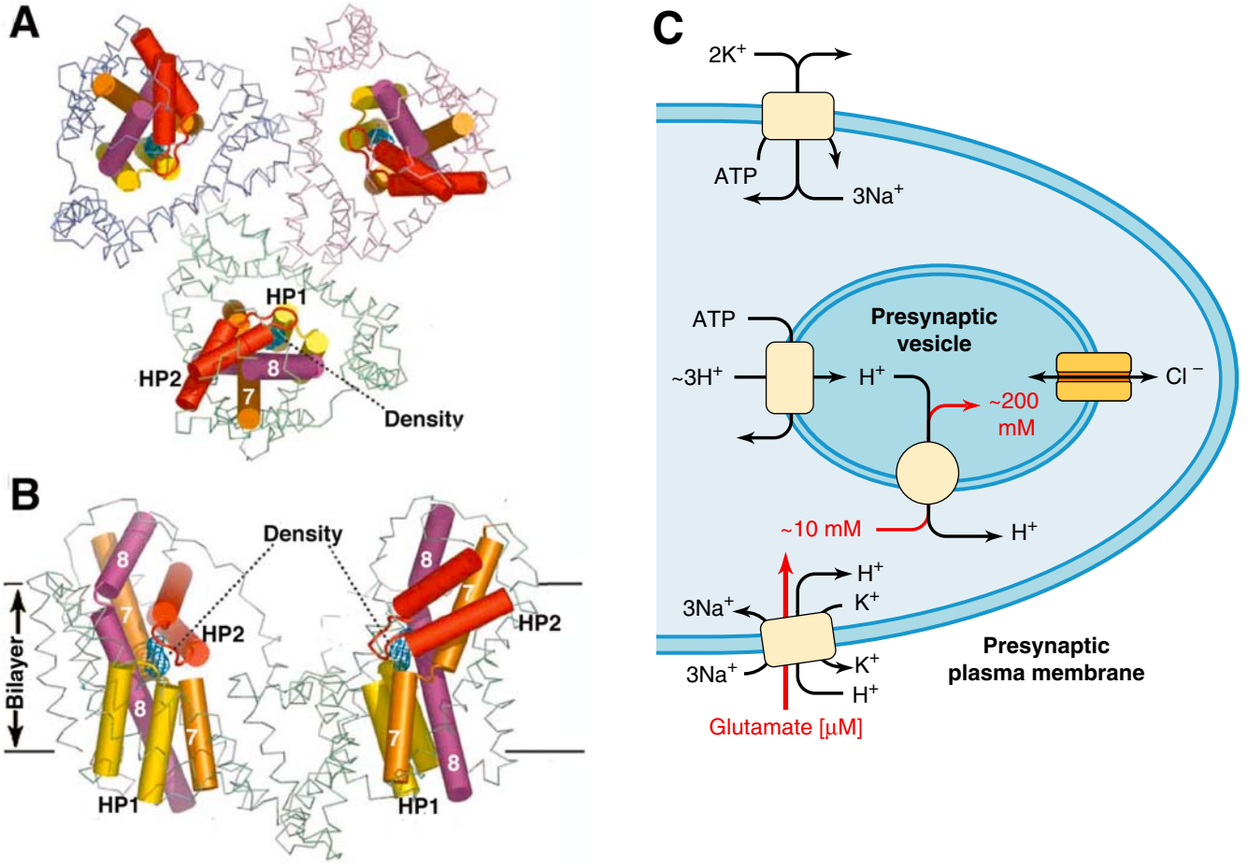

FIGURE 5-13 (**A, B**) Structure of a glutamate transporter. This bacterial glutamate transporter provides the first high-resolution model of a glutamate transporter [88]. The X-ray data indicate a trimeric structure. (**A**) A view of the trimer extracellularly and perpendicular to the bilayer. (**B**) A view from the bilayer plane. The trimer forms a deep bowl that allows extracellular solutes to access the extracellular glutamate-binding site located near the center of the bilayer plane. The helices represented by colored cylinders are those involved in the structure of the ionophoric sites, which involve transmembrane helices 7 and 8 and two re-entrant loops, HP1 (yellow) and HP2 (red), that enter from opposite sides of the bowl. The objects marked 'Density' appear to be glutamates bound at the ionophoric sites. The authors discuss this structure in terms of a model in which each bound glutamate is carried into the cytoplasm by obligatory coupling to the symport of 3 Na^+ and 1 H^+. (With permission from reference [88].) (**C**) Processes involved in neurotransmitter uptake and packaging at nerve endings. Concentration into the cytoplasm is achieved by means of Na^+ symporters with high affinity and specificity for the neurotransmitter in the plasmalemma. As indicated in red, μM extracellular glutamate is concentrated in mM levels in cytoplasm and to about 200 mM in the vesicles. Synaptic vesicle membranes contain the V-ATPase proton pump (Fig. 5-9) and chloride channels, which together acidify the internal vesicle space and generate a proton gradient that drives the neurotransmitter-selective antiporters.

increased metabolism associated with neuronal activity [42] (see Ch. 31). There are two distinct subfamilies of Na^+-dependent transporters that function in plasma membranes. One subfamily, SLC6, is characterized by 12 transmembrane segments and includes the (Na^+,Cl^-)-dependent transporters for gamma-amino butyric acid (GABA), glycine, norepinephrine, dopamine, serotonin and histamine.

The other subfamily, SLC1, includes the Na^+-dependent glutamate transporters. It encompasses some amino- and carboxylic-acid transporters including glutamate transporters that are expressed in bacteria. X-ray diffraction data have been obtained from crystals of one of these [43] (Fig. 5-13). Analysis of multiple sequence alignments indicates that this molecule has a high degree of structural similarity to the glutamate transporters that are expressed in brain. Each subunit appears to contain an ionophoric site that can bind glutamate, probably in association with three Na^+ and one H^+. This site is located near the central plane of the bilayer and involves two of the eight transmembrane helices plus two 're-entrant loops', one from each side, which may act as 'access gates' that open sequentially to permit binding and dissociation within each transport cycle.

Glutamate transporters in brain are coded by five different but closely related genes, *SLC1A1–4* and *SLC1A6*. There are several trivial names for each of the corresponding proteins. The transporters can all symport one Glu, with three Na^+ and one H^+, and antiport one K^+ within each cycle, but they differ in their cellular expression.

Different isoforms have different regulatory interactions and are expressed in different cell types. Astrocytes recover most of the synaptically released glutamate via the transporters GLT1/EAAT2 (SLC1A2) and GLAST/EAAT1 (SLC1A3). They convert much of this to glutamine, which is recycled to neurons (see Chs 15 and 31), probably by Na^+- or H^+-coupled members of the SLC38 gene family. A mutant form of SLC1A2 has been associated with amyotrophic lateral sclerosis (see Ch. 44). Mice [34] and rats [35] deficient in GLAST or GLT-1 have elevated extracellular glutamate and, in the case of mice, have lethal seizures [44].

Regulation of glutamate concentration is of special interest because its extracellular accumulation is a factor in various pathologies (see Ch. 32). While brain total glutamate concentration is about 10 mmol/l, extracellular glutamate, as measured by *in vivo* microdialysis, is normally only 3–4 μmol/l. Glutamate within the synaptic cleft must be maintained at still lower concentrations in the resting state. With 'normal' membrane potentials and ion concentrations, the glutamate transporter can theoretically reduce this value to 0.6 μmol/l or less. However, under depolarizing or anoxic conditions, the membrane potential and the Na^+ and K^+ gradients can decrease to levels that cause the symporter to fail or to operate in reverse, producing 100- to 1,000-fold increases in extracellular glutamate.

In addition to their transporter function, perisynaptic transporters function as buffers to confine the extracellular free glutamate to receptors nearest the postsynaptic release sites. The total concentration of glial transporters in some CNS synaptic regions is estimated to be sufficient to bind the glutamate content of three to five vesicles per synapse [45]. This 'buffering', which occurs mostly in endfeet of astrocytes, is essential for reliable synaptic transmission of high-frequency signals [46].

GABA reuptake is mediated by the secondary transporters, GAT1–4 (SLC6A1–4), that are expressed in GABAergic neurons. The SLC6 family also includes the transporter genes for catecholamines, serotonin, histamine and glycine. All are energized by Na^+ symport. Chloride is cotransported but, because in most cells it is passively distributed across the plasma membrane, chloride diffusion does not supply energy to the system. Many of these transporters are expressed at high densities in perisynaptic neuronal plasma membrane. However, as much as half of the transporters are found within small endocytotic vesicles that recycle in parallel with the neurotransmitter vesicles [47, 48]. Recycling between vesicles and membranes appears to be a mechanism for rapidly regulating the populations of many different integral membrane proteins. The catecholamine and serotonin transporters are inhibited by a variety of drugs, both therapeutic and addictive [49].

The synaptic action of acetylcholine is unique among neurotransmitters in that it is terminated by hydrolysis rather than by transport (Ch. 11). Consequently, cholinergic neurons recover choline, rather than acetylcholine, via a high-affinity choline transporter, CHT-1. A major fraction of these CHT-1 transporters reside in presynaptic vesicles and are only incorporated into the presynaptic membranes concurrently with the release of acetylcholine [50]. This is a form of regulation somewhat analogous to that observed for the SLC6 family noted above. Structurally, CHT-1 is most similar to the Na^+-dependent glucose transporters and is classified within the SLC5 gene family.

Packaging neurotransmitters into presynaptic vesicles is mediated by proton-coupled antiporters. As discussed above, Golgi-derived membranes, including presynaptic vesicle membranes, contain V-type primary transporters that pump protons into the lumen of these membranes and vesicles.

Glutamate packaging antiporters are VGLUT1, -2, and -3. VGLUT1 and -2 are principally localized in glutamatergic neurons and exhibit high specificity, but low affinity (≈1 mmol/l) for cytoplasmic glutamate because the concentration of cytoplasmic glutamate is high. Some indication of the functional significance of different isoforms can be inferred from demonstrations that VGLUT2 is expressed in some dopaminergic neurons whereas VGLUT3 vesicles occur in some serotonergic and GABAergic interneurons. It has been suggested that such neurons may release glutamate as an autoregulator acting on metabotropic presynaptic receptors [51].

The GABA-packaging antiporter, VGAT or VIAAT, was first identified by the characterization of a *Caenorhabditis elegans* mutant gene that produced a phenotype that mimicked ablation of an identified GABA neuron. The mammalian ortholog of this gene functions in both GABAergic and glycinergic neurons. It is a proton antiporter and, like the VGLUTs, has relatively low affinity for its substrates, GABA and glycine.

The various monoamine neurotransmitters are packaged in vesicles by the relatively nonspecific antiporters VMAT1 (SLC18A1) and VMAT2 (SLC18A2). Neurotransmitter selectivity in these cases is evidently determined by the expression of the biosynthetic enzymes. Acetylcholine packaging in synaptic vesicles of both central and peripheral neurons is mediated by the same antiporter, VAChT (SLC18A3) [52]

PHYSIOLOGICAL ASPECTS OF THE NEUROTRANSMITTER TRANSPORTERS

Presynaptic vesicles are estimated to each contain approximately 3,000–5,000 transmitter molecules; the internal volume of a vesicle is such that its internal transmitter concentration may be ≈0.5 mol/l; vesicle contents may be released within less than 1 ms and this occurs within <0.02 μm of the postsynaptic receptors. The space within

a synaptic cleft of ≈1 μm² will be ≈1000-fold greater than the vesicle volume, so that the instantaneous concentration is more than 1 mmol/l. Because only 3–4 free molecules within the volume of such a cleft is effectively ≈1 μmol/l, rapid inactivation of a postsynaptic receptor requires removal of essentially all the transmitter. Glutamate transporters have affinity constants of more than 10 μmol/l, and transport reaction cycles are slow relative to impulse rates. This problem appears to be solved by having an extremely high density of transporters in the astroglial processes immediately adjacent to CNS glutamatergic synapses. The densities of astroglial transporters in processes near glutamatergic synapses are estimated to be 5,000–10,000/μm² [45]; binding of glutamate to transporters is an important factor in the rapid (ms) removal of glutamate and thus the distribution of transporters can regulate the accessibility of glutamate to extrasynaptic receptors [53].

Cytoplasmic chloride must be transported outward to generate the gradient that allows GABA- or glycine-gated channels to hyperpolarize neurons. This transport, in mature neurons, is mediated by a K^+,Cl^- symporter, KCC2. However, immature neurons accumulate chloride via a Na^+,Cl^- symporter, NKCC1, and thus activation of $GABA_A$ channels is depolarizing [54]. The ratio of KCC2/NKCC1 expression increases with maturation in most neurons. However this ratio can be regulated in mature neurons: For example, in suprachiasmatic neurons this ratio is controlled by clock genes and in other neurons by endocrine cycles.

In the 'normal' state of mature neurons, the K^+ diffusion potential is similar to the membrane resting potential and thus has little ability to energize KCC2. However, K^+ released during membrane depolarization may transiently activate KCC2 symport, lower cytoplasmic [Cl^-] and increase the inhibitory postsynaptic current through $GABA_A$ channels. However, excitation also elevates cytoplasmic [Ca^{2+}]; this elevation apparently regulates KCC2 and is the basis of inhibitory 'coincidence detection' that has been demonstrated in hippocampal neurons: this coincidence is that of excitatory impulses arriving 'simultaneously' with $GABA_A$ channel activation at inhibitory synapses [55].

The anion antiporters comprising the SLC8 gene family all transport bicarbonate [56]. CNS energy production derives almost entirely from aerobic glycolysis resulting in a rate of metabolic CO_2 production nearly equal to the rate of oxygen consumption. In adult human brain, this is about 1.5 mmol/l per minute (Ch.31). Because most neurons express only low concentrations of carbonic anhydrase, CO_2 diffuses out of neurons mostly unhydrated, but is converted to HCO_3^- before it enters the blood. The Cl^-/HCO_3^- antiporter, AE1/(SLC4A1), also known as band 3 protein, is a major protein of erythrocytes, where it mediates rapid uptake of HCO_3^- in exchange for Cl^- and functions in the reverse direction in lungs to exchange HCO_3^-. An isoform of this antiporter (AE3) is expressed in neurons, which suggests that significant anion exchange occurs across neuronal and/or glial membranes. Since some neurons express significant concentrations of cytoplasmic carbonic anhydrase, Cl^-/HCO_3^- exchange may have unrecognized functions: its operation in neurons could influence both Cl^- potentials and cytoplasmic pH.

CATION ANTIPORTERS

Na^+,Ca^{2+} exchangers are important for rapidly lowering high pulses of cytoplasmic Ca^{2+}. They can remove cytoplasmic Ca^{2+} up to ten times faster that SERCA or PMCA pumps. Three Na^+,Ca^{2+} antiporter isoforms, NCX1–3, are expressed in brain: NCX1 and NCX3 in neurons, NCX2 predominantly in glia. As discussed above, these exchangers colocalize with the sodium pump isoforms α2 and α3 in neurons and astrocytes to form junctional complexes with the ER (Fig. 5-6). The NCX exchange process is electrogenic with three Na^+ exchanged for one Ca^{2+}.

Intracellular pH in brain is regulated by Na^+, H^+ antiporters, anion antiporters and Na^+,HCO_3^- symporters. In principle the products of cerebral energy metabolism, primarily water and CO_2, may leave the brain and enter the circulation without disturbing the ionic balance. However processes such as incomplete oxidation, CO_2 hydration or Ca^{2+} influx can change local pH. Neuronal activity is accompanied by intracellular acidification and extracellular alkalinization. Cerebral metabolic rate decreases with decreasing pH so that, without adequate pH control, local metabolic deficits can be amplified and propagated [57]. The pH response of astrocytes to membrane depolarization is opposite to the neuronal response: intracellular alkalinization and extracellular acidification occur [58]. The neuronal acidification appears to involve antiporter exchange of HCO_3^- for Cl^-, whereas astrocytic alkalinization is probably mediated by a Na^+, HCO_3^- symporter. This is an oversimplification of CNS pH regulation in view of the fact that additional secondary transporters are involved in various aspects of local pH regulation [59].

Na^+,H^+ antiporters (NHE) occur in synaptosomes, glia and neuroblastoma cells [60] (Fig. 5-8B). They are relatively inactive at neutral pH but with a decrease in intracellular pH they produce an efflux of protons at the expense of the Na^+ gradient. The NHE transport stoichiometry is 1:1. Activation by an internal pH decrement apparently results from protonation of a cytoplasmic site, which allosterically increases the affinity of the proton ionophoric site. In some cells, the NHE is under additional control by receptor mechanisms. Several growth factors and hormones produce transient cytoplasmic alkalinization, probably by mediating a protein kinase

phosphorylation of the antiporter, which increases its affinity for cytoplasmic protons.

Rapid clearance of K^+ from neuronal extracellular space is critical because high extracellular K^+ depolarizes neurons. Neural activity discharges K^+ which, if not rapidly removed, lowers the membrane potential of adjacent neurons. Normal neuronal activity can lead to elevations of 1–3 mmol/l in extracellular K^+ ($[K^+]_e$) but, during epileptogenesis, concentrations can be three to four times higher (see Ch. 37). Usually, the upper limit of axonal impulse-conduction frequency is set by a conduction block that occurs when extracellular K^+ accumulates to a concentration of 25–30 mmol/l (see Ch. 37). Both neurons and astroglia are involved in K^+ uptake but their relative contributions remains controversial. It is clear that neurons accumulate K^+ almost exclusively by means of active transport, but the Na,K pump is slow relative to the channel-mediated K^+ release from neurons and to the rates of K^+ increase in the extracellular space. Moreover, the neuronal Na^+,K^+ pump is saturated at low $[K^+]_e$, making it an ineffective regulator for this purpose.

Glial processes invest nearly all extrasynaptic neuronal surfaces (see Ch. 1), and one of their principal functions is to regulate $[K^+]_e$. The extracellular space of the brain consists primarily of the 150–250 Å clefts separating glia and neurons. Astrocytes have higher K^+ permeability than most neurons, and they are extensively interconnected by gap junctions. K^+ efflux from a neuron can diffuse into the local glial cytoplasm, and compensatory K^+ efflux can occur from more distal reaches of the glial syncytium, where the $[K^+]_e$ is lower (Fig. 5-14). It should be noted that this 'spatial buffering' is a diffusion process that requires no energy input. Measurements on retinal Müller cells have provided relevant data. These are specialized astrocytes that do not form syncytia. Rather, they extend from the deep layers of the retina to form endfeet at the retinal interface with the vitreous body (see Fig. 49-1). The K^+ conductance of these cells is about 30 times higher at the endfeet in contact with the vitreous fluid than elsewhere. Moreover, K^+ currents through plasma membranes other than endfeet are carried by inward-rectifying channels, whereas the K^+ channels at the endfeet are high-conductance and nonrectifying [40]. This seems designed to facilitate diffusive currents of K^+ from the retina into the Müller cell, which are balanced by K^+ efflux into the vitreous space and constitute a relatively 'infinite' sink, equivalent to the syncytium of the generalized astrocytes. Normally Na,K pumps in neurons and glia set the baseline extracellular $[K^+]$, and partial inhibition of these pumps can markedly slow its return to baseline levels following high-frequency neuronal activity [61].

Although spatial buffering can effectively prevent accumulation of moderate concentrations of extracellular K^+, this diffusion process may be inadequate at very high rates of neuronal K^+ efflux. Several K^+ symporters occur in astrocytes and probably broaden the range of effective $[K^+]_e$ regulation. Glial swelling accompanies neural activity. This reflects the operation of an NKCC symporter in response to the volume-regulation mechanism discussed below.

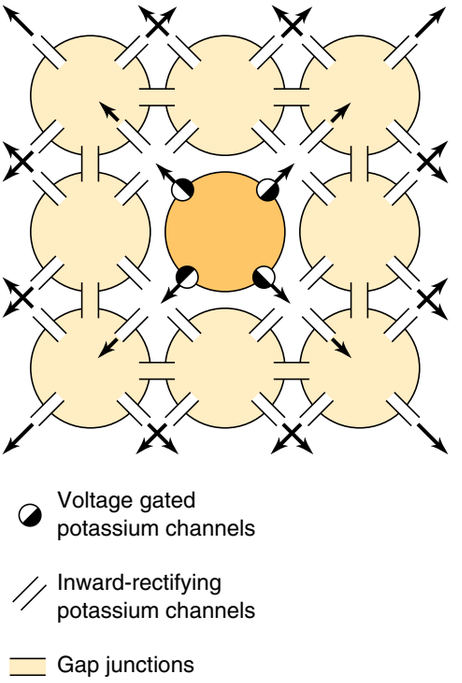

FIGURE 5-14 Spatial buffering by astrocytes. This conceptual diagram indicates the pathways available for potassium ions to diffuse through the glial syncytium (light red) subsequent to their release from neuronal membranes (dark red) during neural activity.

Cell-volume regulation involves control of the content of osmotically active impermeant molecules and ions. Brain oxidative glycolysis produces water at about 10 mmol/l per minute or ≈20 ml per brain-minute. Accordingly, water must be able to move rather freely between cells and blood. Normally most of this flow is through the choroid epithelia and enters the venous blood under positive pressure via valves in the arachnoid villi. Brain volume may change because of altered internal solute content, termed an isotonic response, or because of altered extracellular solute concentration, termed hyper- or hypotonic responses. Impairment of brain energy metabolism, such as may ensue from ischemia, anoxia or trauma, produces an isotonic cellular swelling called 'cytotoxic edema' and other effects (see Chs 32 and 34). Cellular swelling is also produced by extracellular hypotonicity, often caused by hyponatremia, which can result acutely from postsurgical fluid therapies. Chronic hyponatremia can arise from congestive heart failure, Addison's disease

or a 'syndrome of inappropriate vasopressin release'. A general reduction in brain size resulting from acute extracellular hypertonicity may produce neurological disturbances. This condition may be a component of pathology originating outside the brain, such as kidney disease (uremia) and diabetes mellitus (hyperglycemia). Central pontine myelinolysis, which is produced experimentally by acute infusion of hypertonic saline, can occur as a complication of liver transplantation and of too rapid reversal of hyponatremia in clinical situations.

Cells have short-term mechanisms that tend to restore their volume toward 'normal', primarily by shifting the distributions of Na^+, K^+ and Cl^-. The details of the mechanisms appear to vary with cell type. Subsequent to cell swelling, regulatory volume decrease usually involves opening ion channels. Subsequent to hyperosmotic shrinking, regulatory volume increase frequently involves activation of NHE and NKCC. The primary detectors of volume and osmotic changes are presently unknown. Although cell membranes can readily change shape, they do not stretch significantly. However, cytoskeletal proteins attached to membrane proteins might form tensile elements that could mediate volume changes. Cell shape is determined by interactions of cytoskeletal elements, membranes and components of the extracellular matrix. Stretch-activated cation channels have been detected in neuroblastoma cells and in cultured astrocytes. Activation of a Cl^- channel by cellular swelling is part of the regulatory volume decrease process, but the identity of the channel remains elusive [62]. Altered extracellular tonicity can induce compensatory changes in the concentrations of certain intracellular osmolytes through mechanisms involving gene regulation. Glioma cells in culture have been shown to respond after a few hours of exposure to hypertonic medium by transcribing high levels of the Na^+, inositol symporter. In brain, active osmolytes include taurine, glutamine, glutamate, myo-inositol, creatine, glycerophosphoryl-choline and choline. Na^+-dependent symporters for most of these compounds have been identified [63].

FACILITATORS

Simple diffusion of the polar water molecules through lipid bilayers is too slow to account for observed rates in some cells. Therefore, it was hypothesized that specific transporters or channels regulate rapid water movement across cell membranes. Peter Agre and colleagues in 1992 isolated and identified the first member of a family of water transport molecules, now called aquaporin1 (AQP1). Now, this family is known to have 11 members found in mammals and about 150 members found throughout all biological species. The aquaporin family consists of two subsets of channel proteins, the classical group permeated only by water, and a second group, aquaglyceroporins, permeated by water or glycerol and other small molecules such as urea [64, 65].

Cell membranes or synthetic lipid vesicles with normal low permeability to water will, if reconstituted with AQP1, absorb water, swell and burst upon exposure to hypoosmotic solutions. The water permeability of membranes containing AQP1 can be about 100 times greater than that of membranes without aquaporins. The water permeability conferred by AQP1 (about 3 billion water molecules per subunit per second) is reversibly inhibited by Hg^{2+}, exhibits low activation energy and is not accompanied by ionic currents or translocation of any other solutes, ions or protons. Thus, the movement of water through aquaporins is an example of facilitated diffusion, in this case driven by osmotic gradients.

The most crystallographic and architectural data are available for AQP1, which is a prototype for the other aquaporins. The 28 kDa protein consists of six membrane-spanning α-helixes with both the carboxyl and amino terminals on the cytoplasmic surface. It exists in membranes as a homotetramer. Each monomer contains a central water channel. Water molecules can enter the channel from either the extracellular or intracellular vestibules. The narrowest segment of the channel, near its center, is 2.8 Å in diameter, which is the van der Waals diameter of a single water molecule. Passage of molecules through this pore is controlled by size restriction and electrostatic repulsion. A positively charged arginine residue near the pore obstructs protonated water and other cations while two positively charged dipoles at the midpoint of the pore disrupt hydrogen bonds and prevent protons from passing through the pore.

The aquaporin isoforms have different distributions in various tissues or organs and often the site of localization suggests the function. The aquaporins so far found in brain are: AQP1 in choroid plexus; AQP4 in astrocytic processes; and AQP9, an aquaglycerol transporter, in astrocytes. Neurons have not yet been found to contain any aquaporin.

Cerebrospinal fluid formation and regulation of its rate and composition are dependent on a number of transporters. In choroid plexus, AQP1 is localized to the apical surface of the epithelium where Na,K-ATPase, $Na^+/K^+/2Cl^-$ co-transporter and HCO_3^- channels are also situated. The basolateral membranes contain Cl^-/HCO_3^- and Na^+/H^+ exchangers. The Na,K-ATPase pumping Na^+ out of the epithelial apical surfaces into the ventricle provides the energy driving the osmotic gradient that extrudes water from epithelial cell apical surfaces through the AQP1 transporter.

AQP4 is the major water facilitator in brain and is most concentrated in astrocyte endfeet, but AQP4 has not been found in neurons. It is localized to basolateral surfaces of ependymal cells and along the entire plasmalemma of astrocytes, including processes that ensheath

synapses. It is not found in oligodendrocytes or microglia but is observed in brain endothelial cells. The presence of AQP4 in perivascular and subpial membranes suggest that it functions in the exchange of water between brain and blood and that it may be a component of the blood–brain barrier. In AQP4 knockout mice, brain edema after experimental stroke or hypoosmotic stress is less than in wild type mice, indicating that the influx of water into brain is at least partially through the AQP4 channels, and that AQP4 channels facilitate water influx under conditions that produce edema. But, in contrast, after intraparenchymal fluid infusion, freeze injury or implanted neoplasm, mice lacking AQP4 develop even greater brain edema and have worse clinical outcomes than do wild type mice after similar treatment [66, 67].

The distribution of AQP4 is localized to astrocytic endfeet membranes by anchoring to a complex of proteins, including dystrophin or α-syntrophin (α-syn), that connects the cell cytoskeleton to the extracellular matrix (see Chs 2 and 8). The anchoring proteins maintain the AQP4 in regular orthogonal arrays that can be visualized by electron microscopy in the endfeet membranes. In dystrophin- or α-syn-null mice, AQP4 content is not reduced but it is diffusely distributed in astrocyte membranes, largely reduced in perivascular endfeet but not from subpial endfeet, other astrocytic processes or ependymal cells. In these animals, development of brain edema after hypoosmotic stress or ischemia is delayed and attenuated relative to the edema in wild-type mice. In addition, in hippocampus of α-syn-null mice, in which 90% AQP4 of perivascular end feet is lost, recovery of extracellular K^+ concentrations after stimulation is delayed. This condition can decrease the threshold for seizures. Perivascular endfeet in α-syn-null mice under basal conditions consistently exhibit swelling, suggesting that the AQP4 in endfeet facilitates efflux of the water generated by glucose metabolism. Hence, AQP4 can facilitate water transport in either direction depending on the osmotic gradient. In retinal Mueller glia, AQP4 is colocalized with Kir4.1 channels involved in K^+ clearance, suggesting that these molecules function in concert to promote K^+ and water flow from extracellular fluid through these cells into the peripheral 'sink'. In this view, water can flow from the extracellular compartment through the AQP4 on non-endfeet plasmalemma into astrocytes and through the endfeet pool of AQP4 into the blood circulation [67]). This water flux together with K^+ flux through Kir4.1 channels constitute the K^+- buffering function of astrocytes discussed above and represented in Figure 5-14.

The binding of α-syn to AQP4 is sensitive to ischemia, such that, in the postischemic stage, the othogonal arrays of AQP4 normally characteristic of the endfeet perivascular membranes are lost, although the total level of α-syn is unchanged. Edema is attenuated in the α-syn-null mice. This loss of AQP4 from perivascular endfeet might retard the extent of water influx but would delay the resolution of edema through perivascular membranes. Further information regarding the kinetics of edema formation and resolution under these conditions is necessary to understand the regulation. Since the anchoring protein is critical to the specific localization of the AQP4, its level or binding characteristics also may be involved in regulating the AQP4 activity and function.

AQP4 is also distributed in a nonpolarized pattern in astroglial lamellae of the osmosensitive supraoptic nuclei and circumventricular organs. These glia, when subjected to hypoosmotic stress, release taurine on to glycine receptors on osmosensitive neurons, which, when activated, in turn lead to reduced secretion of antidiuretic hormone [68]. AQP9 is found in third ventricle ependyma, including the tanycytes, while mRNA for AQP9 is also seen in astrocytes and endothelial cells. Its function in brain is not understood.

A number of toxic conditions beside hypoosmotic stress and hypoxia/ischemia produce brain edema and the causes may be related to regulation of the aquaporins (see Ch. 34). AQP1 and AQP4 are intensely upregulated in reactive astrocytes in subarachnoid hemorrhage [69], in human glioma and astrocytoma [70] and AQP4 in endothelia and reactive astrocytes in metastatic carcinoma [71, 72].

Knowledge of the regulation of aquaporins is expected to provide clues as to potential therapy for brain edema. Mannitol is often used clinically as an hyperosmotic agent to reduce cerebral edema. The mannitol effect might be related to its induction of expression of AQP4 and AQP9 in cultured astrocytes and in cortex after intraperitoneal infusion. An inhibitor of p38 MAPK suppressed expression of these aquaporins in cultured astrocytes [73]. Anchoring proteins that regulate aquaporin localization are also targets for therapeutic intervention. New pathways for a molecular classification of brain edema and investigation of possible means for treating brain edema have been opened by these discoveries.

Facilitated diffusion of glucose across the blood–brain barrier and into brain cells is catalyzed by GLUT1, -2 and -3, products of the SLC2 gene superfamily. The SLC2 (solute carrier) superfamily consists of 12 glucose transporters (GLUT1–12) and one H^+-myo-inositol cotransporter (HMIT or GLUT13). They all have 12 transmembrane segments with the N- and C-termini both on the cytoplasmic aspect and a specific N-linked oligosaccharide side-chain on either the first or fourth extracellular loop.

The K_m of GLUT1 is 1–2 mmol/l for glucose influx and 20–30 mmol/l for efflux. A proposed transport mechanism evokes mutually exclusive glucose binding to sites internal and external to the plasmalemma; glucose binding to the extracellular side would induce a conformational change involving transport of the glucose. Possibly GLUT1 exists in membranes as a conformationally asymmetric dimer or tetramer (see [74] for a complete review).

GLUT1 is predominantly expressed in brain endothelial cells of the blood–brain barrier and on the astrocytic endfeet covering the abluminal surface of capillaries. GLUT1 is expressed also in Schwann cells and perineurium. GLUT1 is essential for glucose delivery from blood into brain through the astrocyte endfeet. Astrocytes metabolize glucose to lactate, which can be transferred into neurons via the monocarboxylate transporters, MCT1 and MCT2 (see also Ch. 31). A hereditable mutation in the gene for GLUT1 results in a limited glucose supply to the brain and a syndrome of infantile epilepsy, acquired microcephaly and hypoglycorrhachia. A number of milder phenotypic variants have been described [75]. It is noteworthy that, despite its vital dependence on an adequate glucose supply (Ch. 31), the brain relies for its supply of glucose on facilitators rather than active transport, and this is largely regulated by local blood flow (Chs. 31, 58).

GLUT3 occurs in neurons, mainly in plasmalemma and to a lesser extent in nonsynaptic intracellular vesicles. GLUT2 and glucokinase are believed to be part of a glucose-sensor system in certain neurons that modulate feeding behavior and regulate glucose uptake by peripheral tissues: their mRNAs are expressed in human ventromedial and arcuate nuclei of the hypothalamus [76]. GLUT8 is expressed in certain brain regions, including hippocampus, amygdala, olfactory cortex, hypothalamus, brainstem and posterior pituitary nerve endings. It is mainly observed in intracellular vesicles and may be translocated to the plasmalemma by as yet unknown regulatory stimuli. This endocytic recycling model of regulation may apply to others of the GLUT family. For example, GLUT 4, in skeletal and cardiac muscle and adipose tissue, occurs mostly in an intracellular vesicle compartment. Insulin activates its rapid translocation to the cell surface resulting in increased cellular uptake of glucose. Exercise also regulates glucose uptake through trafficking of GLUT4 to the cell surface, but by an insulin-independent mechanism. Deficiencies in GLUT4 may underlie some variants of diabetes type 2 and insulin resistance as well as some skeletal or cardiac muscle disorders [74].

HMIT is a H^+-coupled myo-inositol symporter. High levels of its expression are observed in neurons and glia of hippocampus, hypothalamus, cerebellum and brainstem. Since *myo*-inositol is a precursor for phosphatidyl inositol, which itself is a critical regulator of many neuronal processes (Ch. 20), HMIT regulation is possibly involved in various mood and behavior patterns that are affected by inositol metabolism and by pharmacologic agents that modify inositol metabolism (see Chs 54 and 55).

Brain capillary endothelial cells and some neurons also express a Na^+-dependent D-glucose symporter, SGLT1. SGLT1 (SLC5A1) was the first characterized of the large SLC5 family of Na-dependent symporters (SSSF) which transport various solutes and ions into cells [77, 78]. SGLT1 is found mainly in the intestine, trachea, kidney and heart. It cotransports both glucose and galactose. A genetic disease producing glucose–galactose malabsorption and severe diarrhea is related to mutations in the SGLT1 gene [79]. In brain endothelium, SGLT1 expression is limited to the luminal membranes of brain capillary endothelial cells. This suggests that SGLT1 and GLUT1 are both involved in blood glucose transport into capillary endothelia whereas glucose efflux from the endothelia into astrocytes and neurons depends primarily on GLUT1. The low affinity of GLUT1 for intracellular glucose ($K_m \approx 25$ mmol/l) may require SGLT1 to accumulate sufficiently high intracellular glucose to maintain an adequate rate of supply to the astrocytic endfeet [77]. It is of interest that SGLT1 expressed in oocytes transports water into cells in stoichiometric relation to the glucose symport. This is calculated to account for half of the 8 liters of water absorbed by human small intestine per day. This provides a rationale for the treatment of secretory diarrhea with oral salt solutions containing glucose. It has been proposed that cotransport of water is a general property of symporters [80].

REFERENCES

1. Glitsch, H. G. Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol. Rev.* 81: 1791–1826, 2001.
2. Besirli, C. G., Gong, T. W. and Lomax, M. I. Novel beta 3 isoform of the Na,K-ATPase beta subunit from mouse retina. *Biochim. Biophys. Acta* 1350: 21–26, 1997.
3. Peng, L., Martin, V. P. and Sweadner, K. J. Isoforms of Na,K-ATPase alpha and beta subunits in the rat cerebellum and in granule cell cultures. *J. Neurosci.* 17: 3488–3502, 1997.
4. Lecuon, E., Luquin, S., Avila, J., Garcia-Segura, L. M. and Martin-Vasallo, P. Expression of the beta 1 and beta2(AMOG) subunits of the Na,K-ATPase in neural tissues: cellular and developmental distribution patterns. *Brain Res. Bull.* 40: 167–174, 1996.
5. Feschenko, M. S., Donnet, C., Wetzel, R. K., Asinovski, N. K., Jones, L. R. and Sweadner, K. J. Phospholemman, a single-span membrane protein, is an accessory protein of Na,K-ATPase in cerebellum and choroid plexus. *J. Neurosci.* 23: 2161–2169, 2003.
6. Béguin, P., Crambert, G., Guennoun, S. *et al.* CHIF, a member of the FXYD protein family, is a regulator of Na,K-ATPase distinct from the γ-subunit. *EMBO J.* 20: 3993–4002, 2001.
7. Knapp, P. E., Itkis, O. S. and Mata, M. Neuronal interaction determines the expression of the alpha-2 isoform of Na, K-ATPase in oligodendrocytes. *Brain Res. Dev. Brain Res.* 125: 89–97, 2000.
8. Banerjee, B. and Chaudhurym, S. Thyroidal regulation of different isoforms of Na,K-ATPase in glial cells of developing rat brain. *Life Sci.* 69: 2409–2417, 2001.
9. Potaman, V. N., Ussery, D. W. and Sinden, R. R. Formation of a combined H-DNA/open TATA box structure in the promoter sequence of the human Na,K-ATPase alpha2 gene. *J. Biol. Chem.* 271: 13441–13447, 1996.
10. Murakami, Y., Ikeda, U., Shimada, K. and Kawakami, K. Promoter of the Na,K-ATPase alpha3 subunit gene is

composed of cis elements to which NF-Y and Sp1/Sp3 bind in rat cardiocytes. *Biochim. Biophys. Acta* 1352: 311–324, 1997.
11. Pathak, B. G., Neumann, J. C., Croyle, M. L. and Lingrel, J. B. The presence of both negative and positive elements in the 5′-flanking sequence of the rat Na,K-ATPase alpha 3 subunit gene are required for brain expression in transgenic mice. *Nucleic Acids Res.* 22: 4748–4755, 1994.
12. Nomoto, M., Gonzalez, F. J., Mita, T., Inoue, N. and Kawamura, M. Analysis of *cis*-acting regions upstream of the rat Na^+/K^+-ATPase alpha 1 subunit gene by in vivo footprinting. *Biochim. Biophys. Acta* 1995: 68–39, 1995.
13. Kobayashi, M., Shimomura, A., Hagiwara, M. and Kawakami, K. Phosphorylation of ATF-1 enhances its DNA binding and transcription of the Na,K-ATPase alpha 1 subunit gene promoter. *Nucleic Acids Res.* 25: 877–882, 1997.
14. Farman, N., Bonvalet, J. P. and Seckl, J. R. Aldosterone selectively increases Na^+-K^+-ATPase alpha 3-subunit mRNA expression in rat hippocampus. *Am. J. Physiol.* 266: C423-C428, 1994.
15. Ahmad, M. and Medford, R. M. Evidence for the regulation of Na^+, K^+-ATPase alpha 1 gene expression through the interaction of aldosterone and cAMP-inducible transcriptional factors. *Steroids* 60: 147–152, 1995.
16. Seok, J. H., Hong, J. H., Jeon, J. R. *et al.* Aldosterone directly induces Na, K-ATPase α1-subunit mRNA in the renal cortex of rat. *Biochem. Mol. Biol. Int.* 47: 251–254, 1999.
17. Grillo, C., Piroli, G., Lima, A., McEwen, B. S. and DeNicola, A. F. Aldosterone up-regulates mRNA for the alpha3 and beta1 isoforms of (Na,K)-ATPase in several brain regions from adrenalectomized rats. *Brain Res.* 767: 120–127, 1997.
18. Peng, M., Huang, L., Xie, Z., Huang, W. H. and Askari, A. Partial inhibition of Na^+/K^+-ATPase by ouabain induces the Ca^{2+}-dependent expressions of early-response genes in cardiac myocytes. *J. Biol. Chem.* 271: 10372–10378, 1996.
19. Narkar, V. A., Hussain, T. and Lokhandwala, M. F. Activation of D2-like receptors causes recruitment of tyrosine-phosphorylated NKA a1-subunits in kidney. *Am. J. Physiol. Renal Physiol.* 283: F1290–F1295, 2002.
20. Teixeira, V. L., Katz, A. I., Pedemonte, C. H. and Bertorello, A. M. Isoform-specific regulation of Na^+,K^+-ATPase endocytosis and recruitment to the plasma membrane. *Ann. N.Y. Acad. Sci.* 986: 587–594, 2003.
21. Arystarkhova, E., Wetzel, R. K., Asinovski, N.K. and Sweadner, K. J. The gamma subunit modulates Na^+ and K^+ affinity of the renal Na,K-ATPase. *J. Biol. Chem.* 274: 33183–33185, 1999.
22. Munzer, J. S., Daly, S. E., Jewell-Motz, E. A., Lingrel, J. B. and Blostein, R. Tissue- and isoform-specific kinetic behavior of the Na,K-ATPase. *J. Biol. Chem.* 269: 16668–16676, 1994.
23. Juhaszova, M. and Blaustein, M. P. Na^+ pump low and high ouabain affinity α subunit isoforms are differently distributed in cells. *Proc. Natl Acad. Sci. U.S.A.* 94: 1800–1805, 1997.
24. Schoner, W. Endogenous cardiac glycosides, a new class of steroid hormones. *Eur. J. Biochem.* 269: 2440–2448, 2002.
25. Lingrel, J., Moseley, A., Dostanic, I. *et al.* Functional roles of the alpha isoforms of the Na,K-ATPase. *Ann. N.Y. Acad. Sci.* 986: 354–359, 2003.
26. Hartford, A. K., Messer, M. L., Moseley, A. E., Lingrell, J. B. and Delamere, N. A. Na,K-ATPase alpha-2 inhibition alters calcium responses in optic nerve astrocytes. *Glia* 45: 229–237, 2004.
27. Hamlyn, J. M., *et al.* 11-hydroxylation in the biosynthesis of endogenous ouabain. *Ann. N.Y. Acad. Sci.* 986: 685–693, 2003.
28. Liu, L., Mohammadi, K., Aynafshar, B. *et al.* Role of caveolae in the signal transducing function of cardiac Na^+/K^+-ATPase. *Am. J. Physiol. Cell Physiol.* 284: C1550–C1560, 2003.
29. Froehlich, J. P., Bamberg, E. *et al.* Contribution of quaternary protein interactions to the mechanism of energy transduction in Na^+/K^+-ATPase. In K. Taniguchi and S. Kaya (eds.), *Na/K-ATPase and Related ATPases.* Amsterdam: Elsevier, 2000.
30. Mahaney, J. E., Thomas, D. D. and Froehlich, J. P. The time-dependent distribution of phosphorylated intermediates in native sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle is not compatible with a linear kinetic model. *Biochemistry* 43: 4400–4416, 2004.
31. Laughery, M., Todd, M. and Kaplan, J. H. Oligomerization of the Na,K-ATPase in cell membranes. *J. Biol. Chem.* 279: 36339–36348, 2004.
32. Harris, Z. L. and Gitlin, J. D. Genetic and molecular basis for copper toxicity. *Am. J. Clin. Nutr.* 63: 836S–841S, 1996.
33. Arata, Y., Nish, T., Kawasaki-Nishi, S., Shao, E., Wilkens, S. and Forgac, M. Structure, subunit function and regulation of the coated vesicle and yeast vacuolar H′-ATPases. *Biochim. Biophys. Acta* 1555: 71–74, 2002.
34. Janas, E., Hofacker, M., Chen, M., Gompf, S., van der Does, C. and Tampe, R. The ATP hydrolysis cycle of the nucleotide-binding domain of the mitochondrial ATP-binding cassette transporter Mdl1p. *J. Biol. Chem.* 278: 26862–26869, 2003.
35. Gong, J.-S., Kobayashi, M., Hayashi, H. *et al.* Apolipoprotein E (apoE) isoform-dependent lipid release from astrocytes prepared from human apoE3 and apoE4 knock-in mice. *J. Biol. Chem.* 277: 29919–29926, 2002.
36. Arakawa, R., Hayashi, M., Remaley, A. T., Brewer, B. H., Yamauchi, Y. and Yokoyama, S. Phosphorylation and stabilization of ATP binding cassette transporter A1 by synthetic amphiphilic helical peptides. *J. Biol. Chem.* 279: 6217–6220, 2004.
37. Tanaka, Y., Yamada, K., Zhou, C. J., Ban, N., Shioda, S. and Inagaki, N. Temporal and spatial profiles of ABCA2-expressing oligodendrocytes in the developing rat brain. *J. Comp. Neurol.* 455: 353–367, 2003.
38. Higgins, C. F. and Gottesman, M. M. Is the multidrug transporter a flippase? *Trends Biochem. Sci.* 17: 18–21, 1992.
39. Van Helvoort, A., Smith, A. J., Sprong, H. *et al.* MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* 87: 507–17, 1996.
40. Schlachetzki, F. and Pardridge, W. M. P-glycoprotein and caveolin-1a in endothelium and astrocytes of primate brain. *Neuroreport* 14: 2041–2046, 2003.
41. Membrane Transport Classification Databases. Available on line at: http://tcdb.ucsd.edu/tcdb http://www.gene.ucl.ac.uk/nomenclature/genefamily.shtml.
42. Attwell, D. and Laughlin, S. B. An energy budget for signaling in the grey matter of the brain. *J. Cereb. Blood Flow Metab.* 21: 1133–1145, 2001.

43. Yernool, D., Boudker, O., Jin, Y. and Gouaux, E. Structure of a glutamate transporter homologue from *Pyococcus horikoshii. Nature* 431:811–818, 2004.
44. Kanai, Y. and Hediger, M. A. The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. *Eur. J. Physiol.* 447: 469–479, 2004.
45. Lehre, K. P. and Danbolt, N. C. The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. *J. Neurosci.* 18: 8751–8757, 1998.
46. Bergles, D. E., Tzingounis, A. V. and Jahr, C. E. Comparison of coupled and uncoupled currents during glutamate uptake by GLT-1 transporters. *J. Neurosci.* 22: 10153–10162, 2002.
47. Deken, S. L., Wang, D. and Quick, M. W. Plasma membrane GABA transporters reside on distinct vesicles and undergo rapid regulated recycling. *J. Neurosci.*: 23: 1563–1568, 2003.
48. Gonzalo, E., Torres, R., Gainetdinov, R. and Caron, M. G. Plasma membrane monoamine transporters: structure, regulation and function. *Nat. Rev. Neurosci.* 4: 13–24, 2003.
49. Chen, N.-H., Reith, M. E. A. and Quick, M. W. Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6. *Eur. J. Physiol.* 447: 519–531, 2004.
50. Ferguson, S. M., Savchenko V., Apparsundaram, S. *et al.* Vesicular localization and activity-dependent trafficking of presynaptic choline transporters. *J. Neurosci.* 23: 9697–9709, 2003.
51. Somogyi, J., Baude, A., Omori, Y. *et al.* GABAergic basket cells expressing cholecysokinin contain vesicular glutamate transporter type 3 (VGLUT3). *Eur J. Neurosci.* 19: 552–569, 2004.
52. Eiden, L. E., Schäfer, M. K.-H., Weihe, E. and Schütz, B. The vesicular amine transporter family (SLC18): amine/proton antiporters required for vesicular accumulation and regulated exocytotic secretion of monoamines and acetylcholine. *Eur. J. Physiol.* 447: 636–640, 2004.
53. Rusakov, D. A. and Kullmann, D. M. Extrasynaptic glutamate diffusion in the hippocampus. *J. Neurosci.* 18: 3158–3170, 1998.
54. Yamada, J., Okabe, A., Toyoda, H., Kilb, W., Luhmann, H. J. and Fukuda, A. Cl^- uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. *J. Physiol.* 557: 829–841, 2004.
55. Woodin, M. A., Ganguly, K. and Poo, M.-M. Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl^- transporter activity. *Neuron* 39: 807–820, 2003.
56. Romero, M. F., Fulton C. M. and Boron W. F. The SLC4 family of HCO_3^- transporters. *Eur. J. Physiol.* 447: 495–509, 2004.
57. Siejö, B. K., von Hanwehr, R., Nergelius, G., Nevander, G. and Ingvar, M. Extra- and intra cellular pH in the brain during seizures and in the recovery period following the arrest of seizure activity. *J. Cereb. Blood Flow Metab.* 5: 47–57, 1985.
58. Deitmer, J. and Rose, C. pH regulation and proton signalling by glial cells. *Prog. Neurobiol.* 48:73–103, 1996.
59. Xue, J., Douglas, R. M., Zhou, D., Lim, J. Y., Boron, W. F. and Haddad, G. G. Expression of Na^+/H^+ and HCO_3^- dependent transporters in Na^+/H^+ exchanger isoform-1 null mutant mouse brain. *Neuroscience* 122: 37–46, 2003.
60. Orlowski, J. and Grinstein, S. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Eur. J. Physiol.* 447: 549–565, 2004.
61. D'Ambrosio, R., Gordon, D. S. and Winn, H. R. Differential role of K_{IR} channel and Na^+/K^+-pump in the regulation of extracellular K^+ in rat hippocampus. *J. Neurophysiol.* 87: 87–102, 2002.
62. Sardini, A., Amey, J. S., Weylandt, K.-H., Nobles, M., Valverde, M. A. and Higgins, C. F. Cell volume regulation and swelling-activated chloride channels. *Biochim. Biophys. Acta* 1618: 153– 162, 2003.
63. Strange, K. Regulation of solute and water balance and cell volume in the central nervous system (editorial). *J. Am. Soc. Nephrol.* 3: 12–27, 1992.
64. Amiry-Moghaddam, M. and Ottersen, O. P. The molecular basis of water transport in the brain. *Nat. Rev. Neurosci.* 4: 991–1001, 2003.
65. King, L. S., Kozono, D. and Agre, P. From structure to disease: the evolving tale of aquaporin biology. *Nat. Rev. Mol. Cell. Biol.* 5: 687–698, 2004.
66. Papadopoulos, M. C., Manley, G. T., Krishna, S. and Verkman, A. S. Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. *FASEB J.* 18: 1291–1293, 2004.
67. Amiry-Moghaddam, M. Ottersen, O. P., Hurn, P. D. *et al.* An alpha-syntrophin-dependent pool of AQP4 in astroglial end-feet confers bidirectional water flow between blood and brain. *Proc. Natl Acad. Sci. U.S.A.* 100: 2106–11, 2003.
68. Hussy, N., Deleuze, C., Desarmenien, M. G. and Moos, F. C. Osmotic regulation of neuronal activity: a new role for taurine and glial cells in a hypothalamic neuroendocrine structure. *Prog. Neurobiol.* 62: 113–134, 2000.
69. Badaut, J., Brunet, J. F., Grollimund, L. *et al.* Aquaporin 1 and aquaporin 4 expression in human brain after subarachnoid hemorrhage and in peritumoral tissue. *Acta Neurochir.* 86(Suppl): 495–8, 2003.
70. Oshio, K., Binder, D. K., Bollen, A., Verkman, A. S., Berger, M. S. and Manley, G. T. Aquaporin-1 expression in human glial tumors suggests a potential novel therapeutic target for tumor-associated edema. *Acta Neurochir.* 86(Suppl.): 499–502, 2003.
71. Saadoun, S., Papadopoulos, M. C., Davies, D. C., Bell, B. A. and Krishna, S. Increased aquaporin 1 water channel expression in human brain tumours. *Br. J. Cancer* 87: 621–3, 2002.
72. Davies, D. C. blood–brain barrier breakdown in septic encephalopathy and brain tumours. *J. Anat.* 200: 639–46, 2002.
73. Arima, H., Yamamoto, N., Sobue, K. *et al.* Hyperosmolar mannitol simulates expression of aquaporins 4 and 9 through a p38 mitogen-activated protein kinase-dependent pathway in rat astrocytes. *J. Biol. Chem.* 278: 44525–44534, 2003.
74. Uldry, M. and Thorens, B. The SLC2 family of facilitated hexose and polyol transporters. *Eur. J. Physiol.* 447: 480–489, 2004.
75. Pascua, J. M., Wang, D., Lecumberri, B. *et al.* GLUT1 deficiency and other glucose transporter diseases. *Eur. J. Endocrinol.* 150: 627–633, 2004.

76. Roncero, I., Alvarez, E., Chowen, J. A. *et al.* Expression of glucose transporter isoform GLUT-2 and glucokinase genes in human brain. *J. Neurochem.* 88: 1203–1210, 2004.
77. Elfeber, K., Köhler, A., Lutzenburg, M. *et al.* Localization of the Na^+-D-glucose cotransporter SGLT1 in the blood–brain barrier. *Histochem. Cell Biol.* 121: 201–207, 2004
78. Poppe, R., Karbach, U., Gambaryan, S. *et al.* Expression of the Na^+,D-g1ucose cotransporter SGLT1 in neurons. *J. Neurochem.* 69: 84–94, 1997
79. Wright, E. M. and Turk, E. The sodium/glucose cotransport family, SLC5. *Eur. J. Physiol.* 447: 510–518, 2004.
80. Loo, D. D. F., Wright, E. M. and Zeuthen, T. Water pumps. *J. Physiol.* 542: 53–60, 2002
81. Ernst, S. A., Palacios, J. R. and Siegel, G. J. Immunocytochemical localization of Na,K-ATPase in mouse choroid plexus. *J. Histochem.* 66: 1742–1751, 1986.
82. Chauhan, N. B., Lee, J. M. and Siegel, G. J. Na,K-ATPase mRNA levels and plaque load in Alzheimer's disease. *J. Mol. Neurosci.* 9: 151–166, 1997.
83. Yudowski, G. A., Efendiev, R., Pedemonte, C. H., Katz, A. I., Berggren, P.-O. and Bertorello, A. M. Phosphoinositide-3 kinase binds to a proline-rich motif in the Na^+,K^+-ATPase α subunit and regulates its trafficking. *Proc. Natl Acad. Sci. U.S.A.* 97: 6556–6561, 2000.
84. Toyoshima, C., Nomura, H. and Sugita, Y. Structural basis of ion pumping by Ca^{2+}-ATPase of sarcoplasmic reticulum. *FEBS Lett.* 555: 106–110, 2003.
85. Guex, N., Diemand, A., Peitsch, M. C., and Schwede, T. *Deep View Swiss Pdb Viewer.* Basel: Swiss Institute of Bioinformatics, 2001. Available on line at: www.expasy.org/spdbv/.
86. Morel, N. Neurotransmitter release: the dark side of the vacuolar-H^+ATPase. *Biol Cell.* 95: 453–457, 2003.
87. Chang, G. Structure of MsbA from *Vibrio cholerae*: a multidrug resistance ABC transporter homolog in a closed conformation. *J. Mol. Biol.* 330: 419–430, 2003.
88. Jones, P. M. and George, A. M. The ABC transporter structure and mechanism: perspectives on recent research. *Cell. Mol. Life Sci.* 61: 682–699, 2004.

CHAPTER

6

Electrical Excitability and Ion Channels

Bertil Hille

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The nervous system enables animals to receive and act on internal and external stimuli with speed and coordination. Activities of the nervous system are reflected in ongoing electrical and chemical signals. Consider a simple reflex arc mediating reflex withdrawal of the leg from a painful stimulus. Several cell types are involved in a network shown diagrammatically in Figure 6-1. Messages travel from skin receptors through the network as a volley of electrical disturbances, terminating in contraction of some muscles. This chapter concerns the origin of electrical potentials in such excitable cells, potentials generated by the passive diffusion of Na^+, K^+, Ca^{2+} and Cl^- ions through highly selective molecular pores in the cell plasma membrane called ion channels. Such channels play a role in membrane excitation as central as the role of enzymes in metabolism. The opening and closing of specific ion channels shape the membrane potential changes. (The interested reader is referred to Hodgkin [1], Armstrong [2], Nicholls *et al.* [3] and Hille [4, 5] for more detailed treatment of this subject.) Over 500 human genes coding for subunits of ion channels have been identified.

MEMBRANE POTENTIALS AND ELECTRICAL SIGNALS IN EXCITABLE CELLS

Excitable cells have a negative membrane potential. Before examining the variety of electrical signals, let us consider the electrochemical theory behind their

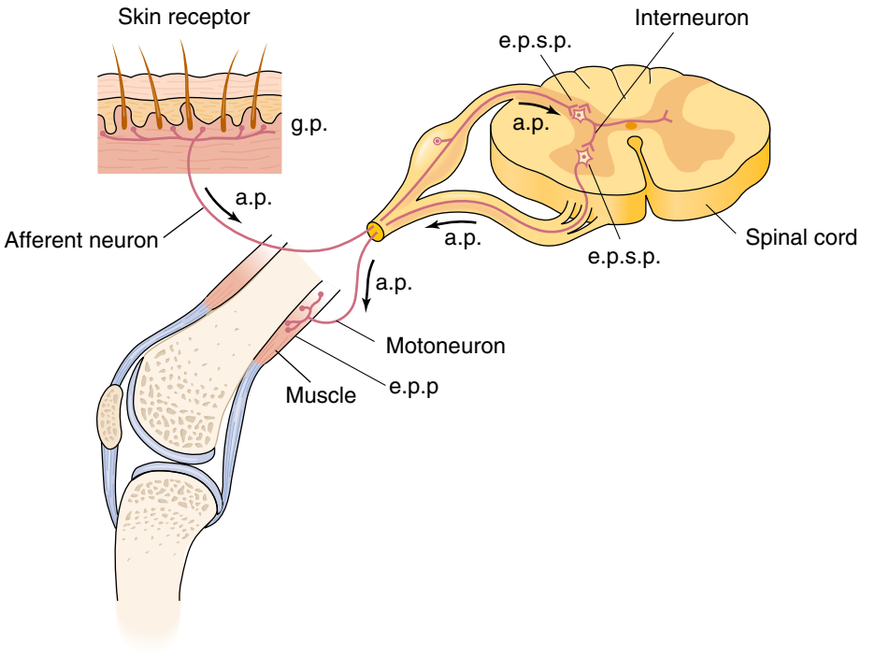

FIGURE 6-1 Path of excitation in a simplified spinal reflex that mediates withdrawal of the leg from a painful stimulus. In each of the three neurons and in the muscle cell, excitation starts with a localized slow potential and is propagated via an action potential (*a.p.*). Slow potentials are generator potential (*g.p.*) at the skin receptor; the excitatory postsynaptic potentials (*e.p.s.p.*) in the interneuron and the motoneuron and end-plate potential (*e.p.p.*) at the neuromuscular junction. Each neuron makes additional connections to other pathways that are not shown.

generation. At rest, the cytoplasm is electrically more negative than the external bathing fluid by 30–100 mV. This potential drop appears across the cell's plasma membrane, as is revealed by recording with an electrolyte-filled glass pipette microelectrode. When the narrow tip of such an electrode passes through the plasma membrane, one sees a sudden negative drop, the resting potential of the cell. By convention, the membrane potential is defined as 'inside' minus 'outside,' so the resting potential is a negative number. Signals that make the cytoplasm more positive than at rest are said to depolarize the membrane, and those making it more negative are said to hyperpolarize the membrane.

Membrane potentials arise from the diffusion of ions [3–5]. Consider the electrolyte system represented in Figure 6-2 (left), where a porous membrane separates aqueous solutions of unequal concentrations of a fictitious salt KA. Assume that the membrane pores are permeable exclusively to K^+ so that K^+ begins to diffuse across the membrane but A^- does not. Initially the movement of K^+ from the concentrated side to the dilute side exceeds the movement in the reverse direction, so a few K^+ ions flow down their concentration gradient. This process does not continue long because each K^+ ion carries a positive charge from one compartment to the other and leaves a net negative charge behind. The growing separation of charge creates an electrical potential difference (the membrane potential) between the two solutions. The accumulated positive charge on the side into which the K^+ ions diffuse sets up an electrical force that opposes further net movement of K^+, and soon the membrane potential stops changing.

The membrane potential reached in a system with only one permeant ion and no perturbing forces is called the equilibrium, or Nernst, potential for that ion; thus, the final membrane potential for the system in Figure 6-2 is

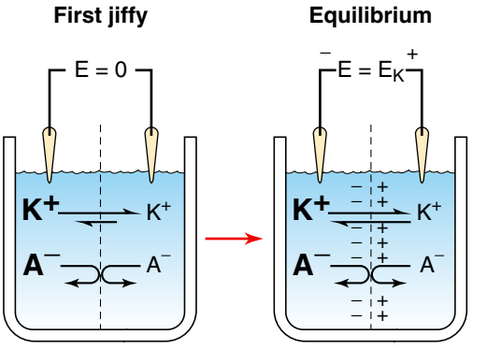

FIGURE 6-2 Origin of the membrane potential in a purely K^+-permeable membrane. The porous membrane separates unequal concentrations of the dissociated salt K^+A^-. In the first 'jiffy', the membrane potential, E, recorded by the electrodes above is zero and K^+ diffuses to the right down the concentration gradient. The anion A^- cannot cross the membrane, so a net positive charge builds up on the right and a negative charge on the left. At equilibrium, the membrane potential, caused by the charge separation, has built up to the Nernst potential, E_K, where the fluxes of K^+ become equal in the two directions.

the K^+ equilibrium potential E_K. At that potential, there is no further net movement of K^+, and unless otherwise disturbed, the membrane potential and ion gradient will remain stable indefinitely. The value of the Nernst potential is derived from thermodynamics by recognizing that the change of electrochemical potential $\Delta\mu_j$, for moving the permeant ion j^{+z} across the membrane, must be zero at equilibrium:

$$\Delta\mu_j = 0 = RT \ln \frac{[j]_o}{[j]_i} - zFE \qquad (1)$$

where R is the gas constant (8.31 J)/°/mol, T is absolute temperature in kelvin (°C + 273.2), and F is Faraday's constant (96,500 C/mol). Using terms appropriate to biology, $[j]_o$ and $[j]_i$ represent activities of ion j^{+z} outside and inside a cell; z is the ionic valence and E the membrane potential defined as 'inside minus outside'. Solving for E, and calling it E_j to denote the ion at equilibrium gives the Nernst equation for j:

$$E_j = \frac{RT}{zF} \ln \frac{[j]_o}{[j]_i}. \qquad (2)$$

For practical use at 20°C, the Nernst equation can be rewritten

$$E_j = \frac{58\,\text{mV}}{z} \log_{10} \frac{[j]_o}{[j]_i}, \qquad (3)$$

showing that for a 10:1 transmembrane gradient, a monovalent ion can give 58 mV of membrane potential. Table 6-1 gives approximate intracellular and extracellular concentrations of the four electrically most important ions in a mammalian skeletal muscle cell and the Nernst potentials calculated from these numbers at 37°C (neglecting possible activity coefficient corrections). Experimentally it is found that the resting muscle membrane is primarily permeable to K^+ and Cl^- and, therefore, the resting potential in muscle is −90 mV, close to the equilibrium potentials E_K and E_{Cl}. To summarize, membrane potentials arise by diffusion of a small number of ions down their concentration gradient across a permselective membrane.

TABLE 6-1 Approximate free ion concentrations in mammalian skeletal muscle

Ion	Extracellular concentration (mmol/l)	Intracellular concentration (mmol/l)	$[Ion]_o/[Ion]_i$	Nernst potential* (mV)
Na^+	145	12	12	+66
K^+	4	155	0.026	−97
Ca^{2+}	1.5	$<10^{-3}$	>1,500	>97
Cl^-	120	4†	30†	−90†

*Equilibrium potentials calculated at 37°C from the Nernst equation.
†Calculated assuming a −90 mV resting potential for the muscle membrane and assuming that chloride ions are at equilibrium at rest.

Real cells are not at equilibrium. Although the concept of equilibrium potentials is essential to understanding and predicting the membrane potentials generated by ion permeability, real cells actually never are at equilibrium, because different ion channels open and close during excitation and even at rest several types of channels are open simultaneously. Under these circumstances, the ion gradients are dissipated constantly, albeit slowly, and ion pumps are always needed in the long run to maintain a steady state (see Ch. 5). When the membrane is permeable to several ions, the steady-state potential is given by the sum of contributions of the permeant ions, weighted according to their relative permeabilities P_j:

$$E = \frac{RT}{F} \ln \frac{P_{Na}[Na^+]_o + P_K[K^+]_o + P_{Cl}[Cl^-]_i}{P_{Na}[Na^+]_i + P_K[K^+]_i + P_{Cl}[Cl^-]_o}. \qquad (4)$$

This Goldman–Hodgkin–Katz voltage equation is often used to determine the relative permeabilities of ions from experiments where the bathing ion concentrations are varied and changes in the membrane potential are recorded [5].

Transport systems may also produce membrane potentials. The equations just discussed describe passive electrodiffusion in ion channels where the only motive forces on ions are thermal and electrical, and they explain almost all the potentials of excitable cells. However, another class of electric current source in cells can generate potentials: the ion pumps and other membrane transporters that couple ion movements to the movements of other molecules. In excitable cells, the most prominent is the Na^+/K^+ pump (see Ch. 5), which gives a net export of positive charge and hence can hyperpolarize the plasma membrane slightly in proportion to the rate of pumping [3]; but hyperpolarization from this electrogenic pumping is at most a few millivolts. By contrast, mitochondria (and plant, algal and fungal cells) have powerful current sources in their proton transport system. Their membrane potentials are often dominated by this electrogenic system and cannot be described in terms of diffusion in passive ion channels.

Electrical signals recorded from cells are of two types: stereotyped action potentials and a variety of slow potentials. The action potential of axons is a brief, spike-like depolarization that propagates regeneratively as an electrical wave without decrement and at a high, constant velocity from one end of the axon to the other [1, 3]. It is used for all rapid signaling over a distance. For example, in the reflex arc of Figure 6-1 action potentials in motor axons would carry the message from spinal cord to leg, telling some muscle fibers of the biceps femoris to contract. In large mammalian axons at body temperature, the action potential at any one patch of membrane may last only 0.4 ms as it propagates at a speed of 100 m/s.

The action potential is normally elicited when the cell membrane is depolarized beyond a threshold level; and it is said to arise in an all-or-nothing manner because a subthreshold stimulus gives no propagated response, whereas every suprathreshold stimulus elicits the stereotyped propagating wave. Underlying the propagated action potential is a regenerative wave of opening and closing of voltage-gated ion channels that sweeps along the axon. Action potentials are also frequently referred to as spikes, or impulses. Most nerve and muscle cells and many endocrine cells can make action potentials.

By contrast, slow potentials are localized membrane depolarizations and hyperpolarizations, with time courses ranging from several milliseconds to minutes. They are associated with a variety of transduction mechanisms. For example, slow potentials arise postsynaptically at the sites of action of neurotransmitter molecules (see Ch. 10) and in transduction sites of sensory receptors (see Chs 49–51). Slow potentials are graded in relation to their stimulus and sum with each other within the cell while decaying passively over a distance of no more than a few millimeters from their site of generation. Underlying the slow potential is a graded and local opening or closing of ion channels reflecting the intensity of the stimulus. These channels are gated by stimuli other than voltage. The natural stimulus for the initiation of propagated action potentials is a depolarizing slow potential exceeding the firing threshold. In each of the cell types involved in the reflex arc in Figure 6-1, depolarizing slow potentials initiate propagating action potentials as the message moves forward.

ACTION POTENTIALS IN ELECTRICALLY EXCITABLE CELLS

During excitation, ion channels open and close and a few ions flow. The membrane potential does not change unless there is a net charge movement across the membrane. During a propagated action potential, ion channels permeable to Na^+ open, some Na^+ enters the fiber, and the membrane potential swings transiently toward E_{Na}. When these pores close again, the membrane potential returns to rest near E_K and E_{Cl}. During activity the extra ion fluxes impose an extra load on the Na^+/K^+ pump and the Ca^{2+} pump, consuming ATP and stimulating an extra burst of cellular oxygen consumption until the original gradients are restored. How large are these fluxes? The physical minimum, calculated from the rules of electricity, is very small. Only 10^{-12} equivalents of charge need be moved to polarize 1 cm^2 of membrane by 100 mV, meaning that, ideally, the movement of 1 pmol/cm^2 of monovalent ion would be enough to depolarize the membrane fully. With this kind of Na^+ gain, a squid giant axon of 1 mm diameter could be stimulated 10^5 times and a mammalian unmyelinated fiber of 0.2 µm diameter only 10–15 times before the internal Na^+ concentration would be doubled, assuming that the Na^+/K^+ pump had been blocked.

Gating mechanisms for Na^+ and K^+ channels in the axolemma are voltage-dependent. The breakthrough in explaining the action potential came with an electrical method to study the kinetics of ion permeability changes directly. In a classic series of experiments, Hodgkin, Huxley and Katz [1, 3–6] studied squid giant-axon membranes by a method they called the voltage clamp. Their method controls the membrane voltage electrically while ion movements are recorded as electric current flowing across the membrane. The recorded current can be resolved into individual ionic components by changing the ion content of the solutions that bathe the membrane. The voltage clamp is a rapid and sensitive assay for studying the opening and closing of ion channels. Today, a widely used miniature version of the voltage clamp is the patch clamp, which has sufficient sensitivity to study the current flow in a single ion channel [7]. A glass micropipette, the patch pipette, with a tip diameter <1 µm, is fire polished at the tip and then pressed against the membrane of a cell. Because the tip is smooth, it seals to the membrane in the annular contact zone, rather than piercing the membrane, and defines a tiny patch of the cell surface whose few ion channels can be detected easily by the currents flowing through them. The patch clamp readily measures fluxes of as little as 10^{-20} mol of ion in less than 1 ms.

With the voltage clamp, Hodgkin and Huxley [1, 6, 8] discovered that the opening and closing conformational changes of axonal Na^+ and K^+ channels are directly controlled by the membrane potential. Hence, channel gating (these conformational changes) derives its energy from the work done by the electric field on charges associated with the channel macromolecule. Hodgkin and Huxley [6] identified currents from two types of ion-selective channel – Na^+ channels and K^+ channels – that account for almost all the current in axon membranes. Then they made a kinetic model of the opening and closing steps, simplified in Figure 6-3. Depolarization of the membrane

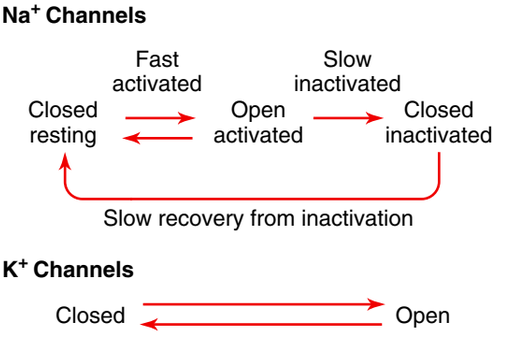

FIGURE 6-3 Simplified kinetic model for opening and closing steps of Na^+ and K^+ channels. (Adapted from Hodgkin and Huxley [6].)

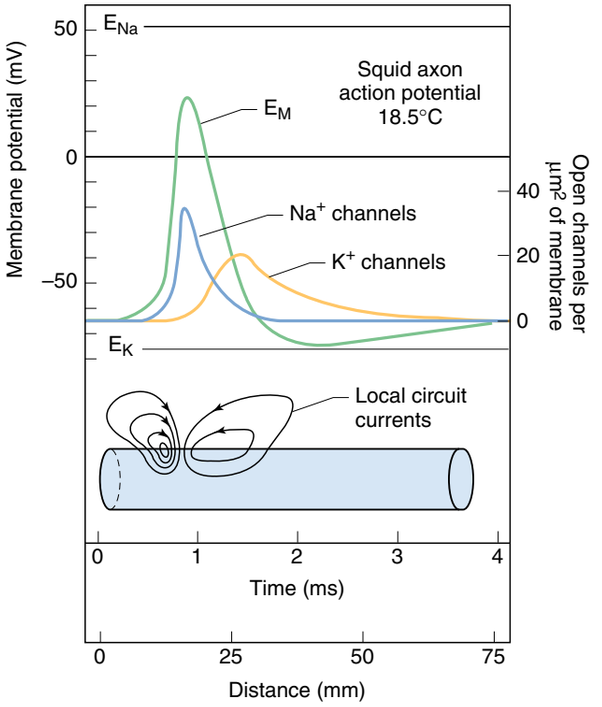

FIGURE 6-4 Events of the propagated action potential calculated from the Hodgkin and Huxley [6] kinetic model. Because the action potential is a nondecrementing wave, the diagram shows equivalently the time course of events at one point in the axon, or the spatial distribution of events at one time as the excitation propagates to the left. **Upper.** Action potential (E_M) and the opening and closing of Na^+ and K^+ channels. The Nernst potentials for Na^+ and K^+ are indicated by E_{Na} and E_K. **Lower.** Local circuit currents. The intense loop on the left spreads the depolarization to the left into the unexcited membrane.

is sensed by the voltage sensors of each channel and causes the conformational reactions to proceed to the right. Repolarization or hyperpolarization causes them to proceed to the left. We can understand the action potential in these terms. The action potential, caused by a depolarizing stimulus, begins with a transient, voltage-gated opening of Na^+ channels that allows Na^+ to enter the fiber and depolarize the membrane fully, followed by a transient, voltage-gated opening of K^+ channels that allows K^+ to leave and repolarize the membrane. Figure 6-4 shows a calculation of the temporal relation between channel-opening and membrane-potential changes in an axon at 18.5°C, using the full kinetic model of Hodgkin and Huxley [6].

The action potential is propagated by local spread of depolarization. How does the action potential propagate smoothly down an axon, bringing new channels into play ahead of it? Any electrical depolarization or hyperpolarization of a cell membrane spreads a small distance in either direction from its source by a purely passive process often called cable or electrotonic spread. The spread occurs because the intracellular and extracellular media are much better conductors than is the membrane, so that any charges injected at one point across the membrane repel each other and disperse along the membrane surface. The lower part of Figure 6-4 shows diagrammatically the so-called local circuit currents that spread the depolarization forward. In this way, an excited depolarized membrane area smoothly depolarizes the next unexcited region ahead of the action potential, bringing it above firing threshold, opening Na^+ channels there, and advancing the wave of excitation. The action potential in the upper part of Figure 6-4 is calculated by combining the known geometry of the squid giant axon with the rules of ionic electricity and the Hodgkin and Huxley kinetic equations for the voltage-dependent gating of Na^+ and K^+ channels. The success of the calculations means that the factors described are sufficient to account for action-potential propagation.

Membranes at nodes of Ranvier have high concentrations of Na^+ channels. A wide variety of cells have now been studied by voltage clamp methods, and quantitative descriptions of their permeability changes are available. All axons, whether vertebrate or invertebrate, operate on the same principles: They have a small background permeability, primarily to K^+, that sets the resting potential and display brief, dramatic openings of Na^+ and K^+ channels in sequence to shape the action potentials. Chapter 4 describes myelin, a special adaptation of large (1–20 μm diameter) vertebrate nerve fibers for higher conduction speed. In myelinated nerves, like unmyelinated ones, the depolarization spreads from one excitable membrane patch to another by local circuit currents; but, because of the insulating properties of the coating myelin, the excitable patches of axon membrane (the nodes of Ranvier) may be more than 1 mm apart, so the rate of progression of the impulse is faster. Nodes of Ranvier have at least ten times as many Na^+ channels per unit area as other axons to depolarize the long, passive internodal myelin. The Na^+/K^+ pump is also concentrated at nodes (see Ch. 5). Between nodes, the internodal axon membrane has K^+ channels but far fewer Na^+ channels.

FUNCTIONAL PROPERTIES OF VOLTAGE-GATED ION CHANNELS

Ion channels are macromolecular complexes that form aqueous pores in the lipid membrane. We have learned much about ion channel function from voltage clamp and patch clamp studies on channels still imbedded in native cell membranes [1–6, 8]. A diversity of channel types was discovered in the different cells in the body, where the repertoire of functioning channels is adapted to the special roles each cell plays [5]. The principal voltage-gated ones are the Na^+, K^+ and Ca^{2+} channels, and most of these are opened by membrane depolarizations. Figure 6-5A summarizes the major functional properties of a voltage-gated

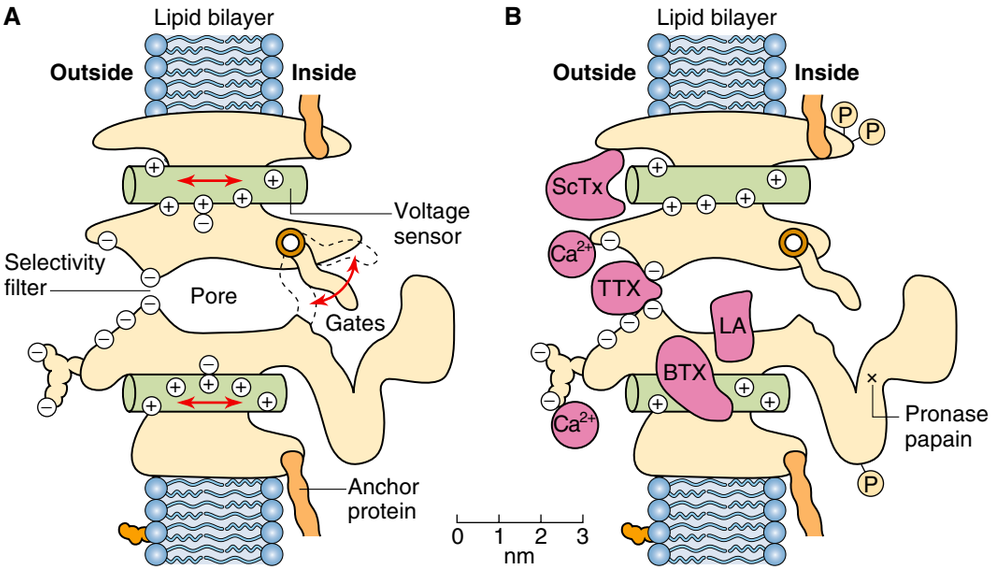

FIGURE 6-5 (**A**) Diagram of the functional units of a voltage-gated ion channel and (**B**) the hypothesized binding sites for several drugs and toxins affecting Na^+ channels. The drawing is fanciful, and the dimension and shapes of many parts are not known. Drug receptors: *TTX*, tetrodotoxin and saxitoxin; *ScTx*, scorpion toxins and anemone toxins; *BTX*, batrachotoxin, aconitine, veratridine and grayanotoxin; *LA*, local anesthetics; *Ca^{2+}*, divalent ions screening and associating with surface negative charge.

channel in terms of a fanciful cartoon drawn before the availability of crystal structures. The pore is narrow enough in one place, the ionic selectivity filter, to 'feel' each ion and to distinguish among Na^+, K^+, Ca^{2+} and Cl^-. The channel also contains charged components that sense the electric field in the membrane and drive conformational changes that open and close gates controlling the permeability of the pore. In voltage-gated Na^+, K^+ and Ca^{2+} channels, the gates close the axoplasmic mouth of the pore and the selectivity filter is near the outer end of the pore.

How did the voltage clamp show that a channel is a pore? The most convincing evidence was the large ion flux a single channel can handle. In patch-clamp studies, it is common to observe ionic currents of 2–10 pA flowing each time one channel in the patch is open. This would correspond to $12–60\times10^6$ monovalent ions moving per second. Such a turnover number is several orders of magnitude faster than known carrier mechanisms and agrees well with the theoretical properties of a pore of atomic dimensions.

Water molecules break and make hydrogen bonds with other waters 10^{11}–10^{12} times per second, and alkali ions exchange water molecules or other oxygen ligands at least 10^9 times per second. In these terms, the progress of an ion across the membrane is not the movement of a fixed hydrated complex; rather it is a continual exchange of oxygen ligands as the ion dances through the sea of relatively free water molecules and polar groups that form the wall of the pore. Dipoles and charged groups in the pore provide stabilization energy to the permeating ion, compensating for those water molecules that must be left behind as the ion enters into the pore. Evidence for important negative charges in the selectivity filter of Na^+, K^+ and Ca^{2+} channels comes from a block of their permeability as the pH of the external medium is lowered below pH 5.5 [5] and from site-directed mutagenesis of aspartate and glutamate residues in cloned channels.

The minimum pore size of ion channels was first determined from the van der Waals dimensions of ions that will pass through them [5]. The voltage-gated channels, which show considerable ion selectivity, are so narrow that ions need to shed several (not all) water molecules to pass through. The ion fluxes are often described by models with temporary binding to attractive sites and jumps over energy barriers. Formally, the kinetics of flux through channels can be formulated similarly to enzyme kinetics. It is assumed that the channel passes through a sequence of 'channel-ion complexes' as it catalyzes the progression of an ion across the membrane. Such theories also can describe other properties of ion channels, such as selectivity, saturation, competition and block by permeant ions [5].

Voltage-dependent gating requires voltage-dependent conformational changes in the protein component(s) of ion channels. On theoretical grounds, a membrane protein that responds to a change in membrane potential must have charged or dipolar amino acid residues, located within the membrane electric field, acting as voltage sensors as illustrated in Figure 6-5A [5, 6, 8]. Changes in the membrane potential then exert a force on these charged residues. If the energy of the field-charge interactions is

great enough, the protein may be induced to undergo a change to a new, stable conformational state in which the net charge or the location of charge within the membrane electric field has been altered. For such a voltage-driven change of state, the steepness of the state function versus membrane potential curve defines the equivalent number of charges that move, according to a Boltzmann distribution. On this basis, activation of Na^+ channels would require the movement of as many as 12 positive charges from the intracellular to the extracellular side of the membrane. The movement of a larger number of charges through a proportionately smaller fraction of the membrane electrical field would be equivalent.

Such movements of membrane-bound charge give rise to tiny 'gating' currents that can be detected electrophysiologically [9]. Their voltage and time dependence are consistent with the multistep changes of channel state from resting to active. In contrast to activation, fast inactivation from the open state of Na^+ channels and certain K^+ channels does not seem to be a strongly voltage-sensitive process. This inactivation can be blocked irreversibly by proteolytic enzymes acting from the intracellular side of the channel and it requires regions of the channel that are exposed at the intracellular surface of the membrane.

Pharmacological agents acting on ion channels help define their functions. The Na^+ channel is so essential to successful body function that it has become the target in evolution of several potent poisons. The pharmacology of these agents has provided important insights into the further definition of functional regions of the channel [2, 5, 10]. Figure 6-5B shows the supposed sites of action of four prominent classes of Na^+ channel agents. At the outer end of the channel pore is a site where the puffer fish poison, tetrodotoxin (TTX), a small, lipid-insoluble charged molecule binds with a K_i of 1–10 nmol/l and blocks Na^+ permeability. A second important class of Na^+ channel blockers includes such clinically useful local anesthetics as lidocaine and procaine and related antiarrhythmic agents. They are lipid-soluble amines with a hydrophobic end and a polar end, and they bind to a hydrophobic site on the channel protein where they also interact with the inactivation gating machinery. The relevant clinical actions of local anesthetics are fully explained by their mode of blocking Na^+ channels. Two other classes of toxins either open Na^+ channels spontaneously or prevent them from closing normally once they have opened. These are lipid-soluble steroids, such as the frog-skin poison batrachotoxin (BTX), the plant alkaloids aconitine and veratridine, all three acting at a site within the membrane, and peptide toxins from scorpion and anemone venoms, which act at two sites on the outer surface of the membrane. Most scorpion and anemone toxins block the inactivation gating step specifically. Interestingly, the affinity of the channel for each of these classes of toxins depends on the gating conformational state of the channel.

Similarly specific agents affect K^+ channels or Ca^{2+} channels. Most K^+ channels can be blocked by tetraethylammonium ion, Cs^{2+}, Ba^{2+}, and 4-aminopyridine [2, 5]. Except for 4-aminopyridine, there is good evidence that these ions become lodged within the channel at a narrow place from which they may be dislodged by K^+ coming from the other side. In addition, certain K^+ channels can be distinguished by their ability to be blocked near the outer mouth by polypeptide toxins from scorpion (charybdotoxin), bee (apamin), or snake (dendrotoxin) venoms. Ca^{2+} channels can be blocked by externally applied divalent ions, including Mn^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+}. Different Ca^{2+} channel subtypes can be distinguished by their block by dihydropyridines (nifedipine), cone snail toxins (ω-conotoxins) and spider toxins (agatoxins).

THE VOLTAGE-GATED ION CHANNEL SUPERFAMILY

Why should we study the structural properties of the channel macromolecules themselves? Although biophysical techniques define the functional properties of voltage-sensitive ion channels clearly, it is important to relate those functional properties to the structure of the channel proteins. We focus first on the discovery of the ion channel proteins, in which three different experimental approaches were used

Na^+ channels were identified by neurotoxin labeling and their primary structures were established by cDNA cloning. Neurotoxins act at several different sites on Na^+ channels to modify their properties (Figs 6-5B, 6-6A, B. Photoreactive derivatives of the polypeptide toxins of scorpion venom were covalently attached to Na^+ channels in intact cell membranes allowing direct identification of channel components. Reversible binding of saxitoxin and tetrodotoxin to their common receptor was used as a biochemical assay for the channel protein. Solubilization of excitable membranes with nonionic detergents released the Na^+ channel, and the solubilized channel was purified by chromatographic techniques that separate glycoproteins by size, charge and composition of covalently attached carbohydrate [11–13]. Na^+ channels consist of a large α-subunit, a glycoprotein with molecular mass of 260,000, usually in association with glycoprotein β-subunits with molecular masses of 33,000–36,000 (Fig. 6-6A). An important step in the study of a purified membrane transport protein is to reconstitute its function in the pure state. This was accomplished in two ways for the Na^+ channel. In the first approach, purified channels were incorporated into vesicles of pure phospholipid. Activation of the reconstituted channels by treatment with the neurotoxins veratridine or batrachotoxin markedly increased the permeability of the vesicles to Na^+. The purified channels retained the ion selectivity and pharmacological properties of native channels. In the second

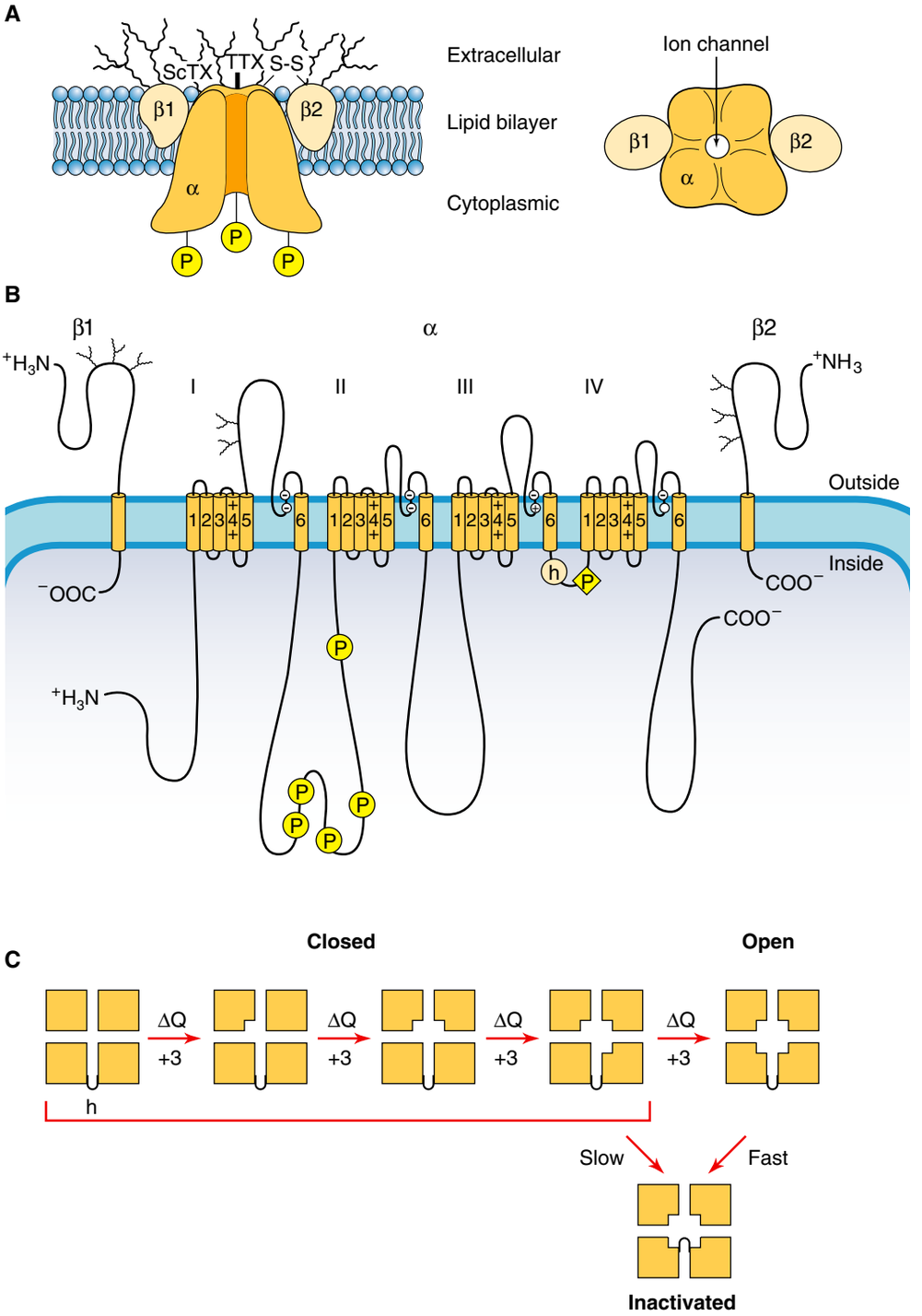

FIGURE 6-6 Structural model of the Na^+ channel. (**A**) **Left.** A topological model of the rat brain Na^+ channel illustrating the probable transmembrane orientation of the three subunits, the binding sites for tetrodotoxin (*TTX*) and scorpion toxin (*ScTX*), oligosaccharide chains (*wavy lines*) and cAMP-dependent phosphorylation sites (*P*). **Right.** An *en face* view of the protein from the extracellular side illustrating the formation of a transmembrane ion pore in the midst of a square array of four transmembrane domains of the α subunit. (**B**) A transmembrane folding model of the α and β subunits of the Na^+ channel. The amino acid sequence is illustrated as a *narrow line*, with each segment approximately proportional to its length in the molecule. Transmembrane α-helices are illustrated as cylinders. The positions of amino acids required for specific functions of Na^+ channels are indicated: ++, positively charged voltage sensors in the S4 transmembrane segments; *O*, residues required for high affinity binding of TTX with their charge characteristics indicated by –, + or open field; *h*, residues required for fast inactivation; *P in a circle*, sites for phosphorylation by cAMP-dependent protein kinase; and *P in a diamond*, sites for phosphorylation by protein kinase C. (**C**) Sequential gating of the Na^+ channel. A reaction pathway from closed to open Na^+ channels is depicted. Each square represents one homologous domain of the α subunit. Each domain undergoes a conformational change initiated by a voltage-driven movement of its S4 segment, leading eventually to an open channel. Inactivation of the channel occurs from the final closed state and the open state, by folding of the intracellular loop connecting domains III and IV into the intracellular mouth of the transmembrane pore.

approach, ion conductance mediated by single purified channels was measured electrically. Channels reconstituted in phospholipid vesicles were studied directly using patch clamp methods or incorporated into planar, phospholipid bilayer membranes by fusion. The individual purified channels observed retained the single-channel conductance, ion selectivity, and voltage dependence of activation and inactivation that are characteristic of native Na^+ channels.

The amino acid sequences of the Na^+ channel α, β1 and β2 subunits were determined by cloning DNA complementary to their mRNAs, using antibodies and oligonucleotides developed from work on purified Na^+ channels [14, 15, 16]. The primary structures of these subunits are illustrated as transmembrane folding models in Figure 6-6B. The large α subunits are composed of 1,800–2,000 amino acids and contain four repeated domains having greater than 50% internal sequence identity. Each domain contains six segments that form transmembrane α-helices and an additional membrane reentrant loop that forms the outer mouth of the transmembrane pore (see below). In contrast, the smaller β1 and β2 subunits consist of a large extracellular N-terminal segment having a structure similar to antigen-binding regions of immunoglobulin, a single transmembrane segment and a short intracellular segment (Fig. 6-6B).

Ca^{2+} channels have a structure similar to Na^+ channels. The same general experimental strategy was successfully applied to voltage-gated Ca^{2+} channels. Drugs and neurotoxins that act on Ca^{2+} channels were used to identify and purify their protein components, and the ion transport function of the purified channels was restored by reconstitution (Fig. 6-7A) [17, 18]. Like Na^+ channels, Ca^{2+} channels have a principal subunit, designated α1, which is structurally homologous to the Na^+ channel α subunit. They have associated α2 and δ subunits, which form a disulfide-linked transmembrane glycoprotein complex, and β subunits, which are intracellular (Fig. 6-7A). In addition, Ca^{2+} channels in skeletal muscle have a transmembrane γ subunit. The auxiliary subunits of Ca^{2+} channels are not related in primary structure to the Na^+ channel β subunits.

Voltage-gated K^+ channels were identified by genetic means. Genes that harbor mutations causing an easily detectable altered phenotype can be cloned directly from genomic DNA without information about the protein they encode. The *Shaker* mutation in *Drosophila* causes flies to shake when under ether anesthesia and is accompanied by loss of a specific K^+ current in the nerve and muscle of the mutant flies. Cloning successive pieces of genomic DNA from the region of the chromosome that specifies this mutation allowed isolation of DNA clones encoding a protein related in amino acid sequence to the α subunit of Na^+ channels [19]. The K^+ channel protein is analogous to one of the homologous domains of Na^+ or Ca^{2+} channels [19] and functions as a tetramer of four separate subunits, analogous to the structure of Na^+ and Ca^{2+} channels (Fig. 6-7B). Like the Na^+ channels and the Ca^{2+} channels, the K^+ channels have auxiliary subunits, which include intracellularly located β subunits as well as minK or minK-related subunits having a single transmembrane segment [19, 20].

Inwardly rectifying K^+ channels were cloned by expression methods. A third experimental approach was used to clone and characterize inwardly rectifying K^+ channels, the simplest member of the ion channel protein family [19, 21]. DNA complementary to mRNAs from brain or heart was prepared and separated into pools. mRNA was prepared from each pool and its ability to direct the synthesis of K^+ channels in *Xenopus* oocytes was measured. The active cDNA was isolated by repeatedly dividing the pool of cDNAs until only a single one remained. The amino acid sequence that it encoded showed that inwardly rectifying K^+ channels have only two transmembrane segments (Fig. 6-7B), analogous to the S5 and S6 segments of voltage-gated Na^+, Ca^{2+} or K^+ channels. Four subunits are required to form an ion channel. Thus, these two segments must also form the transmembrane pores of voltage-gated Na^+, Ca^{2+} or K^+ channels.

THE MOLECULAR BASIS FOR ION CHANNEL FUNCTION

The cloning of the cDNA encoding the ion channel subunits permitted detailed tests of the functional properties of the polypeptides. cDNA clones can be expressed in recipient cells and the resulting ion channels can be studied by voltage-clamp methods. Only the principal α subunits of the Na^+, Ca^{2+} or K^+ channels are required for function [22, 23]. However, co-expression of the auxiliary subunits increases the level of expression and modifies the voltage-dependent gating, conferring more physiologically correct functional properties on the expressed channels [15]. These results indicate that the principal subunits of the voltage-gated ion channels are functionally autonomous, but the auxiliary subunits improve expression and modulate physiological properties.

We begin to know structural determinants of the ion selectivity filter and pore. Which amino acid sequences are involved in forming the ion selectivity filter and pore? Insight into this question first came from studies of the amino acid residues required for binding of tetrodotoxin, which blocks the ion selectivity filter at the outer mouth of the pore (Fig. 6-5B). Site-directed mutagenesis experiments showed that pairs of amino acid residues required for high-affinity tetrodotoxin binding are located in analogous positions in all four domains near the carboxyl ends of the short membrane-re-entrant segments between

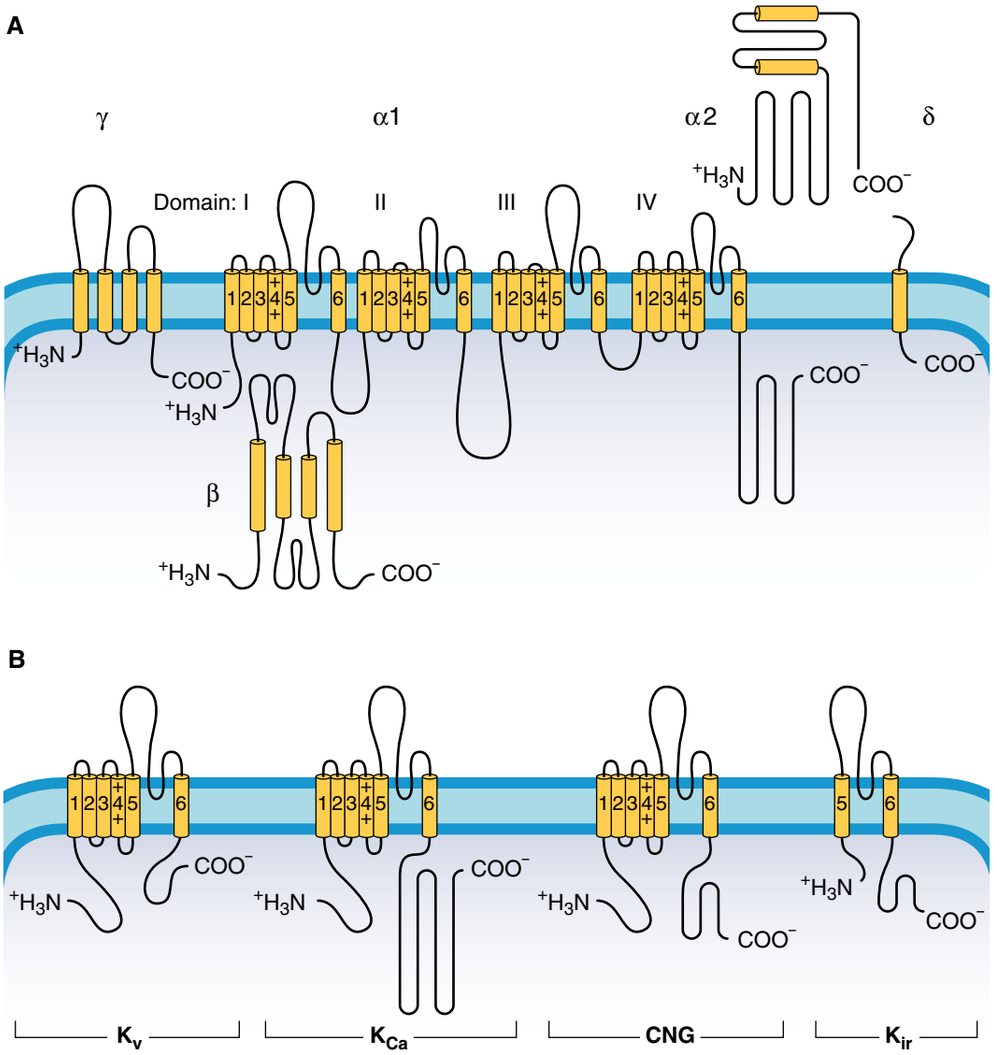

FIGURE 6-7 Transmembrane organization of voltage-gated Ca^{2+} channels, K^+ channels and relatives. (**A**) The primary structures of the subunits of voltage-gated Ca^{2+} channels are illustrated. *Cylinders* represent probable α-helical transmembrane segments. *Bold lines* represent the polypeptide chains of each subunit, with length approximately proportional to the number of amino acid residues. **B:** The primary structures of the plasma membrane cation channels related to K^+ channels are illustrated as transmembrane folding diagrams based on analysis of the hydrophobicity of the amino acid sequence. Predicted transmembrane α-helices are illustrated as *cylinders*. The remainder of the polypeptide chain is illustrated as a *bold line*, with the length of each segment approximately proportional to the length of its amino acid sequence. K_v, voltage-gated K^+ channel; K_{Ca}, Ca^{2+}-activated K^+ channel types 2 and 3; *CNG*, cyclic nucleotide-gated channel; K_{ir}, inward rectifying K^+ channel.

transmembrane α-helices S5 and S6 [24] (Fig. 6-6B). Six of these eight residues are negatively charged and may interact with permeant ions as they approach and move through the channel. In agreement with this idea, mutation of the only two of these residues that are not negatively charged (see domains III and IV, Fig. 6-6B) to glutamic acid residues, as present in the analogous positions in the Ca^{2+} channel, confers Ca^{2+} selectivity on the Na^+ channel [25]. Parallel results implicated these same regions of the K^+ channel in determining ion selectivity and conductance [26]. These results led to the idea that these segments, often called the P loops, form the ion selectivity filter. The three-dimensional structure of the pore formed from the S5 and S6 segments and the intervening P loop was elegantly determined by X-ray crystallography of a bacterial K^+ channel [27]. As in inward rectifier K^+ channels (Fig. 6-7B), the subunits of this bacterial channel have just two transmembrane segments (analogous to S5 and S6), which form α-helices that cross the membrane at an angle to form an 'inverted teepee' structure (Fig. 6-8A, left). The P loops are cradled in this structure to form the narrow ion selectivity filter at the extracellular end of the pore. This remarkable structure elucidates the molecular basis for the formation of a transmembrane pore and suggests a mechanism for ion conduction (see below).

How does the pore open? The structure illustrated in Figure 6-8A (left) may represent the closed state because

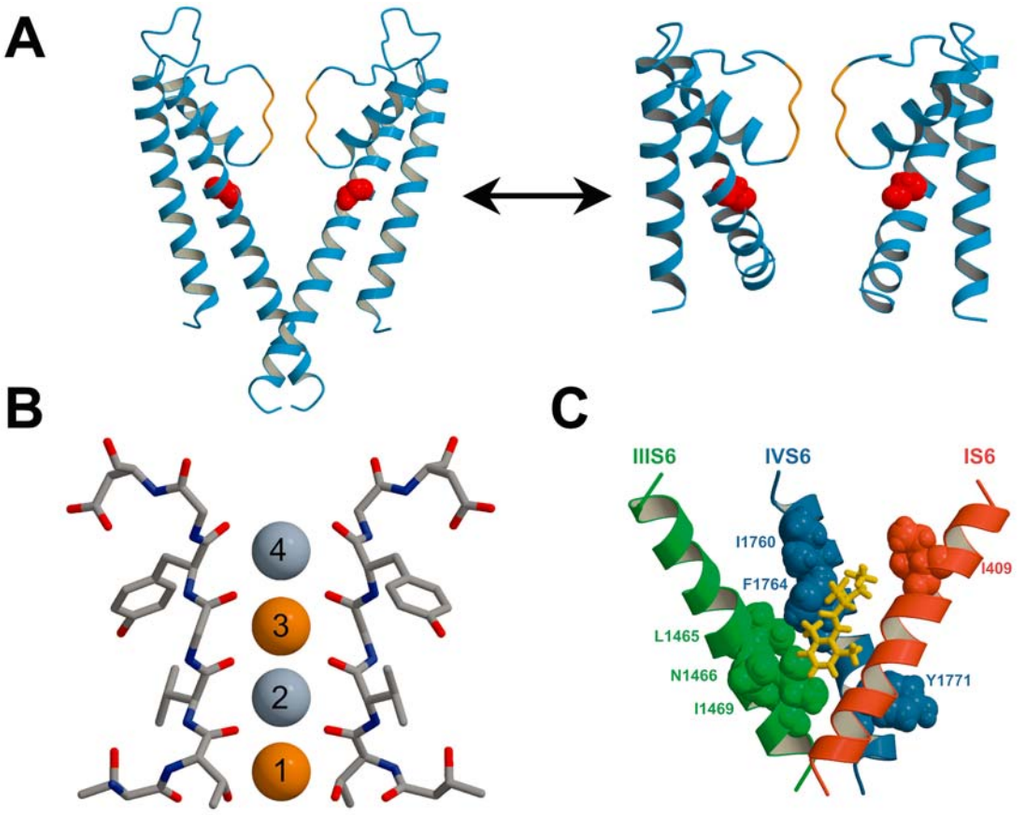

FIGURE 6-8 The ion selectivity filter and pore of Na^+ and K^+ channel illustrated with the extracellular side upwards. (**A**) A drawing of the three-dimensional structures of K^+ channels in the closed and open states, adapted from the three-dimensional structures of the K^+ channel from the bacterium *Streptococcus lividans* (closed, left) and *Methanococcus thermolytiyum* (open, right). Only two subunits are shown for clarity. (**B**) A drawing of K^+ ions moving single file through the ion selectivity filter of the K^+ channel from *S. lividans*. The four ion-coordination sites are thought to work in pairs. In one cycle of outward K^+ conductance, K^+ ions occupy sites 1 and 3 (orange), shift to sites 2 and 4 (gray), and then the K^+ ion in site 4 moves into the extracellular space while a new K^+ ion occupies site 1 and the K^+ ion in site 2 moves to site 3, re-establishing the initial state. (**C**) A drawing of the local anesthetic receptor site in the pore of the Na^+ channel. The amino acid residues that are thought to contact a bound etidocaine molecule (yellow) are indicated in a space-filling format for the S6 segments in domains I (red), III (green) and IV (blue).

the α-helices lining the inner pore cross at their intracellular ends, apparently closing the pore. In contrast, the structure of a distantly related bacterial K^+ channel [28], which is gated open by binding of Ca^{2+}, has a bend of approximately 30° in the inner pore helix (analogous to S6) at the position of a highly conserved glycine residue (Fig. 6-8A, right). This conformational change appears to open the pore at its intracellular end by splaying open the bundle of inner pore helices. Mutational studies of Na^+ channels support this pore-opening model because replacement of the conserved glycine with proline, which strongly favors a bend in the α-helix, also strongly favors pore opening and greatly slows closure [29].

Once the pore is open, K^+ ions appear to move outward in single file. Analysis of crystals shows four K^+ ions interacting with the backbone carbonyl groups of the amino acid residues that form the ion selectivity filter (Fig. 6-8B). It is thought that two K^+ ions occupy sites 1 and 3 (Fig. 6-8B, orange) and then switch to sites 2 and 4 (gray). The K^+ ion in site 4 would dissociate into the extracellular space, the K^+ ion in site 2 would move to site 3 and another K^+ ion from the intracellular space would bind to site 1. In this way, the pore remains occupied by two ions, during steady outward conduction.

Local anesthetics bind in the inner pore of Na^+ channels and block them (Fig. 6-5B). What is the structure of the local anesthetic binding site? A combination of site-directed mutagenesis and molecular modeling [16] reveals that local anesthetics and related antiepileptic drugs bind to a receptor formed by amino acid residues at specific positions in the S6 segments in domains I, III and IV of the Na^+ channel, as illustrated in Figure 6-8C. The aromatic and hydrophobic side chains of these amino acids contact the aromatic and substituted amino groups of the drug molecules and hold them in the receptor site, where they block ion movement through the pore.

Voltage-dependent activation requires moving charges. Structural models for voltage-dependent gating of ion channels must identify the voltage-sensors or gating charges (Fig. 6-5A) within the channel structure and suggest a plausible mechanism for transmembrane movement

of gating charge and its coupling to the opening of a transmembrane pore. The S4 segments of the homologous domains have been proposed as voltage sensors [13, 30]. These segments, which are conserved among Na^+, Ca^{2+} and K^+ channels, consist of repeated triplets of two hydrophobic amino acids followed by a positively charged residue. In the α-helical configuration, these segments would form a spiral of positive charge across the membrane, a structure that is well suited for transmembrane movement of gating charge (Fig. 6-5A). The positive charges are thought to be neutralized by negative charges in the nearby S2 and S3 segments. Much direct evidence in favor of designating the S4 segments as voltage sensors comes from mutagenesis studies [8, 19, 31]. Neutralization of positive charges results in progressive reduction of the steepness of voltage-dependent gating and of the apparent gating charge, as expected if indeed the S4 segments are the voltage sensors. At the resting membrane potential, the force of the electric field would pull the positive charges inward. Depolarization would abolish this force and allow an outward movement of the S4 helix. This outward movement has been detected in clever experiments that measure the movement of chemically reactive cysteine residues substituted for the native amino acids in S4 by analyzing the functional effects of specific chemical reactions at those substituted cysteines or the fluorescence of chemical probes located there [8, 32, 33]. This movement of the S4 helix is proposed to initiate a more general conformational change in each domain. After conformational changes have occurred in all four domains, the transmembrane pore can open and conduct ions (Fig. 6-6C).

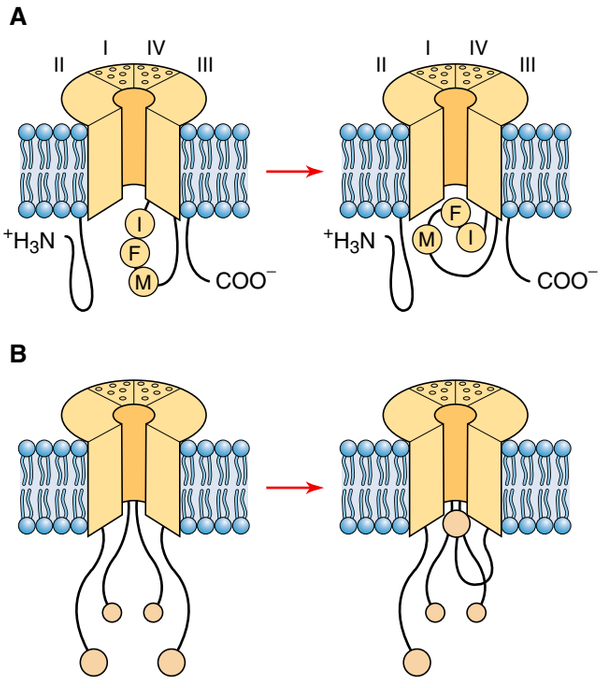

FIGURE 6-9 Mechanisms of inactivation of Na^+ and K^+ channels. (**A**) A hinged-lid model for Na^+ channel inactivation, illustrating the inactivation gate formed by the intracellular segment connecting domains III and IV and the critical cluster of hydrophobic residues that forms a latch holding the inactivation gate closed. *IFM*, isoleucine-phenylalanine-methionine. (**B**) A ball-and-chain model of K^+ channel inactivation. Each of the four subunits of a K^+ channel has a ball-and-chain structure at its N-terminus. Any one of the four can bind to the intracellular mouth of the open channel and inactivate it.

The fast inactivation gate is on the inside. Shortly after opening, many voltage-gated ion channels inactivate. The inactivation process of Na^+ channels can be prevented by treatment of the intracellular surface of the channel with proteolytic enzymes [2] or antibodies against the intracellular segment connecting domains III and IV (h, Fig. 6-6B) [23, 34], and expression of the Na^+ channel as two pieces with a cut between domains III and IV greatly slows inactivation [31]. A single cluster of three hydrophobic residues in this intracellular loop is required for fast inactivation [35] and inactivation is eliminated if these three hydrophobic residues are mutated to hydrophilic ones. Mutation of a single phenylalanine in the center of this motif nearly completely blocks fast inactivation of the channel. The segment of the Na^+ channel between domains III and IV is therefore proposed to serve as the inactivation gate by forming a hinged lid which folds over the intracellular mouth of the pore after activation [16] (Figs 6-6C and 6-9A). The cluster of hydrophobic residues may bind to the intracellular mouth of the pore like a latch to keep the channel inactivated.

A detailed model of K^+ channel inactivation has been derived from mutagenesis experiments on the original *Shaker* K^+ channels from *Drosophila* [36, 37]. The N-terminal of the K^+ channel serves as an inactivation particle and both charged and hydrophobic residues are involved. A ball-and-chain mechanism [9, 36, 37] has been proposed in which the N-terminal segment serves as a loosely tethered ball and inactivates the channel by diffusion and binding in the intracellular mouth of the pore (Fig. 6-9B). Consistent with this mechanism, synthetic peptides whose amino acid sequences correspond to that of the inactivation particle region can restore inactivation to channel mutants whose N-terminus has been removed. The mechanisms of inactivation of Na^+ and K^+ channels are similar in that in each case hydrophobic amino acid residues seem to mediate binding of an inactivation particle to the intracellular mouth of the pore. They differ in that charged amino acid residues are important in the inactivation particle of K^+ channels but not Na^+ channels, and that the inactivation particle is located over 200 residues away from the membrane at the N-terminus of the K^+ channel, compared to only 12 residues away from the membrane between domains III and IV of the Na^+ channel. It is likely that the hinged-lid mechanism of Na^+ channel inactivation evolved from the ball-and-chain mechanism of K^+ channels.

ION CHANNEL DIVERSITY

There are a surprisingly large number of evolutionarily and structurally related ion channel proteins— in the human genome more than 140 genes encode these proteins (Table 6-2) [5, 38]. What do they all do? Experiments in progress in many laboratories are beginning to reveal the meaning of this exceptional ion channel diversity.

Na^+ channels are primarily a single family. There are ten human genes encoding voltage-gated Na^+ channels (Table 6-2), and at least nine of the ten encode members of a single family ($Na_v1.1$–1.9) [38]. These Na^+ channels are expressed in different tissues and cells but their function is almost always to initiate action potentials in response to membrane depolarization.

Three subfamilies of Ca^{2+} channels serve distinct functions. In many cell types, it is not uncommon to find several Ca^{2+} channels that open with depolarization. The inflow of Ca^{2+} can assist in depolarizing cells but it also performs an important messenger role. The entering Ca^{2+} may activate exocytosis (secretion), contraction, gating of other channels, ciliary reorientation, metabolic pathways, gene expression, etc. (see Ch. 22). Indeed, whenever an electrical message activates any non-electrical event, a change of the intracellular free Ca^{2+} concentration acts as an intermediary. Genes that encode voltage-gated Ca^{2+} channels (Table 6-2) are grouped in three subfamilies that have distinct functions. In general, the members of a subfamily are greater than 75% identical in amino acid sequence in their conserved transmembrane regions while there is only 25–50% amino acid sequence identity between subfamilies [38]. The Ca_v1 subfamily of Ca channels conducts L-type Ca currents that initiate excitation–contraction coupling in muscle cells and secretion in endocrine cells, control gene transcription and regulate many enzymes. The Ca_v2 subfamily conducts N-, P/Q-, and R-type Ca^{2+} currents and are particularly concentrated in nerve terminals where a Ca^{2+} influx is required for release of chemical neurotransmitters (see Ch. 10). The Ca_v3 subfamily conducts T-type Ca currents that are activated at negative membrane potentials and are transient. These channels are important in repetitively firing cells, like the sinoatrial nodal cells that serve as pacemakers in the heart and the neurons in the thalamus that generate sleep rhythms. This division of Ca^{2+} channels is ancient – the worm *Caenorhabditis elegans* has a single member of each of these Ca^{2+} channel subfamilies. Evidently, specialization of Ca^{2+} signaling is crucial for even simple nervous systems.

There are many families of K^+ channels. K^+ channels have many different roles in cells. For example, in neurons they terminate the action potential by repolarizing cells, set the resting membrane potential by dominating the resting membrane conductance, determine the length and frequency of bursts of action potentials and respond to neurotransmitters by opening or closing and causing prolonged changes in membrane potential [5]. These channels are regulated by a combination of voltage, G proteins and intracellular second messengers. Many K^+ channels are gated primarily by voltage (K_v, Table 6-2). They can be divided into 12 subfamilies based on their amino acid sequence relationships [38]. In neurons, all the K_v channels are primarily involved in repolarizing the membrane to terminate action potentials.

In addition to the K_v family, the inward rectifier family, K_{ir} (Table 6-2), comprises seven subfamilies of channels [19, 38] that are primarily involved in setting the resting membrane potential. The $K_{ir}3$ subfamily is strikingly activated by G protein βγ subunits released by activation of G-protein-coupled receptors (see Ch. 19). This has an important regulatory influence on the resting membrane potential in many neurons. The $K_{ir}6$ subfamily forms a complex with a large nucleotide binding protein (the sulfonylurea receptor, SUR) to form ATP-gated K^+ channels that couple changes in intracellular ATP and ADP to changes in the resting membrane potential.

TABLE 6-2 The voltage-gated ion channel protein superfamily

Channel family	Symbol	No. in human genome	Primary activators	Primary physiological roles in neurons
Voltage-gated Na^+ channel	Na_v	10	Depolarization	Initiate action potentials
Voltage-gated Ca^{2+} channel	Ca_v	10	Depolarization	Couple depolarization to Ca^{2+} entry; repetitive firing
Voltage-gated K^+ channel	K_v	40	Depolarization	Repolarization
Inwardly rectifying K^+ channels	K_{ir}	15	G protein βγ-subunits, ATP	Set and regulate resting membrane potential
Two-pore K^+ channels	K_{2P}	15	Constitutively open	Set resting membrane potential
Calcium-activated K^+ channel	K_{Ca}	8	Depolarization and Ca^{2+}	Repolarization; regulation of repetitive firing
Cyclic nucleotide-gated channels	CNG	6	Cyclic AMP and cyclic GMP	Visual and olfactory sensory transduction
Hyperpolarization and cyclic nucleotide-gated channels	HCN	4	Hyperpolarization; cyclic AMP and cyclic GMP	Regulation of firing rate and pattern; regulation of pacemaking
Transient receptor potential channels	TRP	32	Lipid messengers; heat and cold	Ca^{2+} homeostasis, thermal and pain sensation

A third type of K^+ channel, K_{2P}, has a structure similar to two fused K_{ir} subunits, and only two subunits are required to form a pore (Table 6-2) [39, 40]. These channels are often called leak channels or open rectifiers because they are continuously open. Like the K_{ir} channels they are important in setting the resting membrane potential. Their activity is often regulated by kinases.

More ion channels are related to the Na_v, Ca_v and K_v families. Remarkably, evolution has created an even more diverse array of ion channels based on variations of the structure of voltage-gated K^+ channels (Fig. 6-7B) [38]. Ca^{2+}-activated K^+ channels (K_{Ca}) have a core, six-transmembrane-segment architecture similar to K_v channels, and they also contain regions in their C-terminal domain that can bind Ca^{2+} itself or the ubiquitous regulatory protein calmodulin. Binding of Ca^{2+} or Ca^{2+}/calmodulin to the C-terminal domain acts synergistically with membrane depolarization to activate the channel. These channels couple changes in intracellular Ca^{2+} concentration to repolarization of the membrane potential.

Cyclic nucleotide-modulated ion channels (Table 6-2) are not K^+-selective. Nevertheless, their inward current of Na^+ and Ca^{2+} ions is conducted through a channel that is similar in overall architecture to *Shaker* K^+ channels. This protein family includes the CNG channels, which respond only to cyclic nucleotides, and the HCN channels, which are activated synergistically by hyperpolarization and cyclic nucleotide binding [38, 40]. The CNG channels are involved in signaling of visual and olfactory information and serve as cyclic nucleotide-gated Ca^{2+} channels. In contrast, the HCN channels are required for normal rhythmic electrical discharges by the sinoatrial node in the heart and the pacemaker cells of the thalamus.

There is also a large family of transient receptor potential (TRP) channels [38, 41] (Table 6-2). These channels, first discovered as the target of a mutation in the *Drosophila* eye, also resemble K_v channels in architecture. However, they are nonselective in most cases, allowing both Na^+ and Ca^{2+} to enter cells when they are active, and they are not strongly affected by membrane potential, even though they have an S4 segment with some positive charges. The activity of this diverse group of channels is regulated in numerous ways, including by lipid messengers, protons and temperature. These channels conduct Ca^{2+} into cells in response to activation of G-protein-coupled receptors, initiate physiological responses to heat and cold, and may serve as mechanosensory channels under some circumstances. It is likely that we have much more to learn about the physiological roles of this interesting family of ion channels

There are many other kinds of ion channels with different structural backbones and topologies. The channels used in the action potential contrast with those generating slow potentials at synapses and sensory receptors by having strongly voltage-dependent gating. The other channels have gates controlled by chemical transmitters, intracellular messengers or other energies such as mechanical deformations in touch and hearing. In general, less is known about these channels than about Na^+, Ca^{2+} and K^+ channels of action potentials, with the exception of the nicotinic acetylcholine receptor channel of the neuromuscular junction (see Ch. 10). The ionic selectivity of these channels includes a very broad, monovalent anion permeability at inhibitory synapses, a cation permeability (about equal for Na^+ and K^+) at excitatory synapses at the neuromuscular junction and at many sensory transducers, and other more selective K^+ and Na^+ permeabilities in other synapses. The acetylcholine receptors of the neuromuscular junction and brain, the excitatory glutamate receptors and the inhibitory GABA and glycine receptors all have been solubilized and chemically purified, and the amino-acid sequences of their subunits have been determined by methods of molecular genetics (see Ch. 10). The structural features that are responsible for the function of these ligand-gated channels are being elucidated rapidly.

Recent research shows there is a great diversity of ion channels playing a great diversity of roles in cells throughout the body. We know now that hundreds of genes code for structural components of channels. Beyond their functions in the nervous system, channel activity in endocrine cells regulates the episodes of secretion of insulin from the pancreas and epinephrine from the adrenal gland. Channels initiate and regulate muscle contraction and cell motility. Channels form part of the regulated pathway for the ion movements underlying absorption and secretion of electrolytes by epithelia. Channels also participate in cellular signaling pathways in many other electrically inexcitable cells. Thus while they are especially prominent in the function of the nervous system, ion channels are actually a basic and ancient component of all cellular life, even bacteria [5].

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REFERENCES

1. Hodgkin, A. L. *The Conduction of the Nervous Impulse.* Springfield, IL: Charles C. Thomas, 1964.
2. Armstrong, C. M. Voltage gated K channels. *Sci. STKE* 2003(188), re10, 2003.
3. Nicholls, J. G., Martin, A. R., Wallace, B. G. and Fuchs, P. A. *From Neuron to Brain*, 4th edn. Sunderland, MA: Sinauer Associates, 2001.
4. Hille, B. Ionic basis of resting and action potentials. In J. M. Brookhart *et al.* (eds.), *Handbook of Physiology.* Washington, DC: American Physiological Society, 1977, vol. 1, pp. 99–136.

5. Hille, B. *Ion Channels of Excitable Membranes*, 3rd edn. Sunderland, MA: Sinauer Associates, 2001.
6. Hodgkin, A. L. and Huxley, A. F. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.)* 117, 500–544, 1952.
7. Hamill, O. P., Marty, A., Neher, E. *et al.* Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391, 85–100, 1981.
8. Bezanilla, F. The voltage sensor in voltage dependent ion channels. *Physiol. Rev.* 80, 555–592, 2000.
9. Armstrong, C. M. Sodium channels and gating currents. *Physiol. Rev.* 61, 644–683, 1981.
10. Catterall, W. A. Neurotoxins acting on sodium channels. *Annu. Rev. Pharmacol. Toxicol.* 20, 15–43, 1980.
11. Agnew, W. S. Voltage-regulated sodium channel molecules. *Annu. Rev. Biochem.* 46, 517–530, 1984.
12. Barchi, R. L. Voltage-sensitive sodium ion channels. Molecular properties and functional reconstitution. *Trends Biochem. Sci.* 9, 358–361, 1984.
13. Catterall, W. A. Molecular properties of voltage-sensitive sodium channels. *Annu. Rev. Biochem.* 55, 953–985, 1986.
14. Noda, M., Ikeda, T., Kayano, T. *et al.* Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* 320, 188–192, 1986.
15. Isom, L. L., De Jongh, K. S. and Catterall, W. A. Auxiliary subunits of voltage-gated ion channels. *Neuron* 12, 1183–1194, 1994.
16. Catterall, W. A. From ionic currents to molecular mechanisms: the struture and function of voltage-gated sodium channels. *Neuron* 26, 13–25, 2000.
17. Catterall, W. A. Structure and regulation of voltage-gated calcium channels. *Annu. Rev. Cell Dev. Biol.* 16, 521–555, 2000.
18. Hofmann, F., Biel, M. and Flockerzi, V. Molecular basis for Ca^{2+} channel diversity. *Annu. Rev. Neurosci.* 17, 399–418, 1994.
19. Jan, L. Y. and Jan, Y. N. Voltage-gated and inwardly rectifying potassium channels. *J. Physiol.* 505, 267–282, 1997.
20. Pongs, O. Voltage-gated potassium channels: from hyperexcitability to excitement. *FEBS Lett.* 452, 31–35, 1999.
21. Doupnik, C. A., Davidson, N. and Lester, H. A. The inward rectifier potassium channel family. *Curr. Opin. Neurobiol.* 5, 268–277, 1995.
22. Noda, M., Ikeda, T., Suzuki, H. *et al.* Expression of functional sodium channels from cloned cDNA. *Nature* 322, 826–828, 1986.
23. Goldin, A. L., Snutch, T., Lubbert, H. *et al.* Messenger RNA coding for only the α-subunit of the rat brain Na channel is sufficient for expression of functional channels of *Xenopus* oocytes. *Proc. Natl Acad. Sci. U.S.A.* 83, 7503–7507, 1986.
24. Terlau, H., Heinemann, S. H., Stühmer, W. *et al.* Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. *FEBS Lett.* 293, 93–96, 1991.
25. Heinemann, S. H., Terlau, H., Stühmer, W. *et al.* Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature* 356, 441–443, 1992.
26. Miller, C. *Annus mirabilis* for potassium channels. *Science* 252, 1092–1096, 1990.
27. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A. *et al.* The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* 280, 69–77, 1998.
28. Jiang, Y., Lee, A., Chen, J. *et al.* Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* 417, 515–522, 2002.
29. Zhao, Y., Yarov-Yarovoy, V., Scheuer, T. *et al.* A gating hinge in Na^+ channels: a molecular switch for electrical signaling. *Neuron*, 41, 859–865, 2004.
30. Guy, H. R. and Conti, F. Pursuing the structure and function of voltage-gated channels. *Trends Neurosci.* 13, 201–206, 1990.
31. Stühmer, W., Conti, F., Suzuki, H. *et al.* Structural parts involved in activation and inactivation of the sodium channel. *Nature* 339, 597–603, 1989.
32. Yang, N. B., George, A. L. Jr. and Horn, R. Molecular basis of charge movement in voltage-gated sodium channels. *Neuron* 16, 113–122, 1996.
33. Gandhi, C. S. and Isacoff, E. Y. Molecular models of voltage sensing. *J. Gen. Physiol.* 120, 455–463, 2002.
34. Vassilev, P., Scheuer, T. and Catterall, W. A. Identification of an intracellular peptide segment involved in sodium channel inactivation. *Science* 241, 1658–1661, 1988.
35. West, J. W., Patton, D. E., Scheuer, T. *et al.* A cluser of hydrophobic amino acid residues required for fast sodium channel inactivation. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10910–10914, 1992.
36. Hoshi, T., Zagotta, W. and Aldrich, R. W. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science* 250, 533–538, 1990.
37. Zagotta, W., Hoshi, T. and Aldrich, R. W. Restoration of inactivation in mutants of *Shaker* potassium channels by a peptide derived from Sh B. *Science* 250, 568–571, 1990.
38. Catterall, W. A., Gutman, G. A. and Chandy, K. G. The IUPHAR compendium of voltage-gated ion channels. Leeds, UK: IUPHAR Media, 2002; www.iuphar-db.org/iuphar-ic.
39. Goldstein, S. A., Bockenhauer, D., O'Kelly, I. *et al.* Potassium leak channels and the KCNK family of two-P-domain subunits. *Nat. Rev. Neurosci.* 2, 175–184, 2001.
40. Matulef, K. and Zagotta, W. N. Cyclic nucleotide-gated ion channels. *Annu. Rev. Cell Dev. Biol.* 19, 23–44, 2003.
41. Clapham, D. E. TRP channels as cellular sensors. *Nature* 426, 517–524, 2003.

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CHAPTER

7

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SUMMARY 120

OVERVIEW

Cell adhesion molecules (CAMs) play critical roles in all facets of nervous system development and maintenance. Important phenomena in which CAMs are involved include initial formation of the neural tube and the neural crest, migration of all neurons and glial cells, axonal outgrowth and guidance, target selection, synaptic stabilization and plasticity, myelination and nerve regeneration after injury (see Chs 4, 24, 28–30 and 53). Adhesion molecules interact with each other and with nonadhesive cell-surface and/or cytoplasmic molecules, and, in the two most extreme situations, may produce diametrically opposite results. For example, under certain circumstances, adhesion can vigorously encourage movement or growth along a preferred pathway, or, in contrast, it can completely immobilize a cell or membrane and prevent further movement or growth. How can membrane adhesion molecules mediate these very different effects? The answer to this question is not straightforward but, to reduce the answer to simple terms, a number of factors sum to yield the net effect of all adhesive forces acting on cell surfaces. There are always many adhesion molecules expressed at the same time on a given cell surface but in different concentrations. Furthermore, each adhesion molecule subtype is unique in terms of adhesive preference and adhesive strength. An additional complication is that, in some cases, an individual adhesion molecule may display more than one binding site, with each site specific for a different peptide ligand protruding from an opposing cell surface or membrane. Also, the post-translational addition of certain charged carbohydrate moieties, for example sialic acid, to adhesion proteins may interfere with the adhesive properties of the polypeptide. The post-translationally modified protein is rendered nonadhesive through charge repulsion and may actually repel abutting membrane surfaces. The reality in many systems is that the net result of all adhesion molecule interactions on the cell surface at a given time yields an averaged effect.

Cell adhesion molecules comprise several 'families'. These families are defined by individual members, which are related to each other by common primary sequences, structural motifs and binding properties (e.g. Fig. 7-1). In spite of differences in the biochemistry and function of each CAM, we can make certain generalizations about how they function in nervous tissue. First, CAMs act at

the cell surface, where they interact with either identical molecules, termed *homophilic interaction,* or different molecules, termed *heterophilic interaction*, expressed on an opposing cell surface or in the extracellular matrix (ECM). Second, adhesion has consequences for the cells involved, usually by virtue of adhesion molecule interactions with the underlying cytoskeleton [1]. Third, adhesive events between cells vary in strength, 'strong' vs. 'weak' adhesion, and the strength of adhesion is influenced by a wide range of factors. For example, the sum of all adhesive forces acting on a growing neurite is likely to be weaker than that of all the forces causing permanent adherence of pre- and postsynaptic membranes. Fourth, intercellular adhesion may be modulated, depending on the situation (Fig. 7-2). Last, because the nervous system is not a homogeneous organ, it is important to consider when and where each CAM is expressed. In other words, the temporal and spatial expression patterns of CAMs sharply focus and limit their interactions.

The three major groups of CAMs found in the nervous system are the members of the immunoglobulin (Ig) gene superfamily (IgCAMs, **Figs 7-1, 7-2**), the integrins (Fig. 7-3) and the cadherins (Fig. 7-4). Another family of carbohydrate-binding CAMs, the selectins, is important in other tissues, particularly in the immune system [2]. However, the selectins seem not to be expressed by neurons or glia. To date, these proteins have been detected only in the nervous system when expressed by invading immune cells in pathological states. The cadherins and IgCAMs engage exclusively in cell–cell or membrane–membrane interactions, while integrins, for the most part, interact with components of the ECM. Integrins appear to be more important in the PNS than the CNS, but play a critical role in early axonal growth in both CNS and PNS [3, 4]. In adult CNS, ECM is less well developed; this might explain the low profile integrins have in the mature CNS. However, it should be noted that within the CNS, integrins do operate to maintain the integrity of the cerebrovascular system, and when the functions of these adhesion molecules are compromised, severe cerebral hemorrhage is the result [5].

Within each of these adhesion molecule families, membership has been defined largely by amino-acid sequence similarity, which is reflected in common structural features. Consequently, distinct binding requirements also characterize each family. For example, cadherins interact in a Ca^{2+}-dependent, usually homophilic manner. Binding of the members of the Ig family is Ca^{2+}-independent and, although frequently homophilic, can be heterophilic. Integrin binding is also divalent cation-dependent (Ca^{2+}, Mg^{2+}) but always heterophilic.

Another, but less well defined, class of molecules, some of whose members mediate adhesion interaction, is the four-transmembrane domain family, which shares similar hydropathy plots and may have similar dispositions with respect to the phospholipid bilayer, for example the myelin proteolipid proteins, the connexins of gap junctions, the ryanodine receptor and others.

THE IMMUNOGLOBULIN GENE SUPERFAMILY

The best studied group of recognition/adhesion molecules expressed in the nervous system is that of the IgCAMs (Fig. 7-1), which are defined by regions that have sequence similarity with Igs, termed the Ig domains [6, 7]. Ig domains contain alternating hydrophobic and hydrophilic stretches of residues, which form a series of antiparallel β strands. The β strands come together to form two β sheets. The folding of the β sheets is, in most cases, stabilized by the formation of a disulfide bond between the sheets. There are three subclasses of Ig-like domains, which are defined by their similarity to variable (V) or constant (C) regions of Igs. For V-like domains, there are 70–110 amino acids spanning the two cysteines that form the disulfide bond, allowing formation of seven to nine β strands. C-like domains have about 50 amino acids spanning the stabilizing cysteines and, consequently, carry seven β sheets. The third class of Ig-like domains is termed a C2 domain. This class of IgCAM has the β strand distribution of a C-like domain but bears more sequence similarity to V-like domains.

The formation of immunoglobulin-like domains may confer characteristics important for extracellular presentation and interaction with other molecules. First, because of the folding pattern, Ig domains are stabilized by both inward-pointing hydrophobic amino acids and the intersheet disulfide bond, making them relatively resistant to proteolysis and, hence, ideal molecules to present to the external cellular environment. Second, the folding of the β strands provides a good platform for the presentation of amino acids, carried in the loops between the strands, for interaction with an opposing molecule. The loops between β strands, in antibodies, carry the antigen-recognition sites and, in Ig-like domains, contain the regions of greatest variability, allowing for distinct and specific interactions.

Members of this family of molecules may have only one Ig-like domain, as is the case for the myelin protein P_0, or, as for most of the family, have many Ig domains. In addition to the subclassification of Ig domains into V-, C- and C2-like domains, Ig family members can be broadly divided into three general classes [8]: (a) those that have only Ig-like domains; (b) those that have Ig domains and additional domains that resemble regions of the ECM component fibronectin, termed FN-like domains; and (c) those that have Ig domains and motifs other than FN-like domains. Moreover, any one Ig family member may have many isoforms, which may differ in the length of the cytoplasmic domain, in their post-translational modifications and whether they are membrane-spanning or glycosylphosphatidylinositol (GPI)-anchored proteins (see Box 3-1). Also, additional amino acid sequences inserted in the extracellular domain may distinguish isoforms of a particular IgCAM. While it is not known how the majority

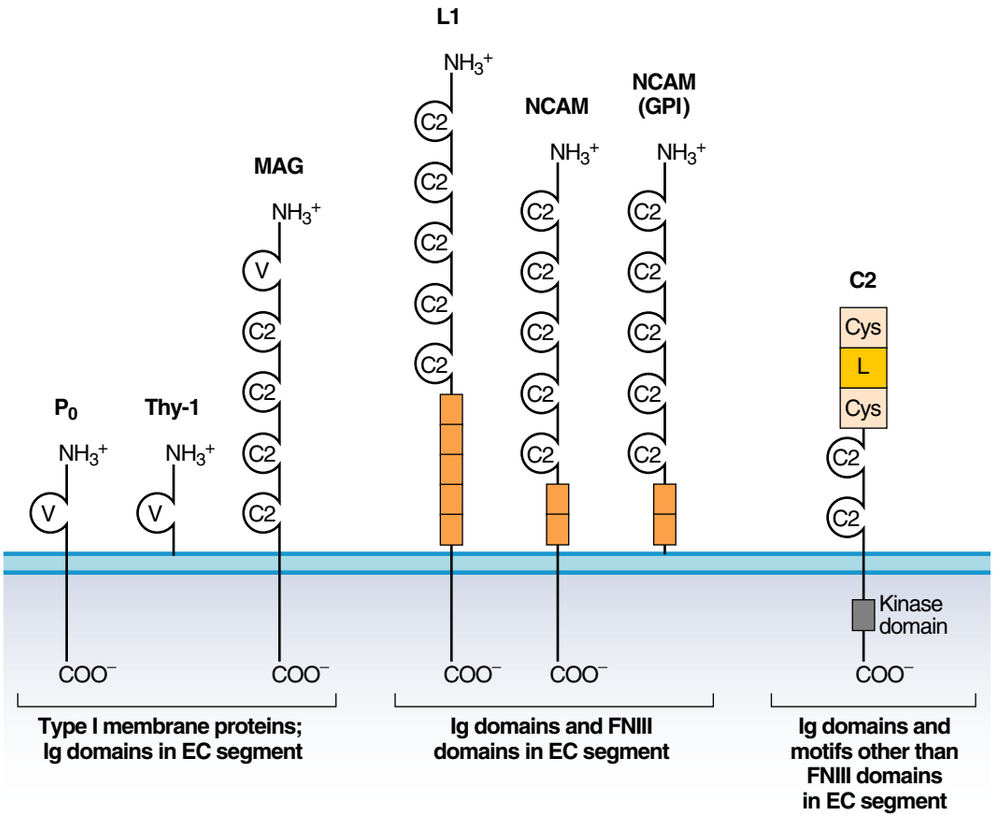

FIGURE 7-1 The immunoglobulin (Ig) gene family of molecules. Several varieties of Ig domain-containing molecules are contained within the Ig gene superfamily. Most are type I membrane proteins; some have only Ig domains or other moieties that may convey function (see text). *V*, variable Ig domain; *C*, constant Ig domain; *MAG*, myelin-associated glycoprotein; *NCAM*, neural cell adhesion molecule; *GPI*, glycosylphosphatidyl-inositol; *EC*, extracellular domain; *FN*, fibronectin.

of different isoforms of a particular IgCAM affect its functioning, differences in effect have been described for molecules that carry some of the isoform-distinguishing amino acid sequences in the extracellular domain of the neural cell adhesion molecule (NCAM). For example, a sequence of ten amino acids, termed the variable alternative spliced exon (VASE) sequence, in the fourth Ig domain of some isoforms of NCAM alters the response of axonal growth to this adhesion molecule; NCAM proteins with the VASE sequence are much less effective at promoting axonal growth than are NCAM proteins without this sequence. However, a puzzling question is: How do IgCAMs that have identical extracellular domains but are either GPI-linked or membrane-spanning, differ in function? Similarly, how differences in the cytoplasmic domain affect function is still not known. Presumably, the cytoplasmic domains interact with signal-transduction cascades and cytoskeletal proteins and in this way transduce adhesion into an intracellular response.

The siglecs constitute a novel subfamily of immunoglobulin-like molecules that bind to sialosides. These molecules, previously termed sialoadhesins, share considerable sequence similarity among the first four amino-terminal Ig domains [8, 9]. More importantly, all members of this subfamily bind to sialoglycoconjugates. To date, only two siglecs have been identified in the nervous system: the myelin-associated glycoprotein (MAG) (see Ch. 4) and the Schwann cell myelin protein (SMP). All other family members are specific to the immune system. An additional common feature of this IgCAM subfamily is that they bind sialic acid with relatively low affinity. Because of this, it is suggested that siglecs must be clustered within the membrane and that the molecule(s) with which siglecs interact must either be clustered or carry multimeric sialic acid residues to be effective. It should be noted, however, that, although both MAG and SMP have been shown to be sialic acid-binding proteins, the identity of a possible sialoglycoconjugate(s) with which they interact and the functional relevance of such an interaction have yet to be described.

Immunoglobulin-like cell adhesion molecules signal to the cytoplasm. In some instances, adhesion may act primarily to bind membranes to surfaces but it now seems clear that some IgCAMs act via the cytoplasmic domain after engaging with a cognate partner molecule to initiate a signal-transduction cascade as a direct consequence of an adhesive interaction. A good example of this is the Trk receptors, which have two Ig domains in their

extracellular sequences, or the fibroblast growth factor (FGF) receptor with four Ig-like domains, which first bind a neurotrophin, such as nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin 3 (NT3) or FGF (Ch. 27), after which signal transduction is triggered by dimerization and autophosphorylation of the cytoplasmic domains by endogenous tyrosine kinases (see Ch. 24). In contrast, in molecules such as NCAM and L1, which have multiple Ig domains, and P_0, which has a single Ig domain, all of which are known to interact homophilically, there is no obvious mechanism whereby a signaling cascade could be initiated after interaction. None of these proteins carries endogenous tyrosine kinase activity or any motifs that might indicate an interaction with G proteins. A novel mechanism for signaling [10, 11] has been suggested for NCAM, L1 and N-cadherin (see below) in that these molecules are believed, in certain circumstances, to cluster with the FGF receptor and induce autophosphorylation of that receptor in the absence of its usual ligand, FGF (Fig. 7-2). Each of these molecules has been implicated in neurite outgrowth [10–13]. In contrast, the myelin P_0 protein, although it has been suggested [14] to cluster within its membrane and interact, initially, with the cytoskeleton, is unlikely to initiate a signal-transduction cascade. The primary, if not the only, role of P_0 is to hold the myelin membranes in a tightly compacted state (Ch. 4).

THE CADHERIN FAMILY

The cadherins (Fig. 7-4) are a superfamily of adhesion molecules that function in cell recognition [15, 16], tissue morphogenesis and tumor suppression [17]. As a group, the cadherins are Ca^{2+}-dependent adhesion molecules and, within the group, the classic cadherins are the best studied. These include neural cadherin (N-cadherin), epithelial cadherin (E-cadherin), placental cadherin (P-cadherin) and retinal cadherin (R-cadherin). Although the classic cadherins were originally named for the tissue in which they were first found, their distributions are in no way limited to these tissues. Brain tissue expresses at least 20 [18, 19], and possibly many more, individual cadherins, which are used in differential neurite outgrowth, cell–cell interactions and 'locking in' pre- and postsynaptic membranes at the synaptic junctional complex.

In general, the cadherins have a common primary structure in that they all contain an amino-terminal

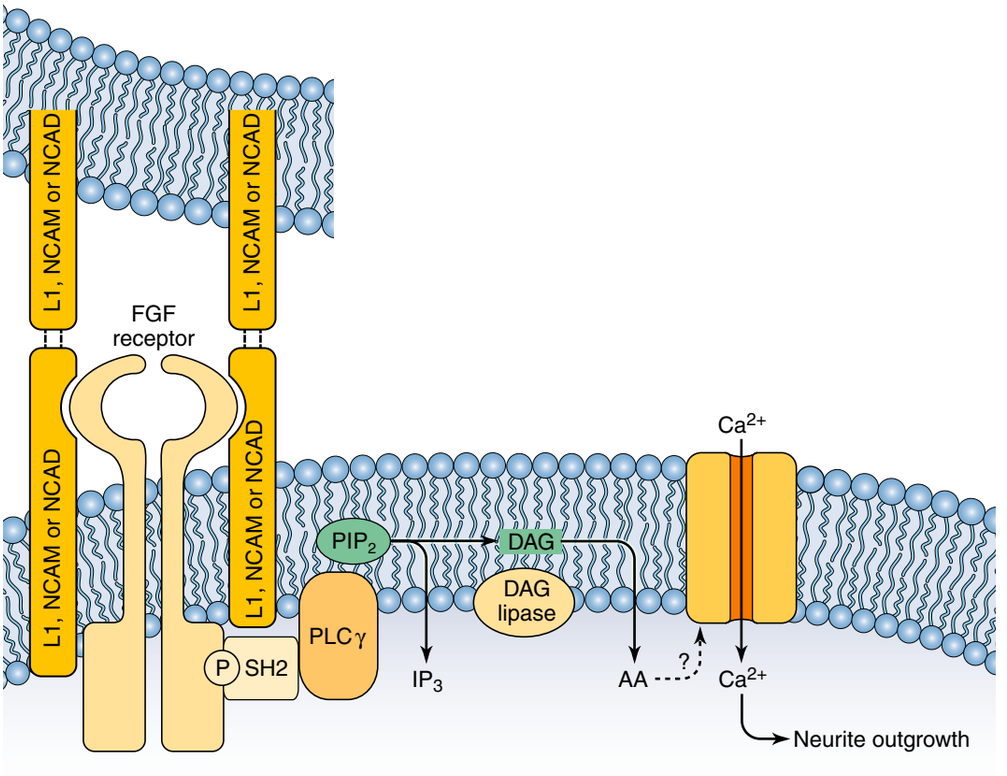

FIGURE 7-2 Signaling events in cell adhesion molecule (*CAM*)-stimulated neurite outgrowth. It has been postulated that a signaling cascade is stimulated by homophilic interactions of neural cell adhesion molecule (*NCAM*), N-cadherin (*NCAD*) or L1 adhesion molecule, which dimerizes the fibroblast growth factor (*FGF*) receptor and activates phospholipase C-γ (*PLCγ*) to generate diacylglycerol (*DAG*), conversion of DAG to arachidonic acid (*AA*) by a DAG lipase and an AA-induced increase in Ca^{2+} influx into the neurons via Ca^{2+} channels. *SH2*, src homology 2 domain; *PIP_2*, phosphatidyl-inositol 4,5-bisphosphate; *IP_3*, inositol (1,4,5)-trisphosphate.

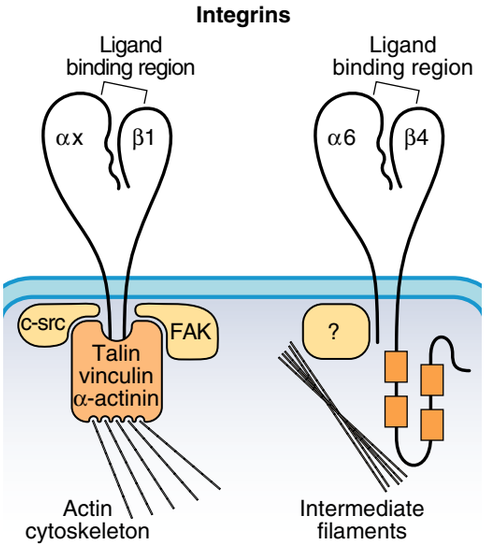

FIGURE 7-3 Integrins are heterodimers. Integrins consist of α and β subunits noncovalently linked, which interact via their cytoplasmic domains with a number of cytoplasmic proteins. The α*x*β1 integrin complex on the left binds to a complex which includes focal adhesion kinase (*FAK*). The α6β4 integrin complex contains fibronectin *(FN)* III repeats, which mediate binding to intermediate filaments.

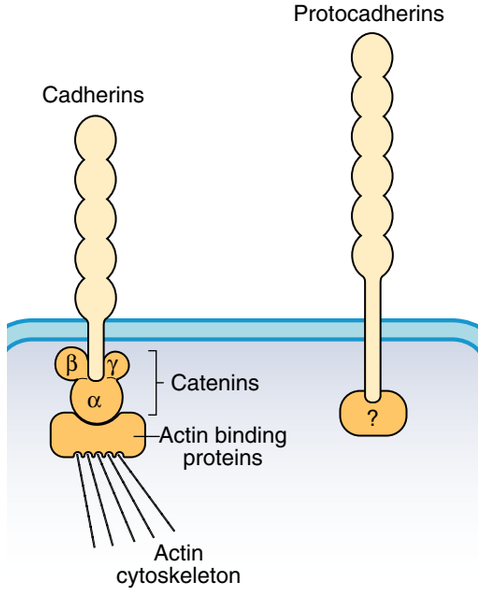

FIGURE 7-4 Basic cadherin structure. Two types of cadherin are present in the nervous system: the classic cadherins with five extracellular domains and the protocadherins with six extracellular domains. For the classic cadherins, the binding partners on the cytoplasmic side include the α, β and γ catenins. The binding partners for the protocadherins have not yet been identified.

extracellular domain, a single transmembrane domain and a conserved carboxyl-terminal cytoplasmic region, which is responsible for interactions with signaling pathways and with cytoplasmic and cytoskeletal proteins. The extracellular domain can be divided into five homologous regions, referred to as extracellular domains 1–5 (EC1–EC5). All of the extracellular domains are highly homologous to one another and, between the extracellular domains, Ca^{2+} articulates with binding sites and serves to rigidify the extracellular domain into a rod-like conformation.

The classic cadherins are homophilic adhesion molecules. That is, E-cadherin expressed on one cell surface binds to E-cadherin expressed on an apposed cell surface, N-cadherin binds to N, P to P and so forth. It was originally thought that all cadherins would behave completely homophilically but it is now clear that, for example, N-cadherin can bind to R-cadherin, although perhaps more weakly than it would to N-cadherin.

A recent crystal structure based model [20] for the structure of C-cadherin postulates that the five extracellular domains EC1–EC5 protrude from the cell surface as a curved rod. The structural analysis of C-cadherin reveals that the molecules facing each other across apposed cell surfaces are antiparallel to one another, forming a dimeric interaction termed a 'strand dimer' (Fig. 7-5). This forms the functional unit that is likely to mediate adhesion between cell surfaces. The structure from this recent paper allows the prediction of both *cis* and *trans* interfaces that together result in a lattice and not, as previously believed, an adhesion zipper. This new model allows for a mechanism by which adhesion 'plates' or puncta might be generated, such as are formed at CNS synapses [21, 22], adherens junctions and desmosomes [23], all cadherin based organelles.

The first cadherin to be expressed in the nervous system is N-cadherin. This molecule appears around the time the neural tube closes [24]. As the neural tube expands and develops, a variety of cadherins can be detected in cell bodies, outgrowing neurites and synaptic endings [25]. Many adhesion/recognition molecules have been implicated in guiding axons to their appropriate targets. Once the target axon has arrived in the vicinity of its ultimate destination, it has been suggested [26, 27] that expression of identical cadherin molecules on both pre- and postsynaptic membranes ultimately links up and locks in these membranes to the synaptic junctional complex. According to this proposed mechanism, neurons in the brain would utilize the same mechanisms to produce pre- and postsynaptic membrane adhesion as do other cells in forming junctions, such as the cadherin-based mechanisms of the epithelial adherens junction. In the brain, neural transmitter release mechanisms, neurotransmitter receptors, second-messenger systems and other uniquely synaptic elements are superimposed on this adhesive scaffold.

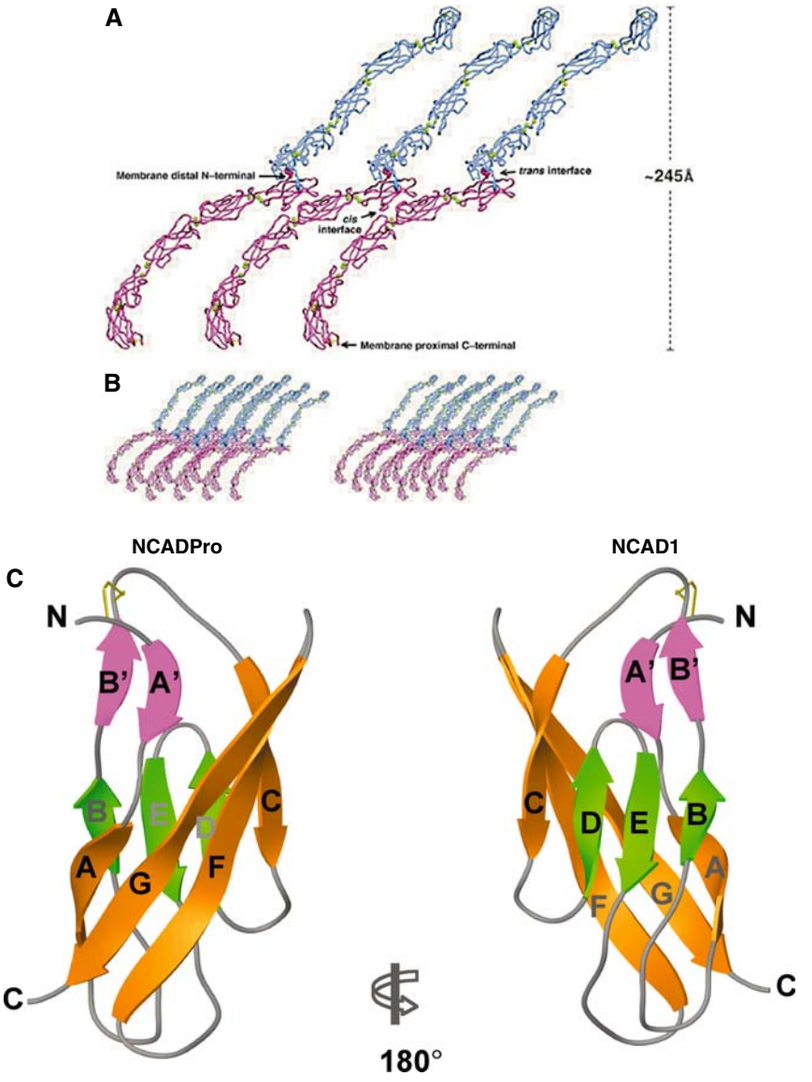

FIGURE 7-5 Cadherin structure. (**A**) Cadherin extracellular domains are illustrated as if protruding from two conjoined cell surfaces (a *pink* and a *blue* surface). The *green* dots are calcium ions. The *cis* and *trans* interfaces together determine the lattice. (**B**) Stereo view of the three-dimensional cadherin lattice. (**A** and **B** from reference [18].) (**C**) This is a ribbon diagram of a representative low-energy NMR structure of NPro (residues 31–124). β strands of the three sheets are depicted as *magenta*, *green* and *orange* arrows and the disulfide bridge between Cys32 and Cys63 is depicted in *yellow*. Loop regions are shown in *gray* and N- and C-termini are indicated.

These complex elements expand the function of this 'neural adherens junction'.

CELL ADHESION MOLECULES AND AXONAL OUTGROWTH

Cell adhesion molecules influence axonal outgrowth. During development, axons extend from the neuronal cell body and grow, frequently over long distances, in order to make very precise connections with a target. Despite the complexity of the nervous system, where over 10^{12} axons must find their targets, the pattern of axonal outgrowth for any one axon is highly reproducible from one individual to another. The flattened tip of a growing axon is called the growth cone, which resembles the palm of the hand, with processes extending from it like fingers (see Chs 8 and 25). It is the growth cone that responds to the environment and determines what direction the growing axon will take. Environmental cues that the growth cone encounters can be either fixed or diffusible, and it is the integration of these signals that determines the final direction. The majority of fixed signals are CAMs, expressed by glia, other cells or older axons that have already traversed that particular pathway. In addition, growth cones use integrins to select a pathway of ECM molecules on which to extend processes.

This has been demonstrated from both tissue culture studies and studies *in vivo* [28–30]. In culture, members of all three families of adhesion molecules found in the nervous system have been shown to promote axonal growth. This was carried out by transfecting the cDNA for the molecule to be tested in a cell line that does not normally express it, usually a fibroblast cell line. Isolated neurons are then grown on a monolayer of the CAM-expressing cells, and neurite length is compared to that obtained by contact with cells not expressing the adhesion molecule. Experiments like these have shown that CAMs, including the cadherins, the integrins and members of the Ig family, in particular NCAM and L1, are very potent promoters of axonal growth from a variety of primary neurons. The same neuron has been shown to respond to different CAMs, indicating that there is not any one unique 'CAM cue' for a particular neuron. Instead, it is likely that a variety of CAMs contribute to the effect on axonal growth. As well as being able to promote axonal growth when fixed in the ECM, some CAMs, such as L1, have been shown to promote axonal growth when added in a soluble form to neurons in culture. This strengthens the suggestion that CAMs are not just 'sticky' molecules but can exert their effects by activation of a signal-transduction pathway. Indeed, a number of soluble forms of various CAMs, including L1, NCAM, MAG and cadherins, have been found in the extracellular milieu of living cells. It remains to be determined whether these soluble CAMs in fact influence axonal growth *in vivo.*

In addition to CAM-expressing fibroblasts, another more physiological cell substrate has been used to demonstrate the effects of CAMs on axonal growth in culture. When dorsal root ganglion neurons are grown on a monolayer of nonmyelinating Schwann cells, which are very permissive for growth *in vivo* when not synthesizing myelin proteins, there is robust axonal outgrowth. Only when a combination of antibodies against NCAM, L1, cadherins and integrins was added to the cultures was outgrowth significantly reduced. Together, these experiments indicate that axonal outgrowth at any one time can be influenced by several CAMs.

This concept is supported by observations *in vivo.* Knockout mice lacking NCAM expression are viable and display subtle abnormalities in the nervous system. These abnormalities include a reduction in the size of the olfactory bulb, which is attributed to a decrease in migration of the neuronal cells that form this structure. Possibly, the inability to migrate normally reflects an increase in cellular adhesiveness, due to the absence of the highly sialylated form of NCAM, which in the wild-type or normal animal tends to repel apposed membranes on which it is expressed. Also, a reduction in the density of mossy fibers in the hippocampus has been noted in the NCAM knockout mouse, revealing a defect in axonal growth. The subtle phenotype in these mice, relative to the dramatic effect of NCAM on axonal growth in culture, suggests that there may be a certain amount of redundancy in nervous tissue, whereby another CAM or combination of CAMs can in part compensate for the absence of NCAM.

Cell adhesion molecules are responsible for axonal fasciculation. Usually, when growing toward their target, axons fasciculate (Fig. 7-6) and grow in bundles. However, there are points along the pathway where axons must defasciculate and different axons must take different paths. Hence, in addition to promoting axonal outgrowth, CAMs can affect the direction an axon takes at key decision points along the way. One model that has been proposed for explaining how an axon is selected to remain fasciculated with respect to other axons or to separate from the bundle and grow alone is based on the relative adhesiveness of the substrate vs. the adhesiveness of other axons. This model was proposed based on observations of adhesion and axonal growth with two forms of NCAM: one form that carries the highly charged sialic acid and polysialic acid (PSA) and another form with very little or no PSA. It has been suggested that the high negative charge on PSA–NCAM renders it less adhesive than its unsialylated counterpart [31]. Because the negatively charged sialic acid moieties tend to sequester water, occupy a large volume in the extracellular space and strongly repel one another across the bilayer, they probably keep plasma membranes sufficiently far apart so as to prevent other adhesion molecules, such as L1 and the cadherins, from

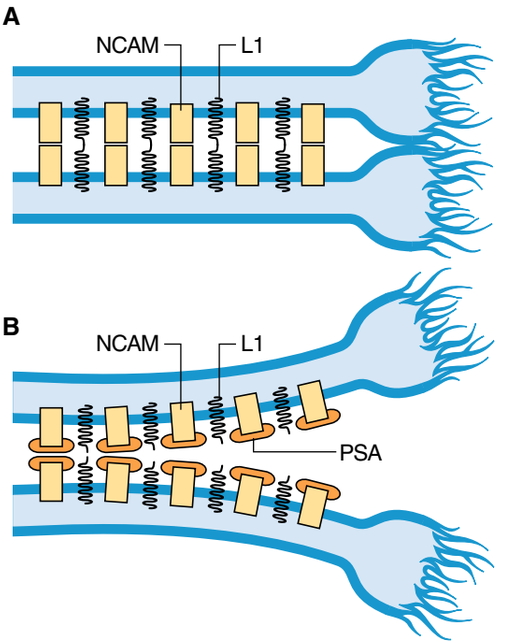

FIGURE 7-6 Neural cell adhesion molecule (*NCAM*) carries different levels of polysialic acid. (**A**) NCAM is depicted on a growing axon, without polysialic acid. Under these conditions, NCAM and L1 molecules interact homophilically and axons fasciculate. (**B**) NCAM carries polysialic acid (*PSA*), and NCAM and L1 molecules are far apart and cannot, therefore, interact. Growing axons do not fasciculate under these circumstances.

getting close enough to interact. Following from this, fasciculated axons, which adhere tightly to each other, express the adhesive form of NCAM that does not carry PSA. In contrast, the PSA form of NCAM is more effective than the unsialylated form at promoting axonal growth.

As stated above, the complexity and precision of connections within the nervous system would appear to require a very rigid set of rules or cues. The ubiquitous expression of individual CAMs throughout the nervous system and during development implies that they could not provide the specificity required for such a precise signal for individual neurons. However, the full repertoire of CAMs used *in vivo* for any single axon to reach its target has yet to be characterized. It is likely, as suggested from both *in vivo* and *in vitro* results, that many CAMs affect the growth of any one axon. In addition, as demonstrated by studies with different isoforms of NCAM, i.e. with and without the VASE sequence or with and without PSA, it is highly likely that subtle differences in isoform expression of any single CAM can have dramatic effects on axonal response. What has been determined regarding the role of adhesion molecules in axonal guidance during development to date is most probably an underestimate of their full involvement.

Cell adhesion molecules may also function in regeneration. As well as growing during development, axons grow and regenerate under certain conditions after injury [32]. Axonal regeneration is often successful in the PNS, where it may be accompanied by full restoration of function. In contrast, in the mammalian CNS, there is usually little or no regrowth and function is often lost. The main difference between axonal growth during development and during regeneration is in whether the damaged axon is allowed by the existing conditions to regrow, not whether it is able (Ch. 30). In the PNS, after injury, damaged tissue, consisting mostly of myelin debris, is cleared away by macrophages. Coincident with this event, Schwann cells downregulate the expression of the myelin-specific proteins and upregulate a number of growth-promoting adhesion molecules, including L1 and NCAM. These Schwann cells now resemble Schwann cells in the developing nervous system and are very permissive for axonal growth (Fig. 7-7). In contrast, myelin is not cleared from the CNS after injury and the myelin-forming cells in the CNS, oligodendrocytes, continue to express myelin proteins and do not upregulate any growth-promoting adhesion molecules. There is now substantial evidence to support the idea that the presence of myelin and the absence of growth-promoting molecules are two factors largely responsible for the lack of regeneration in the mammalian CNS [33]. Myelin membranes have been shown to be inhibitory for regeneration both *in vitro* and *in vivo*, and a number of myelin-specific molecules have been shown to be potent inhibitors of axonal growth. One of these is MAG, which is a member of the siglec subfamily. MAG can promote as well as inhibit axonal growth depending on the age and type of neuron. The ability of MAG to inhibit or promote regeneration when presented to the neuron either as a substrate or in a soluble form strongly suggests that MAG binds to a neuronal receptor and activates a signal-transduction mechanism in the neuron that effects changes in axonal growth [34, 35].

CELL ADHESION MOLECULES IN MYELINATION

Myelin is formed by the compaction of oligodendrocyte plasma membranes in the CNS or Schwann cell plasma membranes in the PNS as the plasma membrane spirals around the axon. For myelin to function efficiently, a tight apposition of these membranes must be maintained, which, when viewed by electron microscopy, displays a uniform and reproducible spacing between the layers (see Ch. 4). Given the close association of myelin membranes, it is not surprising that CAMs play major roles in the formation and maintenance of this plasma membrane organelle. There is strong evidence supporting a role for CAMs in the initiation of myelination, in the compaction of myelin membranes and in the stability of the noncompacted as well as the compact layers of myelin, the axon–myelin interface and the nodes of Ranvier.

The myelinating cell, when brought into contact with a large axon, begins to synthesize vast amounts of plasma membrane. The molecular trigger that starts this process is not yet known but the interaction of MAG with some axonal component has been suggested to play an important role. The next steps in myelin formation are membrane synthesis, axonal wrapping and compaction. While MAG in both the CNS and PNS is located at the axon–myelin interface and is therefore likely to play a role in membrane spiraling, different molecules are responsible for membrane compaction in the two systems. In PNS myelin, the most abundant protein is P_0, a small protein containing a single extracellular Ig domain [36] (see Ch. 4). The P_0 protein is responsible for adhesion at the apposition of extracellular surfaces, or intraperiod line, via homophilic interactions of its Ig domain and at the major dense line, where cytoplasmic surfaces come together via interactions of the highly negatively charged cytoplasmic domain of P_0 with the acidic lipids, mostly phosphatidylserine, of the opposing membrane. Therefore, P_0 protein can be regarded as a 'bifunctional' adhesion molecule. It is interesting that P_0 is an 'obligatory' adhesion molecule in that it induces strong cell–cell adhesion between any cells in which it is expressed [37–39].

In the CNS of mammals, two proteins are needed to accomplish the dual role of adhesion at both myelin bilayer surfaces that P_0 effects in the PNS. The four-transmembrane-domain proteolipid protein (PLP) is likely to be responsible for adhesion of the extracellular surfaces, while the very basic cytoplasmic myelin basic protein

(MBP) holds membranes together at the inner cytoplasmic surfaces in the CNS. Although MBP is also present in the PNS, it is much less abundant than in the CNS and its absence does not have a dramatic effect on PNS myelin compaction.

The importance of P_0 in PNS myelin has been clearly demonstrated. In P_0 gene knockout experiments in mice [40], severe hypomyelination and a virtual absence of compact myelin in the PNS is observed. In humans, there are two disease states associated with mutations in the P_0 gene: Charcot–Marie–Tooth type I disease (see Ch. 38) and Dejerine–Sottas disease, both dysmyelinating diseases that exhibit a spectrum of severity depending on the particular mutation.

The crystal structure of the extracellular domain of P_0 has also been determined [41]. The arrangement of molecules in the crystal indicates that P_0 may exist on the membrane surface as a tetramer (Fig. 7-7) that can link to other tetramers from the opposing membrane to form an adhesive lattice, like a 'molecular Velcro'. The structure also suggests that P_0 mediates adhesion through the direct interaction of apically directed tryptophan side chains with the opposing membrane [42], in addition to homophilic protein–protein interaction.

Functional myelin not only depends on compact myelin in the internodes but also requires maintenance of a stable structure in the membranes adjacent to the nodes of Ranvier, termed the paranodes, which are sinuous, open cytoplasmic channels that are continuous with each other throughout the myelin sheath. In the plasma membrane subdomains that surround these channels, a number of adhesion molecules have been localized, namely MAG, certain integrins [43] and cadherins. The paranodal loops interact across their extracellular surfaces and it is clear that certain cadherins at least participate in holding them firmly in place against each other [44]. In particular, E-cadherin is expressed by Schwann cells and is localized to these cytoplasmic compartments. This cadherin is not present in the underlying axon. Thus, in peripheral nerve, E-cadherin is unusual in that it does not mediate adhesion between two cells but instead mediates adhesion between two regions of a single plasma membrane elaborated by a single Schwann cell.

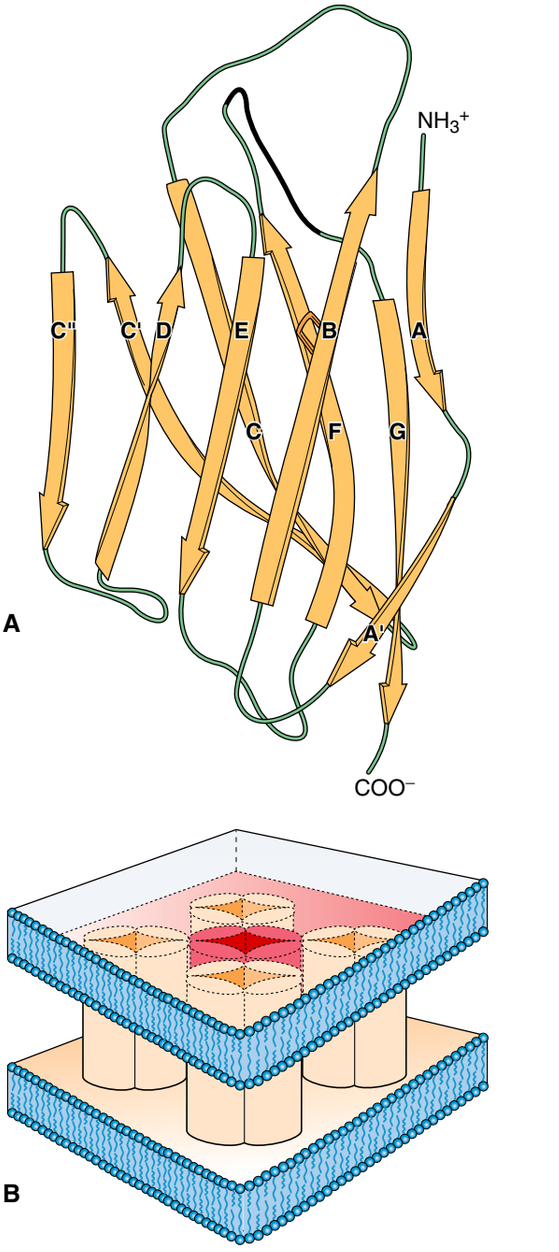

FIGURE 7-7 Structure of the P_0 glycoprotein protomer. (**A**) In this ribbon diagram of the extracellular domain of P_0, each β strand is labeled with a letter and two antiparallel β sheets are formed. The disulfide bridge is indicated in *dark orange* and a hypothetical path for disordered amino acids 103–106 is shown in *black* in the FG loop. (**B**) Lattice formation by P_0. A view of the intraperiod line, or extracellular apposition, of myelin in the PNS. The *orange* tetramer sets emanate from one bilayer and the *blue* tetramer interacts with all four of them. This is a view perpendicular to the plane of the myelin membrane.

SUMMARY

Adhesion molecules are indispensable components of nervous tissue. They adhere cell membranes to each other with varying degrees of strength and 'translate' adhesion into cellular responses via signal-transduction pathways (see, for example, [45]). The major classes of adhesion molecules, the integrins, IgCAMs and cadherins, act cooperatively [46] and in concert to coordinate brain development and maturation and, in adulthood, to maintain the normal tissue architecture.

REFERENCES

1. Gumbiner, B. M. Proteins associated with the cytoplasmic surface of adhesion molecules. *Neuron* 11: 551–564, 1993.
2. Springer, T. A. Adhesion receptors of the immune system. *Nature* 346: 425–434, 1990.
3. Milner, R. and Campbell, I. L. The integrin family of cell adhesion molecules has multiple functions within the CNS. *J. Neurosci. Res.* 69: 286–291, 2002.
4. Clegg, D. O., Wingerd, K. L., Hikita, S. T. and Tolhurst, E. C. Integrins in the development, function and dysfunction of the nervous system. *Front. Biosci.* 8: d723–750, 2003.
5. McCarty, J. H, Lacy-Hulber, A., Charest, A., Bronson, R. T., Crowley, D., Housman, D., Savill, J., Roes, J. and Hynes, R. O. Selective ablation of (alpha)V integrins in the central nervous system leads to cerebral hemorrhage, seizures, axonal degeneration and premature death. *Development* 132: 165–176, 2005.
6. Williams, A. F. and Barclay, A. N. The immunoglobulin superfamily – domains for cell surface recognition. *Annu. Rev. Immunol.* 6: 381–405, 1988.
7. Edelman, G. M. CAMs and Igs: cell adhesion and the evolutionary origins of immunity. *Immunol. Rev.* 100: 11–45, 1987.
8. Kelm, S., Pelz, A., Schauer, R. *et al.* Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. *Curr. Biol.* 4: 965–972, 1994.
9. Brummendorf, T. and Rathjen, F. G. Axonal glycoproteins with immunoglobulin and fibronectin type II-related domains in vertebrates: Structural features, binding activities and signal transduction. *J. Neurochem.* 61: 1207–1219, 1993.
10. Walsh, F. S. and Doherty, P. Neural cell adhesion molecules of the immunoglobulin superfamily: role in axon growth and guidance. *Annu. Rev. Cell. Dev. Biol.* 13: 425–456, 1997.
11. Doherty, P. and Walsh, F. CAM–FGF interactions: a model for axonal growth. *Mol. Cell. Neurosci.* 8: 99–111, 1996.
12. Neiindam, J. L., Kohler, L. B., Christensen, C., Li, S. Pedersen, M. V., Ditlevsen, D. K., Kornum, M. K., Kiselyov, V. V., Berezin, V. and Bock, E. An NCAM-derived FGF-receptor agonist, the FGL-peptide, induces neurite outgrowth and neuronal survival in primary rat neurons. 1. *J. Neurochem.* 91: 920–935, 2004.
13. Adcock, K. H., Brown, D. J., Shearer, M. C., Shewan, D., Schachner, M., Smith, G. M., Geller, H. M. and Fawcett, J. W. Axon behavior at Schwann cell-astrocyte boundaries: manipulation of axonal signaling pathways and the neural adhesion molecule L1 can enable axons to cross. *Eur. J. Neurosci.* 20: 1425–1435, 2004.
14. Filbin, M. T., D'Urso, D., Zhang, K., Wong, M., Doyle, J. P. and Colman, D. R. Protein zero of peripheral nerve myelin: adhesion properties and functional models. *Adv. Mol. Cell. Biol.* 16: 159–192, 1996.
15. Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 25: 1451–1455, 1993.
16. Geiger, B. and Ayalon, O. Cadherins. *Annu. Rev. Cell Biol.* 8: 307–322, 1993.
17. Takeichi, M. Cadherins in cancer: implications for invasion and metastasis. *Curr. Opin. Cell Biol.* 5: 806–811, 1993.
18. Suzuki, S., Sano, K. and Tanihara, H. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. *Cell. Regul.* 2: 261–270, 1991.
19. Sano, K., Tanihara, H., Heimark, R. L. *et al.* Protocadherins: a large family of cadherin-related molecules in central nervous system. *EMBO J.* 12: 2249–2256, 1993.
20. Boggon, T. J., Murray, J., Chapuis-Flament, S., Wong, E., Gumbiner, B. M. and Shapiro, L. C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* 296(5571): 1308–1313, 2002
21. Serafini, T. An old friend in a new home: cadherins at the synapse. *Trends Neurosci.* 20: 322–323, 1997.
22. Colman, D. R. Neurites, synapses and cadherins reconciled. *Mol. Cell Neurosci.* 10: 1–6, 1997.
23. He, W., Cowin, P. and Stokes, D. L. Untangling desmosomal knots with electron tomography. *Science* 302: 109–113, 2003.
24. Kintner, C. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell* 69: 225–236, 1997.
25. Jontes, J. D., Emond, M. R. and Smith, S. J. In vivo trafficking and targeting of N-cadherin to nascent presynaptic terminals. *J. Neurosci.* 17: 423–434, 2004.
26. Fannon, A. M. and Colman, D. R. A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins. *Neuron* 17: 423–434, 1996.
27. Uchida, N., Honjo, Y., Johnson, K. R., Wheelock, M. J. and Takeichi, M. The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. *J. Cell Biol.* 135: 767–779, 1996.
28. Tomaselli, K. J., Neugebauer, K. N., Bixby, J. L., Lilien, J. and Reichardt, L. F. N-cadherin and integrins: two receptor systems that mediate neurite outgrowth on astrocyte surfaces. *Neuron* 1: 33–43, 1988.
29. Matasunaga, M., Hatta, K., Nagafuchi, A. and Takeichi, M. Guidance of optic nerve fibers by N-cadherin adhesion molecules. *Nature* 334: 62–64, 1988.
30. Inouye, A. and Sanes, J. R. Lamina-specific connectivity in the brain: regulation by N-cadherin, neurotrophins, and glycoconjugates. *Science* 276: 1428–1431, 1997.
31. Rutishauser, U. NCAM and its polysialic acid moiety: a mechanism for pull/push regulation of cell interactions during development? *Dev. Suppl.* 99–104, 1992.
32. Fawcett, J. W. and Keynes, R. J. Peripheral nerve regeneration. *Annu. Rev. Neurosci.* 13: 43–60, 1990.
33. Keynes, R. J. and Cook, G. M. W. Repulsive and inhibitory signals. *Curr. Opin. Neurobiol.* 5: 75–82, 1995.
34. Filbin, M. T. Myelin-associated glycoprotein: a role in myelination and in the inhibition of axonal regeneration? *Curr. Opin. Neurobiol.* 5: 588–595, 1995.
35. Filbin, M. T. The muddle with MAG. *Mol. Cell. Neurosci.* 8: 84–92, 1996.
36. Lemke, G., Lamar, E. and Patterson, J. Isolation and analysis of the gene encoding peripheral myelin-protein zero. *Neuron* 1: 73–83, 1988.
37. Filbin, M., Walsh, F. S., Trapp, B. D., Pizzy, J. A. and Tennekoon, G. I. Role of myelin P_0 protein as a homophilic adhesion molecule. *Nature* 344: 871–872, 1990.

38. D'Urso, D., Brophy, P. J., Staugaitis, S. M. *et al.* Protein zero of peripheral myelin: biosynthesis, membrane insertion, and evidence for homotypic interactions. *Neuron* 4: 449–460, 1990.
39. Filbin, M. T. and Tennekoon, G. I. Myelin P_0-protein, more than just a structural protein? *Bioessays* 14: 541–546, 1992.
40. Giese, K. P., Martini, R., Lemke, G., Soriano, P. and Schachner, M. Mouse P_0 gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons. *Cell* 71: 565–576, 1992.
41. Shapiro, L., Doyle, J. P., Hensley, P., Colman, D. R. and Hendrickson, W. A. Crystal structure of the extracellular domain from P_0, the major structural protein of peripheral nerve myelin. *Neuron* 17: 435–449, 1996.
42. Wells, C. A., Saavedra, R. A., Inouye, H. and Kirschner, D. A. Myelin P_0-glycoprotein: predicted structure and interactions of extracellular domain. *J. Neurochem.* 61: 1987–1995, 1993.
43. Einheber, S., Milner, T., Giancotti, F. and Salzer, J. Axonal regulation of Schwann cell integrin expression suggests a role for $\alpha 6\,\beta 4$ in myelination. *J. Cell. Biol.* 123: 1223–1235, 1993.
44. Fannon, A. M., Sherman, D. L., Ilyina-Gragerova, G., Brophy, P. J., Friedrich, V. L. Jr and Colman, D. R. Novel E-cadherin mediated adhesion in peripheral nerve: Schwann cell architecture is stabilized by intracellular signals elicited by autotypic adherens junctions. *J. Cell Biol.* 129: 189–202, 1995.
45. Brady-Kalnay, S., Rimm, D. and Tonks, N. Receptor protein tyrosine phosphatase PTPm associates with cadherins and catenins *in vivo*. *J. Cell Biol.* 130: 977–986, 1995.
46. Monier-Gavell, F. and Duban, J.-L. Cross talk between adhesion molecules: control of N-cadherin activity by intracellular signals elicited by $\beta 1$ and $\beta 3$ integrins in migrating neural crest cells. *J. Cell Biol.* 137: 1663–1681, 1997.

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CHAPTER

8

The Cytoskeleton of Neurons and Glia

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Neurons and glia exhibit a remarkable diversity of shapes. These different morphologies are so characteristic and distinctive that they have been used since the time of Cajal to define functional neural functions. For example, Purkinje cells in the cerebellum have such distinctive morphologies that they are readily identifiable in any vertebrate. Neurons do not divide, so their distinctive morphologies are maintained throughout life. Biochemical and immunological markers are used to delineate neuronal populations. Their distributions are found to correlate well with populations previously defined on morphological grounds. This evidence indicates that the shape of cells in the nervous system is closely connected to their functions. Understanding the proteins and cellular structures that underlie cell morphology is essential for understanding the neural functions.

Proteins of the cytoskeleton play a central role in the creation and maintenance of cell shapes in all tissues. They serve multiple roles in eukaryotic cells. First, they provide structural organization for the cell interior, helping to establish metabolic compartments. Second, cytoskeletal structures serve as tracks for intracellular transport, which creates and maintains differentiated cellular functions. Finally, the cytoskeleton comprises the core framework of cellular morphologies.

Methods for visualizing individual neurons and glia *in vivo* have depended for more than 100 years on histochemical reactions with cytoskeletal elements and even now these methods have not been surpassed. Because cytoskeletal structures play a particularly prominent role in the nervous system, cytoskeletal proteins represent a large fraction of total brain protein, comprising perhaps a third or more of the total. In fact, much of our knowledge about cytoskeletal biochemistry is based on studies of proteins purified from brain. The aims of this chapter are twofold: first to provide an introduction to the cytoskeletal elements themselves and second to examine their role in neuronal function. Throughout, the emphasis will be on the cytoskeleton as a vital, dynamic component of the nervous system.

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MOLECULAR COMPONENTS OF THE NEURONAL CYTOSKELETON

The cytoskeleton is one of several biological elements that define eukaryotic cells (others include the nucleus and mitochondria). The term 'cytoskeleton' is often used as if it described a single, unified structure, but the cytoskeleton of neurons and other eukaryotic cells comprises three distinct, interacting structural complexes that have very different properties: microtubules (MTs), neurofilaments (NFs) and microfilaments (MFs). Each has a characteristic composition, structure and organization that may be further specialized in a particular cell type or subcellular domain. The defining structural elements have long been identifiable in electron micrographs (Fig. 8-1), and a considerable amount is known about the detailed organization of these components in neurons and glia. Each set of cytoskeletal structures will be considered in turn.

Microtubules act as both dynamic structural elements and tracks for organelle traffic. Neuronal MTs are structurally similar to those found in other eukaryotic cells (reviewed in [1]). The core structure is a polymer of 50 kDa tubulin subunits. Heterodimers of α- and β-tubulin align end-to-end to form protofilaments, 13 of which join laterally to form a hollow tube with an outer diameter of 25 nm (Fig. 8-2). Examples also exist of MTs with 12 and 14 protofilaments. The α- and β-tubulins are the best-known members of a unique protein family that have significant sequence similarity [2]. There is approximately 40% sequence identity between α- and β-tubulins, and even greater identity within the α and β gene subfamilies [3]. Conservation of primary sequence for tubulins is also high across species so that tubulins from yeast can readily coassemble with tubulins from human brain. Tubulin dimers bind two GTPs and exhibit GTPase activity that is closely linked to assembly and disassembly of microtubules [1]. While many questions remain about tubulin and its interactions, the structure of

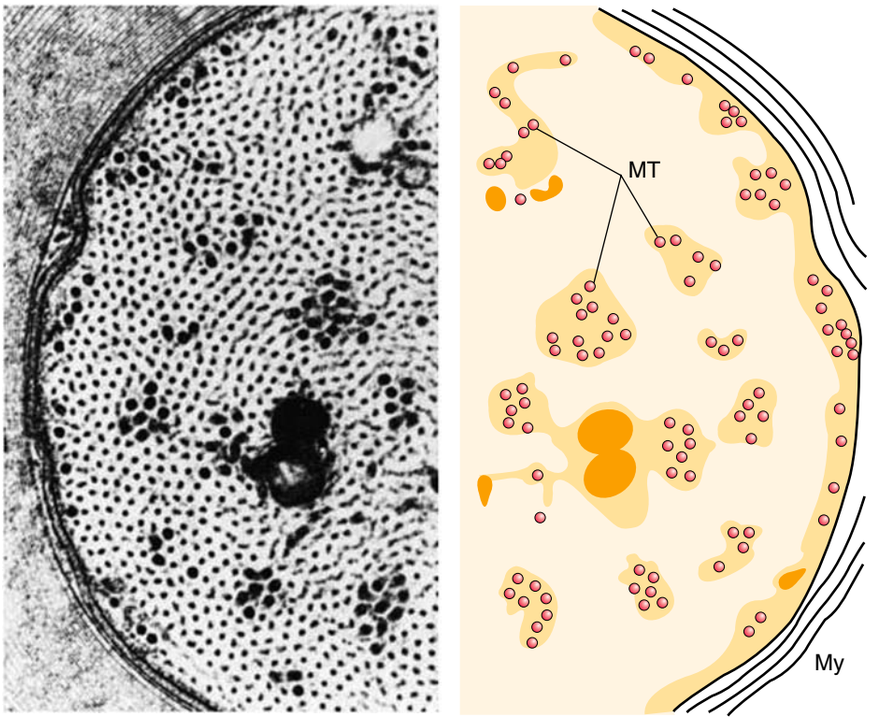

FIGURE 8-1 The cytoskeleton and organization of the axon in cross-section. **Left.** Electron micrograph of a myelinated toad axon in cross-section taken near a Schmidt–Lanterman cleft; axon diameter is slightly reduced and the different domains within the axoplasm are emphasized. **Right.** Diagram highlighting key features of the axoplasm. Portions of the myelin sheath surrounding the axon can be seen (*My*). Most of the axonal diameter is taken up by the neurofilaments (*clear area*). There is a minimum distance between neurofilaments and other cytoskeletal structures that is determined by the side arms of the neurofilaments. (These side arms are visible between some of the neurofilaments in the electron micrograph, **left.**) The microtubules (*MT*) tend to be found in bundles and are more irregularly spaced. They are surrounded by a fuzzy material that is also visible in the region just below the plasma membrane (*stippled areas*, **right**). These areas are thought to be enriched in actin microfilaments and presumably contain other slow component b (SCb) proteins as well. The *stippled regions* with embedded microtubules are also the location of membranous organelles in fast axonal transport (*larger, filled, irregular shapes*, **right**). Both microtubule and microfilament networks need to be intact for the efficient movement of organelles in fast transport. (Electron micrograph provided by Dr Alan Hodge. From Hodge, A. and Adelman, W. In: *Structure and Function in Excitable Cells*. New York: Plenum, 1983, pp. 75-111, with permission.)

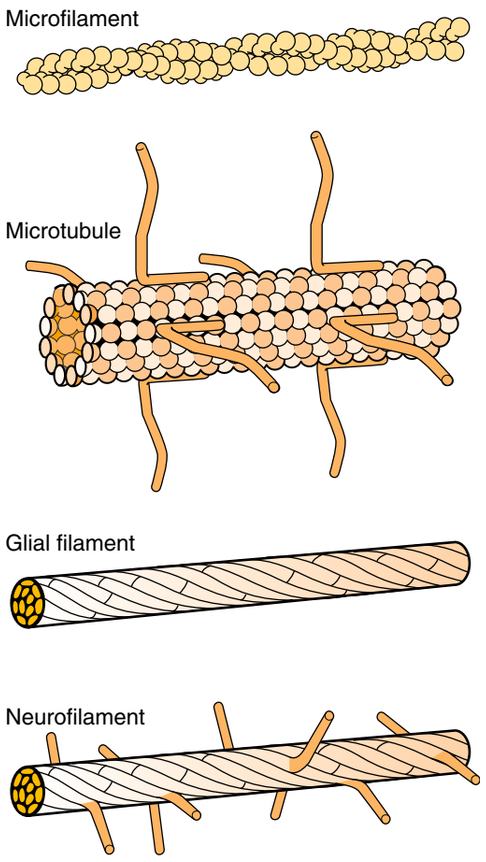

FIGURE 8-2 Microfilaments, microtubules and intermediate filaments in the nervous system. Each cytoskeletal structure has a distinctive ultrastructure. This schematic illustrates the major features of the core fibrils. The microfilament consists of two strands of actin subunits twisted around each other like strings of pearls. The individual subunits are asymmetrical, globular proteins that give the microfilament its polarity. The microtubule is also made from globular subunits, but in this case the basic building block is a heterodimer of α- and β-tubulins. These αβ dimers are organized into linear strands, or protofilaments, with β-tubulin subunits oriented toward the plus end of the microtubule. Protofilaments form sheets *in vitro* that roll up into a cylinder with 13 protofilaments forming the wall of the microtubule. Assembly of both microfilaments and microtubules is coupled to slow nucleotide hydrolysis, ATP for microfilaments and GTP for microtubules. The subunits of both glial filaments and neurofilaments are rod-shaped molecules that will self-assemble without nucleotides. The core filament structure is thought to be a ropelike arrangement of individual subunits. Glial filaments are typical type III intermediate filaments in that they form homopolymers without side arms. In contrast, neurofilaments are heteropolymers formed from three subunits, NFH, NFM and NFL for the high-, medium- and low-molecular-weight subunits. The NFH and NFM subunits have extended carboxy-terminal tails that project from the sides of the core filament and may be heavily phosphorylated.

the αβ-tubulin dimer has recently been derived from electron diffraction studies [4], providing a basis for dissection of MT functional architecture.

Heterodimers in a MT are oriented in the same direction, so the resulting MT has asymmetric ends that differ in their assembly properties [5]. The β-tubulin subunit is exposed at the 'plus' end, which is the preferred end for addition of tubulin dimers. The opposite 'minus' end grows more slowly at physiological concentrations of tubulin. In the case of free microtubules, the balance between assembly and disassembly at each end defines a critical concentration for net microtubule growth. MT assembly under *in vitro* conditions involves a slow nucleation step followed by a more rapid, net growth phase; a kinetic pattern described as dynamic instability. In glia and most other non-neuronal cells, however, the minus-ends of MTs are usually bound at the site of nucleation which is associated with the centrosome or pericentriolar complex of the cell [6], a site often called the microtubule-organizing center (MTOC) [7]. Anchoring of MT minus-ends helps establish and maintain the polarity of cellular MTs. Anchoring and nucleation of microtubules appears to require a third class of tubulin, γ-tubulin, which is detectable only as part of the pericentriolar complex [7, 8].

The organization of MTs in neurons differs several ways from that seen in non-neuronal cells (Fig. 8-3) [9]. Axonal and dendritic MTs are not continuous back to the cell body nor are they associated with any visible MTOC. Axonal MTs can be more than 100 μm long, but they have uniform polarity, with all plus-ends distal to the cell body. Dendritic MTs are typically shorter and often exhibit mixed polarity, with only about 50% oriented plus-end distal. Recent work suggests that MTs in both axons and dendrites are nucleated normally at the MTOC, but they are then released from the MTOC and delivered to neurites [10].

While MTs in neurons are composed of the same basic constituents as those in non-neuronal cells, they are strikingly more diverse (Table 8-1). Brain MTs contain tubulins of many different isotypes, with many different post-translational modifications, and also have a variety of associated proteins (MAPs) [11]. MT composition varies according to location, such as in axons or dendrites, suggesting that brain MTs exist in specialized forms to perform designated tasks in the unique environments of the neuron. For example, axonal MTs contain stable segments that are unusually resistant to treatments that depolymerize MTs in other cells [12]. Such stable domains are preserved as short MT segments and may serve to nucleate or organize MTs in axons [13], particularly during regeneration. This and other specializations of axonal microtubules (see below) may reflect the unusual requirements of the neuronal cytoskeleton, where remarkably long microtubules are maintained at considerable distances from sites of new protein synthesis in the cell body.

Multiple genes exist for both α- and β-tubulins. Tubulin isotypes differ primarily at the carboxy-terminus, the region where most post-translational modifications and MAP interactions occur. While most α- and β-tubulin isotypes are expressed in all tissues, some are expressed preferentially in different tissues. For example, class III and IVa β-tubulins are neuron-specific (reviewed in [3, 14]). It is not known if such examples of tissue-specific

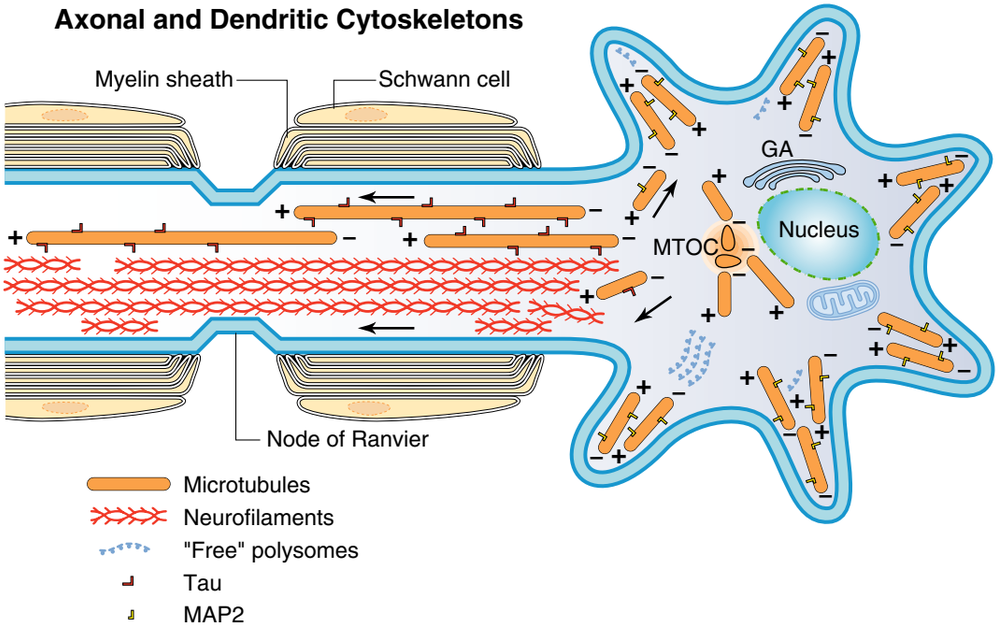

FIGURE 8-3 The axonal and dendritic cytoskeletons differ in both composition and organization. The major differences are illustrated diagrammatically in this diagram. With one exception, all cytoskeletal proteins are synthesized on free polysomes in the cell body, then transported to their different cellular compartments. The exception is MAP2, which is the major microtubule-associated protein of dendrites. While some MAP2 is synthesized in the cell body, MAP2 mRNA is specifically enriched in the dendritic compartment and a significant fraction is thought to be synthesized there. The microtubules of cell bodies, dendrites and axons are thought to be nucleated at the microtubule-organizing center (*MTOC*), then released and delivered to either the dendrites or axon. In the dendrite, microtubules often have mixed polarities with both plus and minus ends distal to the cell body. The functional consequence of this organization is uncertain but may help explain why dendrites taper with distance from the cell body. In contrast, all axonal microtubules are oriented with the plus end distal to the cell body and exhibit uniform distribution across the axon. Although some tau protein can be detected in cell bodies and dendrites, axonal microtubules are enriched in tau and axonal tau is differentially phosphorylated. MAP2 appears to be absent from the axon. Neurofilaments are largely excluded from the dendritic compartments but are abundant in large axons. The spacing of neurofilaments is sensitive to the level of phosphorylation. Microtubules and neurofilaments both stop and start in the axon rather than being continuous back to the cell body. The microfilaments are more dispersed in their organization and may be difficult to visualize in the mature neuron. They are most abundant near the plasma membrane but are also enriched in presynaptic terminals and dendritic spines. *GA*, Golgi apparatus.

TABLE 8-1 Major microtubule cytoskeletal proteins of the nervous system

Protein	Expression pattern and distribution	Modifications
α–tubulin (multigene family)	In all cells, but some isoforms are preferentially expressed in brain	Acetylation Tyrosination
β–tubulin (multigene family)	In all cells, but some isoforms are preferentially expressed in brain	Phosphorylation Polyglutamylation
γ-tubulin	In all cells, pericentriolar region/MTOC	
MAP1a	Appears late, preferentially in dendrites	Phosphorylation
MAP1b (multigene family)	Appears early then declines, enriched in axons	Phosphorylation
MAP2a	High molecular weight, dendritic in mature neurons	Phosphorylation
MAP2b	High molecular weight, dendritic expressed throughout lifetime	Phosphorylation
MAP2c (single gene)	Low molecular weight, dendritic in developing neurons	Phosphorylation
Tau – high MW – low MW (single gene)	Peripheral axons with distinctive phosphorylation pattern Enriched in CNS axons with distinctive phosphorylation pattern	Phosphorylation Phosphorylation
MAP4	Primarily nonneuronal, multiple forms, widespread distribution	Phosphorylation at mitosis
Katanin	Widespread distribution, enriched at MTOC, severs MT	
Stathmin	Widespread distribution, destabilizes MT	Phosphorylation

expression imply that different isotypes are structurally suited to function in different tissues, or merely that different tubulin genes are part of different tissue-specific developmental programs. Where it can be evaluated, all isotypes available in a given cell appear capable of coassembly and typically can be detected in all cellular MTs.

Brain MTs also contain a variety of post-translational modifications. When purified mammalian tubulin is analyzed by isoelectric focusing, over 20 different isoforms can be seen [3]. These are explained by a combination of multiple genes and various post-translational modifications. The two best-studied post-translational modifications of tubulin are α-tubulin detyrosination and acetylation (reviewed in [3]). Most α-tubulins are expressed with a carboxy-terminal tyrosine residue. This tyrosine can be removed by tubulin carboxypeptidase and then replaced by tubulin tyrosine ligase, in a rapid cycle that occurs on the majority of available tubulin. Since the carboxypeptidase acts only on assembled tubulin and the ligase acts on unassembled tubulin, this tyrosination/detyrosination cycle is linked to MT dynamics. Typically, newly assembled MTs will contain Tyr-tubulin. The longer a MT remains polymerized, the higher its content of de-Tyr-tubulin, or Glu-tubulin. A second prominent post-translational modification of neuronal α-tubulin is lysine-ε-acetylation. The enzyme responsible for acetylation, tubulin acetyltransferase, like tubulin carboxypeptidase, acts only on assembled tubulin. Neuronal β-tubulin isotypes appear to be subject to fewer post-translational modifications [3]. The β_{III} isotype of brain is phosphorylated, and while the function of this modification is not clear, correlations with neurite outgrowth in neuroblastoma cells have been made. Another modification of β–tubulin is polyglutamylation, the addition of 1–5 glutamyl units to the γ-carboxyl group of a glutamate residue near the C-terminus [15]. Despite the plethora of neuronal tubulin post-translational modifications, none are known to have a direct effect on MT properties although they correlate with specific states (i.e. assembly, microtubule age, etc.).

MTs *in vivo* invariably include members of a heterogeneous set of proteins known as microtubule-associated proteins [11, 16]. MAPs interact with MTs rather than with free tubulin and maintain constant stoichiometry with the tubulin in MTs through cycles of assembly and disassembly. Several categories of brain MAPs can be distinguished: the high-molecular-weight MAPs, which include MAPs 1A, 1B, 2A and 2B (>270 kDa); the tau proteins; MAPs of intermediate molecular weight, such as MAP3 and MAP4, and the molecular motor MAPs kinesin and dynein, which drive the intracellular transport of membrane-bounded organelles along MT tracts (see Ch. 28). The other groups of MAPs are collectively known as 'fibrous' or 'structural' MAPs because they have been observed to form lateral extensions between adjacent MTs and between MTs and other cytoskeletal elements [16]. Such extensions do not generally represent stable cross-links but rather reflect transient interactions that facilitate MT function. One exception is the formation of stably crosslinked axonemal microtubules of cilia and flagella; such as occur on ependymal cells in the CNS.

The high molar ratio of MAPs to tubulin in brain suggests that MAPs may play an important role in determining MT properties. Some MAPs are differentially distributed in neurons [11]. For example, MAP 2 is found primarily in cell bodies and dendrites, and tau is enriched in axons. Additionally, changes in MAP expression and MAP phosphorylation during development suggest they may play a role in modulating MT function in the developing brain [16]. For example, MAPs 1A and 1B occur in both axons and somatodendritic domains, but MAP 1B is preferentially phosphorylated in axons and especially in developing axons. As many as six different tau proteins may be derived by alternative splicing from a single tau gene [17]. Both the expression and phosphorylation of the different tau isoforms are regulated throughout development. While MAPs affect nucleation and stability of MTs *in vitro*, these may not be their primary functions *in vivo*. Other likely functions include roles in MT spacing and organization, compartmentation, scaffolds for signaling molecules and interaction with other cellular structures.

Recently, another class of MAPs has been identified that lead to depolymerization of MT, stathmin and katanin [11]. Stathmin destabilizes MT either by reducing available tubulin dimer or by altering the frequency of catastrophe at MT plus ends [18]. In contrast, katanin is an ATP-ase that severs MT [19]. In neurons, one important role of katanin is to release the MT from the MTOC and allow transport of the assembled MT into axons and dendrites.

MTs serve multiple roles in neurons. Besides acting as the substrate for the transport of membrane-bounded organelles, MTs are necessary for the extension of neurites during development; they provide the structural basis for maintaining neurites after extension and they also help maintain the definition and integrity of intracellular compartments. The diversity of these functions is reflected in differences in the biochemistry and metabolic stability of different MTs.

Neuronal and glial intermediate filaments provide support for neuronal and glial morphologies. As a class of cytoskeletal structures, intermediate filaments (IFs) display an unusual degree of cell specificity and are often used as markers of cellular differentiation. They comprise a family of related genes that have been classified in five types [20]. All five share homology in a core rod domain, which contains multiple α-helical domains that can form coiled/coils. The sequence homology in this conserved domain is sufficient that some antibodies recognize all known IFs from mammals through a wide range of invertebrates. IFs are also ultrastructurally similar regardless of type, forming 8–10 nm rope-like filaments that may be

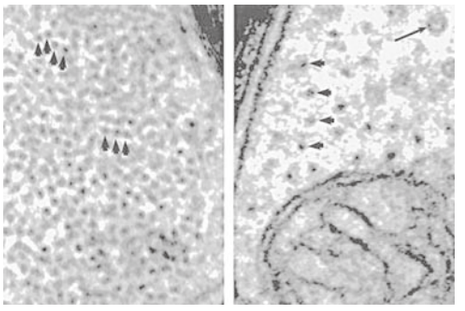

FIGURE 8-4 Glial filaments and neurofilaments are easily recognized in electron micrographs. The glial filaments lack side arms and often appear to be densely packed, with neighboring subunits almost touching (*large arrows* in **left** panel). In contrast, the spacing between neurofilaments is typically much greater (*large arrows* in **right** panel). This spacing is due to the side arms of neurofilaments formed by the tails of the high- and medium-molecular-weight neurofilaments (NFH and NFM, respectively). These tails are heavily phosphorylated in large axons such that NFH tails may have 50 or more phosphates added to the multiple repeats of a consensus phosphorylation site. NFM tails have fewer sites and typically only ten to 12 phosphates. Charges on the surface of phosphorylated neurofilaments are thought to repel neighboring filaments, creating the large spacing. For comparison, a 25 nm microtubule is indicated in the **right** panel (*thin arrow*). (Micrograph provided by H. Ross Payne and Scott Brady.)

several micrometers long. However, NFs differ from the other IFs because they have sidearms that project from their surface. The result is that IFs in non-neuronal cells are often seen in densely packed bundles, while NFs are widely spaced (Fig. 8-4).

Types I and II are the keratins that are found in various combinations in epithelial cells throughout the body. Keratin IFs must include representative subunits of both type I and type II IF subunits with each tissue having a characteristic combination. In contrast, type III IF subunits typically form homopolymers. They include IFs that are characteristic of less differentiated cells like glial or neuronal precursors, as well as those seen in more specialized cell types like smooth muscle cells and mature astrocytes. These three types of IF not only share sequence homology within their rod domains but their genes exhibit similar exon and intron structure. Type IV IFs are all neuron-specific and have a common pattern of exons and introns that differs from that seen in types I–III. Finally, type V polypeptides are the nuclear lamins, which form the walls of the nuclear envelope structure rather than typical IF structures. Although all eukaryotes express type V lamins, plants, many unicellular organisms and arthropods do not express other IF types. Cytoplasmic IFs are nearly ubiquitous in vertebrate cells and some cells contain more than one type of IF in their cytoplasm. Curiously, oligodendrocyte precursors contain vimentin IFs, but these are lost during differentiation, making mature oligodendrocytes one of the few vertebrate cell types that lack cytoplasmic IFs.

The nervous system contains an unusually diverse set of intermediate filaments (Table 8-2) with distinctive cellular distributions and developmental expression [21, 22]. Despite their molecular heterogeneity, all intermediate filaments appear as solid, rope-like fibers 8–12 nm in diameter. Neuronal intermediate filaments (NFs) can be hundreds of micrometers long and have characteristic sidearm projections, while filaments in glia or other non-neuronal cells are shorter and lack sidearms (Fig. 8-2). The existence of NFs was established long before much was known about their biochemistry or properties. As stable cytoskeletal structures, NFs were noted in early electron micrographs, and many traditional histological procedures that visualize neurons are based on a specific interaction of metal stains with NFs.

The primary type of IF in large myelinated axons is formed from three subunit proteins known as the neurofilament triplet: NF high-molecular-weight subunit (NFH, 180–200 kDa); NF middle-molecular-weight subunit (NFM, 130–170 kDa); and NF low-molecular-weight subunit (NFL, 60–70 kDa) [21–23]. A separate gene encodes each of the subunit proteins. The NF triplet proteins are type IV IF proteins that are expressed only in neurons and have a characteristic domain structure. The amino-terminal regions of all three subunits interact via α-helical coiled-coils to form the core of the filament. NFM and NFH also have long carboxy-terminal regions, which project from the core filaments as sidearms.

TABLE 8-2 IF proteins of the nervous system

IF Type	Subunit	Cell type
Type III	Vimentin	Neural and glial precursors
	GFAP	Astrocytes, some Schwann cells
	peripherin	Subset of neurons, particularly in PNS, may co-assemble with NFH/NFM/NFL
	Desmin	Smooth muscle cells in vasculature
Type IV	NFH	
	NFM	
	NFL	Most neurons, most abundant in large neurons
	α–internexin	Subset of neurons, particularly parallel fibers in cerebellum, may also coassemble with NFH/NFM/NFL
Type V	Nuclear lamins	Nuclear envelope
Type VI	nestin	Neuroectodermal precursors in developing brain

NFH, and to a lesser extent NFM, has a large number of consensus phosphorylation sites for proline-directed kinases in this carboxy-terminal extension (>50 on NFH and >10 on NFM in many species). In large myelinated axons, most, if not all, of these sites are phosphorylated [21, 22]. This phosphorylation of NFH and NFM sidearms alters the charge density on the NF surface, repelling adjacent NFs with similar charge. Such mutual repulsion by the sidearms of NFs is thought to be a major determinant of axonal caliber [24].

Other IFs are also found in the nervous system [21, 22]. Vimentin is the most widely expressed type III IF and is found in a variety of cell types like fibroblasts, microglia and smooth muscle cells as well as in embryonic cell types including neuronal and glia precursors. Astrocytes and some Schwann cells contain the type III IF glial fibrillary acidic protein (GFAP). This distribution has led to the widespread use of GFAP immunoreactivity to identify astrocytes in culture and in tissue. In contrast to NFs, type III IFs like GFAP lack sidearms and often appear to be tightly bundled.

At least three other IFs occur in selected neurons or neuronal precursors: α-internexin, peripherin and nestin. All of these are most prominently expressed during development, then downregulated. Based on sequence and gene structure, peripherin is a type III IF, while α-internexin is a type IV IF. Nestin is the most divergent of the IFs that form filaments and nestin sequence has characteristics of both type III and type IV IFs [21]. As a result, nestin is sometimes considered to be a distinct IF type (type VI). While the NF triplet proteins and α-internexin may be components of both CNS and PNS neurons, peripherin appears to be expressed preferentially in PNS neurons. Both can co-assemble with the NF triplet proteins *in vitro* and may do so *in vivo*, but can also form homopolymeric filaments. Both are expressed at higher levels in a variety of developing neurons, and expression becomes more restricted as neurons mature. Many neurons cease to express α-internexin during maturation. However, a few neurons retain the expression of IFs containing α-internexin. Notably, IFs of parallel fibers in the cerebellar cortex contain only α-internexin subunits. Peripherin continues to be expressed in some peripheral neurons in maturity. Although nestin is specific to the nervous system, it is expressed in multipotent neuroectodermal precursors and suppressed during subsequent development. For this reason it can be used as an early marker for differentiation of precursor cells.

Not all neurons have NFs. Indeed, one entire phylum in the animal kingdom, arthropods, expresses only type V nuclear lamins so arthropod cells have no IF cytoskeletal structures at all. In addition, mature oligodendrocytes lack IFs although their embryonic precursors contain vimentin. Clearly, the IFs are not essential for cell survival. Yet, in large myelinated fibers, NFs make up the bulk of axonal volume and represent a substantial fraction of the total protein in brain. In most organisms, IFs in both glia and neurons contribute to the distinctive morphologies of these cells. They are thought to provide mechanical strength and a stable cytoskeletal framework. In neurons, NFs play an important role in regulating cellular and axonal volumes and are a primary determinant of axonal caliber in large fibers. Finally, NFs exhibit an unusual degree of metabolic stability, which makes them well suited for a role in stabilizing and maintaining neuronal morphology [25].

Actin microfilaments and the membrane cytoskeleton play critical roles in neuronal growth and secretion. This major class of cytoskeletal elements is perhaps the oldest. Certainly, the actin cytoskeleton has the most diverse composition and organization. MFs are formed from 43 kDa actin monomers that are arranged like two strings of pearls intertwined into fibrils 4–6 nm diameter (Fig. 8-2) [26]. A remarkable variety of proteins have been found to interact with actin MFs, ranging from myosin motors to cross-linkers, bundling proteins to anchoring proteins, and sequestration proteins to small GTPase regulatory proteins [27, 28].

Actin MFs are found throughout neurons and glia, but they are enriched in cortical regions near the plasmalemma and are particularly concentrated in presynaptic terminals, dendritic spines and growth cones. Under most circumstances in the nervous system, MFs are short oligomers organized into a meshwork most apparent near the plasma membrane and in the vicinity of axonal MTs. MFs are the main components of the membrane cytoskeleton and this may be their primary role in mature neurons. The actin cytoskeleton also plays an important role in the Golgi complex (GC) morphology. Actin and actin-associated proteins, like spectrin and myosin, are associated with the GC, although their exact functions are not fully understood. However, the cytoplasmic arrangement of actin filaments is directly involved in the subcellular localization and the shape of GC [29, 30]. Spectrin is thought to provide a cross-linked matrix surrounding GC membranes, similar to its role at the plasma membrane, providing structural integrity, as well as defining putative GC microdomains and possibly mediating interactions with dynactin and dynein [31, 32]. The prominent actin bundles (stress fibers) seen in fibroblasts and many other non-neuronal cells in culture are not characteristic of neurons *in vivo* or *in vitro*. Most neuronal MFs are less than a micron in length. However growth cones contain many longer MFs, with bundles of MFs in the filopodia and lamellipodia in addition to the typical more dispersed actin network (Fig. 8-4) [33]. The role of actin MFs in the growth cone will be considered in greater detail below.

Many MF-associated proteins [28] have been described in the nervous system (Table 8-3). In general, a good deal is known about their distribution and function in primary cultures of neurons and glia, but less is known about their role in the mature nervous system. Two that have been characterized more extensively are the major non-actin

TABLE 8-3 Selected microfilament-associated proteins expressed in the nervous system

Protein	Activity	Cellular location
Actin	Core subunit of MFs	Throughout neurons and glia, enriched in growth cones and in membrane cytoskeleton
Tropomyosin	Stabilize MFs	Co-distributed with most MFs
Spectrin/fodrin	Cross-link MFs in membrane cytoskeleton	Enriched in membrane cytoskeleton
Ankyrin	Links MF/spectrin to membrane proteins	Membrane cytoskeleton, distinct forms in axon, dendrite and nodes of Ranvier
Fimbrin	MF bundling and cross-linking	Growing neurites
Gelsolin	Fragments MFs and nucleates assembly, regulated by Ca^{2+}	Growing neurites, glia, mature neurons
β–thymosins	Binds actin monomers and regulates MF assembly	Growing neurites
Profilin	Binds actin monomers, inhibits MF formation, regulated by selected signal transduction pathways	Growing neurites, glia, mature neurons
Arp2/3 complex	Nucleation of actin MF assembly in cortex and initiation of MF branches	Enriched in cell cortex where MF assembly is active
N-WASP	Interacts with Arp2/3 complex to nucleate actin MF assembly	Enriched in cell cortex where MF assembly is active, binds to Cdc42 and Arp2/3 complexes

structural elements of the membrane cytoskeleton: spectrin and ankyrin. Spectrin is a flexible rod-shaped molecule composed of homologous α- and β-subunits that was originally characterized as a component of the erythrocyte membrane cytoskeleton. Neurons were the first cell type, other than erythrocytes, shown to contain spectrins and the brain form was initially called fodrin. Spectrin heterodimers align end-to-end to form tetramers, which are cross-linked by short actin MFs. This spectrin-actin meshwork is tightly coupled to the plasma membrane through direct binding to membrane proteins [34]. Some of these interactions occur via the protein ankyrin, which has separate binding sites for specific membrane proteins and β-spectrin (Fig. 8-5). In neurons, specific isoforms of spectrin and ankyrin are localized to axons, dendrites and paranodal regions. The spectrin and ankyrin isoforms in perikarya and dendrites tend to be highly homologous to the erythrocyte forms and distinct from the spectrin and ankyrin isoforms that occur in axons.

A variety of additional MF bundling or linking proteins have been described in the nervous system. For example, fimbrin may play a role in the formation of microfilament

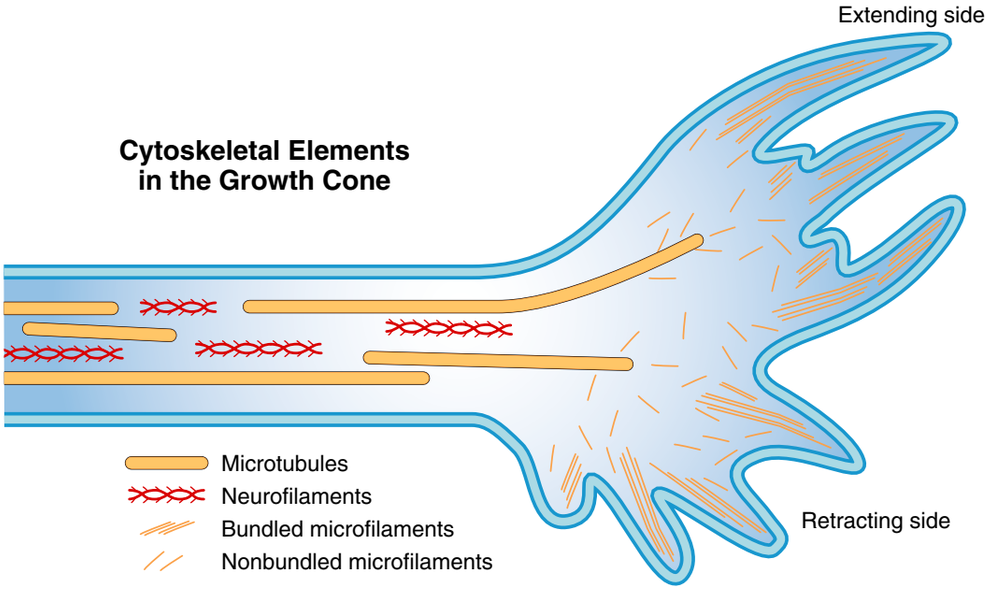

FIGURE 8-5 The cytoskeletal elements of a growth cone are organized for motility. In this diagram of a growth cone, a typical distribution of major cytoskeletal structures is shown. The microfilaments are longer and more prominent in the growth cone than in other regions of a neuron. They are bundled in the lamellipodia and particularly in the filopodia. A combination of actin assembly, microfilament cross-linking and myosin motors (see Ch. 28) is thought to mediate this movement. In the central core of the growth cone, the microfilaments may interact with axonal microtubules which do not extend to the periphery. These microtubules may be pulled toward the preferred direction of growth and appear to be necessary for net advance. In the absence of microtubules, filopodia extend and retract but the growth cone does not advance. Microtubule movements are thought to be a combination of assembly and contractility. Finally, the neurofilaments appear to stabilize the neurite and consolidate advances but appear to be excluded from the growth cone proper.

bundles in growth cone filopodia. Still other actin binding proteins may regulate MF assembly. Gelsolin fragments MFs in a Ca^{2+}-sensitive manner, but also caps and nucleates MFs. In contrast, profilin and β-thymosins bind to actin monomers and may act in part by sequestering actin subunits, although this oversimplifies the effects of these proteins. The list of actin MF-associated proteins has become quite long and this diversity reflects the many forms of MF-based cytoskeletal structures.

Recent studies on mechanisms for regulation of MF assembly have identified a number of components critical for creating and maintaining the actin membrane cytoskeleton including Arp2/3 and the WASp/Scar family [35, 36]. Arp2 and Arp3 are actin-related proteins that form a complex with five other unique polypeptides [28, 36]. The first member of the WASP/Scar family was isolated from hematopoietic cells and was mutated in a rare disease known as Wiskott–Aldrich syndrome. Other family members were expressed at high levels in brain and other tissues [28]. Briefly, members of the Cdc/Rac family of small GTPases activate WASp/Scar proteins. Activated WASp/Scars bind to the Arp2/3 complex to initiate a new MF or a branch off an existing MF [28, 35, 36]. Often actin monomer is sequestered in association with profilin to limit assembly. A second signal (PIP or PIP_2) stimulates dissociation of actin and profilin and rapid MF assembly occurs. This pathway is particularly important in neuronal activities like growth cone motility.

MFs in the nervous system appear to have a variety of functions. The neuronal membrane cytoskeleton plays a role in maintaining the distribution of plasma membrane proteins, establishing cell morphologies and segregating axonal and dendritic proteins into their respective compartments. MFs and the membrane cytoskeleton also mediate the interactions between neurons and the external world, including extracellular matrix components and neighboring cells (see Ch. 7). In neurons and glia, cell adhesion sites such as tight junctions and focal adhesion plaques interact with the MF cytoskeleton either directly or indirectly. The cortical MF meshwork also restricts access of organelles to the plasma membrane and is involved in both regulated and constitutive secretion. Finally, MFs are the basis of filopodia and lamellipodia, which are essential for cell migrations, growth cone motility and myelination.

ULTRASTRUCTURE AND MOLECULAR ORGANIZATION OF NEURONS AND GLIA

A dynamic neuronal cytoskeleton provides for specialized functions in different regions of the neuron. The cytoskeleton in different regions of a neuron differs with regard to both composition and function. While both neurons and glia exhibit distinctive specializations in their cytoskeletal elements, distinct domains are most easily visualized within the neuron. In order to maintain the polarized morphology of a neuron, cytoskeletal, cytoplasmic and membranous elements must be assembled and targeted in a characteristic and consistent manner. The biochemical diversity of each of these elements is striking, as the composition of proteins in the axon differs significantly from that in cell bodies and dendrites. There is diversity even along the length of a single axon. In this section, several examples are given that illustrate how characteristic features of the somatodendritic and axonal cytoskeletons are generated and maintained. A common theme underlying each example is the dynamic nature of the cytoskeleton.

Both the composition and organization of cytoskeletal elements in axons and dendrites become specialized early in differentiation. A brief description of how the cytoskeletal elements within axons and dendrites are established illustrates the dynamic nature of the cytoskeleton. The sequence of events has been described for neurons in primary culture [9, 37]. Postmitotic neurons initially extend and retract multiple neuritic processes. These early short neurites are of comparable length and growth rate. All contain MTs oriented with plus-ends distal to the cell body and all have both MAP2 and tau microtubule-associated proteins. Eventually, in neurons that elaborate an axon, one neurite outgrows the others. This first long neurite continues to grow without tapering and becomes the single axon. Under culture conditions that lack directional information, the choice of neurite that becomes the axon appears to be stochastic. Cues in the local environment are likely to specify a particular direction for neurite outgrowth *in vivo*. As the axon grows, it loses MAP2 while tau is enriched and become differentially phosphorylated. Subsequent to axonal outgrowth, some of the other neurites are stabilized and begin to extend. The number of dendrites varies with type of neuron, ranging from the single dendritic process that partially fuses with the axon to produce the single branched process of dorsal root ganglion pseudo-unipolar neuron to the elaborate branching dendrite of the Purkinje cell and to multipolar motor neurons with both apical and basal dendritic arbors. Most neurites that develop into dendrites become tapered as they grow. When dendrites reach this stage, they begin to contain MTs in both orientations. More or less concurrently, MAP2 becomes enriched in the dendritic processes. Initially, the smallest form of MAP2 is abundant, but a shift to predominantly high molecular weight isoforms occurs with maturation.

As axons and dendrites mature, their differences become more apparent [9]. In axons, MTs have a uniform orientation with plus-ends distal to the cell body, but dendrites contain MTs in both orientations. Dendrites and to a lesser extent perikarya contain MAP2, which is excluded from axons at an early stage. Curiously, MAP2 mRNA is one of several mRNAs that are specifically transported

into the dendrite and translated locally. No other cytoskeletal protein has been found to display a similar expression pattern and protein synthetic machinery is excluded from the axon. Expression of dendritic mRNAs is affected by synaptic activity, but its physiological function is uncertain. In contrast, tau is enriched in axons and NFs similarly occur primarily in axons. The phosphorylation of MAP 1B, tau and NFs is maintained at a high level in axons [16, 23]. Neuron-specific isoforms of spectrin and ankyrin exist only in axons [34].

While tau is not restricted to the growing axonal process, tau expression appears to be critical for the initial elongation event that defines an axon-to-be. If tau expression is blocked before the commitment to axonal outgrowth through use of antisense oligonucleotides, no axon is formed [46]. If tau expression is blocked after commitment, the axon is retracted, suggesting that tau-MT interactions are necessary for axonal differentiation. Similarly, reduction in MAP2 expression inhibits dendrite differentiation. That MAP2 and tau seem to bestow upon MTs the ability to form 'dendrite-like' or 'axon-like' processes respectively has also been suggested by experiments in which these MAPs have been expressed in non-neuronal cells. MAP2 and tau differentially affect the packing density of MTs, so, while these MAPs may not trigger the initial polarization of neurons, they no doubt contribute to maintenance of the polarized phenotype [37]. For both axons and dendrites, cytoskeletal composition and organization are carefully orchestrated during differentiation and maturation.

Although axonal and dendritic microtubules differ in their associated proteins and organization, both are thought to originate in the cell body. Microtubules are likely to continue to grow after entry in the axon or dendrite, but there is little evidence for *de novo* formation of microtubules in either region. The evidence suggests that both are nucleated at the MTOC as another cell types. Instead of remaining associated with the MTOC, neuritic MTs are released from the MTOC and transported into the neurite. The sites where most elongation occurs and where specific microtubule-associated proteins are added remains a matter of dispute. Similarly, the molecular mechanisms by which different cytoskeletal compositions are maintained in different neuronal compartments are unknown.

As the size and shape of neurons change during development, the composition of the IF cytoskeleton varies coordinately. Different types of neurons and neurons in different stages of development vary in the number and composition of their IFs [23]. For example, in many neurons peripherin and α-internexin are expressed very early in neuronal differentiation, then downregulated. NFL and NFM are detectable during initial neurite outgrowth, while NFH is not expressed at significant levels until much later. Phosphorylation of NFH and NFM occurs even later in development, and only reaches its full extent in large myelinated axons. Additionally, NFs in different neuronal populations may not contain the same subunit stoichiometry for NFL, NFM and NFH.

CYTOSKELETAL STRUCTURES IN THE NEURON HAVE COMPLEMENTARY DISTRIBUTIONS AND FUNCTIONS

While each set of cytoskeletal elements has a distinctive spectrum of composition, stability and distribution, all three interact with each other. They have complementary functions and may be coordinately regulated. Such interactions can be seen during development of the nervous system, in mature neuron/glia interactions and in neuropathologies.

Microfilament and microtubule dynamics underlie growth cone motility and function. The mechanisms by which neurons make appropriate synaptic connections are a subject of great interest. The first stages of this process are neurite elongation and pathfinding. As the growing tip of a growth cone advances across a substrate, the growth cone must interpret extracellular cues to steer the growing neurite in the right direction [33, 38]. Growth cones receive both attractive and repulsive cues and respond by selectively stabilizing, destabilizing or rearranging actin and MT cytoskeletons to allow for directed growth. This section briefly describes how extracellular signals mediate rearrangements of the cytoskeleton.

Growth cones can be roughly divided into three domains: *filopodia* are long, thin, spike-like projections that grow and retreat rapidly from the growth cone surface; *lamellipodia* are web-like veils of cytoplasm that also spread and retract, often between filopodia; and the *body* itself of the growth cone is the area of cytoplasm beyond the cylindrical neurite that adheres to the substratum. Filopodia and lamellipodia sample the environment for favorable conditions, then the body of the growth cone will move forward as the axon elongates. The distribution of the three cytoskeletal elements in the growth cone is well established (Fig. 8-4) [33, 38]. In neurites MTs occur as bundles, which then splay out into single filaments in the body of the growth cone, but MTs do not extend into the filopodia. NFs are limited to the shaft of the neurite and do not extend beyond the proximal edge of the growth cone body. Actin MFs make up the majority of the growth cone cytoskeleton. They form a complex meshwork that includes a number of actin-associated proteins beneath the entire plasma membrane of lamellipodia and the growth cone body. Bundles of actin MFs form the cores of filopodia. Forces generated by rearrangement of the actin MFs help realign the MTs, and eventually the NFs, to the preferred direction for growth.

The diameter of a growth cone is often much greater than that of the neurite, allowing it to sample a large

volume of the environment. Typically, the shape of a growth cone is constantly changing, with filopodia and lamellipodia extending and retracting, receiving signals from the surface of other cells, the extracellular matrix or the surrounding media. A number of factors may elicit a growth response including soluble neurotrophins and membrane or matrix-bound ligands. In addition, repulsive signals have been identified that lead to collapse of filopodia or lamellipodia and to retraction of growth cones. The extension of filopodia and lamellipodia are regulated in part by members of the Rho and Rac family of low-molecular-weight GTPases [27].

Signals in response to extracellular guidance cues cause growth cones to steer probably involve multiple pathways [38]. When one or a few filopodia receive an attractive cue, the growth cone will turn in that direction. At least two things occur in the region of the growth cone chosen for further growth. First, extracellular signals activate cell surface receptors to recruit a multiprotein complex that links the receptor to the actin meshwork beneath the surface. This is likely to involve a variety of signals including local Ca^{2+} transients, release of PIP and PIP_2, activation of Cdc42/Rac small GTPases, and phosphorylation of cytoskeletal proteins in the vicinity. Rapid polymerization of actin MFs leads to a protrusion of the membrane at the site of growth. This burst of actin polymerization is probably due to the concerted actions of a number of different actin-binding proteins [36] and the membrane protrusion may also involve actin-based motors like myosins [39] (see Ch. 28). More or less concurrent with MF rearrangements, the splayed MTs in the growth cone body begin to invade the selected site [33, 40]. This invasion may involve both MT elongation and MT movements. Once MTs form an ordered bundle oriented in the new direction, the membrane of the growth cone collapses around the MT bundle to create an extension of the neurite cylinder and the NFs are advanced to consolidate new growth. Then the growth cone begins looking for the next signal. While this description is an oversimplification of growth cone steering events, it nevertheless illustrates that growth cones are highly motile and very dynamic entities.

The axonal cytoskeleton may be influenced by glia. In cross sections of large myelinated axons, most of the volume is occupied by NFs separated from each other by sidearm spacers. Spaces between fields of NFs are occupied by one of two specialized regions: MTs with membrane-bounded organelles or electron-dense regions adjacent to MTs and to the plasma membrane cortex (Fig. 8-1). These electron-dense areas are enriched in short actin MFs. Such images suggest a static cross-linked cytoskeleton and do not reveal the underlying dynamics of the axonal cytoskeleton. In fact, fully mature neurons also have a dynamic cytoskeleton that is both engaged in axonal transport (Ch. 28) and responsive to the local environment.

The relationships between an axon and its myelinating glia are both intimate and extensive. Originally, these relationships were thought to be specified by signals from the axon that elicited specific responses in glial cells after contact, including proliferation and myelination [12]. Little thought was given to the possibility of glia influencing neurons. However, more recent studies indicate that the axonal cytoskeleton is also altered locally by glial contacts. Axonal cytoskeletal elements are subject to constant modulation via signals from the axonal environment including both target cells and cells forming the myelin sheath. Such signals appear to influence axonal branching, synapse formation and axonal caliber.

The response of the axon to loss of myelin is instructive. In the *trembler* mutant mouse, the axon undergoes a continuing cycle of partial myelination followed by demyelination. The result is a thin or absent peripheral myelin sheath and a reduction in axonal caliber (Fig. 8-6). Remarkably, this reduction in axonal caliber is highly localized to segments of axon with disrupted myelin.

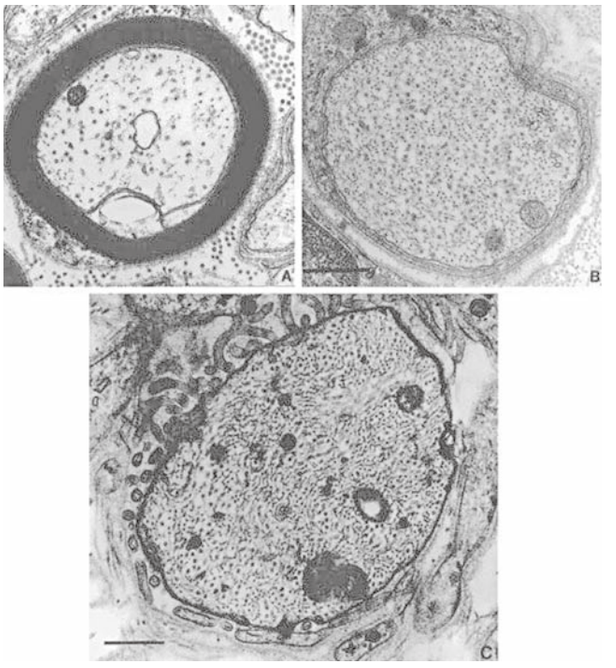

FIGURE 8-6 The local environment can alter the organization of the axonal cytoskeleton. (**A**) In a normal myelinated axon of the sciatic nerve, neurofilaments and microtubules are widely spaced, so they occupy considerable volume. (**B**) In contrast, a comparably sized axon from the sciatic nerve of the demyelinating *trembler* mutant mouse has a denser cytoplasm, with neurofilaments densely packed. This has been shown to result from a shift in the net dephosphorylation of neurofilaments produced by demyelination. This effect on the axonal cytoskeleton is highly localized. (**C**) Similar changes in the organization and phosphorylation of the axonal cytoskeleton occur even over the short gap in the myelin sheath which occurs at the node of Ranvier. Such changes illustrate the dynamic nature of the axonal cytoskeleton. Bars represent 0.5 µm with (**A**) and (**B**) at the same magnification. (Micrographs supplied by Sylvie de Waegh and Scott Brady.)

The local nature of these changes was proven by studies in which regions of *trembler* sciatic nerve were grafted into normal nerves. Only those axon segments surrounded by *trembler* Schwann cells have reduced diameters. More importantly, the reduction in axon caliber is due to an increase in NF density in *trembler* nerves by a factor of two, even though the actual amount of NF protein is not changed. The increased NF packing density means that the same number of NFs now occupies a smaller volume producing a smaller axon. This change in density appears to be due to a reduction in the phosphorylation of NFH and NFM tail domains, which allows the individual NFs to be packed more tightly [24]. Such changes are restricted to axons in contact with *trembler* Schwann cells. Thus, it is the direct interaction between the axons and the Schwann cells that modulates the axonal cytoskeleton. The influence of myelinating Schwann cells is not restricted to NFs, because the stability, organization and composition of the MT cytoskeleton are also altered in *trembler* nerves.

The effects of myelin can be observed in intact normal nerve as well. A similar change in cytoskeletal organization occurs in normal myelinated nerves at nodes of Ranvier [41]. Beginning in the paranodal regions where compact myelin is lost, the diameter of the axon is reduced; the packing density of NFs in increased; and NF phosphorylation is reduced. Thus, the axonal cytoskeleton is constantly influenced by the myelinating glial environment, providing a dramatic example of the dynamic nature of the neuronal cytoskeleton.

Levels of cytoskeletal protein expression change after injury and during regeneration. As described above there are substantial changes in the composition and organization of neuronal and glial cytoskeletal elements during development. Changes are equally dramatic following injury to the nervous system. Some of these changes reflect the switch from maintaining cellular structures to growth or repair modes. The response of PNS neurons during regeneration may fall into this category. In other cases, the response is incomplete or may be a reactive response to the injury. For example, after CNS injury, astrocytes become reactive, rapidly proliferate and frequently form a glial scar. The hallmark of this glial scar is a dramatic increase in GFAP IF bundles, which allows the astrocytes to fill the injured zone and provide mechanical support for surrounding uninjured tissues. Unfortunately, the glial scar often represents a physical barrier to neuronal elongation and repair.

The neuronal response to injury has been studied in some detail to gain a better understanding of why mammalian CNS neurons regenerate poorly or not at all, while PNS neurons regenerate effectively. Although regeneration is often described as paralleling developmental growth of neurons, the distinctive pattern of changes in the neuronal cytoskeleton during regeneration differs in some important ways from changes during development. First, there is a coordinate downregulation in NF subunit expression following injury. NFH, NFM and NFL protein and mRNA levels in the perikarya decline rapidly after injury and do not recover until after reconnection with an appropriate target cell has occurred. Unlike the case during development, where NFH expression lags behind the other two, all three NF subunits decline and recover coordinately during regeneration. Concurrent with the reduction in NF protein levels following injury, both tubulin and actin increase significantly. While NF proteins are low throughout the elongation phase of regeneration, tubulin and actin expression remains high during neurite growth and synaptogenesis. As might be expected, there are associated changes in the expression of MAPs and MF-associated proteins during regeneration as well.

The observation that selected tubulin genes are preferentially upregulated during regeneration has led to proposals that some tubulin isoforms are better suited for neurite growth. While this idea is attractive, there is little evidence for a significant functional difference among the various tubulin genes [3, 14]. Nonetheless, the idea that changes in cytoskeletal protein are required for effective regeneration is consistent with observations that changes in cytoskeletal protein expression after injury are limited or altered in CNS neurons that fail to regenerate after injury. The precise regulation of cytoskeletal gene expression during both development and regeneration suggests the importance of these structures for neuronal growth.

Alterations in the cytoskeleton are frequent hallmarks of neuropathology. A definitive diagnosis for many neurological disorders depends on a histological examination. The identifying characteristic for a number of neuropathologies is a disrupted or aberrant cytoskeleton. This is a feature of many toxic neuropathies and a number of neurodegenerative conditions, including motor neuron disease, frontotemporal dementia (FTDP-17) and Alzheimer's disease. Although other neuronal functions may also be affected and the initial cause of the disease may not directly involve the cytoskeleton, the associated pathogenic disruption of cytoskeletal function maybe a key element in the loss of neurons or neuronal function [42]. In recent years, a new class of neurological disorders captured a lot of attention in the neuroscience community. Both sporadic and familial forms of these puzzling disorders exist, but all of them exhibit a characteristic accumulation of tau protein aggregates and they have come to be known as tauopathies [17]. Several mutations in the tau gene have now been linked to these tauopathies, with differences in the severity of symptoms and the cellular location of tau accumulations depending on the specific mutation. These mutations comprise a heterogeneous group of neurological disorders known as FTDP-17. The pathological hallmark in both sporadic and familial tauopathies is the presence of intracellular tau filamentous inclusions that are typically hyperphosphorylated.

In some cases, the primary pathogenic mechanism is an effect on one or more cytoskeletal structures. For example, some widely used chemotherapeutic agents for treatment

of tumors act by disrupting microtubule function in the spindles of rapidly dividing cancer cells. Such drugs can affect microtubules in both neuronal and non-neuronal cells. Perhaps the two best known examples are vincristine and paclitaxel (taxol). While these two compounds have opposite actions on microtubules, long-term or high-dosage treatment with either leads to a high frequency of associated neuropathies. Vincristine is a classic antimicrotubule drug that destabilizes existing microtubules and blocks assembly of new microtubules. In contrast, paclitaxel stabilizes microtubules and leads to formation of numerous short MTs in inappropriate sites. Despite these differences, peripheral neuropathies are a troublesome side effect of both drugs. Neuronal viability is compromised because microtubule function is compromised. Other environmental toxins or chemicals may interfere with neurofilaments. For example, in rabbits, exposure to some aluminum salts leads to accumulation of neurofilaments in the initial segments of the axon.

In fact, a number of neuropathologies with diverse pathogenic mechanisms have associated disruptions in axonal neurofilaments. Neurofilaments accumulate in the cell bodies of motor neurons in patients with amyotrophic lateral sclerosis and related motor neuron diseases. The example of motor neuron disease is particularly interesting, because the symptoms of motor neuron disease can be produced in animal models in several very different ways. Some of these animal models have little obvious relevance to the cytoskeleton, such as those resulting from point mutations in a superoxide dismutase gene. Others directly involve neurofilaments, such as transgenic mice that express defective NF subunits or overexpress normal NF subunits. Regardless of how these animal models are produced, a characteristic feature of motor neuropathology is the accumulation of poorly phosphorylated NFs in cell bodies and initial segments (reviewed in [22, 23]).

Other diseases with disruptions in neurofilament organization include diabetic neuropathy and Charcot–Marie–Tooth disease. For these diseases, the disruption of neurofilaments may be a secondary effect as in the case of *trembler* axons or a direct effect. For example, some forms of Charcot–Marie–Tooth peripheral neuropathy result from mutations in a neurofilament subunit [22, 43]. In most cases, neuronal degeneration is an eventual consequence, but neuronal function may be impaired prior to substantial loss of neurons. Generally, disruptions of neurofilaments have the most severe consequences in large motor neurons, which is consistent with the fact that the largest neurons have the highest levels of neurofilament expression.

Phosphorylation of cytoskeletal proteins is involved both in normal function and in neuropathology. An entire book could be devoted to research on the phosphorylation of cytoskeletal proteins. Phosphorylation of cytoskeletal components may affect their assembly and organization as well as their associated function. As described above, levels of phosphorylation on NFs are a major determinant in regulation of axonal caliber and a specific set of phosphorylations is a hallmark of tau protein in axons. The phosphorylation of other MAPs is carefully regulated during development and maturation of the nervous system. Similarly, many MF-associated proteins are regulated by phosphorylation, so that the local action of kinases and phosphatases may underlie many changes in the membrane cytoskeleton and growth cone motility.

Phosphorylation of cytoskeletal proteins has also been linked to various neuropathologies. For example, the NF accumulations seen in many diseases have aberrant phosphorylation patterns. A more significant change may be the hyperphosphorylation of brain MAP tau, which is associated with the formation of neurofibrillary tangles in Alzheimer's disease [17]. These tangles contain distinctive paired helical filaments that are extremely resistant to solubilization and analysis. They were initially thought to be aberrant NFs based on their dimensions, but a careful immunochemical and biochemical dissection showed that the primary polypeptide present in tangles was tau, the axonally enriched MAP [44]. The tau in neurofibrillary tangles is differentially phosphorylated and this misphosphorylation is thought to play a role in the formation of tangles. While the precise etiology of neurofibrillary tangles and their relationship to the deposition of amyloid are not yet certain, the appearance of these tangles is closely correlated with loss of neurons and their number is a good indication of how far the disease has advanced. Clearly, alterations in the axonal cytoskeleton are an important component of the Alzheimer's neuropathology [45] (see Ch. 46).

CONCLUSIONS

The architectures of neurons and glia are generated by the diverse specializations of MTs, NFs and MFs. Each of these components forms a set of structures that are constantly changing and subject to the influence of extracellular signals. While the importance of the cytoskeleton for development, maintenance and regeneration of nerve fibers is now well documented, many details about their activities remain to be delineated. Continued exploration of these phenomena will provide the basis for a deeper understanding of neuronal development, regeneration and neuropathology.

REFERENCES

1. Hyams, J. S. and Lloyd, C. W. Microtubules. In J. B. Harford (ed.), *Modern Cell Biology*. New York: Wiley-Liss, 1994, vol. 13, p. 439.
2. Burns, R. G. and Surridge, C. D. Tubulin: conservation and structure. In J. S. Hyams and C. W. Lloyd (eds), *Microtubules. Modern Cell Biology*, vol. 13. New York: Wiley-Liss, 1994, pp. 3–31.

3. Luduena, R. F. Multiple forms of tubulin: different gene products and covalent modifications. *Int. Rev. Cytol.* 178: 207–275, 1998.
4. Nogales, E., Wolf, S. G. and Downing, K. H. Structure of the ab tubulin dimer by electron crystallography. *Nature* 391: 199–203, 1998.
5. Bayley, P. M., Sharma, K. K. and Martin, S. R. Microtubule dynamics *in vitro*. In J. S. Hyams and C. W. Lloyd (eds), *Microtubules. Modern Cell Biology*, vol. 13. New York: Wiley-Liss, 1994, pp. 111–137.
6. Ou, Y. and Rattner, J. B. The centrosome in higher organisms: structure, composition, and duplication. *Int. Rev. Cytol.* 238: 119–182, 2004.
7. Joshi, H. C. Microtubule organizing centers and gamma-tubulin. *Curr. Opin. Cell Biol.* 6: 54–62, 1994.
8. Moritz, M. and Agard, D. A. Gamma-tubulin complexes and microtubule nucleation. *Curr. Opin. Struct. Biol.* 11: 174–181, 2001.
9. Heidemann, S. R. Cytoplasmic mechanisms of axonal and dendritic growth in neurons. *Int. Rev. Cytol.* 165: 235–296, 1996.
10. Baas, P. W. Microtubule transport in the axon. *Int. Rev. Cytol.* 212: 41–62, 2002.
11. Cassimeris, L. and Spittle, C. Regulation of microtubule-associated proteins. *Int. Rev. Cytol.* 210: 163–226, 2001.
12. Brady, S. T. Axonal dynamics and regeneration. In A. Gorio (ed.), *Neuroregeneration*. New York: Raven Press, 1993, pp. 7–36.
13. Dent, E. W., Callaway, J. L., Szebenyi, G., Baas, P. W. and Kalil, K. Reorganization and movement of microtubules in axonal growth cones and developing interstitial branches. *J. Neurosci.* 19: 8894–8908, 1999.
14. McKean, P. G., Vaughan, S. and Gull, K. The extended tubulin superfamily. *J. Cell Sci.* 114: 2723–2733, 2001.
15. Kann, M. L., Soues, S., Levilliers, N. and Fouquet, J. P. Glutamylated tubulin: diversity of expression and distribution of isoforms. *Cell Motil. Cytoskeleton* 55: 14–25, 2003.
16. Schoenfeld, T. A. and Obar, R. A. Diverse distribution and function of fibrous microtubule-associated proteins in the nervous system. *Int. Rev. Cytol.* 151: 67–137, 1994.
17. Goedert, M. Tau protein and neurodegeneration. *Semin. Cell Dev. Biol.* 15: 45–49, 2004.
18. Cassimeris, L. The oncoprotein 18/stathmin family of microtubule destabilizers. *Curr. Opin. Cell Biol.* 14: 18–24, 2002.
19. Karabay, A., Yu, W., Solowska, J. M., Baird, D. H. and Baas, P. W. Axonal growth is sensitive to the levels of katanin, a protein that severs microtubules. *J. Neurosci.* 24: 5778–5788, 2004.
20. Herrmann, H. and Aebi, U. Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular Scaffolds. *Annu. Rev. Biochem.* 73: 749–789, 2004.
21. Herrmann, H. and Aebi, U. Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. *Curr. Opin. Cell Biol.* 12: 79–90, 2000.
22. Lariviere, R. C. and Julien, J. P. Functions of intermediate filaments in neuronal development and disease. *J. Neurobiol.* 58: 131–148, 2004.
23. Lee, M. K. and Cleveland, D. W. Neuronal intermediate filaments. *Ann. Rev. Neurosci.* 19: 187–217, 1996.
24. De Waegh, S. M., Lee, V. M.-Y. and Brady, S. T. Local modulation of neurofilament phosphorylation, axonal caliber, and slow axonal transport by myelinating Schwann cells. *Cell* 68: 451–463, 1992.
25. Lasek, R. J. Studying the intrinsic determinants of neuronal form and function. In R. J. Lasek and M. M. Black (eds), *Intrinsic Determinants of Neuronal Form and Function*. New York: Allan R. Liss, 1988, pp. 1–60.
26. Steinmetz, M. O., Stoffler, D., Hoenger, A., Bremer, A. and Aebi, U. Actin: from cell biology to atomic detail. *J. Struct. Biol.* 119: 295–320, 1997.
27. Burridge, K. and Wennerberg, K. Rho and Rac take center stage. *Cell* 116: 167–179, 2004.
28. Dos Remedios, C. G., Chhabra, D., Kekic, M. *et al.* Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiol. Rev.* 83: 433–473, 2003.
29. Di Campli, A., Valderrama, F., Babia, T., De Matteis, M. A., Luini, A. and Egea, G. Morphological changes in the Golgi complex correlate with actin cytoskeleton rearrangements. *Cell Motil. Cytoskeleton* 43: 334–348, 1999.
30. Valderrama, F., Babia, T., Ayala, I., Kok, J. W., Renau-Piqueras, J. and Egea, G. Actin microfilaments are essential for the cytological positioning and morphology of the Golgi complex. *Eur. J. Cell Biol.* 76: 9–17, 1998.
31. Holleran, E. A. and Holzbaur, E. L. Speculating about spectrin: new insights into the Golgi-associated cytoskeleton. *Trends Cell Biol.* 8: 26–29, 1998.
32. Holleran, E. A., Ligon, L. A., Tokito, M., Stankewich, M. C., Morrow, J. S. and Holzbaur, E. L. Beta III spectrin binds to the Arp1 subunit of dynactin. *J. Biol. Chem.* 276: 36598–36605, 2001.
33. Zhou, F. Q. and Cohan, C. S. How actin filaments and microtubules steer growth cones to their targets. *J. Neurobiol.* 58: 84–91, 2004.
34. Beck, K. A. and Nelson, J. The spectrin-based membrane skeleton as a membrane protein-sorting machine. *Am. J. Physiol.* 270: C1263–C1270, 1996.
35. Bear, J. E., Krause, M. and Gertler, F. B. Regulating cellular actin assembly. *Curr. Opin. Cell Biol.* 13: 158–166, 2001.
36. Millard, T. H., Sharp, S. J. and Machesky, L. M. Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. *Biochem. J.* 380: 1–17, 2004.
37. Mandell, J. W. and Banker, G. A. The microtubule cytoskeleton and the development of neuronal polarity. *Neurobiol. Aging* 16: 229–238, 1995.
38. Gallo, G. and Letourneau, P. C. Regulation of growth cone actin filaments by guidance cues. *J. Neurobiol.* 58: 92–102, 2004.
39. Brown, M. E. and Bridgman, P. C. Myosin function in nervous and sensory systems. *J. Neurobiol.* 58: 118–130, 2004.
40. Gordon-Weeks, P. R. Microtubules and growth cone function. *J. Neurobiol.* 58: 70–83, 2004.
41. Scherer, S. S., Arroyo, E. J. and Peles, E. Functional organization of the nodes of Ranvier. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*, vol. 1. Amsterdam: Elsevier Academic Press, 2004, pp. 89–116.
42. Griffin, J. W., George, E. B., Hsieh, S. and Glass, J. D. Axonal degeneration and disorders of the axonal cytoskeleton. In S. G. Waxman, J. D. Kocsis and P. K. Stys (eds), *The axon: structure, function and pathophysiology*. New York: Oxford University Press, 1995, pp. 375–390.

43. Brownlees, J., Ackerley, S., Grierson, A. J. *et al.* Charcot–Marie–Tooth disease neurofilament mutations disrupt neurofilament assembly and axonal transport. *Hum. Molec. Genet.* 11: 2837–2844, 2002.
44. Kosik, K. S., Orecchio, L. D., Binder, L., Trojanowski, J. Q., Lee, V. and Lee, G. Epitopes that span the tau molecule are shared with paired helical filaments. *Neuron* 1: 817–825, 1988.
45. Lee, V. M., Goedert, M. and Trojanowski, J. Q. Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24: 1121–1159, 2001.
46. Caceres, A. and Kosik, K.S. Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* 343: 461–462, 1990.

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CHAPTER

9

Intracellular Trafficking

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GENERAL MECHANISMS OF INTRACELLULAR MEMBRANE TRAFFICKING IN MAMMALIAN CELLS INCLUDE BOTH UNIVERSAL AND HIGHLY SPECIALIZED PROCESSES

Eukaryotic cells have evolved a complex, intracellular membrane organization. This organization is partially achieved by compartmentalization of cellular processes within specialized membrane-bounded organelles. Each organelle has a unique protein and lipid composition. This internal membrane system allows cells to perform two essential functions: to *sort and deliver* fully processed membrane proteins, lipids and carbohydrates to specific intracellular compartments, the plasma membrane and the cell exterior, and to *uptake* macromolecules from the cell exterior (reviewed in [1, 2]). Both processes are highly developed in cells of the nervous system, playing critical roles in the function and even survival of neurons and glia.

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The biosynthetic, secretory pathway is responsible for protein sorting and delivery and allows, among other functions, for cell–cell communication through secreted products. This delivery process starts at the endoplasmic reticulum (ER), to finish in the cell plasma membrane or, in some cases, in specific intracellular organelles. To accomplish this, specific proteins must be properly directed to the correct destination, while other proteins are retained as residents within specific organelles along the way.

The series of molecular events responsible for the uptake process constitutes the endocytic pathway, which enables cells to internalize macromolecules from the cell exterior, forming an endosome. The endosome is an intermediate organelle that serves as an essential component for many receptor-mediated signaling pathways and as a transport vector for eventual delivery to a specialized organelle known as the lysosome. Once in the lysosomal lumen, digestive enzymes provide essential metabolites from these macromolecules (i.e. free amino acids and lipids) directly to the cytosol for their use.

Intracellular membrane compartments are in a continuous, balanced and dynamic interaction. A constant, directed flow of membrane components among different compartments occurs at all times in a single cell. Transport vesicles of variable shape and composition provide the means by which membrane components and soluble molecules, generically referred as cargoes, move from a donor to a target compartment (Fig. 9-1). Such vesicles first bud off from the donor membrane and later fuse with the target membrane. In order to maintain the biochemical composition and the unique structural identities of each organelle, eukaryotic cells developed specialized retrieval and retention mechanisms. The retrieval process implies that recycling transport vesicles must take only the proteins to be recycled (i.e. proteins that are continuous residents in their original compartment, or resident proteins), while others are left behind. The specific sequence of events observed in any direction within the biosynthetic and the endocytic pathways implies that transport vesicles need to fuse only with the correct target organelle. These mechanisms allow for maintenance and proper balance of membrane flow among compartments, assuring that each protein is targeted for the correct location as well as redirecting resident membrane components to the compartment where they originated.

Neurons constitute the most striking example of membrane polarization. A single neuron typically maintains thousands of discrete, functional microdomains, each with a distinctive protein complement, location and lifetime. Synaptic terminals are highly specialized for the vesicle cycling that underlies neurotransmitter release and neurotrophin uptake. The intracellular trafficking of a specialized type of transport vesicles in the presynaptic terminal, known as synaptic vesicles, underlies the ability of neurons to receive, process and transmit information. The axonal plasma membrane is specialized for transmission of the action potential, whereas the plasma membrane of cell body and dendrites has specialized to receive signals from other nerve cells.

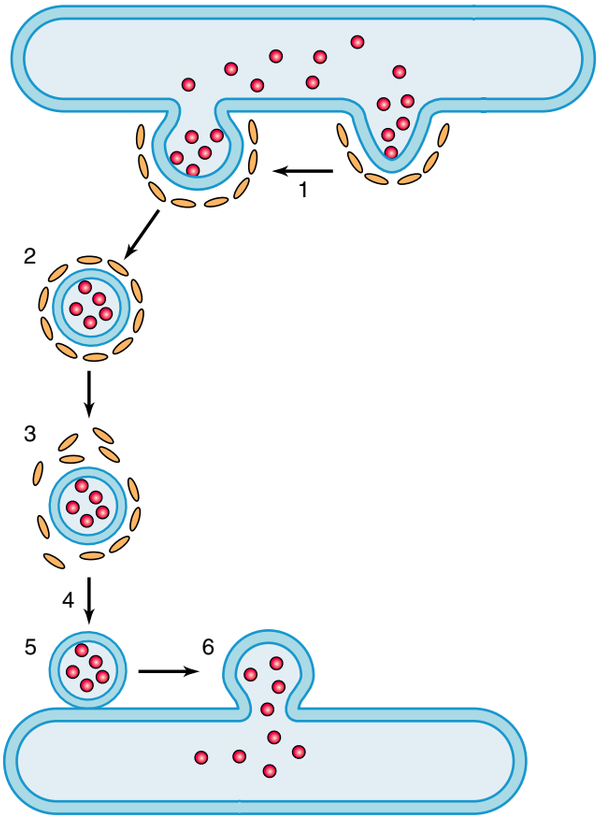

FIGURE 9-1 Fundamental steps of intracellular membrane transport. A series of basic steps allows the transfer of material (generally referred to as cargo) from the lumen and membrane of a donor compartment to a target/acceptor compartment. First, specific cargo is selected for packaging in the donor organelle (*1*). This process is concurrent with the formation of a specific protein coat on the cytosolic surface of the donor membrane, which helps molding a newly formed transport vesicle. Once a transport vesicle is formed, it buds off from the donor organelle (*2*). Transport vesicles shed their coats shortly after budding (*3*) in a an active process that involves either an ATPase/chaperone or a small GTPase. After uncoating, vesicles are actively translocated across the cytoplasm, usually through mechanisms mediated by microtubule-based molecular motors (*4*). Transport vesicles eventually reach, recognize and tether to the appropriate target organelle (*5*). Finally, unloading of the transport vesicle cargo to the target membrane occurs by membrane fusion (*6*).

Glial cells also require extensive, albeit less diverse intracellular membrane trafficking activity. This includes the delivery of myelin proteins during both the generation and maintenance of myelin. In the case of oligodendrocytes, different processes myelinating different axons may require differential trafficking of these components, similar to the situation in neurons (see Ch. 4). Finally, astrocytes must distribute a variety of transporters and channels to different astrocytic domains as part of their role in regulating the neuronal environment and delivering nutrients.

In this chapter, the basic mechanisms of membrane trafficking and the functions of the biosynthetic, secretory

and endocytic pathways in the nervous system will be examined. First, the fundamental steps of vesicle formation will be defined. Second, the diverse biosynthetic, secretory pathways and their functions will be examined, including biogenesis of organelles, delivery of plasma membrane components, and secretion. Third, endocytic pathways will be summarized including receptor-mediated endocytosis, membrane recycling and signaling. Finally, the specialized processes of synaptic vesicle trafficking serve to integrate these two pathways and represent a unique form of membrane trafficking in the nervous system.

FUNDAMENTALS OF MEMBRANE TRAFFICKING ARE BASED ON A SET OF COMMON PRINCIPLES

Although the various membrane compartments utilize a different repertoire of proteins for coat formation, vesicle budding and fusion, the basic principles behind these processes are similar (Fig. 9-1). A number of ordered, regulated molecular events allow for protein segregation into discrete, separate membrane domains [2]. Although these events are essential for functionality and survival of all cell types, they are highly specialized and often more complex in neuronal cells. The process of budding is initiated by the selection and assembly of specific components in donor membranes intended to be packaged and delivered together to the same target membranes. The formation of specific protein coats on the cytosolic face of the donor membrane gathers these common components into a single domain that can be packaged as a transport vesicle. This sequence of events provides for membrane targeting specificity.

Most transport vesicles bud off as coated vesicles, with a unique set of proteins decorating their cytosolic surface. The coat has two major known functions. First, it concentrates and selects specific membrane proteins in a discrete portion of donor organelle membrane that will serve as origin to the transport vesicle. Second, the assembly of coat proteins into curved structures delineates the area of the forming transport vesicle. The size and curvature is a function of the coat composition. Thus, vesicles with similar vesicle coat have closely similar size and shape [3].

There are at least three well-established types of coated vesicles common to all cell types, each one with a characteristic coat protein composition, and each used for different transport steps in the cell. Ultrastructural and biochemical studies first suggested a higher degree of diversity within these vesicle types [4]. In addition to these coated-vesicle types familiar from electron microscopy, video microscopic imaging in living cells identified tubulovesicular carriers, rather than spherical-shaped vesicles, budding off from endosomes, the ER and the *trans*-Golgi network. Transport vesicles important for longer distance trafficking, such as secretory vesicles, synaptic vesicles and other specialized vesicle types, are discussed in detail later in this chapter.

Clathrin-coated vesicles mediate transport from the Golgi apparatus to endosomes, and from the plasma membrane to endosomes. A multi-subunit protein, clathrin, constitutes the major protein of this vesicle type (see Ch. 2). Clathrin is composed of three large and three small polypeptide chains, which assemble to form a triskelion (Fig. 9-2). Regulatory mechanisms control the assembly and formation of a convex, polyhexa-pentagonal basket-like structure by these triskelions [5]. This structure is responsible for the formation of coated pits on the cytosolic face of plasma membranes.

Another major protein component of clathrin-coated vesicles, generally known as adaptins, bind and link clathrin coats to the membrane [5]. Multiple types of adaptins have been described, each type binding a unique set of cargo receptors and associated to a specific membrane organelle. Different sets of adaptins participate in forming the coat assembly complexes for the Golgi (AP1) and the plasma membrane (AP2). Sequential assembly and

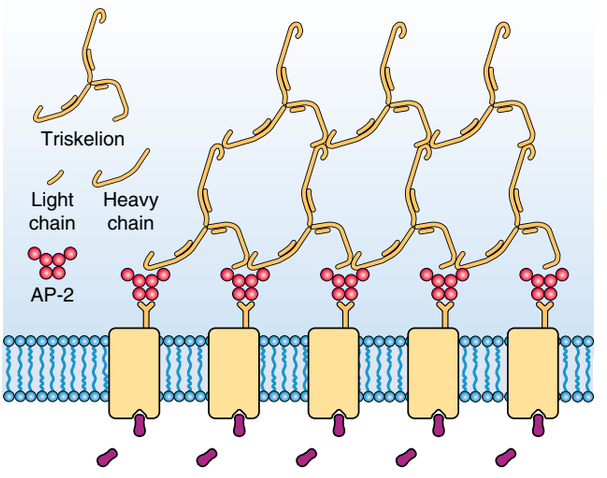

FIGURE 9-2 Endocytosis of membrane components through clathrin-coated pits. Clathrin coats are the best characterized and understood pathway for formation of vesicles either from the plasma membrane or the Golgi complex [5]. Clathrin function was initially worked out by studies of the recycling of membrane receptors for ligands such as transferrin and insulin. Recycling of synaptic vesicle membranes occurs by a similar process. Subsequent studies on Golgi trafficking revealed a parallel clathrin coat pathway. Ligand binding to the receptor appears to induce a conformational change that permits a tyrosine-containing β turn in the cytoplasmic domain to interact with one of the adaptins (AP-2). Clathrin binding to the adaptins then produces the coated pits that develop into endocytotic vesicles. Clathrin consists of three heavy chains (≈190 kDa each) that join near their C-termini to form a triskelion. Three light chains, of undetermined function, associate with the proximal segments of the heavy chains, possibly via an amphipathic α helix (heptad repeats) found in their central domains. Auxillin (not shown) is another component of the triskelion that appears to be important for removal of clathrin coats by a member of the Hsp70 chaperone family.

interactions between adaptins and clathrin aid in bud formation. Adaptins also bind and recruit transmembrane proteins known as cargo receptors, which in turn bind and recruit soluble proteins in the membrane lumen. This provides a means by which proteins may be selectively packaged into a clathrin-coated vesicle.

COPI-coated vesicles mediate intra-Golgi transport and Golgi to ER retrograde transport. The coats of these vesicles do not show the geometric forms seen with clathrin coats and have a more complex protein composition [3]. Coat protein purification first lead to the identification of a complex composed of seven individual coat-protein subunits, known as COPI or coatomer. Some of these subunits bear a sequence similarity to clathrin adaptors. In addition, there is a small GTP-binding protein, Arf1, present on COPI-coated vesicles.

COPII-coated vesicles are anterograde transport vesicles used early in the secretory pathway [3, 6]. COPII components were initially identified by using yeast mutants showing secretion defects and subsequently isolated from mammalian cells. The appearance of COPII coats in electron micrographs is readily distinguished from both clathrin and COPI coats. COPII-coated vesicles bud from the ER for transport to the Golgi, and their coat is composed of four individual coat-protein subunits. One of these is a small GTP-binding protein, SarI, exhibiting some sequence similarity to Arf1.

GTP binding proteins, such as the small monomeric GTPases and heterotrimeric GTPases (G proteins) facilitate membrane transport. GTPase proteins regulate a variety of cellular processes, including membrane trafficking in cells [7]. These proteins act as switches by alternating between an active, GTP-bound state and an inactive, GDP-bound state. Various modulator proteins affect the rate of conversion between these two states. Guanine-nucleotide exchange factors (GEFs), promote the exchange of GDP for GTP, resulting in GTPase activation. On the other hand, GTPase-activating proteins (GAPs), promote GTP to GDP hydrolysis, thus inactivating GTPases.

Although both heterotrimeric G proteins and monomeric GTPases appear to have roles in the regulation of vesicle transport, the role of monomeric GTPases is better understood. In particular, coat protein assembly is regulated by coat-recruitment GTPases, which are monomeric GTPases. In general, coat-recruitment GTPases are abundant in the cytosol, where they exist in an inactive, GDP-bound state. Hydrolysis of GTP by GTPases typically results in their translocation to membranes. Examples of such coat-recruitments GTPases are ARF proteins, which regulate clathrin and COPI coat assembly at Golgi membranes, and the SarI protein, which regulates COPII coat assembly at the ER.

COPI vesicle budding is triggered by the hydrolysis of GTP to GDP by membrane-associated ARF1. Once in the membranes, ARF–GTP recruits pre-assembled coatomers, resulting in membrane deformation. The cytoplasmic tail of an abundant transmembrane protein, known as p24, binds to COPI through an internal KKXX sequence, promoting vesicle formation [8]. As explained below, KKXX sequences represent a motif that is instrumental in retrieval mechanisms leading to retrograde transport of proteins in the secretory pathway.

Another set of proteins regulates the budding of COPII-coated vesicles. Before COPII-coated vesicles bud from the ER, a specific membrane embedded GEF binds to SarI and catalyzes the exchange of GDP to GDP by SarI. A conformational change in SarI is induced by this GTP–GDP exchange, leading SarI to expose a fatty acid tail normally hidden in the SarI core. This structural change allows SarI to insert at the ER membrane and recruit COPII subunits, which in turn starts the membrane deformation and the vesicle budding process [6]. GTP hydrolysis by SarI and other GTPases can eventually reverse this process, albeit at a slow rate in the absence of a GAP activity. This suggested that coat-recruitment GTPases might regulate temporal aspects of vesicle assembly working by hydrolyzing GTP at a slow, but predictable rate.

Once a membrane bud has formed, additional factors are required to form a discrete transport vesicle. The best-known example is seen with clathrin-coated vesicles. As budding proceeds, clathrin-coated vesicles recruit among other proteins, a soluble GTPase known as dynamin [9]. Dynamin and other binding partners form a complex that bends the membrane at the neck of the budding vesicle. This is achieved by locally distorting the membrane or by changing the lipid composition trough the actions of lipid-modifying enzymes, which are recruited to the dynamin complex. Once in close proximity, the two membranes leaflets fuse, resulting in the final pinching-off of the coated vesicle.

Chaperones of the Hsp70 family later remove the clathrin coat once vesicles are released from the donor membrane [5]. These chaperones are ATPases recruited and activated in a regulated manner by specific protein domains within the membrane-associated protein auxillin. In neuronal cell bodies, clathrin coats need to be removed before transport vesicles undergo active translocation to axonal processes from the Golgi apparatus. Presumably, uncoating allows membrane proteins exposed on the surface of vesicles to be recognized by molecular motors of the kinesin and dynein superfamilies (see Ch. 28). COPI and II coats also need to be removed to permit active translocation by molecular motors and to allow fusion of transport vesicles with a target membrane. GTP hydrolysis by the appropriate coat-recruitment GTPase causes COPI and COPII coats to disassemble. This step is catalyzed by the specific GAP protein ARF GAP in the case of COPI vesicles, and sec23 protein promotes in the case of COPII vesicles [4].

The cytoskeleton appears to have a significant role in the localization of different organelles, as well as in the active transport of vesicles (see Chs 8 and 28). Both microtubules (MTs) and actin filaments appear to be involved in these processes. For example, through its association with the minus end of MTs, the Golgi apparatus is

localized in close proximity with the centrosome. MTs also provide the tracks used by molecular motors for transport of vesicles and organelles throughout the neuron (see Ch. 28). Neuronal cells are highly dependent on these MT-based transport systems, because of their high degree of polarization and their architectural dynamics. The actin cytoskeleton also appears to play a role in vesicle tethering and transport, particularly in the event of regulated secretion, and the synaptic vesicle cycle. Finally, cytoplasmic-linker proteins, or CLIPs, appear to mediate certain types of interactions among vesicles and MTs. CLIP-170 for example, links endosomal membranes to MTs.

SNARE proteins and Rabs control recognition of specific target membranes. Rabs are a family of monomeric GTPases normally localized on the cytosolic face of membranes that aid in vesicular transport specificity [10, 11]. There are more than 30 known Rabs, each showing a distinctive organelle distribution. Targeting to specific organelles is though to be mediated by variable carboxyl terminal sequences, which appear to bind proteins specific to the surface of each organelle [12]. In their active, GTP-bound state, Rabs appear to bind other proteins in the membrane (generally known as Rab effectors), through an exposed lipid anchor.

SNAREs provide specificity to a target membrane and participate in the fusion process. SNARE proteins are transmembrane proteins with a cytoplasmic helical domain. There are more than 20 different SNAREs in each cell, each one associated to a specific membranous organelle [13]. SNAREs exist as complementary sets of v-SNAREs (in the transport vesicles) and t-SNAREs (in the target membranes). The SNARE hypothesis proposes that SNARE molecules are the basis for specificity of membrane fusion [14]. When SNAREs bind, their cytoplasmic helical domains interact to form coiled-coil interactions, which are thermodynamically highly stable. These interactions result in the formation of a stable, four-helix bundle complex known as a *trans*-SNARE pair and brings the two membranes together. Recent *in vitro* experiments indicate a high level of specificity in the interaction between v- and t-SNAREs. Three SNARES in one membrane and one in the other appear to be needed to form a functional complex containing four-helix bundle [15]. If different SNARE combinations display different specificities, this explains how a single SNARE can be involved in several transport steps. Inhibitory proteins provide another level of regulation by binding to t-SNARES, limiting their amount and availability for fusion. SNARE complexes are best characterized in neuronal cells, where they mediate the docking and fusion of synaptic vesicles, two critical steps necessary for the process of neurotransmitter release (see below). SNAREs in the nerve terminal can be targeted and cleaved by botulinum neurotoxins, which causes a block in synaptic transmission.

The *trans*-SNARE pair becomes the *cis*-SNARE pair after fusion (this name is given because after fusion, they are in the same membrane) and disassembly of the *cis*-SNARE complex is necessary for the SNAREs to be reused. An ATPase named NSF catalyzes this step. NSF acts as a chaperone, actively unfolding the coiled-coil interaction between SNAREs with the help of adaptor proteins [14]. Once *cis*-SNAREs are disassembled, v-SNAREs may be returned to the donor membrane for reuse. The regulated disassembly of SNARE complexes by NSF provides the means to control the degree and amount of membrane fusion within cell.

Rabs are also thought to regulate v/t-SNARE recognition, and ultimately vesicle fusion. Rabs and their effectors may modulate vesicle trafficking through different means. Although each Rab member has a discrete localization, a common feature of all Rabs is that they contribute to vesicle tethering near the target site and to the release of SNARE control proteins, thus facilitating v- and t-SNARE recognition. Once GTP is hydrolyzed, the inactive Rab protein moves to the cytosol and becomes available for another transport cycle [10]. GEF and GAP proteins regulate the normal intrinsically low GTPase activity of Rabs. In addition, GDP-dissociation inhibitor (GDI) and GDI-displacement factor (GDF) may regulate association of Rabs with membranes. These proteins are effectors of pathways activated by specific extracellular signaling events, thus linking transport steps to extracellular stimuli [16]. Finally, some Rabs appear to directly link specific organelles and transport vesicles to the cytoskeleton.

Unloading of the transport vesicle cargo to the target membrane occurs by membrane fusion. The fusion event need not happen immediately after vesicle docking. Depending on the target, fusion may be constitutive or regulated. Fusion events are often subject to exquisitely sensitive regulatory mechanisms, i.e., localized Ca^{2+} influx at the presynaptic terminal. Although docking indicates sufficient membrane proximity for proteins to interact, fusion requires a much higher degree of proximity. Membranes must be brought as close as 1–2 nm. This proximity eventually allows lipids to move from one membrane to another. Specific proteins facilitate this high-energy-demanding process by removing water molecules from the cytosolic face of closely opposite membranes. SNARE complex formation appears to help repel water as coiled-coil structures wrap around each other. Although *in vitro* experiments indicate that SNAREs could allow for slow membrane fusion by themselves, accessory proteins appear to assist and regulate membrane fusion events *in vivo* [17].

Fusion events may be homotypic or heterotypic. Homotypic fusion indicates fusion between membranes that originate from the same compartment (i.e. fusion of ER-derived vesicles to form tubular vesicular clusters, see below). Heterotypic membrane fusion indicates fusion of membranes originating from different compartments (i.e. synaptic vesicles and the plasma membrane). Triggered fusion like neurotransmitter release is typically heterotypic.

THE BIOSYNTHETIC SECRETORY PATHWAY INCLUDES SYNTHETIC, PROCESSING, TARGETING AND SECRETORY STEPS

The biosynthetic secretory pathway starts at the rough endoplasmic reticulum with synthesis of components, continues through the Golgi apparatus, and eventually finishes in the cell plasma membrane, secretory vesicles, or lysosomes. Along the way, a variety of processing and packaging events must occur. To facilitate understanding of each step, a functional description of each organelle along the biosynthetic pathway is useful before discussing specific transport steps within this pathway.

Historically, endoplasmic reticulum is classified as rough or smooth, based on the presence (RER) or absence (SER) of membrane-associated polysomes. The biosynthetic pathway of membrane trafficking begins with the rough endoplasmic reticulum (RER). Generally, most protein synthesis occurs on the cytosolic surface of the ER (Fig. 9-3). Shortly after initiation of synthesis, proteins destined for secretion, for service as integral membrane proteins or for residence along the biosynthetic pathway are targeted to the ER lumen by a specific amino-terminal sequence. As new polypeptide chains start to protrude from the ribosomes, they are recognized by the signal recognition particle (SRP), which targets the newly formed polypeptide chain to the cytosolic side of the ER membrane, by means of a specific SRP receptor and a protein complex known as Sec61p [18]. Soluble luminal or secretory proteins are cotranslationally translocated into the ER lumen, through the Sec61p membrane channel, while transmembrane proteins are cotranslationally inserted into the ER membrane directly.

Once in the ER lumen, proteins oligomerize and fold. Proper folding in most cases is facilitated by various isomerases and chaperones resident in the ER. Complex mechanisms exist within the ER to ensure that proteins

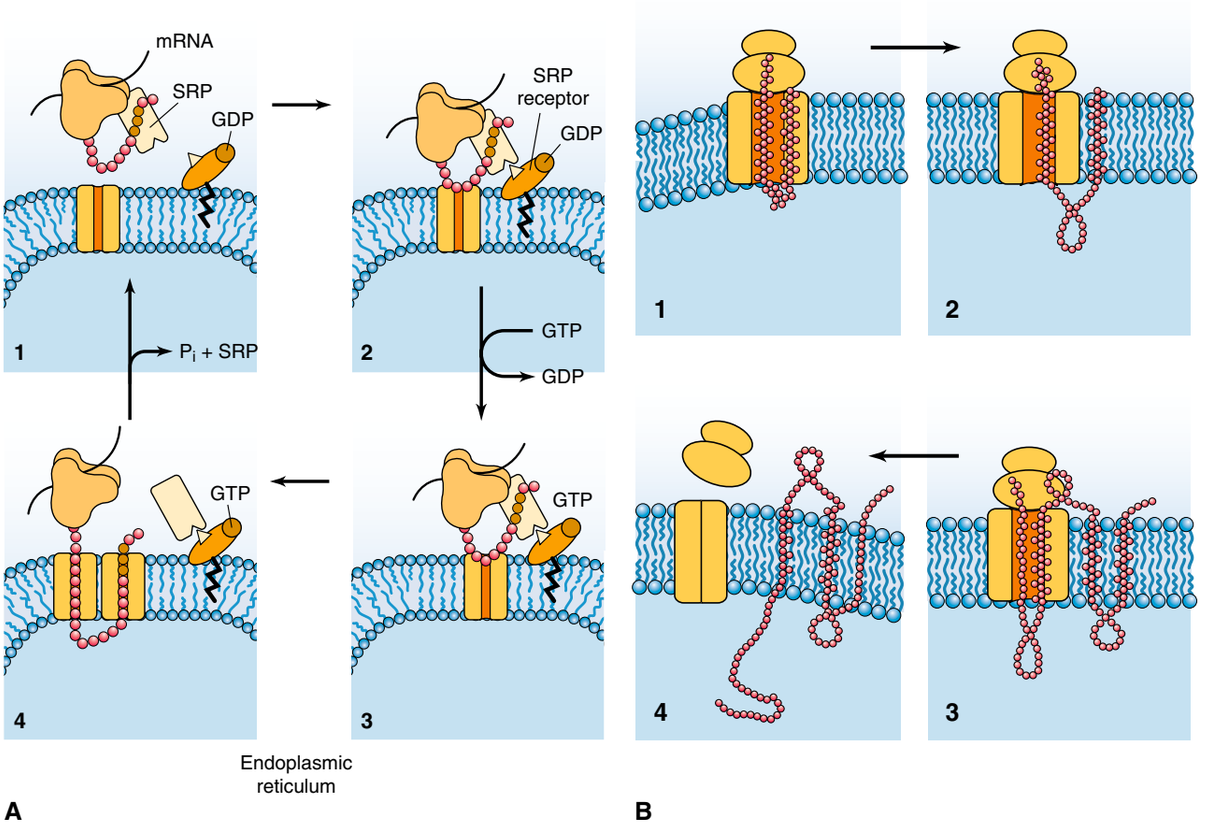

FIGURE 9-3 Insertion of proteins into a membrane. (**A**) Initiation of membrane protein insertion into the endoplasmic reticulum. *1.* Signal-recognition particles (SRP) associate with ribosomes (mRNA), and the signal sequences (darker residue) of nascent membrane proteins. *2.* These complexes associate with SRP receptors in the endoplasmic reticulum membrane. The SRP receptors contain bound GDP. *3.* Bound GDP is exchanged for cytoplasmic GTP, and (*4*) translocation of peptides occurs as GTP is hydrolyzed. The peptides are oriented N- to C-terminal outward as they insert through a membrane. (**B**) The synthesis of polytopic membrane proteins requires multiple sequential steps to generate specific domains including (*1*) the transmembrane segments that involve insertion into the membrane bilayer, (*2*) luminal domains that may eventually project into the extracellular environment and (*3*) cytoplasmic domains. During these steps, the ribosome seals the lumen of the translocator channel. As transmembrane domains are completed, they move laterally into the lipid bilayer, allowing generation of multiple transmembrane domains. *4.* Once translation is complete, the ribosome detaches and the channel closes, leaving an integral membrane protein. Translocation of proteins into the lumen of the endoplasmic reticulum occurs when no transmembrane domains are present.

are correctly folded and multimeric protein complexes correctly assembled before leaving this organelle. For example, some cargo proteins destined to move through the biosynthetic secretory pathway need to be actively recruited into vesicles. This recruitment is mediated by exit signal sequences located on their properly folded surface. ER chaperone proteins, such as Bip, recognize and cover such exit signals in incomplete or improperly folded proteins, thereby impeding their exit from the ER [19]. Such defective proteins may be sent to the cytosol, where they are degraded through the action of the proteasome. In some cases, such as the acetylcholine receptor, 90% or more of the synthesized protein is degraded before it reaches the plasma membrane. Thus, chaperone-mediated quality control mechanisms prevent defective proteins from reaching their normal destinations where they might interfere with the function of normal proteins [20]. A number of human diseases are related to defects in this quality control mechanism, including cystic fibrosis, Tay–Sachs disease (see Ch. 41), and Charcot–Marie–Tooth disease type 1a (see Ch. 36) [21].

The covalent addition of sugars (or glycosylation) is an important part of the proper folding of proteins of many membrane-associated proteins, and this is one of the major functions of the SER (Table 9-1). The majority of proteins destined to the ER itself (membrane and soluble ER proteins), secreted proteins and proteins destined to other organelles in the biosynthetic secretory pathway are in fact sugar-modified proteins, or glycoproteins. Addition of different sugar complexes may be important for the normal function of a protein or for targeting a protein to a particular organelle, but the first stages of glycosylation follow a typical pattern.

Initially, a 14-sugar oligosaccharide containing *N*-acetylglucosamine, mannose and glucose, known as the precursor oligosaccharide, is added *en bloc* to the side chain amino group of an asparagine residue of nascent proteins as they enter the ER lumen. Only asparagines within the consensus sequence NXS/T are modified (where X is any amino acid except proline). This process is also known as N-linked glycosylation, and it is catalyzed by the action of a membrane-bound enzyme known as oligosaccharyl transferase. Proteins in the cytosol are not subject to this kind of modification, because the oligosaccharyl transferase enzyme is oriented in the ER membrane with its catalytically active site facing the ER lumen. Since most proteins are co-translationally imported into the ER, N-linked oligosaccharides are almost always added during protein synthesis. It is thought that N-linked glycosylation is used as a mechanism for recognizing the extent of protein folding, allowing only properly folded proteins to leave the ER.

Most glycoproteins have one mannose and three glucoses removed by specific enzymes while still in the ER. This sugar trimming process has a role in the correct folding of N-linked glycoproteins [22]. For example, specific chaperones such as calnexin and calreticulin bind to monoglucosylated (trimmed) oligosaccharides and assist in their folding. Enzymatic hydrolysis of the remaining glucose in the monoglucosylated protein species leads to dissociation of these chaperones. Proteins not properly folded are enzymatically reglucosylated, recognized again by calnexin or calreticulin and retained in the ER. This process allows the protein to follow the right folding sequence. Improperly folded proteins that cannot be recovered are eventually translocated back to the cytosol, where they are deglycosylated, ubiquitinated and degraded in proteasomes. The abnormal accumulation of unfolded proteins in the ER can eventually trigger a pathway known as unfolded protein response, which leads to the transcription and translation of specific genes that help the ER to continue its appropriate function [23].

TABLE 9-1 Compartmentalization of glycosylation processing steps in the secretory pathway

Compartment	Processing step	Enzymes
Endoplasmic reticulum	N-linked glycosylation	ER mannosidase
	Removal of mannose	Glucosidase I
	Removal of two glucoses	Glucosidase II
cis-Golgi network	Phosphorylation of oligosaccharides in lysosomal luminal proteins	*N*-acetylgalactosaminyl transferase
	O-linked glycosylation	
cis-Golgi cisterna	Removal of mannose	Golgi mannosidase I
medial Golgi cisterna	Removal of mannose	*N*-acetyl glucosamine transferase I
	Addition of N-acetylglucosamine	
trans-Golgi cisterna	Addition of galactose	Golgi mannosidase II
	Addition of NANA	
trans-Golgi network	Sulfation of tyrosines and carbohydrates	α2,6-sialyltransferase
	Addition of sialic acid	
	Selective recognition of M6P by M6P receptor	

Immunoelectron microscopic and biochemical fractionation data on specific processing enzymes have permitted the localization of specific processing steps in the processing pathway for proteins in the Golgi complex. The distribution of many remaining enzymes and steps has not yet been determined. The distribution for each step indicated in this table reflects a significant enrichment rather than an exclusive localization.

Biosynthetic and secretory cargo leaving the ER is packaged in COPII-coated vesicles for delivery to the Golgi complex. These vesicles exit the ER from specialized regions of the ER membrane, devoid of bound ribosomes and known as ER exit sites (Fig. 9-4). These domains are specialized in the generation of COPII-coated transport vesicles. Soon after budding from the ER, COPII-coated vesicles lose their coat and fuse homotypically to form structures known as vesicular tubular clusters, transitional ER, or ER–Golgi intermediate compartment (ERGICs). ERGIC represents an independent ER-derived compartment, which lacks ER-resident proteins.

Two models have been proposed to explain the process of packaging and transport of COPII vesicles from the ER towards the Golgi: the bulk flow transport and selective transport models. Bulk transport proposes that only signals necessary for retention and recycling play a role in the selectivity of cargoes [24]. Proteins without such signals follow a default pathway, which send them to the Golgi apparatus. The selective transport model proposes that proteins are concentrated before packaging by a process involving the recognition of specific exit signals [25]. This model is consistent with the fact that approximately 90% of ERGICs are recycled back to the ER, suggesting active concentration of soluble and membrane proteins from recycling vesicles. However, with some few exceptions, exit signals directing proteins outside ER to Golgi are not well understood. More recent evidence indicates that some cargo proteins are actively recruited to vesicles through exit signals on their surface. These exit signals are recognized by receptor proteins, which also interact with components of the COPII coat. One possibility is that abundant proteins could leave the ER via bulk transport, while certain low-abundance proteins would be packaged by selective transport [26].

Specific retrieval and retention mechanisms allow for the sustained maintenance of the ER's biochemical composition. Shortly after being formed, vesicular tubular clusters start budding COPI-coated vesicles, which retrogradely carry ER-resident proteins and proteins involved in ER budding reaction back to the ER. Thus, the ERGICs sort out proteins by either recycling ER proteins or sending secretory cargo to the *cis*-Golgi. This recycling, known as retrieval transport, is essential for the maintenance of biochemical identity of specific membrane compartments. As a result of retrieval transport, vesicular tubular clusters change their composition as selected proteins are returned to the ER [27]. The retrieval of ER resident proteins depends in turn upon retrieval signals, some of which bind directly to COPI coats, thereby getting packaged in COPI-coated vesicles [28]. Examples of retrieval signals are the KKXX and the H/KDEL amino acid sequences (both names based on the single-letter amino acid code).

The KKXX sequence is present at the carboxyl terminus of ER membrane proteins, while the H/KDEL is present in soluble ER proteins, such as the aforementioned chaperone BiP. Soluble ER proteins containing these sequences bind to receptor proteins such as the Golgi-localized H/KDEL receptor. The KDEL receptor is a transmembrane protein that recognizes and recruits H/KDEL-containing proteins into COPI-coated vesicles that undergo retrograde transport. H/KDEL receptors need to cycle themselves between the ER and the Golgi. To achieve this, H/KDEL receptors affinity towards H/KDEL containing proteins needs to change from very low binding affinity in the ER, to a very high binding affinity while in vesicular tubular clusters and Golgi. Such differences in binding affinities are achieved due to pH-dependent conformational changes in the H/KDEL receptor. Whereas the pH in ER is near neutral, H^+ pumps in the Golgi create slightly acidic conditions and result in high-affinity binding of H/KDEL-containing soluble proteins.

Another potential mechanism for protein retention is that ER proteins bind to each other to form large complexes that cannot be recruited in transport vesicles [27]. This mechanism is known as kin recognition. Because the concentration of resident ER proteins can be very high (some had been estimated in the millimolar range), it can be achieved even with low-affinity interactions among resident proteins.

The Golgi apparatus is a highly polarized structure consisting of a series of flattened cisternae, usually located near the nucleus and the centrosome. Camillo Golgi first discovered the Golgi apparatus while studying neuronal cells, using a staining technique based in heavy metals [29]. Neurons have the most abundant Golgi complement as a result of the high rate of synthesis and processing of membrane proteins in neurons. Golgi localization has been shown to depend on intact microtubules and the action of microtubule motors. Each Golgi stack or cisterna has an entry (or *cis*) face, and an exit (or *trans*) face. The Golgi usually consists of three to eight cisternae, and it can be divided morphologically and functionally in *cis*, medial and *trans*-Golgi. This heterogeneity was demonstrated by the differential distribution of processing enzymes within individual cisterna, particularly for enzymes involved in the glycosylation process (Table 9-1).

The *cis* and *trans* faces of the Golgi apparatus are each associated with a specific compartment composed of interconnected tubular and vesicular structures: the *cis*-Golgi network (CGN) and the *trans*-Golgi network (TGN) respectively. The CGN is thought to originate through the fusion of multiple vesicular clusters. Sorting of proteins is an important process that takes place in these two compartments. After entering the CGN, proteins can either be retrieved to the ER or continue their travel through the Golgi apparatus. Similarly, within the TGN proteins are sorted in different transport packages and sent to their final destinations. Such destinations are specific domains of the plasma membrane (i.e. dendritic or axonal domains), lysosomes, secretory vesicles or earlier compartments in secretory pathway. The TGN is functionally

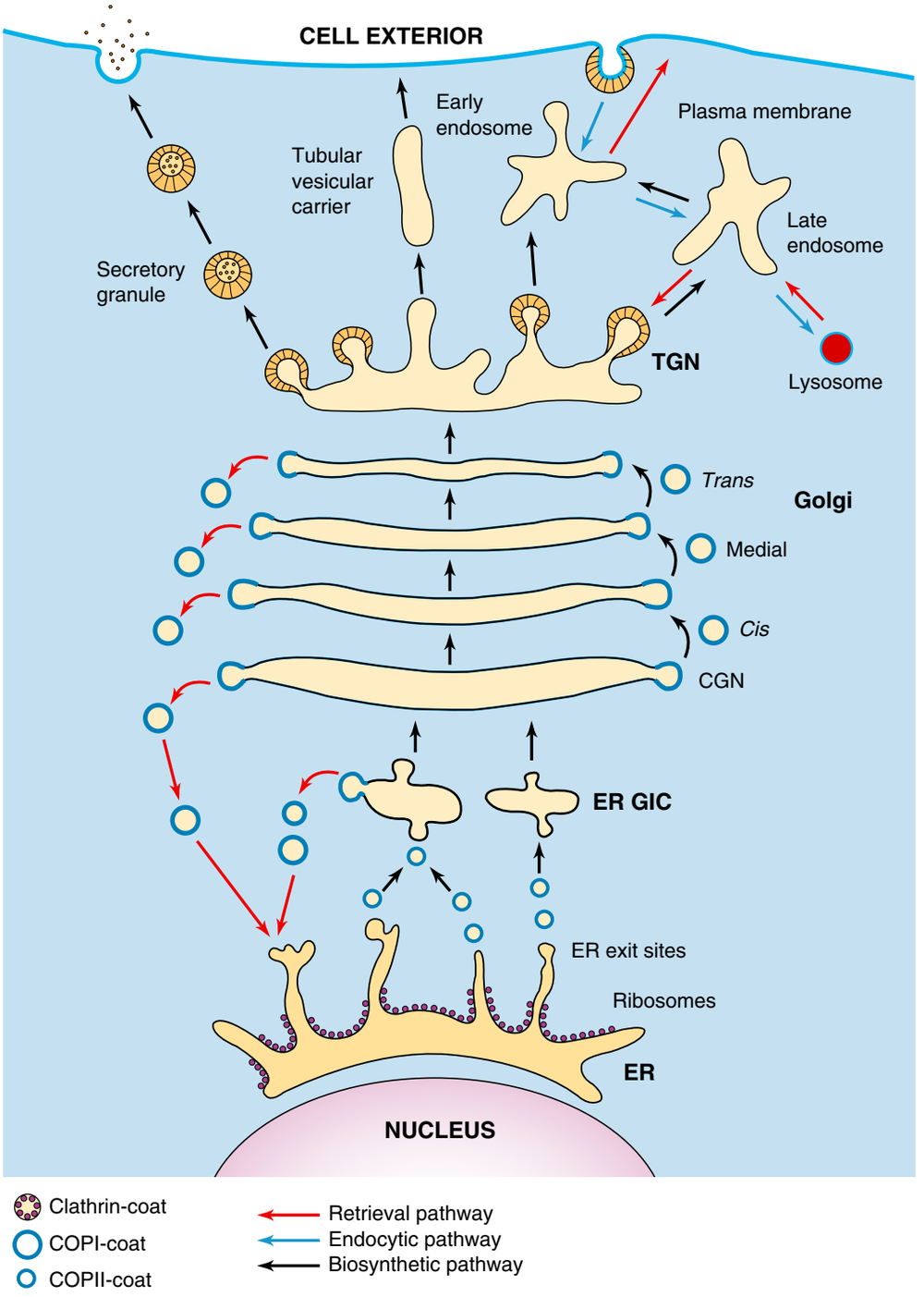

FIGURE 9-4 Schematic illustrating intracellular compartments and major transport steps along the secretory and the endocytic pathways. Intracellular organelles transfer material to each other in the form of transport vesicles. Different coat proteins help in the selection of specific cargoes during different transport steps. In the secretory pathway (black arrows), cargo proteins start their journey by budding off from ER exit sites. These are ribosome-free areas of the endoplasmic reticulum (ER). These proteins are packaged in COPII-coated vesicles, which later fuse to form vesicular clusters (ERGIC). Several ERGICs merge and fuse to form the *cis*-Golgi network (CGN). COPI-coated vesicles mediate the recycling of ER proteins and the transport of cargoes between Golgi stacks. Once in the TGN, proteins get sorted to the plasma membrane, or in some cases to lysosomes. Resident proteins of each organelle along the secretory pathway achieve their localization through specific retrieval mechanisms (*red arrows*). Different types of coated vesicles and tubulovesicular carriers transport cargo to their final destinations. For example, some cargoes reach the plasma membrane and/or the cell exterior by means of secretory granules, while others appear to travel in tubulovesicular structures, or trough recycling endosomes. In the endocytic pathway (*blue arrows*), molecules are internalized in the form of plasma-membrane-derived, clathrin-coated vesicles. These vesicles are delivered to the early endosomes, late endosomes and eventually to lysosomes, where they are degraded. Retrieval pathways allow for the recycling of proteins from the early endosome to the plasma membrane surface, and from the late endosome to the Golgi. Some differentiated cell types have additional pathways in addition to the general ones depicted in this figure, including specialized extremely rapid secretory and recycling pathways for synaptic vesicle proteins in presynaptic terminals.

and morphologically distinct from the stack cisternae in a number of ways. For example, the coat composition of buds derived from TGN (clathrin) differs from those in the Golgi stack (COPI). The TGN is also a location where specific proteins modifications occur, including tyrosine sulfation, proteolytic processing and sialylation [30].

Processing of proteins in the Golgi complex includes sorting and glycosylation of membrane proteins and secretory proteins. Many glycosidases and glycosyltransferases reside within the Golgi apparatus. The great diversity of N-linked oligosaccharide structures on mature glycoproteins results from both the trimming of the original precursor oligosaccharide originally added to proteins in the ER and the addition of further sugars (Fig. 9-3). The result is the formation of complex oligosaccharides and high-mannose oligosaccharides. These two differ in that high-mannose oligosaccharides have no new sugars added to them. High-mannose oligosaccharides contain two *N*-acetylglucosamines and many mannose residues, usually the original number added in the ER. Complex oligosaccharides can contain multiple *N*-acetylglucosamines and a variable number of galactose and sialic acid residues. The formation of both complex and high-mannose oligosaccharides depends largely on their accessibility by processing enzymes in the Golgi [31].

In the Golgi, another mode of protein glycosylation is the O-linked glycosylation. In this process, sugars are added to the OH groups of selected serine or threonine side chains by glycosyl transferase enzymes Typically, *N*-acetyl galactosamine is added first, followed by a variable amount of additional sugar residues, sometimes up to ten.

Finally, the Golgi apparatus is an organelle with an important role in lipid biosynthesis [32]. The enzyme sphingomyelin synthase catalyzes the production of both sphingomyelin and diacylglycerol from the products phosphatidylcholine and ceramide, previously generated in the ER (see Ch. 3). In addition, more complex glycosphingophospholipids are generated in the Golgi.

As these sphingolipids associate with ER-derived cholesterol, they are segregated from unsaturated glycerolipids in the lipid bilayer. These results in a *cis*- to *trans*-Golgi sphingolipid/cholesterol gradient, which translates in increased bilayer thickness. This membrane thickening serves for the selective retention of some Golgi enzymes at the TGN. Golgi-derived vesicles are richer in cholesterol, which causes their lipid bilayer to be thicker than that of the Golgi membrane itself. Thus, only proteins with transmembrane regions long enough to span this thickness can enter transport vesicles at the TGN. This mechanism is consistent with the fact that transmembrane domains of Golgi proteins are approximately five to six amino acids shorter than plasma membrane proteins [33].

The function of protein glycosylation is unknown for most proteins. However, some functional consequences had been established for the glycosylation of certain proteins [34]. In some cases, glycosylation appears to help in the folding process in the ER or to serve a role in protein sorting. For example, carbohydrate binding proteins known as lectins help in ER to Golgi transport, as well as in the sorting process occurring in the TGN [35]. In some cases, specific glycosylation patterns may serve to target a protein to a particular destination, such as the lysosome (see below). Alternatively, glycosylation can influence the stability and or turnover of a protein. Because sugar chains are not very flexible, they tend to protrude from the surface or glycoproteins, potentially limiting the approach of other molecules. For example, the high level of glycosylation on lysosomal membrane proteins makes them resistant to the action of hydrolases.

Glycosylation also plays an important role in cell–cell adhesion (see Ch. 7). Such is the case of glycosylated cell-surface proteins that are recognized by specific lectins known as selectins. The high degree of glycosylation on proteoglycan core proteins also is important on the cell surface. Many proteoglycans are secreted to become components of the extracellular matrix, and others remain attached to the plasma membrane [36].

Proteins and lipids move through Golgi cisternae from the *cis* to the *trans* direction. The addition of sugars to proteins occurs in an organized sequence in the Golgi [37] (Fig. 9-4). Each cisterna is characterized by the abundance of specific processing enzymes. As proteins travel through different cisternae, they are modified successively. In addition, each oligosaccharide-processing enzyme can only accept a glycoprotein as a substrate once a preceding enzyme has processed this protein. Therefore, the processing appears to be ordered both spatially and biochemically. Enzymes catalyzing early steps are concentrated in the *cis* face of the Golgi, with the ones catalyzing downstream processing steps being localized closer to the *trans* face. This localization of specific processing steps in the Golgi apparatus and other organelles in the biosynthetic pathway is shown in Table 9-1. Finally, the lumen of the *trans* compartment appears to be continuous with the TGN, a station where sorting of proteins occurs to target plasma membrane, lysosomes or secretory vesicles.

How the Golgi apparatus maintains its polarized structure while molecules move from one compartment to another is still a matter of debate. Two models were originally proposed based on different experimental evidence (Fig. 9-5): the vesicular transport model and the cisternal maturation model. A third model known as the dual transport model combines elements from both vesicular transport and cisternal maturation models and can better explain intra-Golgi transport.

In the vesicular transport model, individual cisternae represent static structures [38]. Cargo is transported through the Golgi in the form of transport vesicles, which bud from one cisterna and fuse with the next. The vesicular transport model is consistent with the

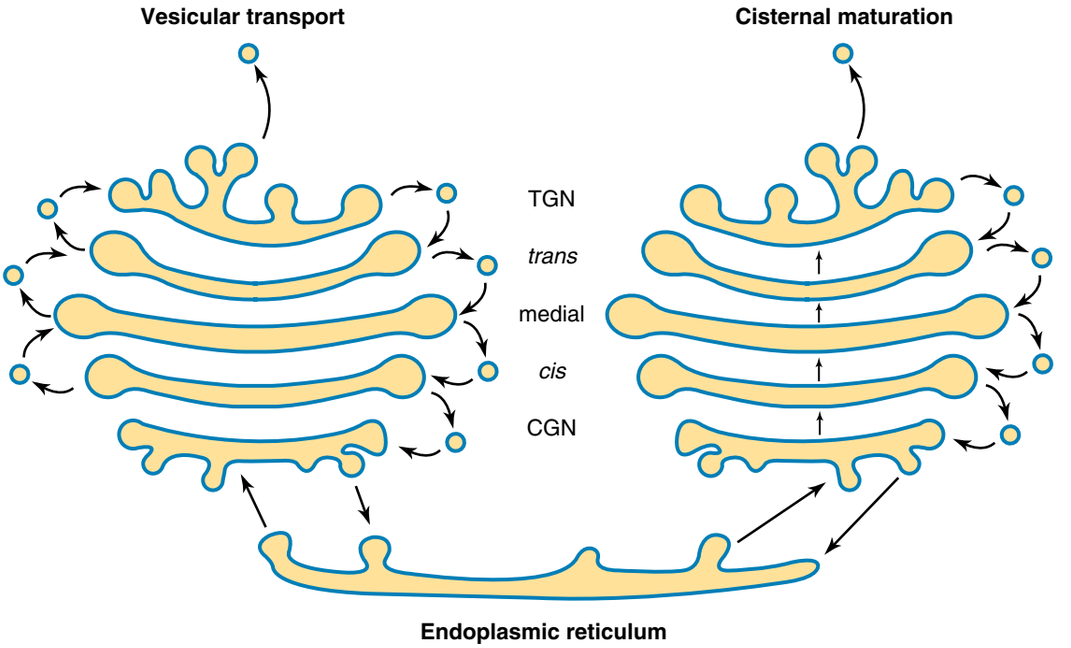

FIGURE 9-5 Two models proposed for intra-Golgi transport. In the vesicular transport model (**left**), cisternae are static, and each cisterna has a unique resident protein composition. Transport vesicles moving forward in the pathway provide the basis for the movement of molecules among cisternae throughout the Golgi. Retrieved proteins are selectively packed and returned in retrograde-moving ones. In the cisternal maturation model (**right**), an individual cisterna matures as it moves forward from the *cis* to the *trans* position in the Golgi. COPI-coated transport vesicles move resident proteins to the preceding cisterna, providing the means for specific localization of processing enzymes within the Golgi apparatus. Notice the absence of forward moving transport vesicles in the cisternal maturation model. An alternative, dual transport model (not depicted here), combines elements of both models and it is likely to represent intra-Golgi transport more accurately.

differential distribution of processing enzymes in each Golgi compartment. Forward moving proteins are selectively packed into forward-moving vesicles, while retrieved proteins are selectively packed and returned in retrograde-moving ones. Alternatively, vesicles move randomly and the flow occurs because of the continuous, specific input at the *cis* cisterna and the corresponding output at the *trans* cisterna. Experimental evidence suggested these cargo-carrying vesicles are COPI-coated vesicles. Movement of vesicles from one cisterna to another is helped by filamentous proteins, which restrict their movement and facilitate their fusion. One problem with this model is that insufficient fusion markers have been identified that would label each compartment. Such markers would be necessary for directional transport among different cisternae in the Golgi. In addition, COPI-coated vesicles appear to be the only type of transport vesicle observed within the Golgi, and they move only retrogradely in the secretory pathway [39]. This evidence is difficult to reconcile with the anterograde movement of vesicles predicted by the vesicular transport model.

These and other inconsistencies fueled the proposal of an alternative model known as the cisternal maturation/progression model [40]. In the cisternal maturation model, cargo is transported in cisternae as they progress through the stacks from *cis* to *trans*-Golgi. Vesicular tubular clusters from the ER fuse to form the *cis* face of the Golgi stack. New *cis* cisternae continuously form and migrate through the stacks as they mature. Although everything moves forward, including enzymes found mainly in early Golgi sacks, a specific distribution of processing enzymes is achieved by a continuous COPI-dependent vesicle retrograde flow [41]. Newly formed cisternae receive their complement of proteins from the one immediately ahead, and later pass them backward to still newer cisternae at the next *cis* position. Documentation of *de novo cis*-Golgi formation, as well as the movement of large molecular structures such as procollagen, which would not fit in a transport vesicle, strongly supported this model [42]. However, a major difficulty faced by the cisternal progression/maturation model is to explain the origin of the TGN, an apparent steady-state compartment that contains a unique set of resident proteins and behaves differently from Golgi stacks in many respects.

The dual-transport model combines aspects of the previous two models [43]. Molecules so large that they would not fit in transport vesicles would move inside maturing cisternae. Bidirectional transport vesicles, on the other hand, would move proteins through the Golgi

more rapidly. Molecular tethers would limit the movement of vesicles, promoting the transfer of cargo between adjacent cisternae, without the requirement for multiple sets of specific SNARE complexes. Regardless of the model proposed, proteins all complete their journey through the Golgi at the TGN, where they are sorted and packaged into specific transport vesicles that target them to specific cell domains and organelles.

Plasma membrane proteins are sorted to their final destinations at the *trans*-Golgi network. Soluble and plasma membrane proteins are delivered to axonal, dendritic or axo-dendritic plasma membrane domains by yet unidentified mechanisms. Axonal and dendritic domains appear to be analogous to the apical and basolateral plasma membrane domain of epithelial cells [44]. The selective enrichment of membrane proteins can be achieved by means of selective protein transport delivery, selective endocytosis and specific retention. Certain proteins appear to be sent directly to each plasma membrane domain by a mechanism involving selective packaging of cargoes at the TGN and attachment to specific molecular motors [44, 45]. Certain apical and basolateral proteins have been observed to leave the TGN in different membrane carriers, and sorting signals have been identified in some protein primary sequences that can direct them to apical or basolateral surfaces of epithelial cells. *In vitro* experiments first suggested that constitutive secretion was carried out entirely in the form of small vesicles. However, more recent imaging experiments using fluorescently tagged proteins in living cells revealed a much more heterogeneous repertoire of membranes moving from the TGN towards the plasma membrane, including large tubulovesicular structures [46].

However, not all proteins proceed directly to their eventual destination. Some proteins relocate from one plasma membrane compartment to another by means of transcytosis. Transcytosis involves endocytosis of selected proteins in one membrane compartment, followed by subsequent transport through early endosomes to recycling endosomes and finally translocation to a different membrane compartment, for example from the apical to the basolateral surfaces. Sorting at the TGN and endosome recycling steps appear to have a primary role in the steady state distribution of proteins in different plasma membrane domains [47]. However, selective retention of proteins at the plasma membrane by scaffolding proteins or selective removal may also contribute to normal distributions. Finally, microtubule-motor regulatory mechanisms have been discovered that might explain the specific delivery of membrane proteins to discrete plasma membrane domains [48].

Lysosomal proteins are also sorted and targeted in the *trans*-Golgi network. Lysosomes are morphologically heterogeneous organelles whose major function is the degradation and digestion of macromolecules (see also Lysosomal Diseases in Ch. 41). They are found in all eukaryotic cells and the lysosomal membrane has a unique composition. ATP-dependent H^+ pumps maintain the low pH of the lysosomal lumen, and transporter proteins move sugars, amino acids and nucleotides resulting from digestion of macromolecules from the lysosomal lumen to the cytosol, where they can be either excreted or reused. The luminal side of this membrane compartment is filled with acid hydrolases, enzymes whose optimal pH is acid to 5.0. The near-neutral pH in cytosol provides a poor environment for acid hydrolases and serves to protect cytosolic components from the activity of any hydrolases that might leak from lysosomes. Nearly 40 different types of lysosomal hydrolase have been described. These include glycosidases, lipases, nucleases, phospholipases and phosphatases. Genetic defects affecting the function of lysosomal hydrolases can lead to human disease, as they usually result in the accumulation of undigested material in lysosomes, often with severe pathological consequences [49]. Examples of these include Hurler's, Niemann–Pick and Tay–Sachs diseases (see Ch. 40).

Several intracellular trafficking pathways converge at lysosomes. Hydrolases and lysosomal membrane proteins are delivered to lysosomes from the TGN (via endosomes), whereas substances destined for digestion are provided to lysosomes by three different pathways: endocytosis, autophagy and phagocytosis (see below). Lysosomal membrane proteins and hydrolases are synthesized first in the rough ER and later transported to the TGN. In the TGN, they are packed in transport vesicles, which are delivered to late endosomes first, and subsequently to lysosomes [50]. Specific targeting mechanisms ensure that these vesicles transport lysosomal proteins while excluding others. In the case of lysosomal hydrolases, this mechanism is based on the selective recognition of a mannose 6-phosphate (M6P) tag, a unique marker for proteins destined to lysosomes [51]. M6P is added specifically to the N-linked oligosaccharides of soluble lysosomal enzymes as they pass through the lumen of the TGN. The signal to add M6P specifically to a glycoprotein is the presence of a motif in its primary sequence known as a signal dispatch. The sequential action of two enzymes then catalyzes the addition of M6P to lysosomal hydrolases. The first is GlcNAc phosphotransferase, which binds the hydrolase and adds GlcNAc-phosphate to one or two mannose residues on each oligosaccharide chain. The second enzyme removes the GlcNAc residue, leaving a M6P behind. Most hydrolases contain multiple oligosaccharides, thus finishing with several M6P. This appears to facilitate the high-affinity signal for the M6P receptor. A M6P receptor in the TGN recognizes and binds to M6P on the luminal side and to adaptins on the cytosolic side of assembling clathrin-coated pits in the Golgi [52]. This allows selective packaging of lysosomal directed

proteins in the TGN for transport to a late endosome in the form of clathrin-coated vesicles.

The retrieval mechanism for the M6P receptor resembles the one previously described for the H/KDEL receptor [53]. Optimal binding of M6P receptor to M6P occurs at pH 6.5–6.7, the pH found in the TGN. When transport vesicles arrive at late endosomes, the pH is lowered by the action of H^+ pumps. The affinity of the M6P receptor for its ligands is reduced at acid pHs, resulting in M6P receptor releasing the M6P in the late endosome. As a result, transport of lysosomal hydrolases occurs unidirectionally. Once the M6P receptor releases M6P-bearing hydrolases, the receptor can be returned to the TGN for reuse. Transport of the M6P receptor to either TGN or late endosome relies on signal peptides on the cytoplasmic tail region of the M6P receptor.

Under some circumstances, lysosomal hydrolases may fail to be properly packaged in the TGN, so they enter the default pathway to the cell surface, where they are secreted. Although these hydrolases do little harm at the nearly neutral pH of most extracellular fluids, they can also be returned to lysosomes by a pathway known as receptor-mediated endocytosis. In this pathway, M6P receptors are sent to the plasma membrane, where they bind escaped lysosomal hydrolases and bring them back to lysosomes through the early and late endosomes. Receptor-mediated endocytosis is a major component of the endocytic pathways for trafficking of membrane proteins and merit more detailed consideration.

Both constitutive and regulated neuroendocrine secretion pathways exist in cells of the nervous system. Vesicular proteins and lipids that are destined for the plasma membrane leave the TGN sorting station continuously. Incorporation into the plasma membrane is typically targeted to a particular membrane domain (dendrite, axon, presynaptic, postsynaptic membrane, etc.) but may or may not be triggered by extracellular stimuli. Exocytosis is the eukaryotic cellular process defined as the fusion of the vesicular membrane with the plasma membrane, leading to continuity between the intravesicular space and the extracellular space. Exocytosis carries out two main functions: it provides membrane proteins and lipids from the vesicle membrane to the plasma membrane and releases the soluble contents of the lumen (proteins, peptides, etc.) to the extracellular milieu. Historically, exocytosis has been subdivided into constitutive and regulated (Fig. 9-6), where release of classical neurotransmitters at the synaptic terminal is a special case of regulated secretion [54].

All eukaryotic cells possess an unspecialized exocytic pathway known as the constitutive secretion. Vesicle membranes in this pathway fuse with the plasma membrane without any extracellular signal. As noted above, proteins destined for the secretory pathway are sorted at the level of the TGN. Proteins to be transported to the plasma membrane are directed into a constitutive secretory pathway.

THE ENDOCYTIC PATHWAY PLAYS MULTIPLE ROLES IN CELLS OF THE NERVOUS SYSTEM

Most pathways in the endocytic system are shared with cells in general, but a special case exists in synaptic vesicle cycling, which is unique to neurons and a keystone in neuronal function. Three categories will be considered here: endocytic processes important for degradation of macromolecules and uptake of nutrients; constitutive and regulated neuroendocrine secretion; and receptor-mediated endocytosis. Synaptic vesicle cycling will then be considered separately and in greater detail.

Endocytosis for degradation of macromolecules and uptake of nutrients involves phagocytosis, pinocytosis and autophagy. The cellular processes by which macromolecules, particulate substances and even other cells may be taken up in a regulated fashion are an important subset of endocytic pathways [55]. These processes may also be important for invasion of the nervous system by viral vectors. There are three general mechanisms for uptake: phagocytosis for engulfing macromolecules, particles and cells or viruses; autophagy for degradation of intracellular organelles and aggregates; and pinocytosis for uptake of smaller molecules and fluids.

Phagocytosis involves the uptake of large materials such as microorganisms or dead cells via large vesicles named phagosomes. The material to be internalized is wrapped up by a special portion of the plasma membrane that invaginates first and then after a regulated molecular mechanism event pinches off forming an endocytic vesicle carrying the phagocytosed material. The usual vesicle diameter size is >200 nm. Phagocytosis of large particles was developed early in the evolution; unicellular organisms use this type of endocytosis as a way to get nutrients and the phenomenon is most familiar in macrophages. Phagocytosed particles via phagosomes fuse with lysosomes; after a digestion process the metabolized products are released into the cytosol to be consumed as nutrients by the organism.

In multicellular organisms, phagocytosis has been developed as a defensive mechanism rather than for feeding purpose and is largely carried out by specialized cells in mammalian tissues. Three different white blood cells commonly exhibit phagocytosis in mammals: macrophages, neutrophils and dendritic cells. These different cell types posses a unique and complex function; they protect us from infections by phagocytosing the invading agents and they also take care of dead or senescent cells throughout the organism. In the nervous system, phagocytosis is normally conducted only by a specific type of glial cell: microglia, which are a specialized type of macrophage [56, 57]. The function of these cells in the normal healthy brain is not very well known, but when these cells detect brain damage they proliferate, migrate to the site of

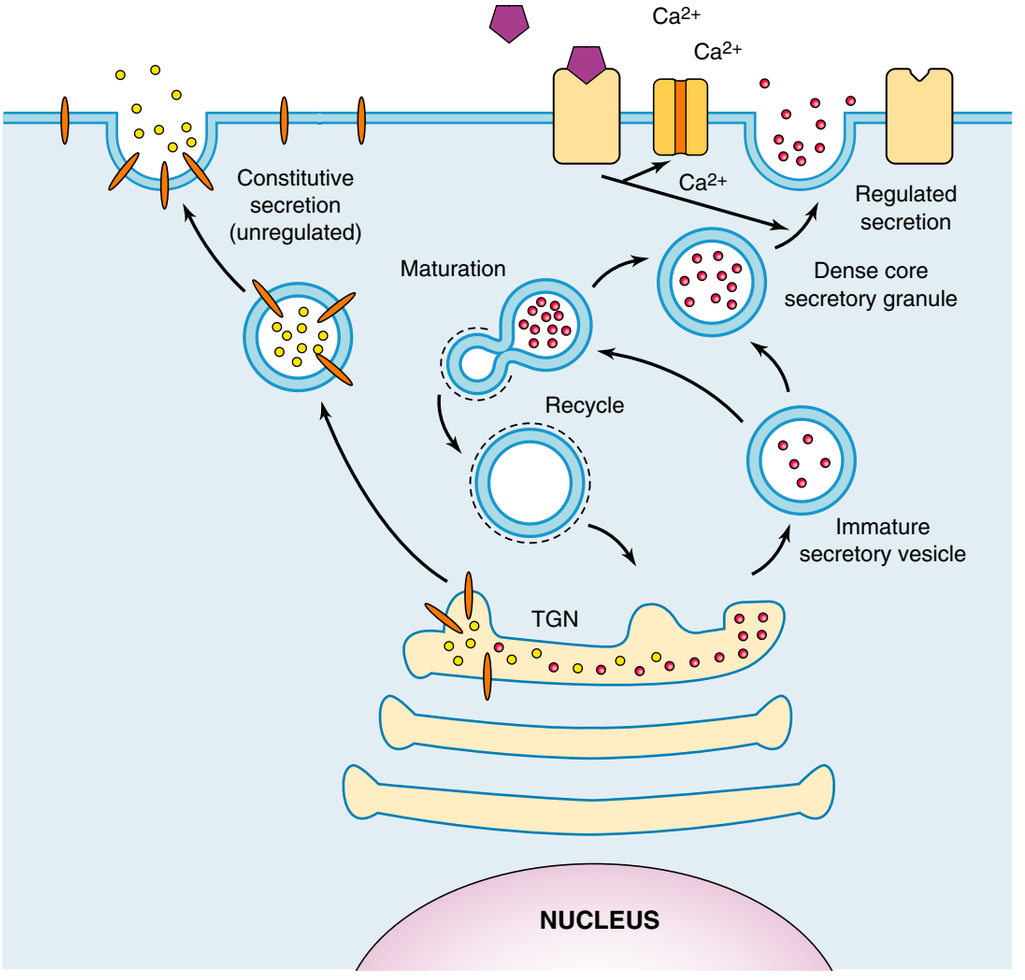

FIGURE 9-6 The constitutive and regulated secretory pathway. Eukaryotic cells determine the fate of newly synthesized proteins and lipids destined to the plasma membrane at the TGN. Eukaryotic cells developed two different exocytic pathways, one called constitutive secretion, which is an unregulated or default pathway, and a second one, which is triggered by extracellular signals, known as regulated secretory pathway because it is tightly coupled to extracellular stimuli. Lipids, secreted proteins and integral membrane proteins lacking a particular sorting signal are packaged into a common type of vesicles in the *trans*-Golgi network (TGN). Once the transport vesicle buds off, it is steadily delivered to a common domain within the plasma membrane via the constitutive secretory pathway. This constitutive pathway typically provides the cellular plasma membrane with newly synthesized lipids and integral membrane proteins (*blue*). In addition, some secreted proteins like extracellular matrix materials may be released constitutively as they are synthesized (*yellow*). Specialized secretory cells such as neurons and endocrine cells to a great extent developed more selective exocytic pathways known collectively as the regulated secretory pathway. These pathways form the basis for interneuronal communication. In this pathway the exocytic event is triggered by a specific extracellular signal, allowing for a tight regulation of secretion. The regulated secretory pathway is mainly responsible for the release of specific cellular products such as hormones, peptides and neurotransmitters. The peptides, hormones and other targeted molecules (*red*) are also sorted and concentrated in the TGN and packaged into immature secretory vesicles with a distinctive polypeptide composition. After the vesicle buds off of the TGN, they undergo a maturation process that is needed to concentrate them further and create a secretion-competent secretory granule. The secretory granules that contain protein or peptides often exhibit a dense core in electron micrographs and are called dense core secretory granules or vesicles. This maturation process often includes a series of steps that require formation of clathrin coats and recycling of membrane and membrane components to the TGN. Release of secretory granule contents requires binding of a ligand (*dark orange pentagon*) to a membrane receptor (*light orange*), which triggers movement of secretory granules closer to the plasma membrane and an influx of Ca^{2+} through a specific channel (*dark orange*) that is required for membrane fusion and release. A specialized variant of these pathways, which is extremely rapid, is found in presynaptic terminals.

injury or disease, turn on their macrophage capabilities and engulf pathogens and cellular debris (see Ch. 1). In addition, vascular macrophages and related cells may on occasion be seen in nervous tissue, particularly in response to disease or damage.

Phagocytosis is a tightly regulated process that is initiated by a specific signal at the plasma membrane that is transmitted to the cell interior, which in turn initiates and regulates the phagocytosis process [55]. There are different signaling initiators to triggers phagocytosis; the best characterized are antibodies. Antibodies bind specifically to the surface of the invader organism leaving the fc region of the antibodies exposed to the exterior. These antibody fc regions are recognized by specific surface receptors on the macrophages and neutrophils, which turn on the phagocytosis machinery. Negative charges on the cell surface of dead cells or cell debris can also trigger phagocytosis. There are also inhibitory signals displayed on the surface of living cells that prevent the activation of phagocytosis pathway. One practical use of phagocytosis is for tracing of neuronal pathways after labeling with specific markers like Fluorogold or horseradish peroxidase.

Pinocytosis involves the internalization of liquids and solutes via small pinocytic vesicles with around 40–100 nm in diameter size [55]. Unlike phagocytosis, pinocytosis is a constitutive process in almost all eukaryotic cells. Cells need to internalize fluids and solutes via small pinocytic vesicles, usually clathrin-coated vesicles around 40 nm in diameter. Many of these small membrane vesicles later return to the plasma membrane by exocytosis in non-growing or stationary cells after passage through a sorting endosome. Cells must keep endocytosis and exocytosis processes tightly balanced, otherwise within time the cell would run out of plasma membrane. Under normal circumstances, the volume and area of the cell in a stationary phase does not change during the endocytic–exocytic events even when rates of exocytosis become quite high, as in the presynaptic terminal. The two linked processes should then be viewed as a continuous endocytic–exocytic cellular cycle. The rate and extent of membrane internalization by pinocytosis varies among cell types. Some are extremely large such as secretory cells and macrophages, which may internalize as much as a quarter of its own volume in one hour. Some unicellular organisms can endocytose their entire plasma membrane even faster, in less than an hour.

Caveolae represent membrane invaginations of cholesterol- and sphingolipid-rich membrane domain. They are thought to mediate the transport of serum proteins into endothelial cells for transcellular transfer across the endothelium and may play a role in signaling pathways including nitrous oxide [55]. The major protein described in association with caveolae is caveolin, although its functions are not fully understood. Curiously, caveolin-null mice are phenotypically normal although subtle changes in specific cell types can be seen. Caveolae are particularly abundant on endothelial cells but can be detected on most cell types. They are not very abundant in neurons but may be important in NO signaling from endothelial cells of the brain.

The bulk of pinocytosis in the nervous system is mediated by clathrin-mediated endocytosis (CME) [55] and this is the best-characterized pathway. More detail about clathrin-mediated pathways will be given when receptor-mediated endocytosis and the synaptic vesicle cycle pathways are considered. Pinocytosis through CME is responsible for uptake of essential nutrients such as cholesterol bound to low density lipoprotein (LDL) and transferring, but also plays a role in regulating the levels of membrane pumps and channels in neurons. Finally, CME is critical for normal synaptic vesicle recycling.

Other pinocytotic pathways also exist that do not depend on either caveolae or clathrin, although these are not as well defined [55]. Specific receptors continue to be internalized in the absence of clathrin or caveolin and these pathways can be monitored by following glycosyl phosphatidylinositol (GPI)-anchored proteins. Nonclathrin, noncaveolin pathways may also be responsible for the reuptake of membrane in neuroendocrine cells after stimulated secretion. Some, but not all, of these pathways appear to require dynamin.

Autophagy involves the disposal of the obsolete part of the cells such as mitochondria, or SER. Three categories of autophagy have been described in cells [58] and all can be seen in the nervous system. Macroautophagy is typically accomplished by envelopment of organelles with an intracellular membrane known as an isolating membrane to form a double membrane structure known as an autophagosome [59]. The engulfment isolates the organelle from the cytoplasm and the compartment may then be acidified. Eventually, the autophagosome fuses or is engulfed by a lysosome for degradation. Macroautophagy is induced by stress and may be seen following nerve or axonal damage [58]. For example, resorption of myelin by Schwann cells in peripheral demyelination or wallerian degeneration is a dramatic example of macroautophagy.

Microautophagy is a constitutive pathway that is normally responsible for turnover of organelles [58]. Unlike macroautophagy, there is no initial isolating membrane. Instead, the organelle fuses or is directly engulfed by the lysosome and degraded. This is an important pathway for recycling amino acids and other biological building blocks. Microautophagy may also be important for degradation of protein aggregates associated with neurodegeneration. This is the only pathway available for degradation of insoluble aggregates and may be neuroprotective. Autophagic structures may be prominent in Parkinson's and Huntington's disease and amyotrophic lateral sclerosis, but the extent to which this is a neuroprotective pathway is unclear [60]. Indeed, a combination of micro- and macroautophagy plays a key role in type II/nonapoptotic programmed cell death [58, 59]. In type II programmed cell death, organelles are isolated and degraded, but the cytoskeleton is spared. Type II programmed cell death is

seen in many developmental programmed cell death events.

Chaperone-mediated autophagy is a mechanism for importing cytoplasmic proteins into lysosomes for degradation [58]. This pathway has only been documented in mammalian cells to date and its cellular functions are not well defined. This process is analogous in some respects to the ubiquitin/proteasome pathway. Proteins targeted for degradation are tagged with a particular KFERQ peptide motif, which is recognized by a chaperone. The chaperone–protein complex binds to a specific lysosomal membrane receptor for import into the lysosome and degradation. Autophagic profiles are common features of the aging brain and include some of the characteristic histological features of the aging brain (lipofuscin, ceroid, multivesicular bodies, etc.) [61]. Neuronal accumulations of autophagic profiles become even more prominent in diseases of the aging brain such as Alzheimer's and Parkinson's disease.

The constitutive pathway is also known as the default pathway because it does not require any type of signal to enter. However, proteins in this pathway are sorted into packages in which the constituents have a common destination. For example, molecular components of the active zone in the presynaptic membrane appear to be transported as a prepackaged dense core vesicle [62]. Thus, active zone scaffold proteins such as piccolo, bassoon and rim are present in the same transport vesicle, but synaptic vesicle proteins such as synaptophysin and synaptobrevin are not present. Components of other functional microdomains in the neuronal plasma membrane such as nodes of Ranvier and the postsynaptic densities may be similarly packaged into discrete vesicles with unique destinations. This is likely to be a general feature of cells with specialized functions and distinct domains, including neurons and glia in the nervous system. However, the full complement of proteins needed in a particular membrane domain may not be packaged together in the TGN and local assembly of functional complexes such as ion channels may still be necessary.

The constitutive pathway has not been studied as intensively as regulated secretion [54]. In particular, relatively little is known about targeting and regulatory mechanisms for these transport vesicles. Clathrin seems not to be directly involved in the constitutive secretory pathway. Antibodies that disrupt clathrin assembly *in vitro* inhibit endocytosis, but constitutive exocytosis is not affected [63].

Secretory cells, including neurons, also possess a specialized regulated secretory pathway. Vesicles in this pathway have soluble proteins, peptides or neurotransmitters stored and concentrated within secretory vesicles. At that point, these vesicles are actively transported to a site for extracellular delivery in response to a specific extracellular signal. Exocytosis through regulated secretion accomplishes different functions, including the release of hormones, peptides and neurotransmitters. Exocytosis through the regulated secretory pathway is the primary basis for intercellular communication in the nervous system.

At least two classes of regulated secretion can be defined [54]. The standard regulated secretion pathway is common to all secretory cells (i.e. adrenal chromaffin cells, pancreatic beta cells, etc.) and works on a time scale of minutes or even longer in terms of both secretory response to a stimulus and reuptake of membranes after secretion. The second, much faster, neuron-specific form of regulated secretion is release of neurotransmitters at the synapse. Release of neurotransmitters may occur within fractions of a second after a stimulus and reuptake is on the order of seconds. Indeed, synaptic vesicles may be recycled and ready for another round of neurotransmitter release within 1–2 minutes [64]. These two classes of regulated secretion will be discussed separately after a consideration of secretory vesicle biogenesis.

Our information about protein secretion was developed through studies with a variety of secretory cells over more than 30 years. Secretion is a multistep process that involves vesicle biogenesis, cargo loading, concentration and processing, vesicle transport and targeting, vesicle docking and Ca^{2+}-dependent vesicular fusion with the plasma membrane. In response to physiological demands, cells synthesize, concentrate and store secretory products into membrane-bounded organelles called variously secretory vesicles, secretory granules or dense core vesicles (named after dense core or electron opaque content in electron micrographs).

Regulated secretion is easily recognized and distinguished from constitutive secretion. First, regulated secretion is linked to an extracellular stimulus. Regulated exocytosis via vesicle membrane fusion is triggered by different stimuli, which in turn results in a transient local rise in intracellular Ca^{+2} that leads to fusion of the vesicle membrane with the plasma membrane and concomitant release of vesicle contents to the extracellular space. Second, the secretory products are concentrated into specialized vesicles that are morphologically distinct and abundant in secretory cells. Finally, the secretory vesicles are transported to cellular locations near release sites and significant numbers are subsequently stored in the cytoplasm as a ready releasable pool for further utilization. This means that secretory cells synthesize, package and store a few specific secretory products that are not released in significant amounts until a specific physiological stimulation is received.

Secretory vesicle biogenesis requires completion of a characteristic sequence of steps before they are competent for secretion. Secretory cells typically invest a substantial fraction of their biosynthetic capacity for generation of secretory vesicles and their secreted products. Neurons devote a particularly high fraction of their biosynthetic activity to the synthesis and assembly of secretory organelles. In secretory cells, the bulk of newly synthesized

membrane and membrane-associated proteins may be devoted to generation of the secretory vesicles, sometimes referred to as secretory or dense core granules. The extensive Nissl substance (mostly RER) and large Golgi complexes seen in neurons reflect this biosynthetic activity and both structures were first described in neurons.

Neurons have multiple types of secretory vesicles whose secretion and biogenesis differ. The typical dense core vesicles (75–95 nm), which deliver protein and neuropeptides to the extracellular space by the classic regulated secretory pathway; and more numerous smaller vesicles (40–50 nm), known as synaptic vesicles specialized in the store and delivery of small neurotransmitters such as acetylcholine, glutamate, glycine, γ amino butyric acid (GABA) and monoamines. Most small, classical synaptic vesicles are electron-lucent, but those containing noradrenalin may also have a dense core in electron micrographs. In contrast, secretory granules in endocrine or neuroendocrine cells may be significantly larger, as much as 165 nm in diameter [65].

The specific molecular signals that drive the packaging and aggregation of secreted proteins or their characteristic integral membrane proteins into distinct secretory vesicles within the TGN are currently unknown. Standard secretory vesicles emerge as immature vesicles from the TGN of the Golgi complex. The size of these immature secretory vesicles is heterogeneous but all have a dense core. This core of aggregated proteins is separated from the vesicle membrane by a space filled with undetermined material, forming a halo-like structure. As secretory vesicles mature, they lose volume and surface area and their contents become concentrated as the result of coordinated retrieval of membrane that is recycled back to the late endosomes and TGN, and the internal vesicle acidification as a consequence of progressive concentration of protons by pumps in the vesicular membrane.

Biogenesis of synaptic vesicles in neurons differs in some important respects from generation of secretory granules [66]. Most vesicles being transported from the neuronal cell body down the axon are actually tubulovesicular structures, 50 nm in diameter and with variable lengths [67] rather than the typical 50 nm synaptic vesicle profiles. Moreover, evidence suggests that not all synaptic vesicle components are transported in the same vesicle [68]. Thus, synaptic vesicle precursors may not be competent to serve as synaptic vesicles. The evidence now suggests that some or all synaptic vesicles are locally assembled in presynaptic terminal regions following an intermediate exocytosis and endocytosis event (see below). Subsequently, they are reconstituted as complete synaptic vesicles in the early endosome compartment. Consistent with this, synaptic vesicles are typically recycled many times and locally filled with neurotransmitter for reuse within minutes, unlike neuropeptide secretory granules, which can be used only once for secretion [64]. However, neurotransmitter vesicles containing noradrenalin are filled with transmitter during transport, leading to their characteristic dense core in electron micrographs. As a result, there may be some heterogeneity in synaptic vesicle biogenesis.

Once secretory vesicles are loaded with their cargo, they pinch off from the TGN and are actively transported to the specific subcellular site, in which they fuse and release the cargo. These sites may be quite far from the site of packaging and processing in the Golgi complex. For example, neurons transport secretory vesicles carrying neuropeptides from the cell body (where peptides are synthesized and packaged into secretory vesicles) down the axon to the nerve terminals, which in some neurons can be a meter or more away. Secretory vesicles are transported to sites of release through the action of microtubule-based motor proteins. In neurons, these transport processes are collectively known as axonal transport (see Ch. 28). The fates of different secretory vesicles once they reach the plasma membrane may vary. Ones carrying cargo to be constitutively secreted will fuse with the plasma membrane once they arrive at their destination. In contrast, those carrying material to be secreted via regulated exocytosis pathways remain near the plasma membrane without fusing until a signal arrives that triggers vesicle fusion with the plasma membrane.

As secretory vesicles mature, many secretory polypeptides undergo post-translational modifications. Many hormones and neuropeptides as well as hydrolytic enzymes are synthesized as inactive polypeptide precursors that need to undergo proteolysis to become active. This maturation process usually starts in the TGN and continues in the secretory vesicles, but may be completed in the extracellular space soon after exocytosis takes place in some cases. The maturation process for neuropeptides is described in Chapter 18.

Retrieval of membrane components in the secretory pathway through receptor-mediated endocytosis (RME) is a clathrin-coat-dependent process [5]. The clathrin coat provides stability to the vesicle core and allows uptake of specific membrane proteins for reuse or degradation. RME shows a remarkable degree of specificity, allowing cells to internalize with astonishing efficiency only those selected molecules independent of their extracellular concentration.

Extracellular ligands (hormones, neurotrophins, carrier protein, adhesion molecules, small molecules, etc.) will bind to specific transmembrane receptors. This binding of specific ligand induces the concentration of the receptor in coated pits and internalization via clathrin-coated vesicles. One of the best studied and characterized examples of RME is the internalization of cholesterol by mammalian cells [69]. In the nervous system, there are a plethora of different membrane receptors that bind extracellular molecules, including neurotrophins, hormones and other cell modulators, being the best studied examples. This type of clathrin-mediated endocytosis is an amazingly efficient process, capable of concentrating

receptors several hundredfold and taking up large quantities of specific molecules. As a result, even a very dilute extracellular ligand can be internalized without taking up a correspondingly large amount of extracellular fluid.

In the CNS, neurons are highly dependent on a continual supply of small polypeptide growth factors with neurotrophic activity (see Ch. 27). This process requires the binding of the secreted polypeptides to specific transmembrane receptors with an intrinsic tyrosine kinase activity on their cytoplasmic domains (Fig. 9-7). Upon binding of peptide growth factor, these receptor tyrosine kinases (RTKs) activate their intrinsic tyrosine kinase activity and induce RME [70]. The sequence of events typically follows a pattern of ligand binding, followed by RTK dimerization and activation of the kinase activity. Once activated, RTKs autophosphorylate on specific cytoplasmic tyrosines and phosphorylate other associated proteins, which recruit proteins containing Src-homology II (SH2) and phosphotyrosine-binding domains. Among these are linking proteins and adaptor proteins for assembly of a clathrin coat. The signaling induces internalization and continues as the RTKs are packaged and returned to the cell body [71].

Cells control the amount of receptors on their surface as well as the level of extracellular ligand by regulating rates of RME, balanced by insertion of new receptors. The internalization of activated receptors through clathrin-coated pits and their translocation to endosomes lowers the number of receptors in the plasma membrane, a process known as downregulation. Cells reduce or attenuate the receptor-mediated intracellular signaling via this downregulation. Alternatively, removing the extracellular ligands via RME will also attenuate intracellular signaling [72]. Thus, intracellular signaling and endocytosis are tightly linked, although many questions remain to be answered [71, 72].

Electron microscopic studies on cells stimulated simultaneously with different labeled ligands indicate that RME can concentrate up to a thousand of different transmembrane receptors in a single clathrin-coated pit. In the canonical model of clathrin-mediated endocytosis, these clathrin-coated pits of the plasma membrane bud off to form clathrin-coated vesicles. These coated vesicles rapidly lose their clathrin coats through the action of a chaperone from the Hsp70 family, an event that facilitates local fusion with early endosomes (EEs). The various endocytic vesicles fuse with the membrane of the same EEs soon after shedding their clathrin coats. However, the fate of various ligand-receptor complexes varies according to their physiological role and cellular demand.

In non-neuronal cells, electron microscopy studies reveal very complex endosomal compartments composed of a highly dynamic array of heterogeneous tubulovesicular-membrane structure extending from close vicinity to the plasma membrane to the cell interior, reaching the boundaries of the Golgi apparatus. Presynaptic terminals have similar endosomal systems, albeit less extensive [73, 74].

The endocytic pathway also contains functionally and physically discrete compartments [75]. EEs are slightly acidic (pH 6.0–6.8), which induces the dissociation of many, but not all, receptor–ligand complexes. Free receptors may then concentrate in the tubular portion of EEs, where they bud off to form recycling vesicles, which transport receptors that are directly or indirectly returned to the plasma membrane. Free ligands are kept in the vesicular portion of the EEs. Depending on the ligand and the cell, ligands that provide essential cellular components (transferrin, etc.) can be actively transported to the perinuclear region of the cell where they fuse with late endosomes (LEs) and the ligand is delivered to the appropriate compartment. Alternatively, once the ligand is transported to the perinuclear region of the cell and LE, the LE will fuse with lysosomes where ligands may be degraded by the lysosomal enzymes in an acidic environment (pH 5.0). Lysosomal enzymes are transported from the TGN to the LEs and lysosomes via the same type of clathrin-coated vesicles. Thus, clathrin-coated vesicles generate vesicular structures designed to fuse with endosomes independent of their origin, either the plasma membrane or the TGN.

EEs with their slightly reduced pH are sorting compartments responsible for the dissociation and sorting of the ligands to the LEs and lysosomes as well as for directing receptors back to the plasma membrane for reuse. So EEs act as the first sorting station in the endocytic pathway. EEs also play an important role in synaptic vesicle recycling (see below). LEs serve to concentrate and sort endocytosed material further, including for delivery to lysosomes where both endogenous and exogenous macromolecules are digested.

Ligand–receptor complexes that do not dissociate in the EEs have different fates. In some cells, they may be returned to the same plasma membrane compartment from which they originated, whereas in polarized cells such as endothelial cells or astrocytes they can be moved to a different plasma membrane domain, resulting in transcytosis. In other cases, the complexes go to LEs and lysosomes for degradation. In neurons, these vesicles may serve as signaling organelles that are transported from the EEs back to the cell body where they influence gene expression [71].

Details of the mechanisms by which endocytosed material moves from the early to the late and lysosomal compartment are still poorly understood. However, portions of the EEs tubulovesicular structures may be actively transported along microtubules towards the perinuclear region of the cell in both neurons and non-neuronal cells. These endosomes on the move may enclose invaginated membranes and also internally bud off vesicles. For that reason, these complex structures are called multivesicular bodies (MVBs) [76]. Material returning by retrograde axonal transport to the neuronal cell body includes many MVBs [67]. The eventual fate of these structures may vary. Some MVBs may fuse with LEs or they may fuse with each

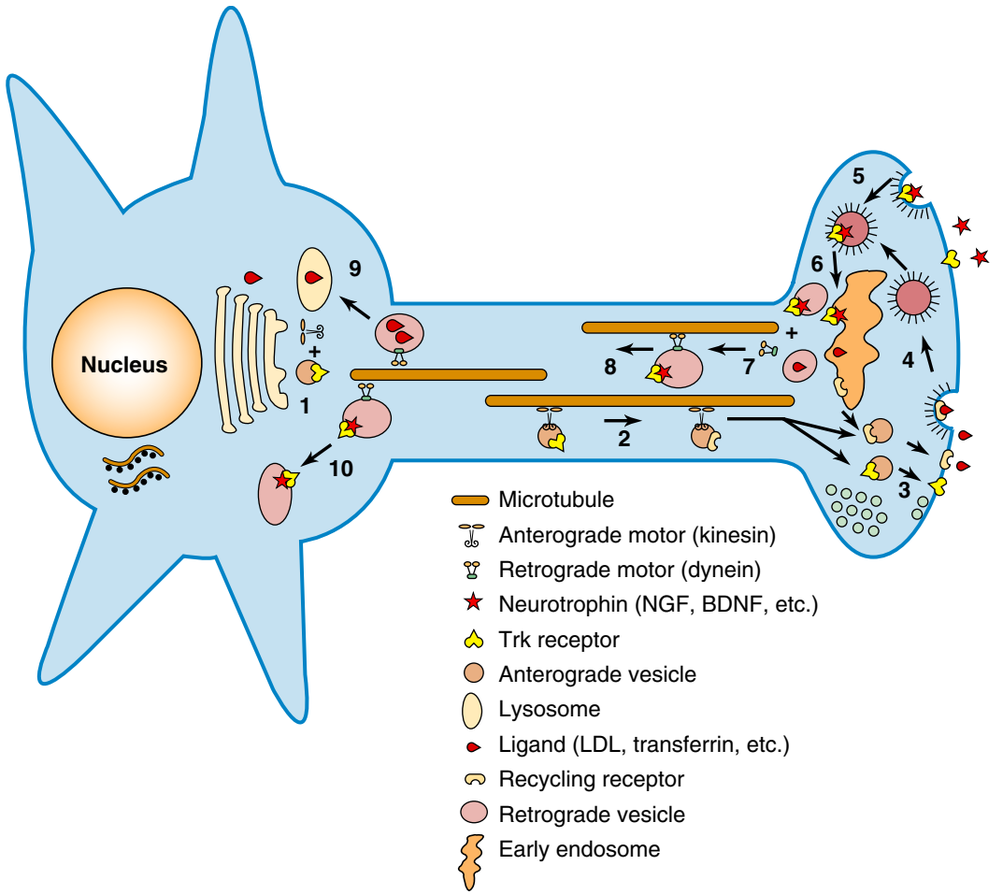

FIGURE 9-7 Receptor-mediated endocytosis. Many kinds of extracellular polypeptides and ligands (including hormones, carrier proteins, adhesion molecules, neurotrophins, etc.) are imported into the cell with a high degree of specificity via a special type of clathrin-mediated endocytosis. A high degree of molecular specificity is achieved through binding of ligands to specific receptors localized within discrete domains of the plasma membrane. The unique dependence on membrane receptors for the internalization of these different extracellular molecules led to the name of receptor-mediated endocytosis. Endocytosis through this pathway ensures the internalization of selected molecules independent of the extracellular concentration of the ligand, As a result, even very dilute extracellular ligands can be internalized without taking up a correspondingly large quantity of extracellular fluid. One of the best-studied receptor-mediated endocytosis pathways is the internalization of neurotrophins along with their specific receptor tyrosine kinase receptors (RTK). The internalization of the neurotrophins and growth factors is achieved through a well-defined sequence of events: *1*. Receptors are synthesized and packaged in the Golgi. Anterograde motors (kinesin) are added to the newly formed vesicles, then (*2*) the vesicles are transported along microtubules to the appropriate membrane domain, where (*3*) the receptors are inserted into the plasma membrane. This typically occurs through a form of targeted constitutive secretion. *4*. Binding of a suitable ligand to a typical recycling receptor like the LDL receptor or the transferrin receptor leads to formation of a coated pit and coated vesicle. The coat is removed and the interior of the endocytosed vesicle is acidified leading to dissociation of receptor and ligand, followed by fusion with an early endosome (EE). *5*. Alternatively, stimulation of cells with a neurotrophin or a growth factor results in the clustering of the growths factor-RTK into clathrin-coated pits. Ligand-RTK complexes are internalized by regulated clathrin-mediated endocytosis but the receptor ligand complex does not dissociate. Clathrin-coated vesicles carrying the growth factor-RTK complexes shed their clathrin coats soon after internalization, before being translocated and fused with EEs. *6*. In the EE, receptors are sorted. If ligands and receptors are dissociated by the slightly acidic pH in the EEs, the receptor is typically recycled back to the plasma membrane to participate in a new cycle of endocytosis. *7*. The ligands are then packaged into a vesicle for return to the cell body. However, neurotrophins remain bound to their RTKs and are sorted into a specialized retrograde endosome that may continue to signal. At this stage, the retrograde motor (dynein) is added to the vesicle. *8*. Retrograde vesicles containing ligands or growth factor-RTK complexes are actively transported returned by retrograde axonal transport (see Ch. 28) to the cell body where (*9*) ligand-containing vesicles and worn-out membrane proteins are fused with lysosomes for eventual degradation and recycling at the end of the journey. *10*. Vesicles containing neurotrophin–RTK complexes continue to signal for a period of time before degradation, leading to changes in gene expression.

other to form LEs. These LEs eventually become lysosomes, a process that requires the fusion of hydrolase-containing vesicles coming from the TGN and acidification of their lumens. In MVBs we can find internalized membrane proteins in their way to be degraded as well as others that have to be recycled and possibly some that are actively signaling organelles [76].

SYNAPTIC VESICLE TRAFFICKING IS A SPECIALIZED FORM OF REGULATED SECRETION AND RECYCLING OPTIMIZED FOR SPEED AND EFFICIENCY

Some neurons can fire several hundred times per second, secreting neurotransmitter each time. Synapses are remarkable secretion machines destined to undergo millions of repeated exocytic cycles in their lifetime. So how does this process work so fast and so efficiently? Answers have gradually emerged from the work of many different laboratories and model systems [73, 74]. An exhaustive description is beyond the scope of this chapter, but a summary of key events and specializations of the synaptic vesicle cycle is useful. This will show how synaptic transmission is optimized spatially and biochemically (see also Chs 6, 10 and 22).

The organization of the presynaptic terminal is one important element for this optimization. The presynaptic terminal is compact so vesicles may rapidly move from one compartment to another. Unlike endocrine secretion, where membrane fusion occurs randomly on a relatively large membrane area, synaptic vesicles are secreted only at active zones after docking. This means that vesicles are primed for membrane fusion in response to highly localized changes in Ca^{2+}. The ability to dock at an active zone is unique to synaptic vesicles. The peptidergic, dense core vesicles also found in most presynaptic terminals are released through unspecialized membrane regions and the kinetics of peptide secretion is similar to release of hormones from endocrine cells. The composition of synaptic vesicles, the active zone and many of the components needed for docking have been identified [73, 74] (see Table 9-2 for a list of proteins and putative functions).

One characteristic of regulated exocytosis is the ability to store secretory vesicles in a reserve pool for utilization upon stimulation. In the presynaptic terminal, this principle is expanded to define multiple pools of synaptic vesicles: a ready releasable pool, a recycled synaptic vesicle pool and a larger reserve pool. This reserve pool assures that neurotransmitter is available for release in response to even the highest physiological demands. Neurons can fire so many times per minute because synaptic vesicles from the ready releasable pool at a given synapse undergo exocytosis in response to a single action potential. Those vesicles have been primed by docking at the active zone and are therefore ready for exocytosis upon arrival of an action potential. However, for the synapse to respond rapidly and repeatedly under heavy physiological demand, these exocytosed vesicles must be rapidly replaced. This is accomplished first from the recycled pool of vesicles and, as the demand increases, from the reserve pool. To be recycled, synaptic vesicles must be reloaded quickly after they release their contents. The sequence of events that is triggered by neurotransmitter exocytosis is known as the synaptic vesicle cycle [73, 74] (Fig. 9-8).

In neurons, an action potential triggers a highly localized rise of synaptic intracellular Ca^{2+} through opening of voltage-gated Ca^{2+} channels closely apposed to the active zones [77]. These Ca^{2+} ions bind proteins that serve as Ca^{2+} sensors, among them isoforms of synaptotagmin, which in turn triggers the vesicular membrane fusion with the plasma membrane for docked synaptic vesicles [78, 79].

In a simplistic model, the exocytosis step of neurotransmission takes place in at least three major different steps. First, synaptic vesicles dock to a special region of the plasma membrane at the synapse, the active zone. Once docked, synaptic vesicle proteins and plasma membrane proteins (v- and t-SNAREs) are closely apposed but not activated. Second, after vesicle docking and prior to a vesicle fusion event, these vesicles must undergo an ATP-dependent priming process requiring the activity of NSF (NEM-sensitive factor) and phosphoinositide transfer proteins. This priming event prepares synaptic vesicles to fuse their membranes with the synaptic plasma membrane within milliseconds after an action potential has reached the synapse. The synaptic vesicle protein Munc13 and the presynaptic protein RIM, localized to the active zone, play critical roles in the priming process. Third, when an action potential reaches the synapse, voltage-gated Ca^{2+} channels open and Ca^{2+} ions flow into the synaptic terminal, raising the local intracellular Ca^{2+} concentration in the vicinity of the active zone and docked vesicles. These free Ca^{2+} ions bind Ca^{2+}-binding proteins, including synaptotagmin, which in turn initiate vesicular membrane fusion with the plasma membrane through a conformational change in both vesicle and plasma membrane proteins.

As noted above, synaptic vesicles are not typically generated at the level of the TGN. Instead, they are assembled from endocytosed material retrieved from the synaptic plasma membrane. Synaptic vesicle and plasma membrane lipids and proteins are synthesized in the endoplasmic reticulum and modified in the Golgi apparatus, where they are then packaged in secretory vesicles. These synaptic precursors are delivered to the plasma membrane from the cell body by the constitutive secretory pathway. Synaptic vesicle proteins must be retrieved by clathrin-mediated synaptic vesicle endocytosis, a variant of RME with some neuron-specific components. Once the vesicle sheds its clathrin coat, the uncoated vesicle fuses with a

TABLE 9-2 A glossary of proteins in the synapse [73, 74]

1. Synaptic vesicle proteins	
Cysteine string protein (CSP)	Peripheral membrane protein that is palmitoylated on >10 cysteines. May have a role in Ca^{2+} sensitivity of exocytosis.
Cytochrome b561	Electron-transport protein required for intravesicular monooxygenases in subsets of secretory vesicles. Required for dopamine-β_2-hydroxylase and peptide amidase activity.
Neurotransmitter transporters	There are probably at least five types of transport protein specific for glutamate, acetylcholine, catecholamines, glycine/GABA and ATP. The type of transporter contributes to determining the transmitter specificity of a synapse.
Rab and Ra1 proteins	Rab3A, Rab3C, Rab5, Rab7 and Ra1. Since Rab proteins cycle between cytosolic and membrane-bound forms, not all synaptic vesicles contain all Rab proteins at the same time. Rab proteins regulate docking and fusion processes.
Rabphilin-3A	Peripheral membrane protein that binds to Rab3A and Rab3C as a function of GTP, is substrate for multiple protein kinases and contains two C-terminal C2 domains that may bind Ca^{2+}.
Secretory carrier membrane proteins (SCAMPs)	Ubiquitous integral membrane proteins of secretory and transport vesicles of unknown function.
SV2s	Highly glycosylated proteins with at least three isoforms (SV2A, B and C) containing 12 transmembrane regions and homology to bacterial and eukaryotic transporters. May help regulate Ca^{2+} levels.
Synapsins Ia, Ib, IIa and IIb	Monotopic membrane proteins with common N-terminal domains, with phosphorylation sites for CaMKI and PKA but diverge C-terminally. Synapsins Ia/b contain C-terminal phosphorylation sites for CaMKII and CDK 5. Interact with microfilaments, neurofilaments, microtubules, SH3 domains, calmodulin and annexin VI *in vitro.*
Synaptobrevins (VAMPs)	Small-membrane proteins that are cleaved by tetanus toxin and by botulinum toxins B, D, F and G.
Synaptogyrin	Polytopic membrane protein that is tyrosine-phosphorylated. Function unknown.
Synaptophysins	Polytopic membrane proteins, including synaptoporin, that are tyrosine-phosphorylated and bind to synaptobrevins. May regulate SNARE function
Synaptotagmins	Membrane proteins with at least 15 isoforms that contain C2 domains; bind Ca^{2+} and phospholipids; and interact with neurexins, AP2 and syntaxins. Synaptotagmins 1 and 2 may function as Ca^{2+} sensors in fast Ca^{2+}-dependent neurotransmitter release
Transport proteins (channels) for chloride and zinc	Components of synaptic vesicles to mediate the chloride flux for glutamate uptake and zinc uptake in most synaptic vesicles. Zinc transporter is homologous to endosomal and plasma membrane zinc transporters; chloride transporters remain to be identified.
Vacuolar proton pump	Protein complex of more than 12 subunits. Constitutes the largest component of synaptic vesicles and establishes electrochemical gradient for neurotransmitter uptake.
2. Proteins that associate with synaptic vesicles or their precursors	
Amphiphysin	Nerve-terminal protein that associates with synaptic vesicles probably via AP2 bound to synaptotagmin. May function in endocytosis.
AP2 and clathrin	AP2 is a protein complex that binds to a specific receptor on synaptic vesicles and plasma membranes to trigger assembly of clathrin for endocytosis.
Ca^{2+},calmodulin-dependent protein kinases I and II (CaMKI and CaMKII)	May transiently associate with synaptic vesicles to phosphorylate synapsins and rabphilin-3A. May regulate various steps in neurotransmitter release.
Dynamin-1	GTPase required for endocytosis that is phosphorylated by protein kinase C and dephosphorylated by calcineurin upon membrane depolarization and binds to AP2. Important for budding and fusion pore closure.
Dynein	Motor protein mediating microtubule-based synaptic vesicle transport. May be involved in retrograde axonal transport to the cell body.
Kinesins	Motor proteins for microtubule-based synaptic vesicle transport. In *Caenorhabditis elegans*, a kinesin encoded by unc-104 is essential for transport of synaptic vesicles to nerve terminals.
GDP-dissociation inhibitors (GDIs)	Bind isoprenylated Rab proteins in the GDP-bound form, resulting in a cytoplasmic complex.
MSS4	Ubiquitous protein that tightly binds to a subgroup of Rab proteins, including Rab1, Rab3 and Rab8. Function unknown.
pp60src	Tyrosine kinase that phosphorylates synaptophysin and synaptogyrin.
3. Presynaptic plasma membrane proteins	
Munc13s	Mammalian homologs of the *C. elegans* unc-13 gene that is essential for exocytosis. Binds phorbol esters but is not a protein kinase. A component of active zones that interact with RIM, syntaxin and other proteins.
Neurexins	Cell surface proteins with more than 1,000 isoforms generated by alternative splicing from three genes. Neurexins include one of the receptors for α_1-atrotoxin and may function in cell–cell recognition between neurons.
SNAP-25	Palmitoylated peripheral membrane protein that is cleaved by botulinum toxins A and E and binds to syntaxins.
Syntaxins	Ubiquitous membrane proteins that are cleaved by botulinum toxin C1 and bind to synaptotagmins, SNAP-25, synaptobrevins, complexins, munc13s, SNAPs, Ca^{2+} channels and munc18s.
Voltage-gated Ca^{2+} channels	Mediate Ca^{2+} influx for neurotransmitter release at the active zone.
RIM	Binds to Rab3 in a GTP-dependent manner and may mediate Rab3 action in regulating fusion.
4. Proteins that reversibly associate with synaptic plasma membrane proteins	
Munc18s	Mammalian homologs of the *C. elegans unc-18* gene and the *sec-1*, *sly-1* and *slp-1* products of yeast. Bind tightly to syntaxins.
N-ethylmaleimide-sensitive factor (NSF)	Trimeric ATPase required for in vitro membrane fusion during vesicular transport. Probably function as chaperones in synaptic vesicle recycling.
$\alpha/\beta/\gamma$-SNAPs	Soluble NSF-attachment proteins required to recruit NSF to membranes in an ATP-dependent manner.

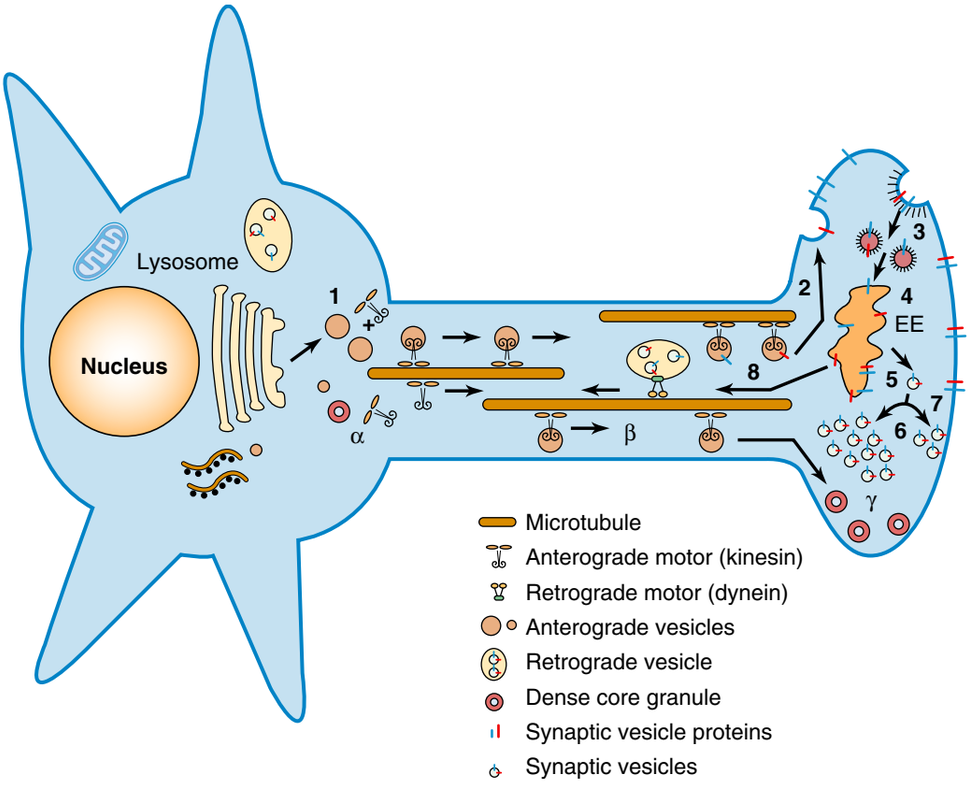

FIGURE 9-8 The life cycle of synaptic vesicles. As with other secretory vesicles, (*1*) membrane components of synaptic vesicles are synthesized in the cell body, packed into membrane-bounded transport vesicles, combined with kinesin motor proteins and actively transported down the axon to the plasma membrane via the constitutive secretory pathway. However, not all synaptic vesicle proteins (*red and blue*) are packaged together, so the synaptic vesicle requires additional steps for reconstitution. Neurons typically release neuropeptides as well as standard neurotransmitters. These are prepared as illustrated in Figure 9-7 for more typical regulated secretory vesicles (α) because their contents must be synthesized in the cell body. *2.* Once a synaptic vesicle precursor has been transported to the presynaptic terminal, where it fuses with the plasma membrane constitutively. Dense core granules are similarly transported down the axon (β). They mature during transport, but are otherwise competent for regulated secretion. *3.* Synaptic vesicle membrane proteins are then gathered efficiently through receptor mediated endocytosis in a clathrin-mediated process. *4.* Soon after the endocytosed vesicle pinches off it sheds its clathrin coat and is transported to the early endosomes (EE) where the components for a synaptic vesicle are sorted and (*5*) then they bud off from the EE to form empty synaptic vesicles. These are rapidly loaded with neurotransmitter via active transport across the membrane. *6.* At this stage, the synaptic vesicle is translocated either to the large reserve pool of synaptic vesicles or back to the plasma membrane, where it docks again to the synaptic active zone. Vesicle exocytosis is a process that requires an ATP-dependent priming process (see text) prior to membrane fusion triggered by Ca^{2+} influx in response to an action potential. The dense core granules (γ) are not associated with active zones or other specialized structures. These peptidergic vesicles typically require higher levels of intracellular Ca^{2+} and have a much slower rate of release. *7.* After release of neurotransmitter, synaptic vesicle membrane components may be recycled by repeating steps 3–6. This recycling can occur very rapidly (see below). *8.* Eventually, some synaptic vesicle components will be repackaged into retrograde vesicles for return to the cell body and degradation.

local EE, where the synaptic vesicle proteins are sorted to form synaptic vesicles. The sorted proteins bud off from the EE as an empty synaptic vesicle and neurotransmitter is taken up into the mature synaptic vesicle in the nerve terminal. The loaded synaptic vesicle is actively transported to the reserve pool of vesicles, where it waits until it is mobilized to the active zone of the synaptic terminal, where it docks with the plasma membrane. After being discharged, synaptic vesicle membrane along with the protein components are retrieved by regulated endocytosis and recycled for additional rounds of secretion.

Many years have passed since the concept of synaptic vesicle recycling was introduced in the early 1970s, but details of the synaptic vesicle cycle continue to be a matter for investigation and debate. The role of clathrin-coated vesicles and a rapid form of endocytosis is well established as a mechanism for synaptic vesicle recycling [64, 80–82] but this classical model was difficult to reconcile with some observations. In recent years, evidence has mounted to support the existence of other pathways that diverge from the classical picture. In particular, membrane fusion may not always be complete. In these cases, the clathrin-mediated endocytosis and sorting endosome steps may be bypassed under certain circumstances. Although these models were originally proposed as an alternative to the classical model, most investigators now acknowledge that these pathways are not mutually exclusive.

In the classic model of synaptic vesicle recycling in nerve terminals, synaptic vesicles fuse completely with the plasma membrane and the integrated vesicle proteins move away from the active zone to adjacent membrane regions (Fig. 9-9A). In these regions, clathrin-mediated synaptic vesicle endocytosis takes place rapidly after neurotransmitter release (within seconds) [64]. The process starts with the formation of a clathrin-coated pit that invaginates toward the interior of the cell and pinches off to form a clathrin-coated vesicle [83]. Coated vesicles are transient organelles that rapidly shed their coats in an ATP/chaperone dependent process. Once uncoated, the recycled vesicle fuses with a local EE for reconstitution as a synaptic vesicle. Subsequently, the recycled synaptic vesicle is filled with neurotransmitter and it returns to the release site ready for use. This may be the normal pathway when neurotransmitter release rates are modest. Clathrin/EE-based pathways become essential when synaptic proteins have been incorporated into the presynaptic plasma membrane.

However, an alternative pathway that bypasses clathrin-mediated endocytosis and EEs appears to be available as well. This model of endocytosis known as 'kiss and run' or its variant 'kiss and stay' have attracted increasing interest in recent years [74] (Fig. 9-9B). Kiss and run has been directly demonstrated with dense-core granules in neuroendocrine cells [84, 85], and this model would explain some observations that are not readily accommodated by the classical pathway. The kiss and run model proposes that neurotransmitters are released by a transient fusion pore, rather than by a complete fusion with integration of the synaptic vesicle components into the plasma membrane. Synaptic membrane proteins never lose their association and the vesicle reforms when the pore closes. As a result, the empty vesicle can be refilled and reused without going through clathrin-mediated endocytosis and sorting in the EEs.

Direct demonstrations of the kiss and run model in mammalian synapses have remained elusive [73], but there is sufficient evidence to suggest that there may be up

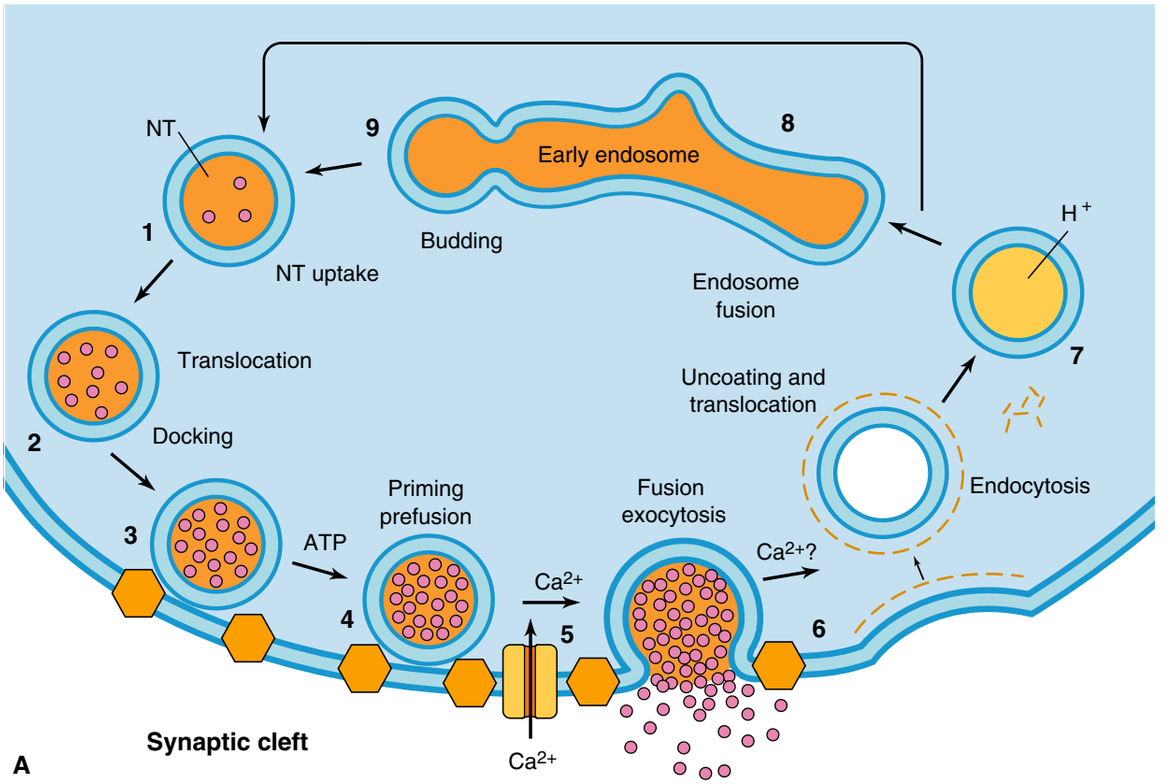

FIGURE 9-9 Release of neurotransmitter from synaptic vesicles is rapid and highly specialized. (**A**) The canonical pathway for neurotransmitter release involves a specific sequence of events. *1.* The first step in neurotransmitter release is to fill the synaptic vesicle (SV) with the appropriate neurotransmitter. This is accomplished by specific transporters in the SV membrane and is ATP-dependent. *2.* After filling, SVs may be moved to a reserve pool or to specialized regions of the presynaptic plasma membrane known as active zones (*black hexagons*). *3.* SVs dock at the active zones where (*4*) they are primed for the fusion event. When an action potential arrives at the terminal (*5*), there is a local influx of Ca^{2+} through voltage-gated channels that triggers fusion of the SV with the plasma membrane and release of neurotransmitter. In the canonical pathway (*6*), SV membrane components are rapidly displaced from the active zone and gathered into coated pits for endocytosis. *7.* The resulting coated vesicle begins to acidify and the clathrin coat is removed by a chaperone. *8.* These endocytosed vesicles fuse with an early endosome for sorting and reconstitution or may be directly refilled. *9.* with neurotransmitter for reuse. Although there is considerable evidence in support of this model, evidence exists for more rapid alternatives [73, 74].

Continued

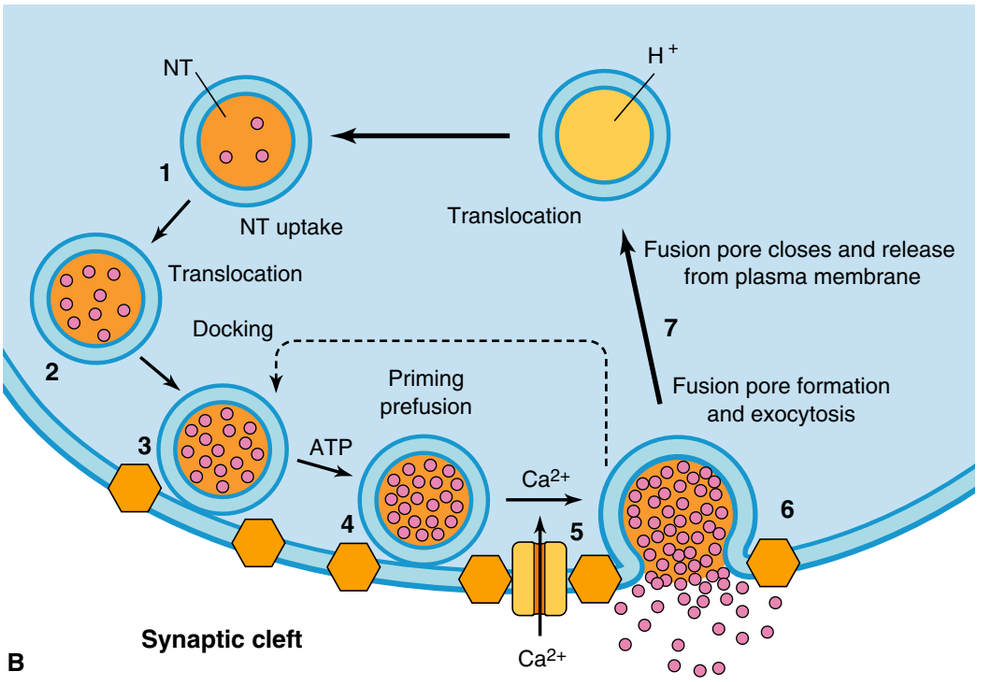

FIGURE 9-9—cont'd (**B**) Two variants of this mechanism have been proposed [74] called respectively ' kiss and run' (*solid arrows*) and 'kiss and stay' (*dashed arrow*). In both of these models, the initial steps of (*1*) filling the SV with neurotransmitter, (*2*) translocation to the active zone, (*3*) docking of the SV and (*4*) priming of the SV prior to fusion occur as in the canonical pathway. However, (*5*) influx of Ca^{2+} leads to the transient formation and rapid closure of a 'fusion pore' for release of neurotransmitter without integration of synaptic vesicle proteins into the plasma membrane. (*6*) Recycling of synaptic vesicles proceeds by a less well-understood mechanism that does not involve clathrin, formation of a coated pit or vesicle, or sorting in an early endosome. Because the SV never loses its integrity (*7*), it may be released from the plasma membrane to reenter at step (1) or may remain docked at the active zone (3) where it is refilled and reprimed (4). These pathways and the classic pathway are not mutually exclusive and both kinds of release may occur in a presynaptic terminal.

to three types of endocytic pathways operating in the presynaptic terminal: the classic, clathrin-mediated endocytic pathway and two faster pathways that do not depend on clathrin coat assembly. In the first, known as kiss and stay, the vesicles remain docked for refilling. In the second, referred to as kiss and run, vesicles recycle locally after forming a transient pore and then releasing from the membrane. Consistent with the existence of one or both of these latter models, elegant studies done with *Drosophila* indicate that flies with mutations in endophilin, a key endocytic protein required for clathrin-mediated endocytosis, have synapses that contain very few vesicles. However, these synapses can sustain synaptic transmission at low frequencies [86], suggesting that multiple pathways are operating in the presynaptic terminal.

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REFERENCES

1. Rothman, J. E. and Wieland, F. T. Protein sorting by transport vesicles. *Science* 272: 227–234, 1996.
2. Van Vliet, C., Thomas, E. C., Merino-Trigo, A., Teasdale, R. D. and Gleeson, P. A. Intracellular sorting and transport of proteins. *Prog. Biophys. Mol. Biol.* 83: 1–45, 2003.
3. Bednarek, S. Y., Orci, L. and Schekman, R. Traffic COPs and the formation of vesicle coats. *Trends Cell Biol.* 6: 468–473, 1996.
4. Kreis, T. E., Lowe, M. and Pepperkok, R. COPs regulating membrane traffic. *Annu. Rev. Cell Dev. Biol.* 11: 677–706, 1995.
5. Schmid, S. L. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66: 511–548, 1997.
6. Barlowe, C. COPII-dependent transport from the endoplasmic reticulum. *Curr. Opin. Cell Biol.* 14: 417–422, 2002.
7. Nuoffer, C. and Balch, W. E. GTPases: multifunctional molecular switches regulating vesicular traffic. *Annu. Rev. Biochem.* 63: 949–990, 1994.
8. Lee, M. C., Miller, E. A., Goldberg, J., Orci, L. and Schekman, R. Bi-directional protein transport between the ER and Golgi. *Annu. Rev. Cell Dev. Biol.* 20: 87–123, 2004.

9. Praefcke, G. J. and McMahon, H. T. The dynamin superfamily: universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* 5: 133–147, 2004.
10. Chavrier, P. and Goud, B. The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol.* 11: 466–475, 1999.
11. Pfeffer, S. R. Rab GTPases: specifying and deciphering organelle identity and function. *Trends Cell Biol.* 11: 487–491, 2001.
12. Zerial, M. and McBride, H. Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* 2: 107–117, 2001.
13. Pelham, H. R., SNAREs and the specificity of membrane fusion. *Trends Cell Biol.* 11: 99–101, 2001.
14. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bormage, H., Geromanos, S., Tempst, P. and Rothman, J. E3. SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362: 318–323, 199.
15. Parlati, F., McNew, J. A., Fukuda, R., Miller, R., Sollner, T. H. and Rothman, J. E. Topological restriction of SNARE-dependent membrane fusion. *Nature* 407: 194–198, 2000.
16. Segev, N. Ypt/rab gtpases: regulators of protein trafficking. *Sci. STKE* 2001: RE11, 2001.
17. Bonifacino, J. S. and Glick, B. S. The mechanisms of vesicle budding and fusion. *Cell* 116: 153–166, 2004.
18. Walter, P. and Johnson, A. E. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell. Biol.* 10: 87–119, 1994.
19. Gething, M. J. Role and regulation of the ER chaperone BiP. *Semin. Cell. Dev. Biol.* 10: 465–472, 1999.
20. Trombetta, E. S. and Parodi, A. J. Quality control and protein folding in the secretory pathway. *Annu. Rev. Cell Dev. Biol.* 19: 649–676, 2003.
21. Aridor, M. and Hannan, L. A. Traffic jam: a compendium of human diseases that affect intracellular transport processes. *Traffic* 1: 836–851, 2000.
22. Parodi, A. J. Protein glucosylation and its role in protein folding. *Annu. Rev. Biochem.* 69: 69–93, 2000.
23. Rutkowski, D. T. and Kaufman, R. J. A trip to the ER: coping with stress. *Trends Cell Biol.* 14: 20–28, 2004.
24. Wieland, F. T., Gleason, M. L., Serafini, T. A. and Rothman, J. E. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell* 50: 289–300, 1987.
25. Kuehn, M. J. and Schekman, R. COPII and secretory cargo capture into transport vesicles. *Curr. Opin. Cell Biol.* 9: 477–483, 1997.
26. Warren, G. and Mellman, I. Bulk flow redux? *Cell* 98: 125–127, 1999.
27. Teasdale, R. D. and Jackson, M. R. Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Ann. Rev. Cell Dev. Biol.* 12: 27–54, 1996.
29. Farquhar, M. G. and Palade, G. E. The Golgi apparatus: 100 years of progress and controversy. *Trends Cell Biol.* 8: 2–10, 1998.
28. Munro, S. and Pelham, H. R. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48: 899–907, 1987.
30. Glick, B. S. Organization of the Golgi apparatus. *Curr. Opin. Cell Biol.* 12: 450–456, 2000.
31. Kornfeld, R. and Kornfeld, S. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54: 631–664, 1985.
32. Van Meer, G. Lipids of the Golgi membrane. *Trends Cell Biol.* 8: 29–33, 1998.
33. Munro, S. Localization of proteins to the Golgi apparatus. *Trends Cell Biol.* 8: 11–15, 1998.
34. Scheiffele, P. and Fullekrug, J. Glycosylation and protein transport. *Essays Biochem.* 36: 27–35, 2000.
35. Zanetta, J. P. Structure and functions of lectins in the central and peripheral nervous system. *Acta Anat. (Basel)* 161: 180–195, 1998.
36. Ruoslahti, E., Structure and biology of proteoglycans. *Annu. Rev. Cell Biol.* 4: 229–255, 1988.
37. Schachter, H. The joys of HexNAc. The synthesis and function of N- and O-glycan branches. *Glycoconj. J.* 17: 465–483, 2000.
38. Orci, L., Glick, B. S. and Rothman, J. E. A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* 46: 171–184, 1986.
39. Cole, N. B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwartz, J. Retrograde transport of Golgi-localized proteins to the ER. *J. Cell Biol.* 140: 1–15, 1998.
40. Saraste, J. and Kuismanen, E. Pathways of protein sorting and membrane traffic between the rough endoplasmic reticulum and the Golgi complex. *Semin. Cell Biol.* 3: 343–355, 1992.
41. Pelham, H. R. Traffic through the Golgi apparatus. *J. Cell Biol.* 155: 1099–1101, 2001.
42. Bonfanti, L., Mironov, A. A. Jr., Martinez-Menarguez, J. A. *et al.* Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. *Cell* 95: 993–1003, 1998.
43. Pelham, H. R. and Rothman, J. E. The debate about transport in the Golgi–two sides of the same coin? *Cell* 102: 713–719, 2000.
44. Higgins, D., Burack, M., Lein, P. and Banker, G. Mechanisms of neuronal polarity. *Curr. Opin. Neurobiol.* 7: 599–604, 1997.
45. Cid-Arregui, A., De Hoop, M. and Dotti, C. G. Mechanisms of neuronal polarity. *Neurobiol. Aging* 16: 239–243; discussion 243–236, 1995.
46. Lippincott-Schwartz, J., Roberts, T. H. and Hirschberg, K. Secretory protein trafficking and organelle dynamics in living cells. *Annu. Rev. Cell Dev. Biol.* 16: 557–589, 2000.
47. Sampo, B., Kaech, S., Kunz, S. and Banker, G. Two distinct mechanisms target membrane proteins to the axonal surface. *Neuron* 37: 611–624, 2003.
48. Morfini, G., Szebenyi, G., Elluru, R., Ratner, N. and Brady, S. T. Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. *EMBO J.* 23: 281–293, 2002.
49. Futerman, A. H. and van Meer, G. The cell biology of lysosomal storage disorders. *Nat. Rev. Mol. Cell Biol.* 5: 554–565, 2004.
50. Peters, C. and von Figura, K. Biogenesis of lysosomal membranes. *FEBS Lett.* 346: 108–114, 1994.
51. Von Figura, K. Molecular recognition and targeting of lysosomal proteins. 3: 642–646, 1991.
52. Ghosh, P., Dahms, N. M. and Kornfeld, S. Mannose 6-phosphate receptors: new twists in the tale. *Nat. Rev. Mol. Cell Biol.* 4: 202–212, 2003.
53. Munier-Lehmann, H., Mauxion, F. and Hoflack, B. Function of the two mannose 6-phosphate receptors in lysosomal enzyme transport. *Biochem. Soc. Trans.* 24: 133–136, 1996.
54. Burgess, T. L. and Kelly, R. B. Constitutive and regulated secretion of proteins. *Annu. Rev. Cell Biol.* 3: 243–293, 1987.

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—II—

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56. Streit, W. J. Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* 40: 133–139, 2002.
57. Van Rossum, D. and Hanisch, U. K. Microglia. *Metab. Brain Dis.* 19: 393–411, 2004.
58. Cuervo, A. M. Autophagy: in sickness and in health. *Trends Cell Biol.* 14: 70–77, 2004.
59. Yoshimori, T. Autophagy: a regulated bulk degradation process inside cells. *Biochem. Biophys. Res. Commun.* 313: 453–458, 2004.
60. Shintani, T. and Klionsky, D. J. Autophagy in health and disease: a double-edged sword. *Science* 306: 990–995, 2004.
61. Keller, J. N., Dimayuga, E., Chen, Q., Thorpe, J., Gee, J. and Ding, Q. Autophagy, proteasomes, lipofuscin and oxidative stress in the aging brain. *Int. J. Biochem. Cell Biol.* 36: 2376–2391, 2004.
62. Ziv, N. E. and Garner, C. C. Cellular and molecular mechanisms of presynaptic assembly. *Nat. Rev. Neurosci.* 5: 385–399, 2004.
63. Doxsey, S. J., Brodsky, F. M., Blank, G. S. and Helenius, A. Inhibition of endocytosis by anti-clathrin antibodies. *Cell* 50: 453–463, 1987.
64. Morris, S. A. and Schmid, S. L. Synaptic vesicle recycling. The Ferrari of endocytosis? *Curr. Biol.* 5: 113–115, 1995.
65. Peters, A., Palay, S. L. and Webster, H. D. *The Fine Structure of the Nervous System: Neurons and their supporting cells.*, 3rd edn. New York: Oxford University Press, 1991.
66. Hannah, M. J., Schmidt, A. A. and Huttner, W. B. Synaptic vesicle biogenesis. *Annu. Rev. Cell Dev. Biol.* 15: 733–798, 1999.
67. Tsukita, S. and Ishikawa, H. The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 84: 513–530, 1980.
68. Yonekawa, Y., Harada, A., Okada, Y. *et al.* Defect in synaptic vesicle precursor transport and neuronal cell death in KIF1A motor protein-deficient mice. *J. Cell Biol.* 141: 431–441, 1998.
69. Herz, J. and Bock, H. H. Lipoprotein receptors in the nervous system. *Annu. Rev. Biochem.* 71: 405–434, 2002.
70. Patapoutian, A. and Reichardt, L. F. Trk receptors: mediators of neurotrophin action. *Curr. Opin. Neurobiol.* 11: 272–280, 2001.
71. Howe, C. L. and Mobley, W. C. Signaling endosome hypothesis: a cellular mechanism for long distance communication. *J. Neurobiol.* 58: 207–216, 2004.
72. Sorkin, A. and Von Zastrow, M. Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* 3: 600–614, 2002.
73. Murthy, V. N. and De Camilli, P. Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.* 26: 701–728, 2003.
74. Sudhof, T. C. The synaptic vesicle cycle. *Annu. Rev. Neurosci.* 27: 509–547, 2004.
75. Mellman, I. Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* 12: 575–625, 1996.
76. Weible, M. W. II and Hendry, I. A. What is the importance of multivesicular bodies in retrograde axonal transport in vivo? *J. Neurobiol.* 58: 230–243, 2004.
77. Meinrenken, C. J., Borst, J. G. and Sakmann, B. Local routes revisited: the space and time dependence of the Ca^{2+} signal for phasic transmitter release at the rat calyx of Held. *J. Physiol.* 547: 665–689, 2003.
78. Chapman, E. R. Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol.* 3: 498–508, 2002.
79. Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H. *et al.* Synaptotagmin I functions as a calcium regulator of release probability. *Nature* 410: 41–49, 2001.
80. Ceccarelli, B., Hurlbut, W. P. and Mauro, A. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57: 499–524, 1973.
81. Heuser, J. E. and Reese, T. S. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57: 315–344, 1973.
82. Holtzman, E., Freeman, A. R. and Kashner, A. Stimulation-dependent alterations in peroxidase uptake at lobster neuromuscular junctions. *Science* 173: 733–736, 1971.
83. Brodin, L., Low, P. and Shupliakov, O. Sequential steps in clathrin-mediated synaptic vesicle endocytosis. *Curr. Opin. Neurobiol.* 10: 312–320, 2000.
84. Breckenridge, L. J. and Almers, W. Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature* 328: 814–817, 1987.
85. Taraska, J. W. and Almers, W. Bilayers merge even when exocytosis is transient. *Proc. Natl Acad. Sci. U.S.A.* 101: 8780–8785, 2004.
86. Verstreken, P., Kjaerulff, O., Lloyd, T. E. *et al.* Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell* 109: 101–112, 2002.

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CHAPTER

10

Synaptic Transmission and Cellular Signaling: An Overview

Ronald W. Holz

Stephen K. Fisher

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SYNAPTIC TRANSMISSION

Chemical transmission between nerve cells involves multiple steps. Until the late 19th century, many physiologists believed that there were direct physical connections between nerves and that an impulse from one nerve was communicated to another through a direct physical connection. However, studies by Golgi, Ramon y Cajal and others convinced many histologists that most connections, which we now know as synapses, were close but not continuous. The pioneering work of Oliver and Shäfer, of Langley and of Elliot beginning in the 1890s provided data that raised the possibility of chemical transmission between nerves. Chemical transmission was convincingly demonstrated in the historic experiments of Otto Loewi. He electrically stimulated the vagus nerve of an isolated frog heart to decrease the strength and rate of contractions. The bathing solution caused a decrease in the strength and rate of contractions when subsequently applied to a second heart. We now know that the inhibition was caused by the neurotransmitter acetylcholine (ACh), which had been released by the nerve terminals of the vagus nerve. (See Davenport [1] for an entertaining and excellent review of the early history of chemical transmission.)

Chemical transmission is the major means by which nerves communicate with one another in the nervous system. The pre- and postsynaptic events are highly regulated and subject to use-dependent changes that are the basis for plasticity and learning in the CNS. Although direct electrical connections also occur, these account for transmission of information between nerves only in specialized cases.

Chemical transmission requires the following steps:

1. Synthesis of the neurotransmitter in the presynaptic nerve terminal
2. Storage of the neurotransmitter in secretory vesicles
3. Regulated release of neurotransmitter into the synaptic space between the pre- and postsynaptic neurons

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4. The presence of specific receptors for the neurotransmitter on the postsynaptic membrane, such that application of the neurotransmitter to the synapse mimics the effects of presynaptic nerve stimulation
5. A means for terminating the action of the released neurotransmitter.

An overview of some of the processes involved in synaptic transmission is shown in Figure 10-1. Many of the processes are discussed below or in other chapters of this book. Many different types of substance are neurotransmitters. 'Classical' neurotransmitters, such as ACh (see Ch. 11) and norepinephrine (NE; see Ch. 12), are low-molecular-weight substances that have no other function but to serve as neurotransmitters. The predominant excitatory neurotransmitter in the brain, glutamate, and the inhibitory neurotransmitter in the spinal cord, glycine, are common and essential amino acids (see Chs 15 and 16). They can function as neurotransmitters because the membranes of secretory vesicles in glutamatergic and glycinergic nerve terminals have specific transport systems that concentrate and store these amino acids so that they can be released by exocytosis in a highly regulated manner. Gamma-aminobutyric acid (GABA), the predominant inhibitory amino acid in brain, ACh and the aminergic neurotransmitters are also concentrated within synaptic vesicles through the actions of specific transport proteins. Synaptic vesicles have an acidic interior, pH ≈5.5, which is maintained by a vacuolar-type proton-translocating ATPase (see Ch. 5). The uptake of low-molecular-weight neurotransmitters is coupled via the transporters to the electrochemical H^+ gradient (for reviews, see [2, 3]).

Neurotransmitter release is a highly specialized form of the secretory process that occurs in virtually all eukaryotic cells. The fundamental similarity between the events in the nerve terminal that control neurotransmitter

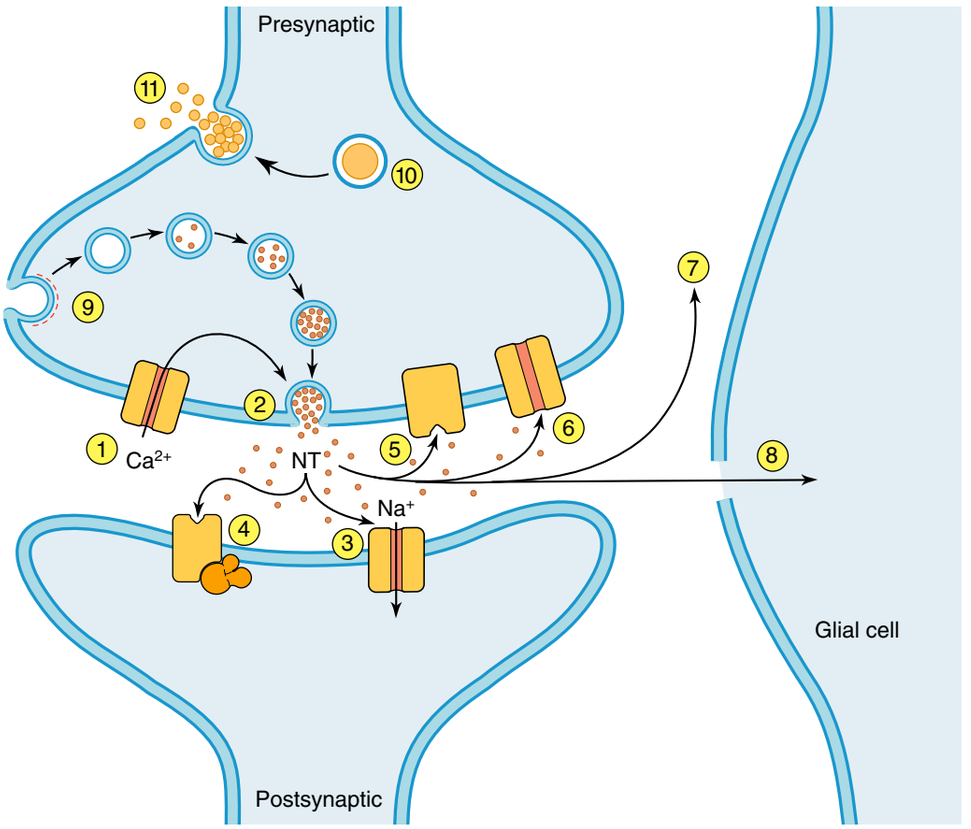

FIGURE 10-1 Depolarization opens voltage-sensitive Ca^{2+} channels in the presynaptic nerve terminal (*1*). The influx of Ca^{2+} and the resulting high Ca^{2+} concentrations at active zones on the plasmalemma trigger (*2*) the exocytosis of small synaptic vesicles that store neurotransmitter (NT) involved in fast neurotransmission. Released neurotransmitter interacts with receptors in the postsynaptic membrane that either couple directly with ion channels (*3*) or act through second messengers, such as (*4*) G-protein-coupled receptors. Neurotransmitter receptors, also in the presynaptic nerve terminal membrane (*5*), either inhibit or enhance exocytosis upon subsequent depolarization. Released neurotransmitter is inactivated by reuptake into the nerve terminal by (*6*) a transport protein coupled to the Na^+ gradient, for example, dopamine, norepinephrine, glutamate and GABA; by (*7*) degradation (acetylcholine, peptides); or by (*8*) uptake and metabolism by glial cells (glutamate). The synaptic vesicle membrane is recycled by (*9*) clathrin-mediated endocytosis. Neuropeptides and proteins are stored in (*10*) larger, dense core granules within the nerve terminal These dense core granules are released from (*11*) sites distinct from active zones after repetitive stimulation.

release and the ubiquitous vesicular trafficking reactions in all eukaryotic cells is described in Chapter 9. This similarity has important implications for the biochemistry of synaptic transmission. Many of the proteins essential for constitutive secretion and endocytosis in yeast and mammalian cells are similar to those involved in the presynaptic events of synaptic transmission (see Ch. 9).

Peptides and proteins can also be released from nerve terminals. Their biosynthetic and storage processes are similar to those in other protein secretory cells [4]. They utilize the endoplasmic reticulum, Golgi and *trans*-Golgi network, which are present in the cell body but not in the nerve terminal. The peptide- and protein-containing vesicles must be transported into the nerve terminal by axonal transport (see Ch. 28). Examples of peptide neurotransmitters include substance P, thyrotropin-releasing hormone (TRH), vasopressin, oxytocin, enkephalins and endorphins (endogenous opiate-like agonists), vasoactive intestinal peptide (VIP) and luteinizing-hormone-releasing hormone (LHRH) (see Ch. 18). The interior of mature secretory granules in neuroendocrine cells in the regulated pathway has a pH of 5.3–5.5, similar to the pH in synaptic vesicles. The low pH influences intravesicular protein processing and the conformation of the stored proteins, as well as the transport of other substances into the granules.

A variety of methods have been developed to study exocytosis. Neurotransmitter and hormone release can be measured by the electrical effects of released neurotransmitter or hormone on postsynaptic membrane receptors, such as the neuromuscular junction (NMJ; see below), and directly by biochemical assay. Another direct measure of exocytosis is the increase in membrane area due to the incorporation of the secretory granule or vesicle membrane into the plasma membrane. This can be measured by increases in membrane capacitance (C_m). C_m is directly proportional to membrane area and is defined as: $C_m = QA_m/V$, where C_m is the membrane capacitance in farads (F), Q is the charge across the membrane in coulombs (C), V is voltage (V) and A_m is the area of the plasma membrane (cm^2). The specific capacitance, Q/V, is the amount of charge that must be deposited across 1 cm^2 of membrane to change the potential by 1 V. The specific capacitance, mainly determined by the thickness and dielectric constant of the phospholipid bilayer membrane, is approximately 1 $\mu F/cm^2$ for intracellular organelles and the plasma membrane. Therefore, the increase in plasma membrane area due to exocytosis is proportional to the increase in C_m.

The electrophysiological technique used to measure changes in membrane capacitance is the patch clamp [5, 6] in the whole-cell recording mode, where the plasma membrane patch in the pipet is ruptured. In another configuration of the patch clamp, the plasma membrane patch is maintained intact. In this case, small currents due to the opening of individual channels can be measured in the membrane patch. The whole-cell patch clamp technique establishes a high resistance seal between the glass rim of the micropipet and the plasma membrane. This seal allows low-noise, high-sensitivity electrical measurements across the entire plasma membrane. An example of the use of membrane capacitance to measure exocytosis in chromaffin cells is shown in **Figure 10-2** [7].

Sensitive electrochemical techniques have also been developed to directly measure the release of oxidizable neurotransmitters such as catecholamines (CAs) and serotonin (5-hydroxytryptamine, 5-HT). Current flows in the circuit when the potential of the electrode is positive enough to withdraw electrons from, i.e. oxidize, the released neurotransmitter. The technique is very sensitive and readily detects the release of individual quanta of neurotransmitter resulting from the fusion of single secretory vesicles to the plasmalemma **(Fig. 10-2)**.

A variety of different types of tissue preparation are used to study neurosecretion and synaptic transmission. A classical preparation is the frog NMJ (discussed below). The brain slice has been used for many years for biochemical studies of CNS metabolism and is a useful preparation for electrophysiological studies of synaptic transmission in the CNS. Slices can be oriented to maintain the local neuronal circuitry and can be thin, ≈0.3 mm, to minimize anoxia. The transverse hippocampal slice is widely used as an electrophysiological preparation to study synaptic plasticity (see Ch. 53). Primary cultures of neurons from selected CNS areas and sympathetic ganglia are also frequently used. They permit excellent visual identification of individual neurons and control of the extracellular milieu, but the normal neuronal connections are disrupted.

Gentle homogenization of brain tissue results in suspensions of intracellular organelles and pinched-off nerve terminals, *synaptosomes*. Homogenization shears off nerve terminals from axons, especially in brain regions with clearly defined anatomical layers such as the cerebral cortex and hippocampus. Synaptosomes can be partially separated from other organelles by centrifugation techniques. Each of these remarkable structures is 0.5–1.0 µm in diameter, contains hundreds of synaptic vesicles and one or more mitochondria and is often associated with postsynaptic membrane fragments. Synaptosomes remain functional for several hours and can be used to study biochemical events, including energy and Ca^{2+} metabolism, neurotransmitter synthesis, transport and secretion. A related preparation is the neurosecretosome, from the posterior pituitary. These nerve terminals originate in the hypothalamus and contain vasopressin and oxytocin in large vesicles that appear as dense core granules. They are obtained in high purity from the neurohypophysis, which does not contain cell bodies. Neurosecretosomes are somewhat larger than synaptosomes and can be used for biochemical and patch clamp studies.

Several types of cells related to sympathetic neurons can be maintained and studied in tissue culture. Adrenal medullary chromaffin cells have the same precursor cells

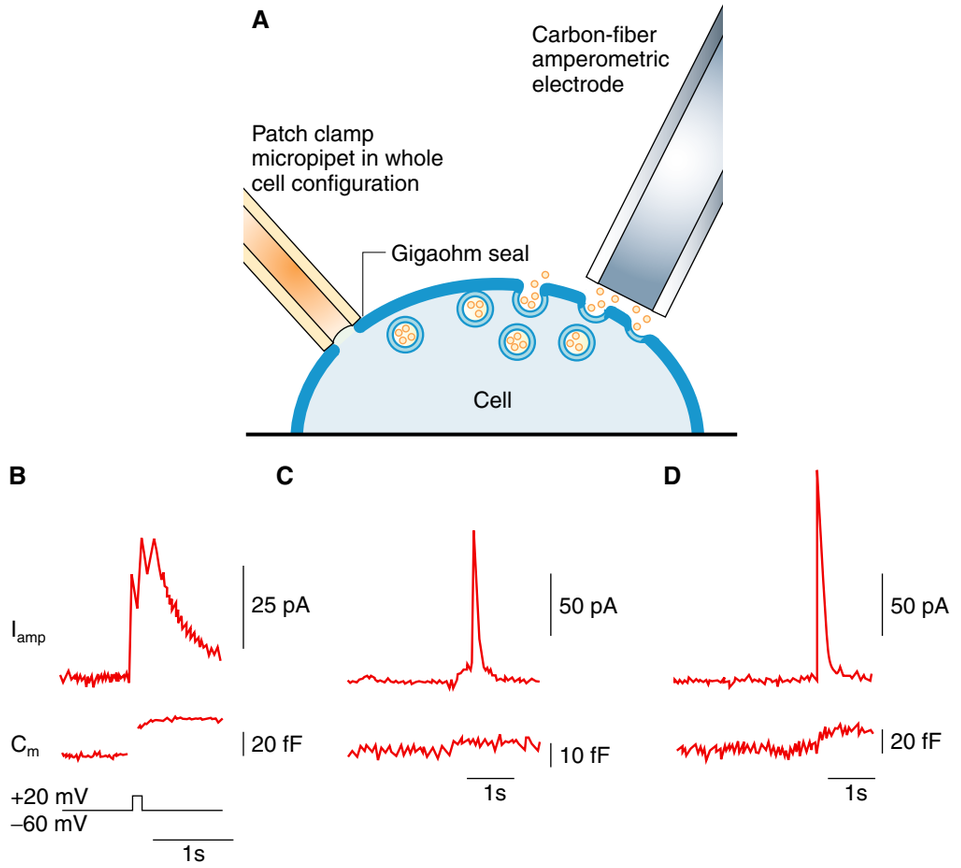

FIGURE 10-2 Secretory events monitored by simultaneous amperometric (I_{amp}) and capacitance (C_m) measurements demonstrate typical patterns of release. (**A**) Configuration of the recording setup. (**B**) Wide amperometric response composed of multiple spikes due to the fusion of several secretory vesicles triggered by a 50 ms depolarization to +20 mV from a holding potential of −60 mV. From the magnitude of the capacitance response (24 fF), it is estimated that between 2 and 24 vesicles fused with the plasmalemma. The exact number of vesicles that fused is not known since there is a distribution in their size. The amperometric response shows at least three to five discernible peaks that lag the capacitance step. However, the broad amperometric response (**B**) is likely to be composed of many more spikes. Two particularly large, isolated fusion events are shown in **C** and **D**. A clear lag between the fusion of a vesicle and the main spike of release can sometimes be observed, resulting in a foot before the spike (**C**); however, it is not always present (**D**).

as postganglionic sympathetic neurons. These excitable neuroendocrine cells store, in large dense core granules called chromaffin granules, epinephrine or NE, together with ATP and a variety of proteins (chromogranins, opiate peptides and their precursors and dopamine β-hydroxylase). Relatively pure primary cultures can be prepared by collagenase digestion of bovine adrenal glands followed by cell-purification techniques. Various aspects of neurotransmitter metabolism and secretion have been extensively studied with these cells. They are amenable to both biochemical and electrophysiological experiments. A clonal cell line, PC12, is derived from a rat pheochromocytoma, a tumor of the adrenal medulla. Upon incubation with nerve growth factor (NGF), PC12 cells differentiate within days into neurons with axons and terminals (see Ch. 27). Thus, they are used not only for biochemical and secretion studies but also for investigation of neuronal differentiation.

The neuromuscular junction is a well-defined structure that mediates the presynaptic release and postsynaptic effects of acetylcholine. The first detailed studies of synaptic transmission were performed with the NMJ. The NMJ is a beautiful example of how structure and function are intimately entwined. The myelinated axon originating from the motor neuron in the spinal cord forms unmyelinated terminals that run longitudinally along the muscle fiber. Specialized transverse release sites, or active zones, occur periodically along the terminals and are oriented opposite to invaginations of the postsynaptic membrane (Fig. 10-3). There are approximately 300 active zones per NMJ. The active zones in the nerve terminal display a cloud of clear vesicles, 50–60 nm in diameter, that contain ACh (Fig. 10-3). The active zone contains aggregates of proteins (active zone material, AZM) composed of discrete structures that link the docked granules to the plasma membrane and probably play a

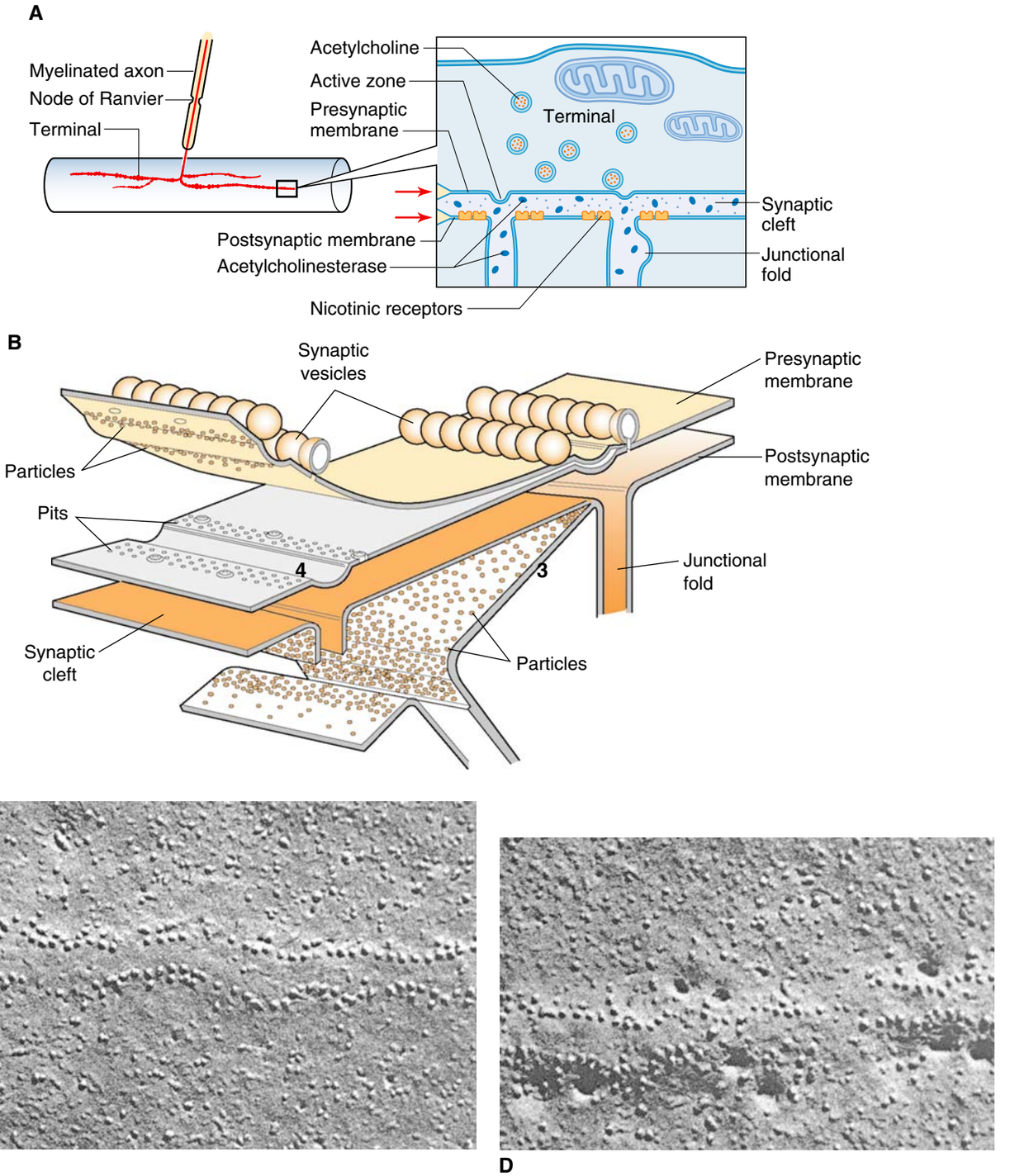

FIGURE 10-3 Synaptic membrane structure. (**A**) Entire frog neuromuscular junction (NMJ, **left**) and longitudinal section through a portion of the nerve terminal (**right**). *Arrows* indicate planes of cleavage during freeze-fracture. (**B**) Three-dimensional view of presynaptic and postsynaptic membranes with active zones and immediately adjacent rows of synaptic vesicles. Plasma membranes are split along planes indicated by the *arrows* in **A** to illustrate structures observed by freeze-fracture. The cytoplasmic half of the presynaptic membrane at the active zone shows on its fracture face protruding particles whose counterparts are seen as pits on the fracture face of the outer membrane leaflet. Vesicles that fuse with the presynaptic membrane give rise to characteristic protrusions and pores in the fracture faces. The fractured postsynaptic membrane in the region of the folds shows a high concentration of particles on the fracture face of the cytoplasmic leaflet; these are probably acetylcholine receptors (AChRs). (Courtesy of U. J. McMahan; with permission from reference [8].) Freeze-fractured active zones from frog resting and stimulated NMJ. (**C**) The active zone is the region of presynaptic membrane surrounding double rows of intramembrane particles, which may be channels for Ca^{2+} entry that initiates transmitter release. (**D**) Holes that appear in active zones during transmitter release are openings of synaptic vesicles engaged in exocytosis. This muscle was prepared by quick-freezing, and transmitter release was augmented with 4-aminopyridine so that the morphological events, such as the opening of synaptic vesicles, could be examined at the exact moment of transmitter release evoked by a single nerve shock (×120,000). (With permission from reference [9].)

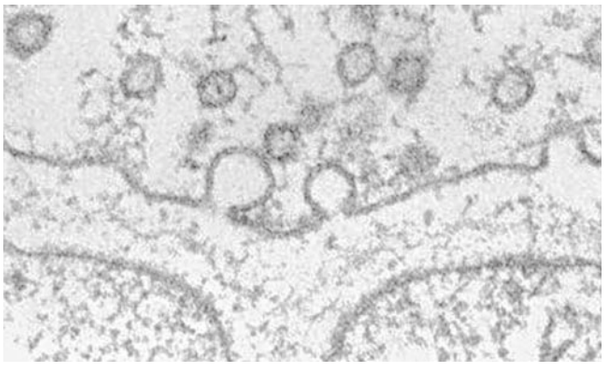

FIGURE 10-4 High-magnification (×145,000) view of freeze-substituted neuromuscular junctions in a muscle frozen during the abnormally large burst of acetylcholine release that is provoked by a single nerve stimulus in the presence of 2 mmol/l 4-aminopyridine. The stimulus was delivered 5.1 ms before the muscle was frozen. The section was cut unusually thin (≈200 Å) to show the fine structure of the presynaptic membrane, which displayed examples of synaptic vesicles apparently caught in the act of exocytosis. In all cases, these open vesicles were found just above the mouths of the postsynaptic folds, hence at the site of the presynaptic active zones. (With permission from reference [10].)

role in exocytosis. There are approximately 500,000 vesicles in all the active zones at one NMJ. It is estimated that on the average a vesicle contains 20,000 ACh molecules. A small subset of the vesicles is attached in rows to the presynaptic membrane (Fig. 10-3A, B). These are thought to be docked vesicles that are able to undergo exocytosis upon Ca^{2+} influx. In freeze fracture, these rows coincide with rows of intramembrane particles that may be Ca^{2+} channels (Fig. 10-3C). Ca^{2+} entry that occurs upon stimulation of the nerve causes exocytosis, which is seen as pits in freeze-fracture micrographs (Fig. 10-3D) or as 'omega' figures in thin-section electron microscopy (Fig. 10-4). The vesicle membranes in the nerve terminal are recycled by endocytosis (see below).

The postsynaptic membrane opposite release sites is also highly specialized, consisting of folds of plasma membrane containing a high density of nicotinic ACh receptors (nAChRs). Basal lamina matrix proteins are important for the formation and maintenance of the NMJ and are concentrated in the cleft. Acetylcholinesterase (AChE), an enzyme that hydrolyzes ACh to acetate and choline to inactivate the neurotransmitter, is associated with the basal lamina (see Ch. 11).

Quantal analysis defines the mechanism of release as exocytosis. Stimulation of the motor neuron causes a large depolarization of the motor end plate. In 1952, Fatt and Katz [11] observed that spontaneous potentials of approximately 1 mV occur at the motor endplate. Each individual potential change has a time course similar to the much larger evoked response of the muscle membrane that results from electrical stimulation of the motor nerve. These small spontaneous potentials were therefore called miniature endplate potentials (MEPPs). Because the MEPPs are reduced by the nicotinic antagonist D-tubocurarine and increased in amplitude and duration by the AChE inhibitor physostigmine, it was concluded that they are initiated by the release of ACh. Because the potential changes are too large to be accounted for by the interaction of individual molecules of ACh with the end plate, Fatt and Katz [11] postulated that they reflect the release of packets, or *quanta*, of ACh molecules from the nerve terminal.

A 'curious effect' was observed by Fatt and Katz [11]: when the Ca^{2+} concentration is reduced and the Mg^{2+} increased, the evoked end-plate potential (EPP) is diminished without altering the size of the spontaneous MEPPs. With sufficiently low Ca^{2+}, the evoked EPP is similar in size to MEPPs and varies in a stepwise manner. A single nerve impulse results in either no EPP or EPPs the approximate size of one, two, three or more MEPPs in an apparently random manner. The results of this type of experiment are displayed in Figure 10-5. The frequency histogram shows that the amplitudes of evoked potentials are clustered in multiples of the mean spontaneous MEPP value. Statistical analysis [14] demonstrates that the release is a random process described by a Poisson distribution. Each event is unaffected by the preceding events. The model assumes n release sites capable of responding to a nerve impulse, each with a probability, p, of releasing a quantum of ACh. The mean number of quanta (m), or quantal content, released per nerve impulse is $m = np$. For a Poisson distribution, p must be small, <0.05, and n large, >100. The probability of evoked release of x quanta is $p_x = (m^x/x)e^{-m}$. (See Martin [15] for a review of the Poisson distribution in the analysis of synaptic transmission.)

One critical test for the validity of the Poisson distribution as a description of release in the presence of reduced Ca^{2+} was the excellent agreement of two measures of m. One was derived empirically from the direct measurement of EPPs and MEPPs:

m = mean amplitude of EPP/mean amplitude of MEPPS.

The other was derived from the Poisson equation and the observed probability of no response, or failures, upon nerve stimulation:

$$p_0 = e^{-m} \text{ and } m = -\ln(p_0).$$

A more stringent test of the model is its ability to predict the histogram in Figure 10-5.

The number of quanta released, m, differs for different types of synapses. For a single impulse at the NMJ, 100–300 quanta are released. The large number of quanta that are released during a single impulse reflects the need for a large safety factor in the all-or-none response of muscle contraction. Where integration of inputs is important, quantal number is often less. At single terminals in sympathetic ganglia, at inhibitory and excitatory inputs on spinal motor neurons and at individual boutons of cultured hippocampal neurons, m is 1–3.

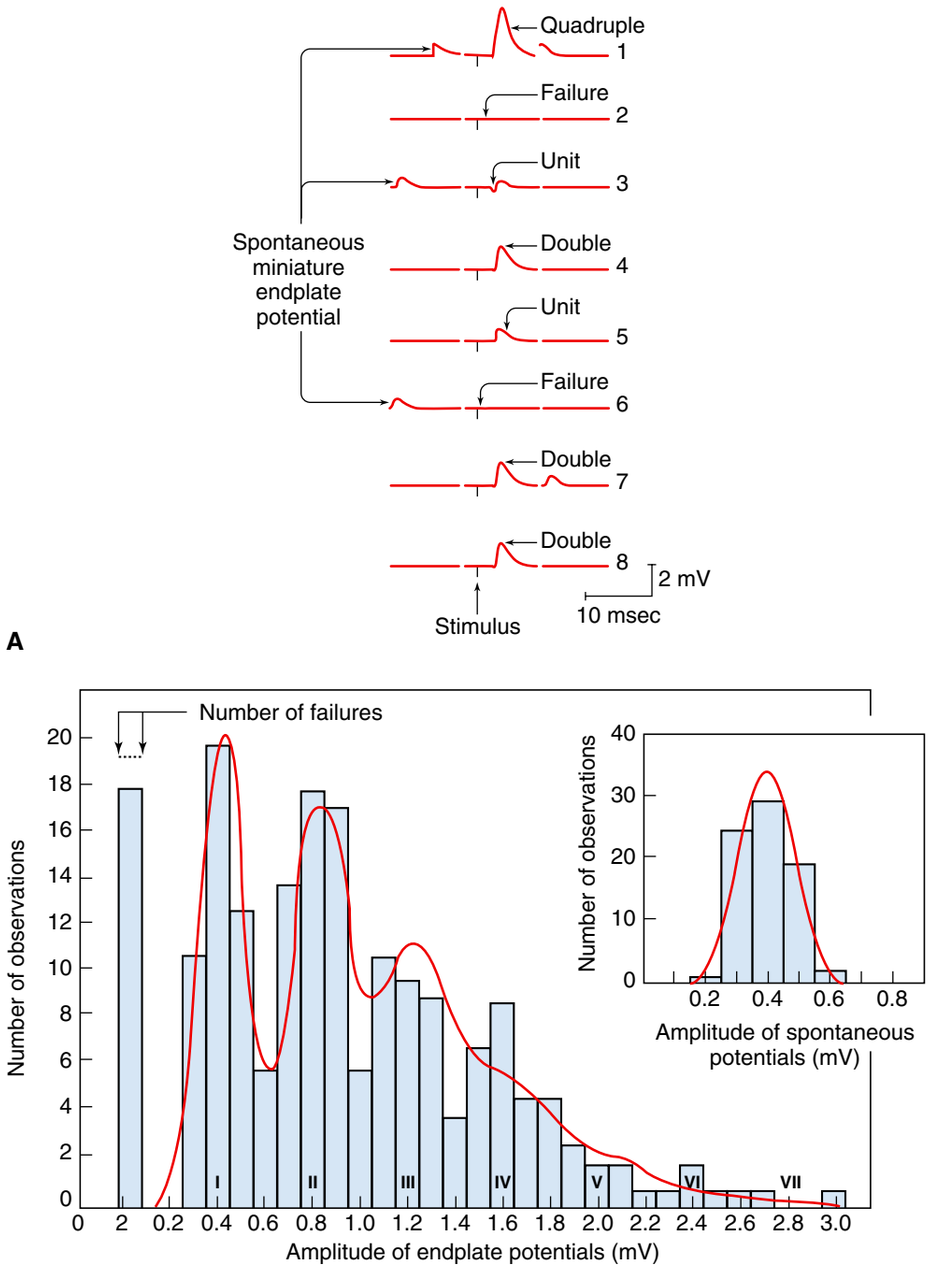

FIGURE 10-5 Comparison of the amplitudes of the spontaneous miniature endplate potentials and the evoked endplate potentials indicates that transmitter is released in quantal packages that are fixed in amplitude but variable in number. (**A**) Intracellular recording from a rat nerve–muscle synapse shows a few spontaneous miniature endplate potentials and the synaptic responses, or endplate potentials, evoked by eight consecutive stimuli to the nerve. The stimulus artifact evident in the records is produced by current flowing between the stimulating and recording electrodes in the bathing solution. In a Ca^{2+}-deficient and Mg^{2+}-rich solution designed to reduce transmitter output, the endplate potentials are small and show considerable fluctuations: two impulses produce complete failures (*2* and *6*); two produce a unit potential (*3* and *5*) and still others produce responses that are two to four times the amplitude of the unit potential. Comparison of the unit potential and the spontaneously occurring miniature endplate potential illustrates that they are the same size. (Adapted with permission from reference [12].) (**B**) Distribution of amplitudes of the spontaneous miniature endplate potentials and the evoked endplate potentials. Synaptic transmission has again been reduced, this time with only a high-Mg^{2+} solution. The histograms of the evoked endplate potential illustrate peaks that occur at 1, 2, 3 and 4 times the mean amplitude of the spontaneous potentials (0.4 mV). The distribution of the spontaneous miniature endplate potentials shown in the **inset** is fitted with a gaussian curve. The gaussian distribution for the spontaneous miniature potentials is used to calculate a theoretical distribution of the evoked endplate potential amplitudes, based on the Poisson equation, that predicts the number of failures, unit potentials, twin and triplet responses and so on. The fit of the data to the theoretical distribution is remarkably good (*solid line*). Thus, the actual number of failures (*dashed line* at 0 mV) was only slightly lower than the theoretically expected number of failures (*arrows above dashed line*). (Adapted with permission from reference [13].)

Ca^{2+} is necessary for transmission at the neuromuscular junction and other synapses and plays a special role in exocytosis. In most cases in the CNS and PNS, chemical transmission does not occur unless Ca^{2+} is present in the extracellular fluid. Katz and Miledi [16] elegantly demonstrated the critical role of Ca^{2+} in neurotransmitter release. The frog NMJ was perfused with salt solution containing Mg^{2+} but deficient in Ca^{2+}. A twin-barrel micropipet, with each barrel filled with 1.0 mmol/l of either $CaCl_2$ or NaCl, was placed immediately adjacent to the terminal. The sodium barrel was used to depolarize the nerve terminal electrically and the calcium barrel to apply Ca^{2+} ionotophoretically. Depolarization without Ca^{2+} failed to elicit an EPP (Fig. 10-6A). If Ca^{2+} was applied just before the depolarization, EPPs were evoked (Fig. 10-6B). In contrast, EPPs could not be elicited if the Ca^{2+} pulse immediately followed the depolarization (Fig. 10-6C). EPPs occurred when a Ca^{2+} pulse as short as 1 ms preceded the start of the depolarizing pulse by as little as 50–100 µs. The experiments demonstrated that Ca^{2+} must be present when a nerve terminal is depolarized in order for neurotransmitter to be released.

The normal extracellular Ca^{2+} concentration is approximately 2 mmol/l. The basal cytosolic Ca^{2+} concentration is 0.1 µmol/l or less. In nerve terminals, the rise of intracellular Ca^{2+} caused by depolarization of the plasma membrane opens voltage-sensitive Ca^{2+} channels. Ca^{2+} influx and the resultant rise in the cytosolic Ca^{2+} concentration adjacent to release sites along the plasma membrane trigger exocytosis. The sites of exocytosis are closely associated with Ca^{2+} channels (Fig. 10-3C). Ca^{2+} channels may, in fact, be components of multimeric protein complexes involved in exocytosis. Intracellular $[Ca^{2+}]$ immediately adjacent to Ca^{2+} channels is probably in the range 50–100 µmol/l [17–19]. It is this high Ca^{2+} concentration that triggers exocytosis. Neuroendocrine cells, such as chromaffin cells from the adrenal medulla, also release hormones, such as epinephrine and opioid peptides, upon Ca^{2+} influx through membrane channels. It is thought that in this type of cell, release sites are usually not closely associated with Ca^{2+} channels and that $[Ca^{2+}]$ in the 0.5–10 µmol/l range can trigger exocytosis. It should be noted that other types of cells, such as exocrine cells (for example, pancreatic acinar cells), also release stored protein by exocytosis upon a rise in cytosolic Ca^{2+}. In many cases, Ca^{2+} is released from intracellular stores by inositol trisphosphate (IP_3), which is generated by the hormonal activation of G protein-linked receptors that activate phosphoinositide-specific phospholipase C (PI-PLC) (see Ch. 20). In this case, extracellular Ca^{2+}

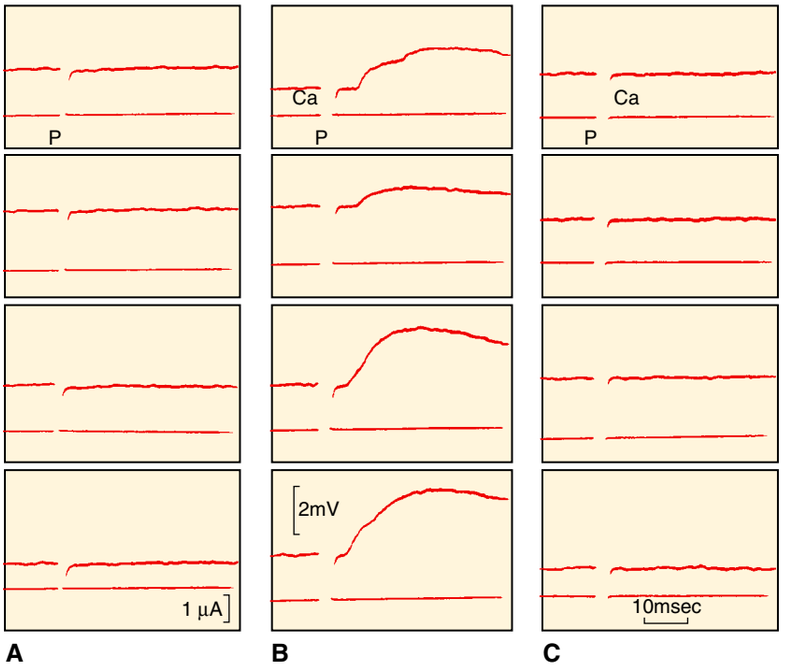

FIGURE 10-6 Synaptic transmission requires that Ca^{2+} be present during the action potential. The effects of iontophoretic pulses of Ca^{2+} on endplate response are shown. Depolarizing pulses (*P*) and Ca^{2+} were applied from a double-barrel micropipet to a small part of a frog sartorius neuromuscular junction. Intracellular recording was from the endplate region of the muscle fiber. **Top traces** show the postsynaptic membrane potential responses. **Bottom traces** show current pulses through the pipet. (**A**) Depolarizing pulses alone. (**B**) Short-duration, approximately 1 ms, Ca^{2+} pulses applied less than 1 ms before the depolarizing pulse. (**C**) Short Ca^{2+} pulses immediately following depolarizing pulses. The acetylcholinesterase inhibitor prostigmin was present to enhance the response. Temperature 3°C. Depolarization elicited endplate potentials only if the Ca^{2+} pulse preceded the depolarizing pulse (**B**). (With permission from reference [16].)

sustains secretion by refilling the intracellular, IP_3-sensitive Ca^{2+} stores rather than by directly triggering secretion.

Presynaptic events during synaptic transmission are rapid, dynamic and interconnected. The time between Ca^{2+} influx and exocytosis in the nerve terminal is very short. At the frog NMJ at room temperature, 0.5–1 ms elapses between the depolarization of the nerve terminal and the beginning of the postsynaptic response. In the squid giant synapse, recordings can be made simultaneously in the presynaptic nerve terminal and in the postsynaptic cell. Voltage-sensitive Ca^{2+} channels open toward the end of the action potential. The time between Ca^{2+} influx and the postsynaptic response as measured by the postsynaptic membrane potential is 200 µs (Fig. 10-7). However, measurements made with optical methods to record presynaptic events indicate a delay of only 60 µs between Ca^{2+} influx and the postsynaptic response at 38°C [21].

The short delays between Ca^{2+} influx and exocytosis have important implications for the mechanism of fusion of synaptic vesicles (see Ch. 9). In this short time, a synaptic vesicle cannot move significant distances and must be already at the release site. From the diffusion constant of Ca^{2+} in squid axoplasm, one can calculate that Ca^{2+} could diffuse a distance of only 850 Å, somewhat greater than the diameter of a synaptic vesicle. Therefore, in fast synapses, vesicle exocytosis sites must be close to the triggering Ca^{2+} channels.. Vesicles are exposed to $[Ca^{2+}]$ of a few hundred micromoles near the cytoplasmic mouth of the channels.

The supply of synaptic vesicles in the nerve terminal is limited. With continuous stimulation of the NMJ, the number of quanta released can be many times the number observed to be available in the nerve terminal. Transport of secretory vesicles from the cell body would be much too slow to maintain fast synaptic transmission in the terminal. Instead, the synaptic vesicle membrane that fuses with the plasmalemma membrane is rapidly recycled via clathrin-mediated endocytosis (reviewed in [22]; see Ch. 9). Hence, the vesicle membrane is a reusable container for neurotransmitter storage and exocytosis. The process of membrane recycling at the nerve terminal is closely related to the general process of endocytosis that occurs in nonneuronal cells. Strong evidence for this process came from electron micrographs of horseradish peroxidase uptake from the extracellular medium into the nerve terminal of the frog NMJ after nerve stimulation [23, 24]. Endocytosis is dispersed along the membrane away from active zones. It was originally proposed that clathrin-coated vesicles bud from the plasma membrane, lose their triskelion clathrin coat and fuse to an intermediary endosomal compartment, from which new synaptic vesicles bud. Synaptic vesicles then take up neurotransmitter and recycle to release sites. However, later studies suggested an alternative pathway that bypasses the intermediate endosomal compartment. It would allow more rapid endocytic recycling of the synaptic vesicle membrane. In this view, clathrin-coated vesicles bud from the plasmalemma, become uncoated, take up neurotransmitter and recycle to the plasmalemma. Strong stimulation of the nerve

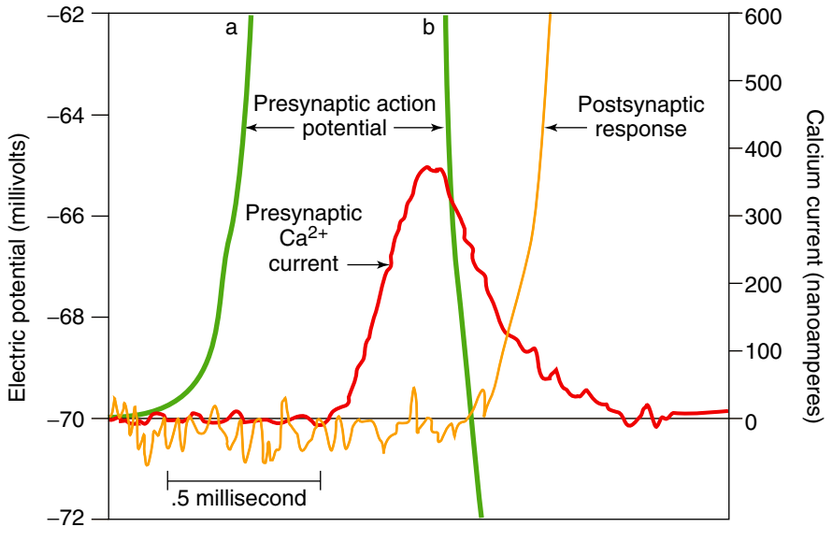

FIGURE 10-7 The delay between Ca^{2+} influx into the nerve terminal and the postsynaptic response is brief. The temporal relationships between the Ca^{2+} current and the action potential in the nerve terminal and the postsynaptic response in the squid giant synapse are shown. The rapid depolarization (*a*) and repolarization (*b*) phases of the action potential are drawn. A major fraction of the synaptic delay results from the slow-opening, voltage-sensitive Ca^{2+} channels. There is a further delay of approximately 200 µs between Ca^{2+} influx and the postsynaptic response. (With permission from reference [20].)

terminal may cause invaginations of the plasmalemma, from which clathrin-coated vesicles can also bud (see Ch. 2).

The development of amphipathic fluorescent dyes that label endocytic vesicles has permitted the study of endocytosis in nerve terminals in real time [25, 26]. The probe FM1-43 equilibrates between the aqueous phase and the membrane but is not membrane-permeating. The plasmalemma becomes fluorescent (Fig. 10-8). Upon endocytosis, the labeled membrane is internalized. When removed from the extracellular medium, the dye is retained by the endocytic vesicles but lost from the plasmalemma. Endocytic vesicles are transformed into synaptic vesicles containing FM1-43. Importantly, recycled synaptic vesicles lose the probe upon exocytosis.

This technique has permitted the dynamics of the exocytic/endocytic cycle to be investigated. At the neuromuscular junction, a readily releasable pool (RRP) and a reserve pool of vesicles coexist, the latter being released after the RRP during high-intensity stimulation. Recycling of the fused vesicle membrane requires seconds for the RRP and approximately 1 minute for the reserve pool, indicating discrete endocytic pathways. A single nerve impulse releases ≈0.1% of the total recycling pool. Readily releasable and reserve vesicle pools also occur in cultured hippocampal neurons [27]. However, in contrast to the neuromuscular junction, there is significant mixing between the pools in the hippocampus. Endocytosis follows approximately 20 seconds after exocytosis. The transformation of the endocytic vesicle into a functioning synaptic vesicle requires about 15 seconds. About 0.5% of the recycling pool is released by a nerve impulse. This corresponds to approximately one vesicle per synaptic bouton.

The importance of endocytosis for the normal function of the nerve terminal is demonstrated by the *shibire* mutant of *Drosophila*. When these mutant flies, bearing a temperature-sensitive allele, are exposed to high temperature,

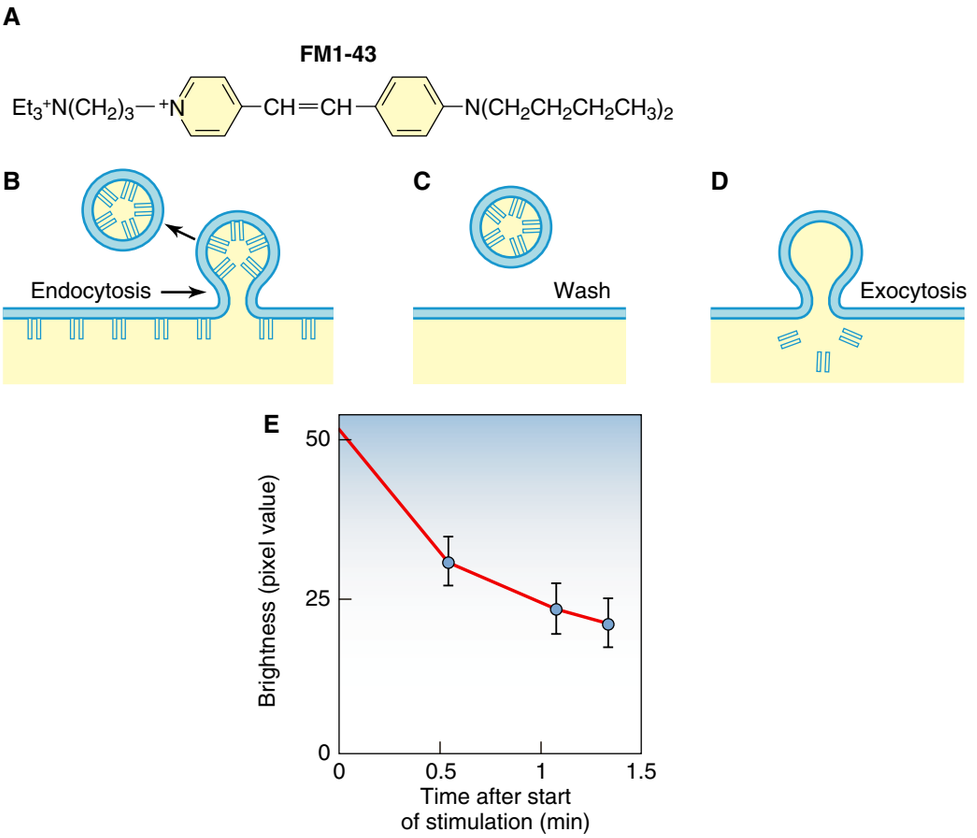

FIGURE 10-8 The probe FM1-43 was used to visualize endocytosis and exocytosis at the neuromuscular junction (NMJ). (**A**) Structure of the amphipathic membrane probe FM1-43. (**B**) Labeling of the plasmalemma by FM1-43 in the extracellular medium. The amphipathic probe is present during electrical stimulation of the NMJ. Note that membrane originating from synaptic vesicles that have undergone exocytosis is labeled. (**C**) A brief wash of the NMJ after electrical stimulation removes FM1-43 from the plasma membrane but not from intracellular endocytic vesicles that had formed following exocytosis in the presence of FM1-43. (**D**, **E**) A second round of exocytosis stimulated by exocytosis in the absence of extracellular FM1-43 results in loss of the fluorescent probe from newly formed synaptic vesicles that have undergone exocytosis. In **E,** the fluorescence intensity of vesicle populations at the NMJ was followed over time during a 10 Hz stimulation. Note the decline of fluorescence as FM1-43-labeled vesicles undergo exocytosis and release the probe into the extracellular medium [25].

they become paralyzed within 1 minute but rapidly recover when returned to the permissive temperature. Electron microscopy demonstrates that the paralysis results from a block of synaptic vesicle endocytosis at the NMJ. The *shibire* allele encodes dynamin, a GTPase that is essential for the fission of the endocytic bud from the plasmalemma.

There are important differences between fast synaptic transmission at nerve terminals and the release of proteins and peptides from nerve terminals and neuroendocrine cells. Because fast synaptic transmission involves recycling vesicles, the neurotransmitter must be replenished locally. Thus, fast synaptic transmission uses neurotransmitters such as ACh, glutamate, GABA, glycine, dopamine (DA) and NE, all of which can be synthesized within the nerve terminal or transported rapidly across the nerve terminal plasmalemma. In contrast, proteins are inserted into secretory granules in the cell body. The secretory granules must then be transported by fast axonal transport into the nerve terminal, a process that can take many hours or days depending on the distance of the nerve terminal from the soma (see Ch. 28). Nerve terminals that are specialized for fast synaptic transmission may have peptidergic granules as well as the recycling vesicles of fast synaptic transmission. For example, nerve terminals can contain VIP as well as ACh, enkephalin as well as NE and substance P as well as 5-HT. The peptidergic granules are usually far less numerous than the smaller vesicles involved in fast exocytosis and are not localized at active zones (Fig. 10-1). The exocytosis of protein-containing granules in nerve terminals may be closely related to exocytosis of protein-containing granules in endocrine and exocrine cells. While a single nerve impulse will release vesicles at active zones, exocytosis of peptidergic granules in nerve terminals can require multiple or high-frequency stimulation. This may reflect the need for sustained elevations of Ca^{2+} that extend into the interior of the nerve terminal. Peptides and proteins released from nerves may have slower and longer-lasting effects on postsynaptic cells than fast neurotransmitters and are then able to modulate the response to fast neurotransmitters.

Discrete steps in the regulated secretory pathway can be defined in neuroendocrine cells. The rapid presynaptic events of synaptic transmission produce closely coordinated exocytosis and endocytosis. Insights into the steps involved in the exocytotic limb of the pathway have come from analyzing the secretion kinetics for protein-containing granules released from adrenal chromaffin cells and PC12 cells. Adrenal chromaffin cells are excitable and contain a large number of secretory or chromaffin granules. In bovine chromaffin cells, neuronal-type nAChRs and voltage-sensitive Ca^{2+} channels permit Ca^{2+} entry, which stimulates secretion. The intracellular milieu of chromaffin cells can be directly controlled by extracellular solutions when their plasmalemmas are rendered leaky by the detergent digitonin, by streptolysin-O [28] or by mechanical disruption of the plasma membrane as cells are passed through a steel cylinder partially blocked by a precision steel bearing [29]. PC12 cells contain far fewer granules than do adrenal chromaffin cells, and many of the granules are closely associated with the plasma membrane. An analysis of the effects of Ca^{2+}, ATP and temperature suggests that ATP hydrolysis occurs before Ca^{2+} is able to cause the granules to secrete. Two distinct Ca^{2+}-dependent steps have been identified. One step enhances the ability of ATP to prime secretion, with maximal effects at approximately 1 µmol/l Ca^{2+}, whereas the other step triggers exocytosis with maximal effects at 30–300 µmol/l Ca^{2+}. Electrophysiological studies have identified additional steps associated with the triggering of exocytosis that may reflect the dynamics of inter-related pools of granules [30].

What is the function of ATP in secretion? While protein phosphorylation can modulate the secretory response, there is compelling evidence that the effect of ATP in priming involves other processes. ATP is necessary for the function of *N*-ethylmaleimide-sensitive factor (NSF). This protein is an ATPase that acts as a molecular chaperone to dissociate complexes of the SNARE proteins VAMP (synaptobrevin), syntaxin and SNAP-25 (see Ch. 9). This may permit their subsequent reassociation as part of the exocytotic response. Another function of ATP in priming exocytosis is the maintenance of the polyphosphoinositides, phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP), by phosphorylation of lipid precursors via phosphatidylinositol 4-kinase and PIP kinase [31–33]. Interestingly, phosphatidylinositol 4-kinase is an integral membrane protein of chromaffin granules and synaptic vesicles. The polyphosphoinositides appear to function in the priming step not as precursors for the formation of IP_3 and diacylglycerol (DAG) but, rather, in some other capacity (see Ch. 20). PIP_2 binds specifically to the vesicle or granule proteins synaptotagmin and rabphilin3. PIP_2 also regulates numerous proteins that control the cytoskeleton, such as profilin, gelsolin, scinderin and myosin I. Therefore, the polyphosphoinositides on the secretory granule membrane may coordinate the secretory functions of several secretory granule proteins and may modulate dynamic changes in the cytoskeletal network. This network and its changes are important for exocytosis. PIP_2 is essential also in endocytosis at presynaptic nerve terminals through the regulation of proteins involved with coating and uncoating of endocytic vesicles and through the regulation of the actin cytoskeleton.

CELLULAR SIGNALING MECHANISMS

Largely as a result of the studies of Langley, the possibility was first entertained that biological tissues possess receptor molecules specific for each neurotransmitter. Langley in the early 1900s noted the high degree of specificity and potency with which some agents

elicit a biological response and postulated the existence of 'receptor' or 'acceptor' molecules. This concept has subsequently been fully validated. Many receptors have been isolated and purified biochemically, and many have also been cloned and sequenced. In several cases, the activity of purified receptors has been reconstituted in artificial systems.

Three phases of receptor-mediated signaling can be identified. The first is the binding component in which an extracellular ligand, usually a polar molecule, forms a complex with a cell-surface receptor, which can be localized on either a pre- or a postsynaptic membrane. The second phase is that in which the activated receptor–ligand complex elicits an increase in either the formation of second messengers, the opening or closing of ion channels or the recruitment of cytoplasmic proteins. The third phase typically involves the activation of enzymes, typically protein kinases or phosphatases, which mediate the biological response. Thus, the initial interaction of a ligand with a receptor results in amplification of the signal by means of a cascade of responses.

Four distinct molecular mechanisms that link agonist occupancy of cell-surface receptors to functional responses have been identified. Traditionally, receptors have been classified according to the mediator to which they respond. The first example, proposed by Sir Henry Dale in 1914, was that the neurotransmitter ACh can interact with two types of AChRs, termed either nicotinic or muscarinic AChRs based on the similarity of action of ACh to the plant alkaloids nicotine and muscarine. Similarly, Ahlquist proposed the division of adrenergic receptors into α and β subtypes, based on the potency of a series of natural and synthetic agonists to elicit a biological response. Although the pharmacological characterization of receptors provided a useful starting point, it rapidly became apparent that extensive subclassification of receptors would be required. For example, it is now recognized that there are both neural and non-neural forms of the nAChR, which can be distinguished pharmacologically and biochemically. In addition, multiple adrenergic receptor subtypes, including α_1, α_2, β_1, β_2 and β_3, can be distinguished based on the ability of selective agonists and antagonists to bind to them. Although the pharmacological division of receptors remains that most commonly employed, an alternative classification is based on the effector mechanism to which receptors are linked.

This classification leads to four distinct groups based on the types of primary effector to which they couple [34] (Fig. 10-9). The first group is comprised of receptors which possess intrinsic ion channels that are composed of multiple subunits. Upon the binding of an agonist to these ligand-gated ion channels, the receptors undergo a conformational change which facilitates opening of the intrinsic ion channel. The permeability to specific ions is

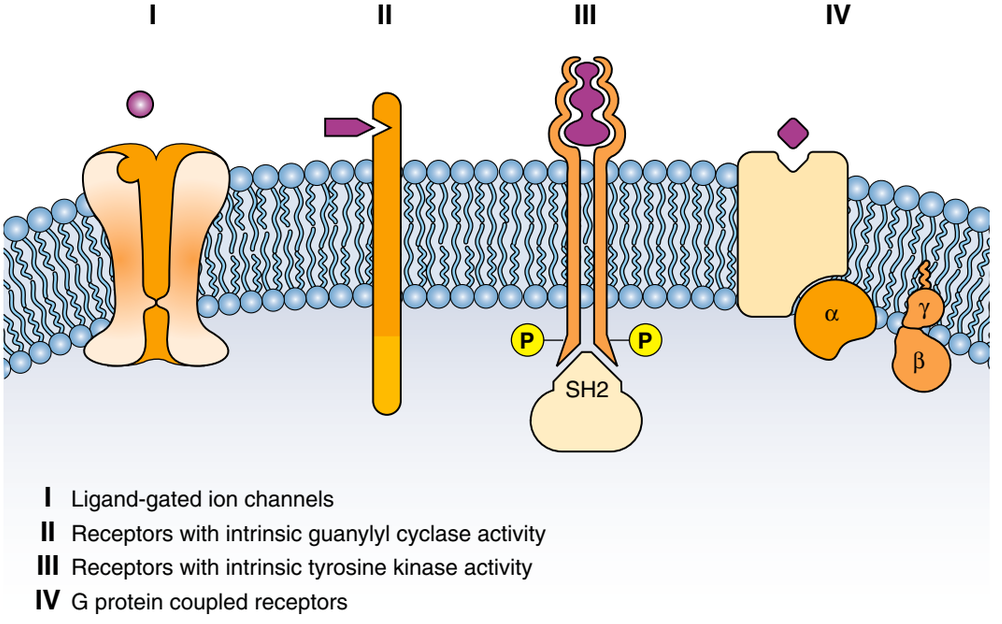

FIGURE 10-9 Cell-surface receptors utilize four distinct molecular mechanisms for transmembrane signaling. *I.* Ligand-gated ion channels. *II.* Receptors which possess intrinsic guanylyl cyclase activity. *III.* Receptors with intrinsic tyrosine kinase activity. *IV.* G-protein-coupled receptors, which are linked to the opening/closing of ion channels, modulation of adenylyl cyclase and phosphoinositide-specific phospholipase C activities. *SH2,* src homology 2 domain.

a characteristic of the receptor; for example, both the neuronal nAChR and NMDA receptors are selectively permeable to Na^+ and Ca^{2+} ions, whereas $GABA_A$ and glycine receptors are primarily permeable to Cl^- ions. As a result of the changes in ion conductance, the membrane potential may become either depolarized, as in nAChR or NMDA receptors, or hyperpolarized, as in $GABA_A$ or glycine receptors. Receptors in this category include those that are activated by synaptically released neurotransmitter and occur on the cell surface. The responses to these cell-surface receptors are extremely rapid, occurring in milliseconds, and do not require either the subsequent generation of second-messenger molecules or protein phosphorylation events. Thus, ligand-gated ion channels mediate 'fast synaptic' transmission events in the CNS. The intracellular ligand-gated receptor for IP_3 also opens within milliseconds upon binding of IP_3 (see Ch. 20).

Receptors in the second group possess intrinsic guanylyl cyclase activity and generate cGMP upon receptor activation, for example, brain natriuretic peptide receptor. These receptors consist of an extracellular binding domain, a single transmembrane-spanning domain (TMD), a protein kinase-like domain and a guanylyl cyclase catalytic domain. Ligand binding results in a conformational change in the receptor and activation of the guanylyl cyclase catalytic region. Membrane-bound guanylyl cyclase activity does not require Ca^{2+} and can be modulated by ligand addition to cell-free preparations. A different, cytoplasmic form of guanylyl cyclase is activated by micromolar concentrations of Ca^{2+}. Receptors with intrinsic guanylyl cyclase activity are often very highly phosphorylated in the absence of agonist and rapidly undergo dephosphorylation upon activation (see Ch. 23).

Receptors in the third group possess intrinsic receptor tyrosine kinase (RTK) activity. RTKs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), are found in all multicellular eukaryotic organisms and are involved in the regulation of cellular growth and differentiation (see Chs 24 and 27). Structurally, RTKs possess an extracellular ligand-binding domain, a single TMD and an intracellular catalytic kinase domain. Three distinct events underlie signal transduction at RTKs. Initially, upon ligand binding to an RTK, the receptor undergoes a dimerization which results in the juxtaposition of the two cytoplasmic domains. Contact between these domains is thought to result in a stimulation of catalytic activity, which in turn results in an intermolecular autophosphorylation of tyrosine residues both within and outside of the kinase domain. The significance of the phosphorylation of tyrosine residues lies in the subsequent ability of the RTK to recruit cytoplasmic proteins which possess src homology 2 (SH2) domains. These regions of the molecule are 60–100 amino acids in length, are globular in structure, protrude from the surface of the protein and permit high-affinity protein–protein interactions to occur. In the case of the SH2 domain, a key arginine residue buried deep in a specific binding pocket interacts with the phosphate group of the tyrosine residue. The presence of an SH2 domain can increase the affinity of a peptide for a phosphorylated tyrosine residue by 1,000-fold. Once autophosphorylated, RTKs can recruit a number of cytoplasmic proteins and initiate a series of reactions involving protein–protein interactions. The best studied pathway of this type is the mitogen-activated protein kinase (MAPK) pathway. RTKs, via the recruitment of an adaptor protein complex such as growth factor receptor-binding protein 2 (Grb2)/son of sevenless (SOS) or SHC (see Ch. 27) can activate Ras, a low-molecular-weight, monomeric G protein. The role of Ras is to recruit and activate Raf (MAPKKK), a serine/threonine kinase, which in turn activates MEK (MAPKK), a dual-specificity tyrosine/ threonine kinase. MEK subsequently activates MAPK, also known as ERK or extracellular signal-regulated kinase, which is a serine/threonine kinase with multiple substrates. ERK can enter the nucleus and regulate gene transcription by phosphorylating nuclear proteins (see Ch. 23).

The fourth group of receptors involves G proteins. Numerically, more diverse types of receptors have been demonstrated to operate via an intervening G protein than by any other mechanism. These G protein-coupled receptors (GPCRs), which have a characteristic seven TMD structure, can be further divided into three categories. Some GPCRs, such as $GABA_B$, α_2-adrenergic, D_2-dopaminergic or muscarinic M_2, regulate the changes in K^+ conductance independently of second-messenger production (see Ch. 19). A second group of GPCRs is linked to the modulation of adenylyl cyclase activity. This regulation may be either positive, as in the case of activation of the β_2-adrenergic receptor, or negative, as occurs following activation of the α_2-adrenergic receptor. Changes in the concentrations of cAMP regulate the activity of protein kinase A (PKA) (see Ch. 21). A third group of GPCRs is linked to the activation of PI-PLC with the attendant breakdown of PIP_2 and formation of IP_3 and DAG (see Ch. 20). These receptors are linked to changes in Ca^{2+} homeostasis and protein phosphorylation via the action of protein kinase C (PKC). Other effector enzymes that may be regulated by IP_3-linked GPCRs include phospholipases A_2 and D.

Cross-talk can occur between intracellular signaling pathways. Most cells possess receptors that operate through each of these four distinct effector mechanisms. Receptors within each class may be subject to further regulation during persistent agonist occupancy. One way that this can occur is via phosphorylation of the receptor, which results in its uncoupling from the effector enzyme, as has been demonstrated for the β-adrenergic receptor (see Ch. 12). This has been called *homologous regulation.* In addition, signaling pathways do not operate in isolation but may regulate and be regulated by one another,

which has been termed *heterologous regulation*. In this way, the output of the cell is fine-tuned via subtle modulation of the relevant intracellular signaling mechanisms. There are numerous examples in which the activity of one receptor can regulate, either positively or negatively, the activity of a second; for example, increases in cAMP mediated by PKA can depress or potentiate PI-PLC activity (reviewed in [35]). Conversely, activation of PI-PLC can result in modulation of the activity of other pathways via the activation of PKC. An additional complexity is that the same ligand may activate multiple pathways in a given tissue. For example, NE can activate β_2-adrenergic receptors, which increase adenylyl cyclase; α_2-adrenergic receptors, which are coupled to inhibition of adenylyl cyclase; and α_1-adrenergic receptors, which are linked to the activation of PI-PLC. Similarly, ACh can activate muscarinic cholinergic receptors, which either inhibit adenylyl cyclase, activate PI-PLC or activate K^+ channels. In addition, ACh can directly activate nAChRs, which are linked to changes in Na^+ and Ca^{2+} permeability. Thus, although individual signaling mechanisms are most frequently studied in isolation, their activity *in vivo* is likely to be highly regulated and integrated by cross-talk and other signal-transduction events.

A further consideration is that receptors which primarily activate one pathway may, on occasion, activate a second pathway (Fig. 10-10). An example is the ability of GPCRs, such as α_2-adrenergic receptors or mAChRs, to activate the MAPK cascade. Activation of adenylyl cyclase-linked receptors results in the release of G protein $\beta\gamma$ subunits, which, probably via an intermediary protein tyrosine kinase (PTK-X), stimulates phosphorylation of the adaptor protein SHC [36]. This in turn recruits the Grb2-SOS complex and activates the MAPK pathway.

Activation of PI-PLC-linked receptors, such as the mAChR, results in increased PKC activity. Since the addition of phorbol esters, which are PKC agonists (see Ch. 20), results in phosphorylation of Raf, this mechanism may provide an explanation for the ability of PI-PLC-coupled receptors to activate MAPK. A recently discovered protein tyrosine kinase PYK2, which is enriched in the CNS, is also activated by PKC. Like PTK-X, PYK2 phosphorylates SHC and recruits the Grb2-SOS complex, which results in activation of the MAPK cascade. PYK2 is also activated by

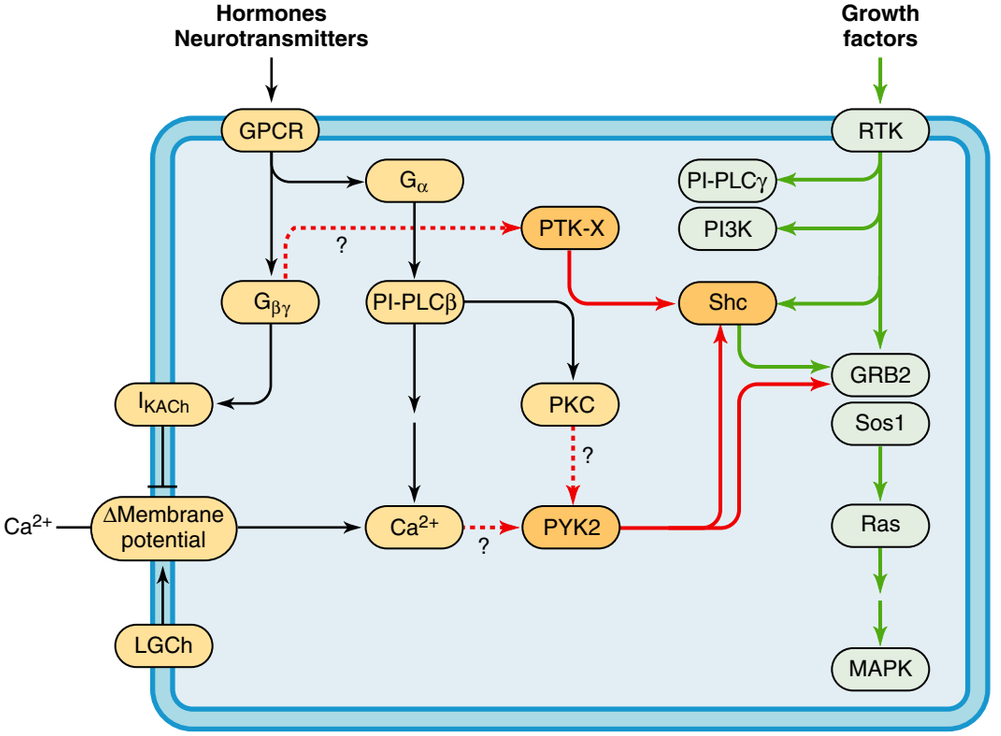

FIGURE 10-10 Cross-talk between G-protein-coupled receptors (*GPCRs*), ligand-gated ion channels (*LGChs*) and receptor protein tyrosine kinases (*RTKs*). GPCRs, via activation of PTK-X or PYK2, can activate the mitogen-activated protein kinase (*MAPK*) pathway. LGChs, via an increase in $[Ca^{2+}]_i$, may also activate PYK2. Note that the key 'go-between' molecule is the adaptor protein Shc, which in turn can interact with growth factor receptor-binding protein 2 (*GRB2*)/son of sevenless (*Sos1*) to activate the MAPK pathway. Question marks indicate that the pathways of activation still need to be firmly established, I_{KACh}, inwardly rectifying, acetylcholine-regulated channel; *PI3K*, phosphatidylinositol 3-kinase; *PKC*, protein kinase C; *PI-PLC*, phosphoinositide-specific phospholipase C. (Adapted with permission from reference [36].)

Ca^{2+}, raising the possibility that the activity of ligand-gated ion channels can also modulate the MAPK pathway. However, to date, there is no evidence to suggest that this regulation is reciprocal, that is, that activation of the MAPK pathway can modulate the activity of either GPCRs or ligand-gated ion channels.

Signaling molecules can activate gene transcription. In addition to their ability to elicit acute effects within cells, second-messenger molecules, such as cAMP, Ca^{2+} and DAG, can regulate gene transcription. The transcription factors cAMP response element-binding (CREB) protein, Fos and Jun, which respond to these signaling molecules, are members of the amphipathic helix family of proteins. Each contains a characteristic 'leucine zipper' that mediates dimerization (see Ch. 26). The best studied of these transcription factors is CREB, which becomes phosphorylated on Ser^{133} in response to increases in the intracellular concentration of cAMP and subsequent activation of PKA. CREB can also be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase IV. In its phosphorylated form, CREB binds to an eight-base *cis*-regulatory sequence called cAMP response element (CRE) and stimulates transcription. CREB has been implicated in long-term potentiation and memory (see Ch. 53). Increased concentrations of cytoplasmic Ca^{2+} and DAG activate PKC, which can also activate gene transcription via *fos* and *jun*. Activation of the MAPK pathway also leads to increased expression of Jun and Fos. Because increases in mRNA for *fos* and *jun* are observed very rapidly following a variety of stimuli, they have been termed *immediate early response genes*. The Fos–Jun heterodimers, termed AP-1, bind to a seven-base pair *cis*-regulatory element called tetradecanoyl-phorbol acetate (TPA) response element (TRE). The latter has been demonstrated to control the transcription of several genes coding for neuropeptide modulators. Thus, despite major differences in the initial molecular mechanisms that underlie activation of RTKs or receptors coupled to either adenylyl cyclase or PI-PLC, these diverse groups of receptors share at least one common end path, that of gene regulation.

Nitric oxide acts as an intercellular signaling molecule in the central nervous system. Because cells rarely act in isolation, a signaling event in one cell may have a significant impact on the activity of neighboring cells. Initial evidence for intercellular, nonsynaptic signaling was obtained from experiments with blood vessels, in which it was observed that the addition of ACh increased cGMP concentrations and resulted in relaxation of the vascular smooth muscle and vasodilation.. The increases in cGMP concentrations were dependent on the presence of Ca^{2+} and could not be demonstrated in tissue homogenates. A key observation was that removal of the endothelium abolished the relaxing effect of ACh, indicating the existence of an *endothelium-derived relaxing factor* (EDRF). Subsequently, it was discovered that EDRF was a short-lived gaseous molecule, nitric oxide (NO). NO is synthesized from arginine via the Ca^{2+} activation of nitric oxide synthase (NOS), an enzyme that requires NADPH as coenzyme and tetrahydrobiopterin as cofactor. Three distinct forms of NOS have been identified: an inducible form (iNOS), which is present in glia; a neuronal form (nNOS), which is widespread in distribution throughout the CNS; and an endothelial enzyme (eNOS) [37]. The activity of both nNOS and eNOS is Ca^{2+}/calmodulin-dependent, whereas that of iNOS is Ca^{2+}-insensitive. The activity of nNOS can be increased by brain lesions or ischemia (see Ch. 32). As a signaling molecule, NO differs from conventional neurotransmitters in that (a) it is not present in synaptic vesicles, (b) it is not released by exocytosis and (c) no specific extracellular synaptic receptors for NO exist. Rather, NO is released upon stimulation and diffuses from its site of production, which can be either neuronal or glial cells, to affect neurons up to 100 μm away. At these locations, NO activates a soluble form of guanylyl cyclase through electron transfer to the heme group on the enzyme. The increase in cGMP concentration subsequently activates cGMP-dependent protein kinases. It has been speculated that, in the CNS, NO plays a role in both long-term potentiation (LTP) and long-term depression, although the precise mechanism remains to be defined. One counterargument against a role of NO in LTP is the observation that the latter persists in transgenic mice lacking nNOS. NO can also lead to post-translational modifications of sulfhydryl groups on synaptic vesicle proteins, thereby facilitating synaptic vesicle exocytosis [38].

It is conceivable that other gaseous molecules may also act as intercellular messengers. One putative candidate is carbon monoxide, which is generated from the conversion of heme to biliverdin, a reaction catalyzed by an oxygenase. One form of the enzyme, heme oxygenase-2, is constitutive and is found in high concentrations in various brain regions. Like NO, CO increases the production of cGMP. Another putative gaseous neuromodulator, hydrogen sulfide, is thought to potentiate NMDA receptors and LTP in the hippocampus [43, 44].

Intercellular signaling may also occur via other mechanisms. For example, cell-to-cell propagation of Ca^{2+} signals can result from the gap junctional transfer of IP_3 [39]. Alternatively, the release of humoral factors such as 5-HT, glutamate and nucleotides can initiate intercellular signaling. Thus, ATP and ADP released from human neuroepithelioma cells in response to a rise in the concentration of intracellular Ca^{2+} serves to further the propagation of intercellular Ca^{2+} signals [40]. Within the CNS, glutamate released from glia can activate receptors on neurons [41]. An additional consideration is that the secreted factor may act in an autocrine fashion. Thus, prostaglandins released from myenteric neurons in response to the addition of bradykinin can modulate the subsequent Ca^{2+}

influx mediated by bradykinin receptors on the same neuron [42].

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REFERENCES

1. Davenport, H. W. Early history of the concept of chemical transmission of the nerve impulse. *Physiologist* 34: 129–190, 1991.
2. Njus, D., Kelley, P. and Harnadek, G. J. Bioenergetics of secretory vesicles. *Biochim. Biophys. Acta* 853: 237–265, 1987.
3. Schuldiner, S., Shirvan, A. and Michal, L. Vesicular neurotransmitter transporters: from bacteria to humans. *Physiol. Rev.* 75: 369–391, 1995.
4. Palade, G. Intracellular aspects of the process of protein synthesis. *Science* 189: 347–358, 1975.
5. Neher, E. and Marty, A. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc. Natl Acad. Sci. U.S.A.* 79: 6712–6716, 1982.
6. Cahalan, M. and Neher, E. Patch clamp techniques: an overview. *Methods Enzymol.* 207: 3–14, 1992.
7. Robinson, I. M., Finnegan, J. M., Monck, J. R., Wightman, R. M. and Fernandez, J. M. Colocalization of calcium entry and exocytotic release sites in adrenal chromaffin cells. *Proc. Natl Acad. Sci. U.S.A.* 92: 2474–2478, 1995. (Full Text in PMC)
8. Nichols, J. G., Martin, A. R. and Wallace, B. G. *From Neuron to Brain.* Sunderland, MA: Sinauer Associates, 1992.
9. Heuser, J. E. and Reese, T. S. Structure of the synapse. In E. R. Kandel (ed.), *Handbook of Physiology. The Nervous System. Cellular Biology of Neurons.* Bethesd, MD: American Physiological Society, 1977, sect. 1, vol. I, pp. 261–294.
10. Heuser, J. E. Synaptic vesicle exocytosis revealed in quick-frozen frog NMJ treated with 4-aminopyridine and given a single electric shock. In W. M. Cowan and J. Ferrendelli (eds.), *Approaches to the Cell Biology of Neurons.* Bethesda, MD: Society for Neuroscience, 1976, pp. 215–239.
11. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. *J. Physiol. (Lond.)* 117: 109–128, 1952.
12. Liley, A. W. The quantal components of the mammalian end-plate potential. *J. Physiol. (Lond.)* 133: 571–587, 1956.
13. Boyd, I. A. and Martin, A. R. The end-plate potential in mammalian muscle. *J. Physiol. (Lond.)* 132: 74–91, 1956.
14. Del Castillo, J. and Katz, B. Quantal components of the end-plate potential. *J. Physiol. (Lond.)* 124: 560–573, 1954.
15. Martin, A. R. Junctional transmission II. Presynaptic mechanisms. In E. R. Kandel (ed.), *Handbook of Physiology. The Nervous System. Cellular Biology of Neurons.* Bethesda, MD: American Physiological Society, 1977, sect. 1, vol. I, pp. 329–355.
16. Katz, B. and Miledi, R. The timing of calcium action during neuromuscular transmission. *J. Physiol. (Lond.)* 189: 535–544, 1967.
17. Simon, S. M. and Llinás, R. R. Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys. J.* 48: 485–498, 1985.
18. Augustine, G. J., Adler, E. M. and Charlton, M. P. The calcium signal for transmitter secretion from presynaptic nerve terminals. *Ann. N.Y. Acad. Sci.* 635: 365–381, 1991.
19. Zucker, R. S. Exocytosis: A molecular and physiological perspective. *Neuron* 17: 1049–1055, 1996.
20. Llinás, R. R. Calcium in synaptic transmission. *Sci. Am.* 247: 56–65, 1982.
21. Sabatini, B. L. and Regehr, W. G. Timing of neurotransmission at fast synapses in mammalian brain. *Nature* 384: 170–172, 1996.
22. De Camilli, P. and Takei, K. Molecular mechanisms in synaptic vesicle endocytosis and recycling. *Neuron* 16: 481–486, 1996.
23. Heuser, J. E. and Reese, T. S. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog NMJ. *J. Cell Biol.* 57: 315–344, 1973.
24. Ceccarelli, B. Hurlbut, P. and Mauro, A. Turnover of transmitter and synaptic vesicles at the frog NMJ. *J. Cell Biol.* 57: 499–524, 1973.
25. Betz, W. J. and Bewick, G. S. Optical analysis of synaptic vesicle recycling at the frog NMJ. *Science* 255: 200–203, 1992.
26. Rizzoli, S. O. and Betz, W. J. Synaptic vesicle pools. *Nat. Rev. Neurosci.* 6: 57–69, 2005.
27. Ryan, T. A. and Smith, S. J. Vesicle pool mobilization during action potential firing at hippocampal synapses. *Neuron* 14: 983–989, 1995.
28. Holz, R. W., Bittner, M. A. and Senter, R. A. Regulated exocytotic fusion I: Chromaffin cells and PC12 cells. *Methods Enzymol.* 219: 165–178, 1992.
29. Martin, T. F. J. and Walent, J. H. A new method for cell permeabilization reveals a cytosolic protein requirement for Ca^{2+}-activated secretion in GH_3 pituitary cells. *J. Biol. Chem.* 264: 10299–10308, 1989.
30. Neher, E. and Zucker, R. S. Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. *Neuron* 10: 21–30, 1993.
31. Eberhard, D. A., Cooper, C. L., Low, M. G. and Holz R. W. Evidence that the inositol phospholipids are necessary for exocytosis: loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. *Biochem. J.* 268: 15–25, 1990.
32. Hay, J. C. and Martin, T. F. J. Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca^{2+}-activated secretion. *Nature* 366: 572–575, 1993.
33. Hay, J. C., Fisette, P. L., Jenkins, G. H. *et al.* ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. *Nature* 374: 173–177, 1995.
34. Neubig, R. R. and Thomsen, W. J. How does a key fit a flexible lock? Structure and dynamics in receptor function. *Bioessays* 11: 136–141, 1989.
35. Fisher, S. K. Homologous and heterologous regulation of receptor-stimulated phosphoinositide hydrolysis. *Eur. J. Pharmacol.* 288: 231–250, 1995.
36. Bourne, H. R. Team blue sees red. *Nature* 376: 727–729, 1995.

37. Bredt, D. S. and Snyder, S. H. Nitric oxide: a physiological messenger molecule. *Annu. Rev. Biochem.* 63: 175–195, 1994.
38. Meffert, M. K., Calakos, N. C., Scheller, R. H. and Schulman, H. Nitric oxide modulates synaptic vesicle docking/fusion reactions. *Neuron* 16: 1229–1236, 1996.
39. Boitano, S., Dirksen, E. R. and Sanderson, M. J. Intercellular propagation of calcium waves mediated by inositol trisphosphate. *Science* 258: 292–295, 1992.
40. Palmer, R. K., Yule, D. I., Shewach, D. S., Williams, J. A. and Fisher, S. K. Paracrine mediation of calcium signaling in human SK-N-MCIXC neuroepithelioma cells. *Am. J. Physiol.* 271 (*Cell Physiol.* 40): C43–C53, 1996.
41. Parpura, V., Basarsky, T. A., Liu, F., Jeftinija, K., Jeftinija, S. and Haydon, P. G. Glutamate-mediated astrocyte–neuron signalling. *Nature* 369: 744–747, 1994.
42. Gelperin, D., Mann, D., Del Valle, J. and Wiley, J. Bradykinin (BK) increases cytosolic calcium in cultured rat myenteric neurons via BK-2 type receptors coupled to mobilization of extracellular and intracellular sources of calcium: Evidence that calcium influx is prostaglandin dependent. *J. Pharmacol. Exp. Ther.* 271: 507–514, 1994.
43. Chen, X., Jhee, K.-H. and Kruger, W. D. Production of the neuromodulator H_2S by cystathionine beta-synthase via the condensation of cysteine and homocysteine. *J. Biol. Chem.* 279: 52082–52086, 2004.
44. Kimura, H. Hydrogen sulfide as a neuromodulator. *Molec. Neurobiol.* 26: 13–19, 2002.

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CHAPTER

11

Acetylcholine

Palmer Taylor

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Acetylcholine (ACh) and its targets of interaction have played a long-standing and critical role in the basic concepts of neurochemistry. At the turn of the last century, ACh emerged as a pivotal mediator in chemical neurotransmission and as a ligand for defining receptors. The natural alkaloids physostigmine (by inhibiting acetylcholinesterase) and nicotine and muscarine (for distinguishing

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receptor subtype) were invaluable tools for identifying ACh as a transmitter and delineating its sites of action. The unusual spatial localization of nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction endplates enabled the discoveries of quantal release of neurotransmitter, receptor desensitization and channel gating properties of individual receptor molecules through single channel and noise analysis.

ACh is widely distributed in the nervous system. It subserves all motor transmission in vertebrates, is the primary transmitter for peripheral ganglia, mediates parasympathetic actions of the autonomic nervous system and is a dominant transmitter in the central nervous system. Toxins from various predatory species have evolved to block motor activity of prey; others from coral and plants use paralysis induced by cholinergic agents for protection from predation. The high affinity and selectivity of these toxins enabled the nAChR to emerge as the first chemically characterized neurotransmitter receptor. Other toxins from snakes and snails have proved useful to identify receptor subtypes in various tissues. In Alzheimer's dementias, ACh-containing nerve terminals are preferentially affected and therapy is directed to extending the surface area and duration of action of released ACh (Ch. 47). Cholinergic nerve terminals are also the site of action in the treatment of certain myasthenias and disorders involving compromised or excessive smooth muscle activity and exocrine secretion in the periphery. Carbamate and organophosphate pesticides act to delay the termination of action of acetylcholine by inhibiting acetylcholinesterase, and volatile organophosphates that have been used in terrorism incidents also act in this manner.

ACh arrived within the evolutionary scheme long before the design of the nervous system and functional synapses. Bacteria, fungi, protozoa and plants store ACh and possess biosynthetic and degradative capacities for turnover of the molecule. Even in higher organisms, ACh distribution is far wider than the nervous system. For example, ACh is found in the cornea, certain ciliated epithelia, the spleen of ungulates and the human placenta [1]. Although definitive evidence is lacking, ACh has been proposed to play a role in development and tissue differentiation.

ACh was first proposed as a mediator of cellular function by Hunt in 1907, and in 1914 Dale [2] pointed out that its action closely mimicked the response of parasympathetic nerve stimulation (see Ch. 10). Loewi, in 1921, provided clear evidence for ACh release by nerve stimulation. Separate receptors that explained the variety of actions of ACh became apparent in Dale's early experiments [2]. The nicotinic ACh receptor was the first transmitter receptor to be purified and to have its primary structure determined [3, 4]. The primary structures of most subtypes of both nicotinic and muscarinic receptors, the cholinesterases (ChE), choline acetyltransferase (ChAT), the choline and ACh transporters have been ascertained. Three-dimensional structures for several of these proteins or surrogates within the same protein family are also known.

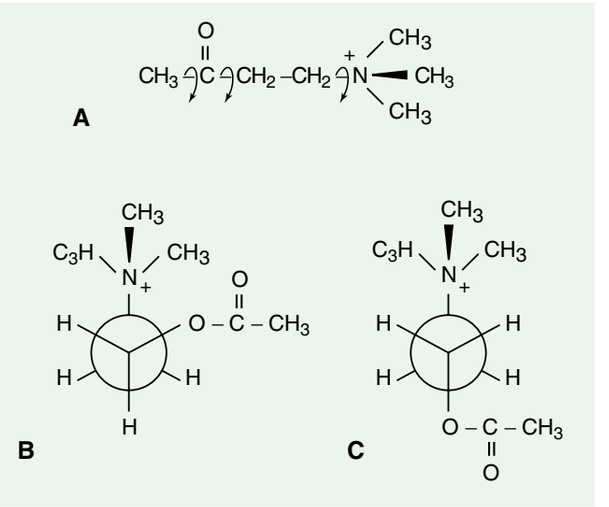


FIGURE 11-1 Structure of acetylcholine. (**A**) The three torsion angles, τ_1, τ_2 and τ_3. (**B**) Newman projection of the *gauche* conformation. (**C**) Newman projection of the *trans* conformation. The molecule is viewed in the plane of the paper from the left side, and the bond angles around τ_2 are compared.

CHEMISTRY OF ACETYLCHOLINE

Torsional rotation in the ACh molecule can occur around bonds τ_1, τ_2 and τ_3 (Fig. 11-1). Since the methyl groups are disposed symmetrically around τ_3 and constraints are placed on τ_1 by the planar acetoxy group, the most important torsion angle determining ACh conformation in solution is τ_2. A view from the β-methylene carbon of the molecule (Fig. 11-1B,C) shows the energy configurations around τ_2. Nuclear magnetic resonance (NMR) studies indicate that the *gauche* conformation is predominant in solution [5, 6]. Studies of the activities of rigid analogs of ACh suggest that the *trans* conformation may be the active conformation at muscarinic receptors [7], while results of NMR and X-ray crystallography studies suggest a change in configuration when ACh is bound to the nicotinic receptor [6, 8]. Hence the bound conformations of this flexible molecule appear to differ substantially with receptor subtype. This finding should not be a great surprise, since structural modifications of ACh that enhance or diminish activity on muscarinic receptors are very different from those modifications that influence activity on nicotinic receptors.

ORGANIZATION OF THE CHOLINERGIC NERVOUS SYSTEM

Acetylcholine receptors have been classified into subtypes based on early studies of pharmacologic selectivity. Long before structures were known, two crude alkaloid fractions, containing nicotine and muscarine (Fig. 11-2), were used to subclassify receptors in the cholinergic nervous system (Fig. 11-3). The greatly different

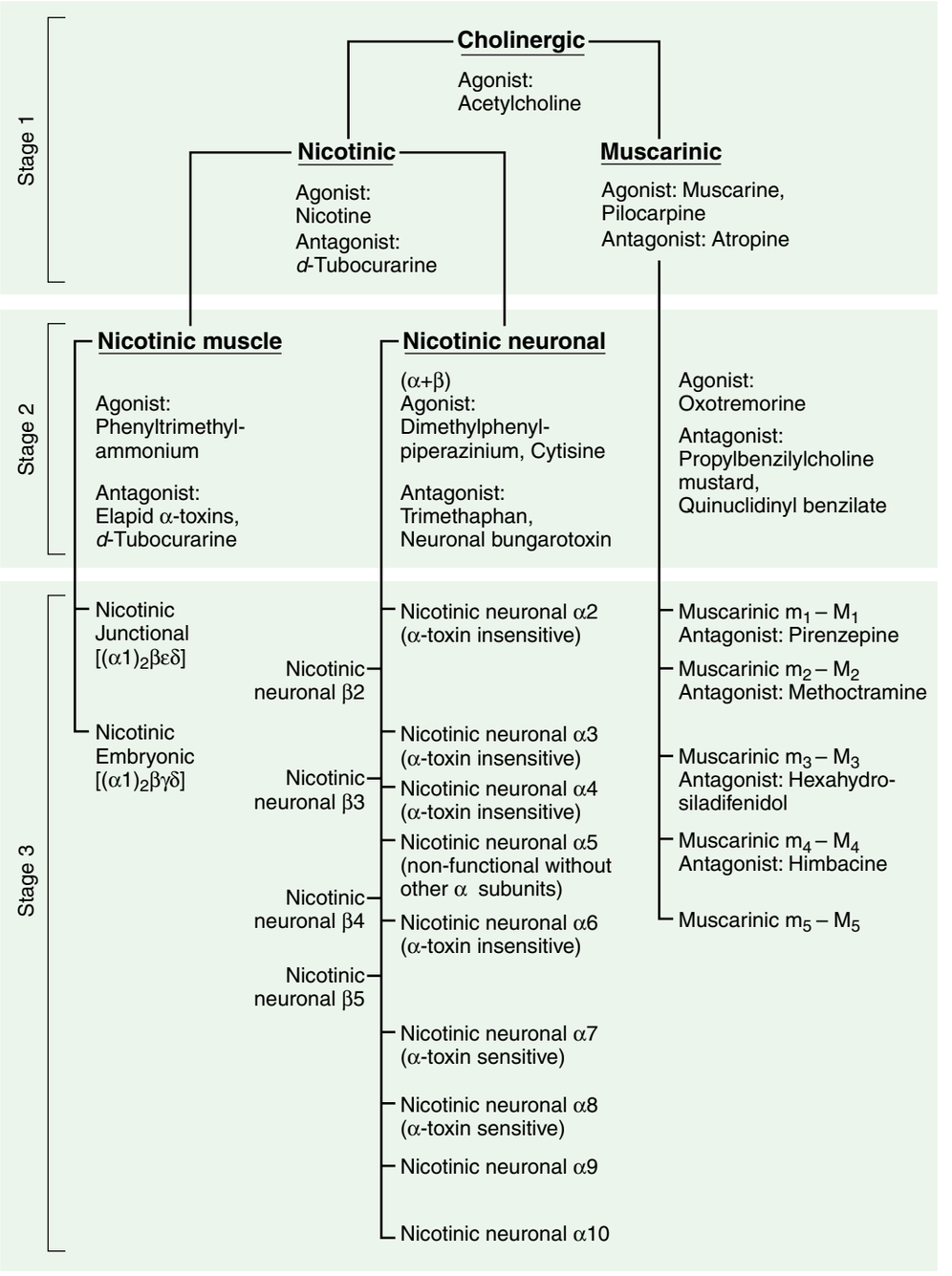


FIGURE 11-2 Classification of cholinergic receptors. The diagram shows a historical classification of receptors analyzed on the basis of distinct responses with crude alkaloids (**stage 1**), the partial resolution of receptor subtypes with chemically synthesized agonists and antagonists (**stage 2**) and the distinction of primary structures of the receptors principally through cloning by recombinant DNA techniques (**stage 3**).

activities of the antagonists atropine on muscarinic receptors and D-tubocurarine on nicotinic receptors further supported the argument that multiple classes of receptors existed for ACh. Subsequently, it was found that all nicotinic receptors are not identical. Nicotinic receptors in the neuromuscular junction, sometimes denoted as N_1 receptors, show selectivity for phenyltrimethylammonium as an agonist; elicit membrane depolarization in response to bisquaternary amines, with decamethonium being the most potent; are preferentially blocked by the competitive antagonist D-tubocurarine and are blocked irreversibly by the snake α-toxins. Nicotinic receptors in ganglia, N_2 receptors, are stimulated preferentially by 1,1-dimethyl-4-phenylpiperazinium, blocked competitively by trimethaphan, blocked noncompetitively by bisquaternary amines, with hexamethonium being the

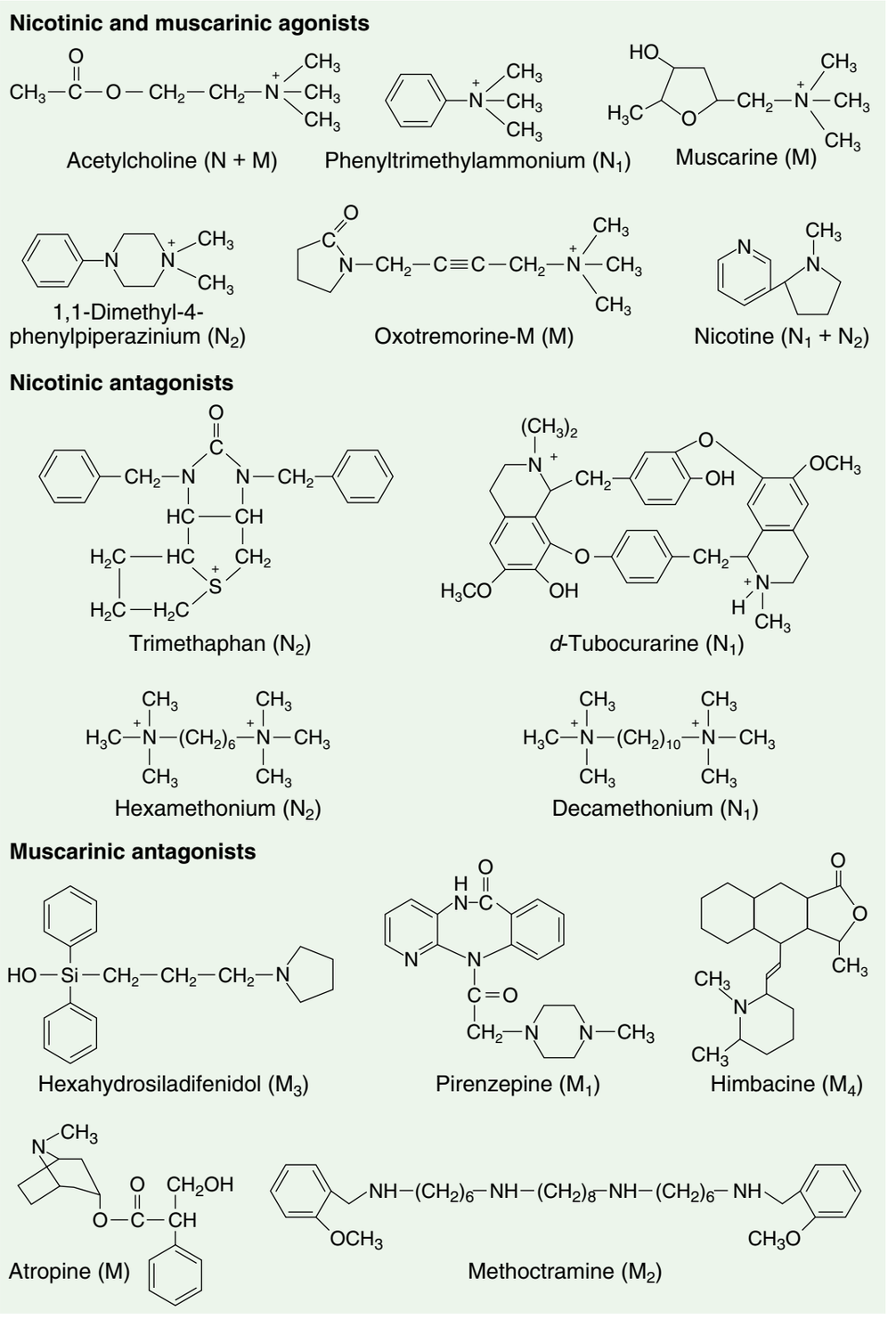


FIGURE 11-3 Structure of compounds important to the classification of receptor subtypes at cholinergic synapses. Compounds are subdivided as nicotinic (*N*) and muscarinic (*M*). The compounds interacting with nicotinic receptors are subdivided further according to whether they are neuromuscular (N_1) or ganglionic (N_2). Compounds with muscarinic subtype selectivity (M_1, M_2, M_3, M_4) are also noted.

most potent, and show resistance to the snake α-toxins [9]. A large number of distinct neuronal nicotinic receptors are found in the CNS; they are closer relatives of the nicotinic receptors in ganglia than of those in muscle.

Muscarinic receptors also exhibit distinct subtypes. The antagonist pirenzepine (PZ) has the highest affinity for one subtype, M_1, which is found mainly in neuronal tissues. Another antagonist, methoctramine, has a higher affinity for M_2 receptors, which are the predominant muscarinic receptor subtype in mammalian heart. Hexahydrosiladifenidol is relatively selective for the M_3 receptors present in smooth muscle and glands, whereas himbacine exhibits high affinity for M_4 receptors. With this level of multiplicity of receptor subtypes, limitations on specificity preclude a single antagonist defining a distinct subtype.

The intrinsic complexity and the multiplicity of cholinergic receptors became evident upon elucidation of their primary structures. In the CNS, at least nine different sequences of α subunits and three different sequences of β subunits of the nicotinic receptor have been identified [10, 11]. Expression of the cloned genes encoding certain subunit combinations yields functional receptors with different sensitivities toward various toxins and agonists.

At least five distinct muscarinic receptor genes have been cloned and sequenced. The genes are called m_1 to m_5. They correlate with the M_1 to M_5 receptors identified pharmacologically. The subtypes differ in their ability to couple to different G proteins and, hence, to elicit cellular signaling events.

Thus, cholinergic receptor classification can be considered in terms of three stages of development. Initially, Dale [2] distinguished nicotinic and muscarinic receptor subtypes with crude alkaloids. Then, chemical synthesis and structure-activity relationships clearly revealed that nicotinic and muscarinic receptors were heterogeneous, but chemical selectivity could not come close to uncovering the true diversity of receptor subtypes. Lastly, analysis of subtypes came from molecular cloning, making possible the classification of receptors on the basis of primary structure (Fig. 11-2).

FUNCTIONAL ASPECTS OF CHOLINERGIC NEUROTRANSMISSION

The individual subtypes of receptors often show discrete anatomical locations in the peripheral nervous system, and this has facilitated their classification. Nicotinic receptors are found in peripheral ganglia and skeletal muscle. Upon innervation of skeletal muscle, receptors congregate in the junctional or postsynaptic endplate area. Upon denervation or in noninnervated embryonic muscle, the receptors are distributed across the surface of the muscle, and these extrajunctional receptors are synthesized and degraded rapidly. Junctional receptors exhibit far slower rates of turnover and are distinguished by an ε subunit replacing a γ subunit in the assembled pentameric receptor.

Ganglionic nicotinic receptors are found on postsynaptic neurons in both parasympathetic and sympathetic ganglia and in the adrenal gland. Ganglionic nicotinic receptors appear in tissues of neural crest embryonic origin and exhibit identical properties in sympathetic and parasympathetic ganglia.

Muscarinic receptors are responsible for postganglionic parasympathetic neurotransmission and thus for control of a wide range of smooth muscle, cardiac muscle and secretory responses. Some responses originating in the sympathetic division of the autonomic nervous system, such as sweating and piloerection, also are mediated through muscarinic receptors.

Both muscarinic and nicotinic responses are elicited in brain and spinal cord. A few specific central cholinergic pathways have been characterized. In higher brain centers, ACh is concentrated in interneurons and long projection neurons. In the striatum, cholinergic interneurons provide critical intercircuitry for extrapyramidal motor control and implicit memory. Four predominant nuclei in the basal forebrain send projecton neurons to the hippocampus, olfactory bulb and cerebral cortex. These include the nucleus basalis of Meynert, a region of primary degeneration in Alzheimer's disease. Four areas of the brain stem send cholinergic projections to the thalamus, interpeduncular nucleus and the superior colliculus. Renshaw cells in the spinal cord play a role in modulating motoneuron activity by a feedback mechanism. Stimulation of Renshaw cells occurs through branches of the motoneuron, and the transmitter is ACh acting on nicotinic receptors.

Both nicotinic and muscarinic receptors are widespread in the CNS. Muscarinic receptors with a high affinity for pirenzepine (PZ), M_1 receptors, predominate in the hippocampus and cerebral cortex, whereas M_2 receptors predominate in the cerebellum and brainstem, and M_4 receptors are most abundant in the striatum. Central muscarinic and nicotinic receptors are targets of intense pharmacological interest for their potential roles in regulating abnormal neurological signaling in Alzheimer's disease, Parkinson's disease and certain seizure disorders. Nicotinic receptors are largely localized at prejunctional sites and control the release of neurotransmitters [10, 11].

Histochemical studies using antibodies selective for receptor subtype, ChAT and presynaptic transport proteins, along with receptor autoradiography with labeled ligands, have produced detailed maps of the CNS. In addition, the nerve cell bodies containing the mRNA encoding these proteins have been defined through *in situ* hybridization with a cDNA or antisense mRNA. Studies involving iontophoretic application of transmitter, local stimulation and intracellular or cell-surface measurements of responses establish appropriate

functional correlates. Recently, knockout mice strains, harboring deletions of M1–M5 mAChRs and various nAChR subunits, have been generated, providing a direct means of determining the localization of function of these receptors [12, 13].

Neurotransmission in autonomic ganglia is more complex than depolarization mediated by a single transmitter. In autonomic ganglia, the primary electrophysiological event following preganglionic nerve stimulation is the rapid depolarization of postsynaptic sites by released ACh acting on nicotinic receptors. This activation gives rise to an initial excitatory postsynaptic potential (EPSP), which is due to an inward current through a cation channel (see Chs 6 and 10). This mechanism is virtually identical to that in the neuromuscular junction, with an immediate onset of the depolarization and decay within a few milliseconds. Nicotinic antagonists such as trimethaphan competitively block ganglionic transmission, whereas agents such as hexamethonium produce blockade by occluding the channel. An action potential is generated in the postganglionic nerve when the initial EPSP attains a threshold amplitude.

Several secondary events amplify or suppress this signal. These include the slow EPSP, the late, slow EPSP and an inhibitory postsynaptic potential (IPSP). The slow EPSP is generated by ACh acting on muscarinic receptors and is blocked by atropine. It has a latency of approximately 1 sec and a duration of 30–60 seconds. The late, slow EPSP can last for several minutes and is mediated by peptides found in ganglia, including substance P, angiotensin, luteinizing-hormone-releasing hormone (LHRH) and the enkephalins. The slow EPSP and late, slow EPSP result from decreased K^+ conductance and are believed to regulate the sensitivity of the postsynaptic neuron to repetitive depolarization [14]. The IPSP seems to be mediated by the catecholamines dopamine and/or norepinephrine and is blocked by α-adrenergic antagonists as well as by atropine. ACh released from presynaptic terminals may act on a catecholamine-containing interneuron to release dopamine and perhaps norepinephrine. Similar to the slow EPSP, the IPSP has a prolonged latency and duration of action. These secondary events vary with the individual ganglia and are believed to modulate the sensitivity to the primary event. Hence, drugs that selectively block the slow EPSP, such as atropine, will diminish the efficiency of ganglionic transmission rather than eliminate it. Similarly, agonists such as muscarine and the ganglion-selective muscarinic agent, McN-A-343, are not thought of as primary ganglionic stimulants. Rather, they enhance the initial EPSP under conditions of repetitive stimulation.

Since parasympathetic and sympathetic ganglia exhibit comparable sensitivities to nicotinic agonists such as nicotine and ACh in producing the initial EPSP, the pharmacological effect of ganglionic stimulation depends on the profile of innervation to particular organs or tissues (Table 11-1). For example, blood vessels are innervated only by the sympathetic nervous system; thus, ganglionic stimulation should produce only vasoconstriction. Similarly, the pharmacological effects of ganglionic blockade will depend on which component of the autonomic nervous system is exerting the predominant tone at the effector organ.

Muscarinic receptors are widely distributed at postsynaptic parasympathetic effector sites. The response to systemically administered ACh is characteristic of stimulation of postganglionic effector sites rather than of ganglia. This is a consequence of the greater abundance of muscarinic receptors at effector sites in innervated tissues and the limited blood flow to ganglia. Muscarinic receptors are found in visceral smooth muscle, cardiac muscle, secretory glands and endothelial cells of the vasculature. Except for endothelial cells, each of these sites receives cholinergic innervation. Responses can be excitatory or inhibitory, depending on the tissue. Even within a single tissue the responses may vary. For example, muscarinic stimulation causes gastrointestinal smooth muscle to depolarize and contract, except at sphincters, where hyperpolarization and relaxation are seen (Table 11-2). In most tissues innervated by the cholinergic nervous system, smooth muscle exhibits intrinsic electrical and/or mechanical activity. This activity is modulated rather than initiated by cholinergic nerve stimulation. Cardiac muscle and smooth muscle exhibit spikes of electrical activity that are propagated between cells. These spikes are initiated by rhythmic fluctuations in resting membrane potential. In intestinal smooth muscle, cholinergic stimulation

TABLE 11-1 Predominance of sympathetic or parasympathetic tone at effector sites: effects of autonomic ganglionic blockade

Site	Predominant tone	Primary effects of ganglionic blockade
Arterioles	Sympathetic (adrenergic)	Vasodilation, increased peripheral blood flow, hypotension
Veins	Sympathetic (adrenergic)	Dilation, pooling of blood, decreased venous return, decreased cardiac output
Heart	Parasympathetic (cholinergic)	Tachycardia
Iris	Parasympathetic (cholinergic)	Mydriasis
Ciliary muscle	Parasympathetic (cholinergic)	Cycloplegia (focus to far vision)
Gastrointestinal tract	Parasympathetic (cholinergic)	Reduced tone and motility of smooth muscle, constipation, decreased gastric and pancreatic secretions
Urinary bladder	Parasympathetic (cholinergic)	Urinary retention
Salivary glands	Parasympathetic (cholinergic)	Xerostomia
Sweat glands	Sympathetic (cholinergic)	Anhidrosis

TABLE 11-2 Effects of acetylcholine (ACh) stimulation on peripheral tissues

Tissue	Effects of ACh
Vasculature (endothelial cells)	Release of endothelium-derived relaxing factor (nitric oxide) and vasodilation
Eye iris (pupillae sphincter muscle)	Contraction and miosis
Ciliary muscle	Contraction and accommodation of lens to near vision
Salivary glands and lacrimal glands	Secretion – thin and watery
Bronchi	Constriction, increased secretions
Heart	Bradycardia, decreased conduction (atrioventricular block at high doses), small negative inotropic action
Gastrointestinal tract	Increased tone, increased gastrointestinal secretions, relaxation at sphincters
Urinary bladder	Contraction of detrusor muscle, relaxation of the sphincter
Sweat glands	Diaphoresis
Reproductive tract, male	Erection
Uterus	Variable, dependent on hormonal influence

will cause a partial depolarization and increase the frequency of spike production. In contrast, cholinergic stimulation of atria will decrease the generation of spikes through hyperpolarization of the membrane.

Membrane depolarization typically results from an increase in Na^+ conductance. In addition, mobilization of intracellular Ca^{2+} from the endoplasmic or sarcoplasmic reticulum and the influx of extracellular Ca^{2+} appear to be elicited by ACh acting on muscarinic receptors (see Ch. 22). The resulting increase in intracellular free Ca^{2+} is involved in activation of contractile, metabolic and secretory events. Stimulation of muscarinic receptors has been linked to changes in cyclic nucleotide concentrations. Reductions in cAMP concentrations and increases in cGMP concentrations are typical responses (see Ch. 21). These cyclic nucleotides may facilitate contraction or relaxation, depending on the particular tissue. Inhibitory responses also are associated with membrane hyperpolarization, and this is a consequence of an increased K^+ conductance. Increases in K^+ conductance may be mediated by a direct receptor linkage to a K^+ channel or by increases in intracellular Ca^{2+}, which in turn activate K^+ channels. Mechanisms by which muscarinic receptors couple to multiple cellular responses are considered later.

Stimulation of the motoneuron releases acetylcholine onto the muscle endplate and results in contraction of the muscle fiber. Contraction and associated electrical events can be produced by intra-arterial injection of ACh close to the muscle. Since skeletal muscle does not possess inherent myogenic tone, the tone of apparently resting muscle is maintained by spontaneous and intermittent release of ACh. The consequences of spontaneous release at the motor endplate of skeletal muscle are small depolarizations from the quantized release of ACh, termed *miniature endplate potentials* (MEPPs) [15] (see Ch. 10). Decay times for the MEPPs range between 1 and 2 ms, a duration similar to the mean channel open time seen with ACh stimulation of individual receptor molecules. Stimulation of the motoneuron results in the release of several hundred quanta of ACh. The summation of MEPPs gives rise to a postsynaptic excitatory potential (PSEP), also termed *motor endplate potential*. A sufficiently large and abrupt potential change at the endplate will elicit an action potential by activating voltage-sensitive Na^+ channels. The action potential propagates in two-dimensional space across the surface of the muscle to release Ca^{2+} and elicit contraction. Therefore, the PSEP may be thought of as a generator potential, found only in junctional regions and arising from the opening of multiple receptor channels. Normal resting potentials in endplates are about −70 mV. The PSEP causes the endplate to depolarize partially to about −55 mV. It is the rapid and transient changes from −70 to −55 mV in localized areas of the endplate that trigger action potential generation [9, 15].

Competitive blocking agents cause muscle paralysis by preventing access of acetylcholine to its binding site on the receptor. Competitive blockade with agents such as D-tubocurarine result in maintenance of the endplate potential at −70 mV. Without frequent PSEPs, action potentials are not triggered and flaccid paralysis of the muscle results. The actions of competitive blocking agents can be surmounted by excess ACh. Depolarizing neuromuscular blocking agents, such as decamethonium and succinylcholine, produce depolarization of the endplate such that the endplate potential is −55 mV. The high concentrations of depolarizing agent that are maintained in this synapse do not allow regions of the endplate to repolarize, as would occur with a labile transmitter such as ACh. Since it is the transition in membrane potential between −70 and −55 mV that triggers the action potential, flaccid paralysis also will occur with a depolarizing block [9]. Excess ACh will not reverse the paralysis by depolarizing blocking agents. As might be expected if depolarization occurs in a non-uniform manner in microscopic areas within individual endplates and in individual motor units, the onset of depolarization blockade is characterized by muscle twitching and fasciculations that are not evident in competitive block. Once paralysis occurs, the overall pharmacological actions of competitive and depolarizing blocking agents are similar, yet intracellular measurements of endplate potential can distinguish these two classes of agent.

SYNTHESIS, STORAGE AND RELEASE OF ACETYLCHOLINE

The biosynthesis and storage of ACh can be divided into three processes that allow for recovery of hydrolyzed transmitter by choline transport back into the nerve ending, conversion by acetylation to active transmitter and then storage in a vesicle for subsequent release [16–21] (Fig. 11-4).

The synthesis reaction is a single step catalyzed by the enzyme choline acetyl transferase (ChAT):

$$\text{Choline} + \text{Acetyl coenzyme A} \rightleftharpoons \text{Acetylcholine} + \text{Coenzyme A}.$$

ChAT, first assayed in a cell-free preparation in 1943, has subsequently been purified and cloned from several sources [16]. The purification of ChAT has allowed production of specific antibodies. Whereas acetylcholinesterase (AChE), the enzyme responsible for degradation of ACh, is produced by cells containing cholinoreceptive sites as well as in cholinergic neurons, ChAT is found in the nervous system specifically at sites where ACh synthesis takes place. Within cholinergic neurons, ChAT is concentrated in nerve terminals, although it is also present in axons, where it is transported from its site of synthesis in the soma. When subcellular fractionation studies are carried out, ChAT is recovered in the synaptosomal fraction, and within synaptosomes it is primarily cytoplasmic. It has been suggested that ChAT also binds to the outside of the storage vesicle under physiological conditions and that ACh synthesized in that location may be situated favorably to enter the vesicle.

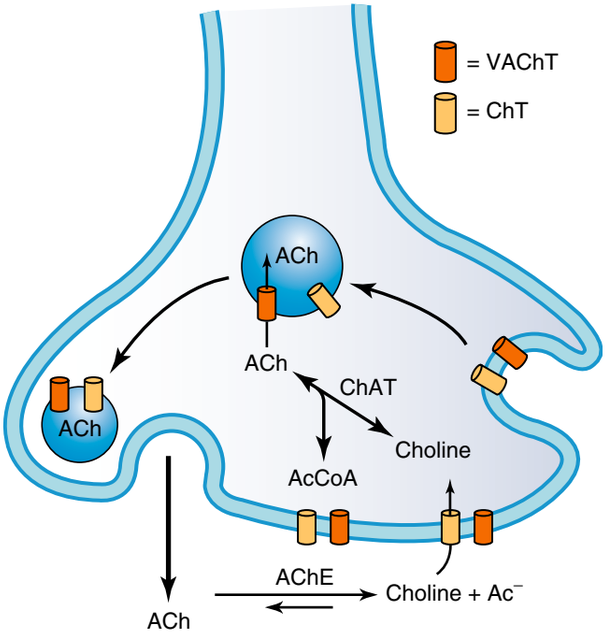


FIGURE 11-4 Transport, synthesis and degradative processes in a cholinergic presynaptic nerve terminal and synapse. The choline transport protein (*ChT*) functions at the nerve ending membrane to transport choline into the cytoplasm, where its acetylation by acetyl CoA is catalyzed by choline acetyltransferase (*ChAT*) to generate acetylcholine (*ACh*) in the vicinity of the synaptic vesicle. The vesicular acetylcholine transporter (*VAChT*) concentrates acetylcholine in the vesicle. ChT is also found on the vesicle but in a functionally inactive state. Upon nerve stimulation, depolarization and Ca^{2+} entry, ACh-containing vesicles fuse with the membrane and release their contents. The fusion of the membrane results in more ChT being exposed to the synaptic gap, where it becomes active. ACh is hydrolyzed to acetate and choline catalyzed by acetylcholinesterase (*AChE*), allowing for recapture of much of the choline by ChT. Because of the differing ionic compositions in the extracellular milieu and within the cell, ChT is thought to be active only when situated on the nerve cell membrane. Similarly, the VAChT may only be active when encapsulated in the synaptic vesicle [19].

Brain ChAT has a K_D for choline of approximately 1 mmol/l and for acetyl coenzyme A (CoA) of approximately 10 µmol/l. The activity of the isolated enzyme, assayed in the presence of optimal concentrations of cofactors and substrates, appears far greater than the rate at which choline is converted to ACh *in vivo*. This suggests that the activity of ChAT is repressed *in vivo*. Surprisingly, inhibitors of ChAT do not decrease ACh synthesis when used *in vivo*; this may reflect a failure to achieve a sufficient local concentration of inhibitor, but also suggests that this step is not rate-limiting in the synthesis of ACh [18–20].

The acetyl CoA used for ACh synthesis in mammalian brain comes from pyruvate formed from glucose. It is unclear how the acetyl CoA, generally thought to be formed at the inner membrane of the mitochondria, accesses the cytoplasmic ChAT. This may also be a regulated, rate-limiting step.

Acetylcholine formation is limited by the intracellular concentration of choline, which is determined by active transport of choline into the nerve ending. Choline is present in the plasma at a concentration of about 10 µmol/l. A 'low-affinity' choline uptake system with a K_m of 10–100 µmol/l is present in all tissues, but cholinergic neurons also have a Na^+-dependent 'high-affinity' choline uptake system, with a K_m for choline of 1–5 µmol/l [17–20]. The high-affinity uptake mechanisms should be saturated at 10 µmol/l choline, so the plasma choline concentration is probably adequate for sustained ACh synthesis even under conditions of high demand, as observed in ganglia. Since the plasma concentration of choline is above the K_m of the high-affinity choline-transport system, it is not expected that choline concentrations in the nerve ending would be increased by increasing the plasma concentration of choline or by changing the K_m of the uptake system. However, neuronal choline content can be influenced by altering the capacity of the high-affinity choline-uptake mechanism, i.e. by changing the maximum velocity (V_{max}) for transport. The cloning of the high affinity choline transporter has provided considerable insight into the mechanisms by which events associated with neuronal

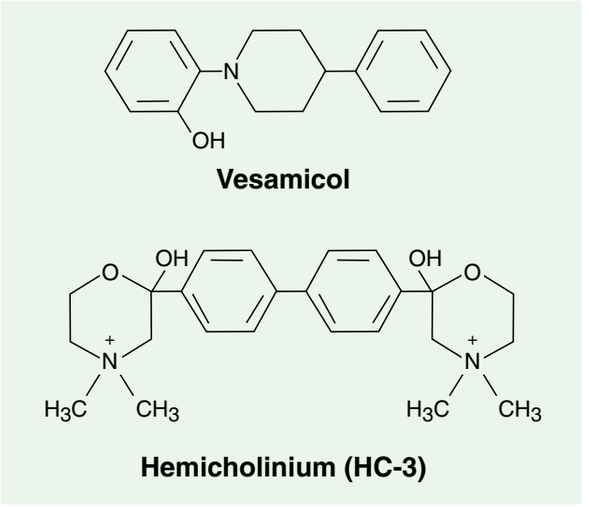


FIGURE 11-5 Structures of hemicholinium (HC-3) and vesamicol.

activity enhance choline entry into neurons [17, 19]. If the K_m of ChAT for choline *in vivo* is as high as that seen with the purified enzyme, one would expect ACh synthesis to increase in proportion to the greater availability of choline. Conversely, ACh synthesis should be diminished when high-affinity choline uptake is blocked. Hemicholinium-3 is a potent inhibitor of the high-affinity choline-uptake system, with a K_i in the submicromolar range (Fig. 11-5). Indeed, treatment with this drug decreases ACh synthesis and leads to a reduction in ACh release during prolonged stimulation; these findings lend support to the notion that choline uptake is the rate-limiting factor in the biosynthesis of ACh [18].

Characterization and regulation of the high affinity choline transporter. The successful cloning of the high affinity choline transporter [17], now referred to as the CHT, was spurred by the discovery of a *Caenorhabditis elegans* transporter exhibiting cholinergic neuron specificity. The homologous rat CHT cDNA conferred high affinity Na^+ and Cl^- dependent choline transport when expressed in *Xenopus* oocytes, and subsequently homologous cDNAs were cloned from numerous species including mice and humans [19]. The gene structure is highly conserved amongst the species, and the encoded protein is a member of a family of Na^+-dependent transporters. The structure reveals 13 transmembrane spans, glycosylation sites on the extracellular surface and several potential sites for phosphorylation on intracellular loops and at the C-terminus. The development of antibodies to CHT revealed the anticipated localization of CHT at presynaptic sites and exclusively in cholinergic neurons. Surprisingly, however, much of the CHT immunoreactivity was found associated with intracellular vesicles rather than at the presynaptic plasma membrane. These vesicles appear to be those involved in ACh synthesis (and its subsequent storage and release) as CHT is co-localized with the vesicular ACh transporter (VAChT) described below. Interestingly, blocking endocytosis leads to CHT accumulation at the plasma membrane and an enhanced capacity for choline uptake [19]. Thus, it appears that the transporter is normally removed from the plasma membrane and recycled. Conversely, stimuli that trigger ACh release have been shown to increase CHT density at the plasma membrane, providing a mechanism by which uptake can be tightly coupled to the rate of ACh release. It is likely that post-translational modification, e.g. phosphorylation, also serves to regulate the transporter in response to altered synaptic activity. Whether this occurs via activation of a resident plasma membrane pool of CHT or alterations in activity or recycling of the vesicular CHT pool remains to be determined. The transporter precursor progresses down the axons by axoplasmic transport and is localized to the vesicular environment by poorly understood mechanisms.

A second transport system concentrates acetylcholine in the synaptic vesicle. The vesicular ACh transporter (VAChT) also has been cloned and expressed (see also Ch. 5). Its sequence places it in the 12-membrane-spanning family characteristic of the other biogenic amine transporters found in adrenergic nerve endings [18, 20, 21]. Interestingly, the gene encoding the transporter is located within an intron of the ChAT gene, suggesting a mechanism for coregulation of gene expression for ChAT and VAChT [20]. ACh uptake into the vesicle is driven by a proton-pumping ATPase. Coupled countertransport of H^+ and ACh allows the vesicle to remain isoosmotic and electroneutral [18].

A selective inhibitor of ACh transport, vesamicol (Fig. 11-5), inhibits vesicular ACh uptake with an IC_{50} of 40 nnol/l [16–18]. Inhibition appears noncompetitive, suggesting that this inhibitor acts on a site other than the ACh-binding site on the transporter. Vesamicol blocks the evoked release of newly synthesized ACh without significantly affecting high-affinity choline uptake, ACh synthesis or Ca^{2+} influx. The fact that ACh release is lost secondary to the blockade of uptake by the vesicle supports the concept that the vesicle is the site of ACh release. The activity of the recombinant transporter also is inhibited by vesamicol [19, 20].

Choline is supplied to the neuron either from plasma or by metabolism of choline-containing compounds. The majority of the choline used in ACh synthesis is thought to come directly from recycling of released ACh, hydrolyzed to choline by acetylcholinesterase (Fig. 11-4). Presumably, uptake of this neurotransmitter-derived choline occurs rapidly, before the choline diffuses away from the synaptic cleft. Another source of choline is the breakdown of phosphatidylcholine, which may be stimulated by locally released ACh. Choline derived from these two sources is then subject to high-affinity uptake into the

nerve ending. In the CNS, these metabolic sources of choline appear to be particularly important because choline in the plasma cannot readily pass the blood–brain barrier. Thus, the high-affinity uptake of choline into cholinergic neurons in the CNS might not always be saturated, and ACh synthesis could be limited by the supply of choline, at least during sustained activity. This would be consistent with the finding that ACh stores in the brain are subject to variation, whereas ACh stores in ganglia and muscles remain relatively constant.

A slow release of acetylcholine from neurons at rest probably occurs at all cholinergic synapses. Quantized ACh release was described first by Fatt and Katz, who recorded small, spontaneous depolarizations at frog neuromuscular junctions that were subthreshold for triggering action potentials. These MEPPs were shown to be due to the release of ACh. When the nerve was stimulated and endplate potentials recorded and analyzed, the magnitude of these potentials always was found to be some multiple of the magnitude of the individual MEPPs. It was suggested that each MEPP resulted from a finite quantity or quantum of released ACh and that the endplate potentials resulted from release of greater numbers of quanta during nerve stimulation (see Ch. 10).

A possible structural basis for these discrete units of transmitter was discovered shortly thereafter when independent electron microscopic and subcellular fractionation studies by de Robertis and Whittaker revealed the presence of vesicles in cholinergic nerve endings. These investigators defined procedures for subcellular fractionation of mammalian brain and *Torpedo* electric organs that yield resealed nerve endings, or synaptosomes, that can be lysed to release a fraction enriched in vesicles. More than half of the ACh in the synaptosome is associated with particles that look like the vesicles seen under an electron microscope. Therefore, it is clear that ACh is associated with a vesicle fraction, and it is likely that it is contained within the vesicle. The origin of the free ACh within the synaptosome is less clear. It may be ACh that is normally in the cytosol of the nerve ending, or it may be an artifact of release from the vesicles during their preparation (see Ch. 10).

The relationship between acetylcholine content in a vesicle and the quanta of acetylcholine released can only be estimated. Estimates of the amount of ACh contained within cholinergic vesicles vary considerably [22]. Whittaker estimated that there are about 2,000 molecules of ACh in a cholinergic vesicle from the CNS. A similar estimate of about 1,600 molecules of ACh per vesicle was made using sympathetic ganglia. The most abundant source of cholinergic synaptic vesicles is the electric organ of *Torpedo*. Vesicles from *Torpedo* are far larger than those from mammalian species and are estimated to contain up to 100 times more ACh per vesicle. The *Torpedo* vesicle also contains ATP and, in its core, a proteoglycan of the heparin sulfate type. Both of these constituents may serve as counter-ions for ACh, which otherwise would be at a hyperosmotic concentration.

The amount of ACh in a quantum has been estimated by comparing the potential changes associated with MEPPs to those obtained by iontophoresis of known quantities of ACh. Based on such analysis, the amount of ACh per quantum at the snake neuromuscular junction was estimated to be something less than 10,000 molecules [22]. Since this compares with estimates of vesicle content, quanta are likely defined by the amount of releasable ACh in the vesicle. An alternative favored by some investigators is that ACh is released directly from the cytoplasm. In this model, definable quanta are evident because channels in the membrane are open for finite periods of time when Ca^{2+} is elevated. A presynaptic membrane protein suggested to mediate Ca^{2+}-dependent translocation of ACh has been isolated [21]. Although some arguments support this model, most investigators favor the notion that the vesicle serves not only as a unit of storage but also as a unit of release. The vesicle hypothesis and release of neurotransmitters are discussed also in Chapter 10.

Depolarization of the nerve terminal by an action potential increases the number of quanta released per unit time. Release of ACh requires the presence of extracellular Ca^{2+}, which enters the neuron when it is depolarized. Most investigators are of the opinion that a voltage-dependent Ca^{2+} current is the initial event responsible for transmitter release, which occurs about 200 μs later. The mechanism through which elevated Ca^{2+} increases the probability of ACh release is not yet known; presumably activation of proteins in the synaptic vesicle membrane fusion complex causes the vesicle to fuse with the neuronal membrane. Dependence on Ca^{2+} is a common feature of all exocytotic release mechanisms and it is likely that exocytosis is a conserved mechanism for transmitter release. There is good evidence that adrenergic vesicles empty their contents into the synaptic cleft because norepinephrine and epinephrine are released along with other contents of the storage vesicle. Although less rigorous data are available for cholinergic systems, cholinergic vesicles contain ATP, and release of ATP has been shown to accompany ACh secretion from these vesicles. Furthermore, Heuser and Reese demonstrated, in electron microscopic studies at frog nerve terminals, that vesicles fuse with the nerve membrane and that vesicular contents appear to be released by exocytosis; it has been difficult to ascertain, however, whether the fusions are sufficiently frequent to account for release on stimulation. The nerve ending also appears to endocytose the outer vesicle membrane to form vesicles that subsequently are refilled with ACh [19, 23].

All the acetylcholine contained within the cholinergic neuron does not behave as if in a single compartment. Results of a variety of neurophysiological and biochemical experiments suggest the presence of two distinguishable

pools of ACh, only one of which is readily available for release. These have been referred to as the 'readily available' or 'depot' pool and the 'reserve' or 'stationary' pool. The reserve pool refills the readily available pool as it is utilized. Unless the rate of mobilization of ACh into the readily available pool is adequate, the amount of ACh that can be released may be limited. It is also likely that newly synthesized ACh is used to fill the readily available pool of ACh because it is the newly synthesized ACh that is released preferentially during nerve stimulation. The precise relationship between these functionally defined pools and ACh storage vesicles is not known. It is possible that the readily available pool resides in vesicles poised for release near the nerve ending membrane, whereas the reserve pool is in more distant vesicles.

ACETYLCHOLINESTERASE AND THE TERMINATION OF ACETYLCHOLINE ACTION

Cholinesterases are widely distributed throughout the body in both neuronal and non-neuronal tissues. Based largely on substrate specificity, the cholinesterases are subdivided into the acetylcholinesterases (AChEs) (EC 3.1.1.7) and the butyrylcholinesterase (BuChE) (EC 3.1.1.8) [24–26]. Choline esters with acyl groups of the size of butyric acid or larger are hydrolyzed very slowly by AChE; selective inhibitors for each enzyme have been identified. AChE and BuChE are encoded by single but distinct genes. BuChE is synthesized primarily in the liver and appears in plasma; however, it is highly unlikely that appreciable concentrations of ACh diffuse from the locality of the synapse and elicit a systemic response. That the population distribution of BuChE mutations correlates with resistance of the enzyme to naturally occurring inhibitors suggests that this enzyme hydrolyzes dietary esters of potential toxicity. Although BuChE is localized in the nervous system during development, the existence of nonexpressing mutations in the BuChE gene within the human population also demonstrates that this enzyme is not essential for nervous system function. In general, AChE distribution correlates with innervation and development in the nervous system. The AChEs also exhibit synaptic localization upon synapse formation. Total knock out of the AChE gene in mice gives rise to an animal with continuous tremors, stunted growth and deficits in neurologic function [27].

The primary and tertiary structures of the cholinesterases are known. The primary structures of the cholinesterases initially defined a large and functionally eclectic superfamily of proteins, the α,β hydrolase fold family, that function not only catalytically as hydrolases but also as surface adhesion molecules forming heterologous cell contacts, as seen in the structurally related proteins neuroligin and the tactins. A sequence homologous to the cholinesterases and a presumed common structural matrix are found in thyroglobulin, in which tyrosine residues become iodinated and conjugated to form thyroid hormone [24, 25].

The initial solution of the crystal structure of the *Torpedo* enzyme [28], followed by the mammalian AChE structure [29], revealed that the active center serine lies at the base of a rather narrow gorge that is lined heavily with aromatic residues (Fig. 11-6). The enzyme carries a net negative charge, and an electrostatic dipole is oriented on the enzyme to facilitate diffusional entry of cationic ligands. Crystal structures of several inhibitors in a complex with AChE also have been elucidated [25].

Acetylcholinesterases exist in several molecular forms governed by alternative splicing. The open reading frame in mammalian AChE genes is encoded by three invariant exons (exons 2, 3 and 4) followed by three splicing alternatives. Continuation through exon 4 gives rise to a monomeric species. Splicing to exon 5 gives rise to the carboxyl-terminal sequence signal for addition of glycophospholipid, while splicing to exon 6 encodes a sequence containing a cysteine that links to other catalytic or structural subunits. These species of AChE differ only in the last 40 residues in their C-termini.

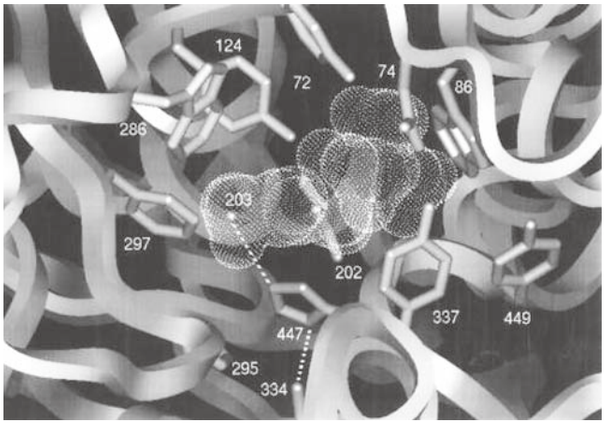


FIGURE 11-6 View of the active center gorge of mammalian acetylcholinesterase looking into the gorge cavity. The gorge is 18–20 Å in depth in a molecule of 40 Å diameter and is heavily lined with aromatic amino acid side chains. Side chains from several sets of critical residues are shown emanating from the α carbon of the α carbon-amide backbone: (a) A catalytic triad between Glu 334, His 447 and Ser 203 is shown by dotted lines to denote the hydrogen-bonding pattern. This renders Ser 203 more nucleophilic to attack the carbon of acetylcholine (shown in white with the van der Waals surface). This leads to formation of an acetyl enzyme, which is deacetylated rapidly. (b) The acyl pocket outline by Phe 295 and 297 is of restricted size in acetylcholinesterase. In butyrylcholinesterase, these side chains are aliphatic, increasing the size and flexibility in the acyl pocket. (c) The choline subsite lined by the aromatic residues Trp 86, Tyr 337 and Tyr 449 and the anionic residue Glu 202. (d) A peripheral site which resides at the rim of the gorge encompasses Trp 286, Tyr 72, Tyr 124 and Asp 74. This site modulates catalysis by binding inhibitors or, at high concentrations, a second substrate molecule.

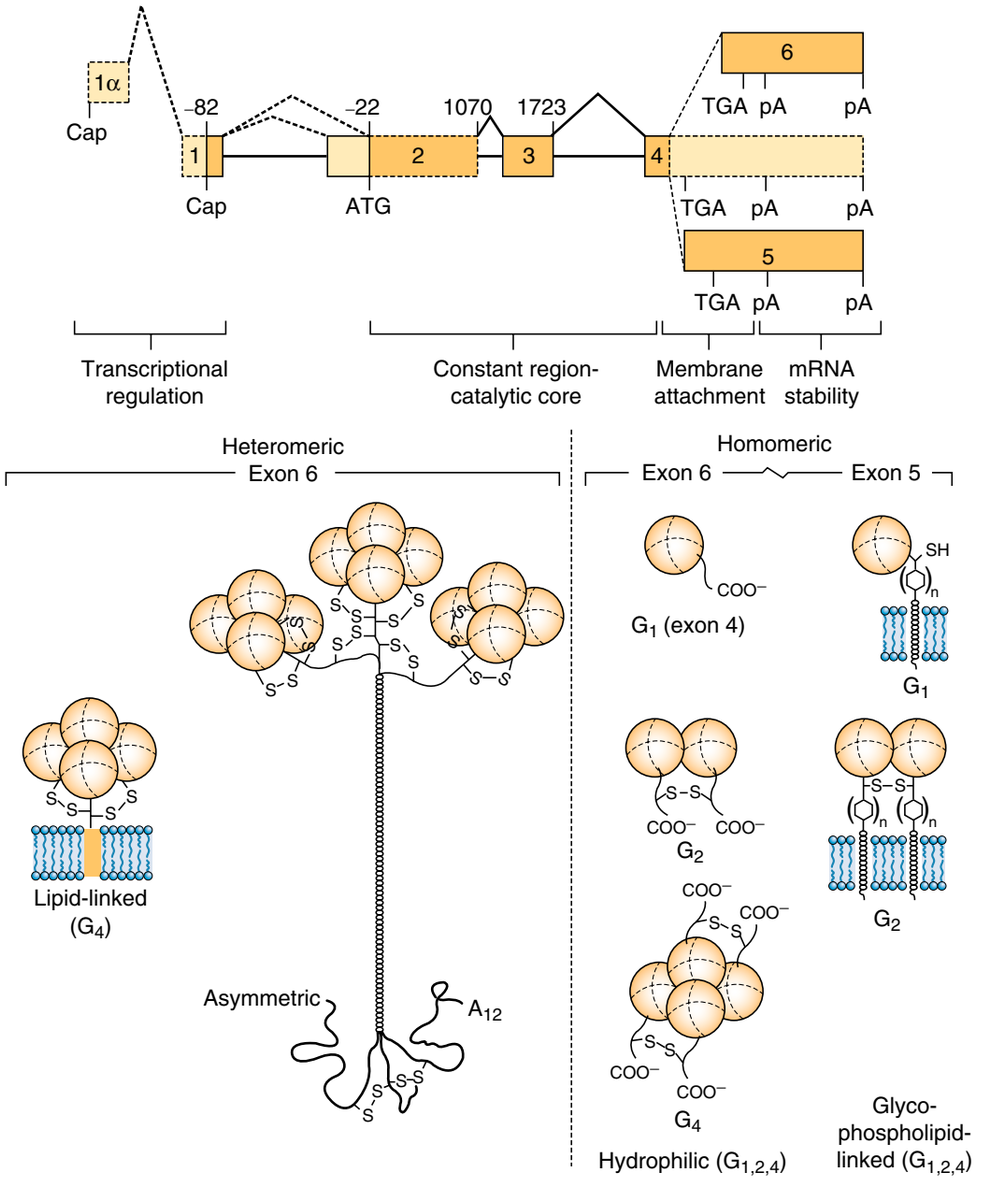


FIGURE 11-7 Gene structure of AChE. Alternative cap sites in the 5′ end of the gene allow for alternative promoter usage in different tissues. Skeletal-muscle-specific regulation is controlled by the intron region between Exons 1 and 2. Exons 2, 3 and 4 encode an invariant core of the molecule that contains the essential catalytic residues. Just prior to the stop codon, three splicing alternatives are evident: *1*, a continuation of exon 4; *2*, the 4–5 splice; and *3*, the 4–6 splice. The catalytic subunits produced differ only in their carboxy-termini and are shown in the lower panel. (Modified with permission from reference [24].)

These AChE forms differ in solubility and mode of membrane attachment rather than in catalytic activity. One class of molecular forms exists as homomeric assemblies of catalytic subunits that appear as monomers, dimers or tetramers (Fig. 11-7). These forms also differ in hydrophobicity, and their amphiphilic character arises from either exposure of an amphipathic helix or post-translational addition of a glycophospholipid on the carboxyl-terminal amino acid. The glycophospholipid allows the enzyme to be tethered on the external surface of the cell membrane.

The second class of AChEs exists as heteromeric assemblies of catalytic and structural subunits. One form consists of up to 12 catalytic subunits linked by disulfide bonds to filamentous, collagen-containing structural subunits. These forms are often termed *asymmetric*, since the tail unit imparts substantial dimensional asymmetry to the molecule. The collagenous tail unit links by disulfide bonding at its proline rich N-terminus through a coiled coil arrangement to the C-terminus of two of the catalytic subunits [30]. The tail unit associates with the basal lamina of the synapse rather than the plasma membrane.

Asymmetric forms are present in high density at the neuromuscular junction. A second type of structural subunit, found primarily in the CNS, has a similar proline-rich attachment domain but contains covalently attached lipid, enabling this form of the enzyme to associate with cell membranes. The different C-termini and attachment modes lead to distinctive extracellular localizations of AChE but do not affect the intrinsic catalytic activities of the individual forms.

Cholinesterase catalysis and inhibition mechanisms involve formation of reversible complexes and covalent conjugates. In ester catalysis, an initial acylation step proceeds through the formation of a tetrahedral transition state; acylation is rapidly followed by deacylation. Alkylphosphate inhibitors are tetrahedral in configuration and their geometric resemblance to the transition state in part accounts for their effectiveness as inhibitors of AChE. Acylation occurs on the active-site Ser 202, which is rendered nucleophilic by proton withdrawal by Glu 334 through His 447. The acetyl enzyme that is formed is short-lived, lasting approximately 10 μs; this accounts for the high catalytic efficiency of the enzyme (Fig. 11-6). The availability of a crystal structure of AChE has enabled investigators to assign residues and domains in the cholinesterase responsible for catalysis and inhibitor specificity [26, 28, 29].

Several AChE inhibitors are used therapeutically in glaucoma, myasthenia gravis and smooth muscle atony, whereas others have proved useful as insecticides. Still others have been produced for more insidious uses in terrorism and chemical warfare. Inhibitors such as edrophonium bind reversibly to the active center of AChE at the base of the gorge (Fig. 11-6). Other reversible inhibitors, such as gallamine, propidium and the three-fingered peptide from snake venom, fasciculin, bind to a peripheral site located at the gorge rim. The carbamoylating agents, such as neostigmine and physostigmine, form a carbamoyl enzyme by reacting with the active-site serine. The carbamoyl enzymes are more stable than the acetyl enzyme; their decarbamoylation occurs over several minutes. Since the carbamoyl enzyme will not hydrolyze ACh, the carbamoylating agents are alternative substrates that are effective inhibitors of ACh hydrolysis. The alkylphosphates, such as diisopropylfluorophosphate or echothiophate, act in a similar manner; however, the alkylphosphorates and alkylphosphonates form extremely stable bonds with the active-site serine on the enzyme. The time required for their hydrolysis often exceeds that for biosynthesis and turnover of the enzyme. Accordingly, inhibition with the alkylphosphates is effectively irreversible.

Consequences of acetylcholinesterase inhibition differ with effector site. At postganglionic parasympathetic effector sites, AChE inhibition enhances or potentiates the action of administered ACh or ACh released by nerve activity. In part, this is a consequence of diffusion of transmitter and stimulation of receptors extending over a larger area from the point of transmitter release. Similarly, ganglionic transmission is enhanced by cholinesterase inhibitors. Since atropine and other muscarinic antagonists are effective antidotes of AChE inhibitor toxicity, some CNS manifestations result largely from excessive muscarinic stimulation.

By prolonging the residence time of ACh in the synapse, AChE inhibition in the neuromuscular junction promotes a persistent depolarization of the motor endplate. The decay of endplate currents or potentials resulting from spontaneous release of ACh is prolonged from 1–2 ms to 5–30 ms. This indicates that the transmitter activates multiple receptors before diffusing from the synapse. Excessive depolarization of the endplate, resulting from slower rates of decay of endplate currents, leads to a diminished capacity to initiate coordinated action potentials. In a fashion similar to depolarizing blocking agents, fasciculations and muscle twitching are observed initially with AChE inhibition, followed by flaccid paralysis.

NICOTINIC RECEPTORS

The nicotinic acetylcholine receptor (nAChR) was the first characterized neurotransmitter receptor. The nAChR was purified a decade earlier than other neurotransmitter receptors. The electric organ of *Torpedo*, consisting of stacks of electrocytes, tissue of common embryonic origin to skeletal muscle, is a rich source of nicotinic receptors. Upon differentiation, the electrogenic bud in the electrocyte proliferates but the contractile elements are not expressed. The excitable membrane encompasses the entire ventral surface of the electrocyte rather than being localized to focal junctional areas covering ≈0.1% of the cell surface as found in skeletal muscle. The electrical discharge in *Torpedo* relies solely on a PSEP resulting from depolarization of the postsynaptic membrane, rather than propagation from an action potential. The density of receptors in the *Torpedo* electric organ approaches 100 pmol/mg protein, which may be compared with 0.1 pmol/mg protein in skeletal muscle.

In the early 1960s, it was established that snake α-toxins, such as α-bungarotoxin, irreversibly inactivate receptor function in intact skeletal muscle, and this finding led directly to the identification and subsequent isolation of the nicotinic ACh receptor from *Torpedo* [3]. By virtue of their high affinity and very slow rates of dissociation, labeled α-toxins serve as markers of the receptor during solubilization and purification. Sufficient amino acid sequence of the receptor was obtained to permit the cloning and sequencing of the genes encoding the individual subunits of the receptor, first in *Torpedo* and then in other species [4]. As a consequence of the high density of nicotinic ACh receptors in the postsynaptic membranes of *Torpedo*, sufficient order of the receptor molecules is achieved in isolated membrane fragments such that image

reconstructions from electron microscopy have allowed a more detailed analysis of structure [31]. Labeling of functional sites, determination of subunit composition and structure modification through mutagenesis contributed to our understanding of the structure of nicotinic receptors [32]. Recently, a soluble acetylcholine binding protein (AChBP) from mollusks was purified and its three dimensional structure determined (Fig. 11-8) [33]). Not only are these pentameric proteins structural surrogates of the extracellular domain of the nAChR [33] but, when appropriately linked to the transmembrane spans of nAChR family, they gate ions in response to agonists and are blocked by antagonists [34].

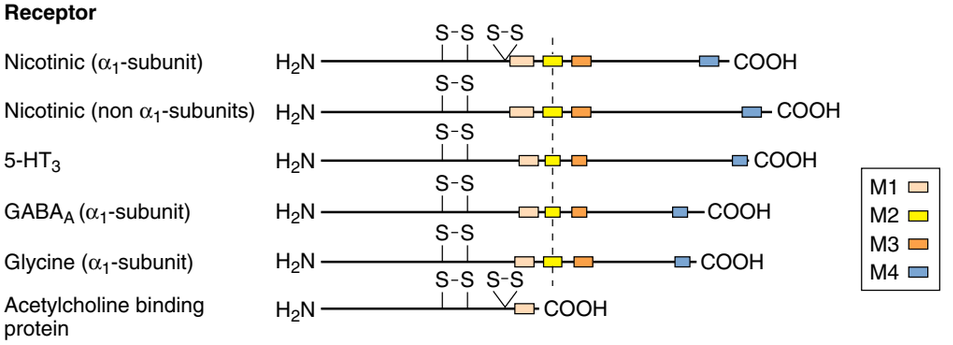
The nAChR consists of five subunits arranged around a pseudoaxis of symmetry. The subunits display homologous amino acid sequences with 30–40% identity of amino acid residues [4, 11, 32]. In muscle, one subunit, designated α, is expressed in two copies; the other three, β, γ and δ, are present as single copies (Fig. 11-9). Thus, the receptor is a pentamer of molecular mass of approximately 280 kDa. Structural studies show the subunits to be arranged around a central cavity, with the largest portion of the protein exposed toward the extracellular surface. The central cavity is believed to lead to the ion channel, which in the resting state is impermeable to ions; upon activation, however, it opens to a diameter of 6.5 Å. The open channel is selective for cations. The two α subunits and the opposing face of the γ and δ subunits form the two sites for binding of agonists and competitive antagonists and provide the primary surfaces with which the larger snake α-toxins associate. A similar pentameric ring of subunits is found for AChBP [8, 33, 34] with the binding sites located at the five interfaces between the identical subunits of this homomeric pentamer (Fig. 11-8). The sites for ligand binding are localized toward the external perimeter of each of the ligand binding subunit interfaces; occupation of multiple sites on the pentamer is necessary for receptor activation. Electrophysiological and ligand-binding measurements together with analysis of the functional states of the receptor indicate positive cooperativity in the association of agonists; Hill coefficients greater than unity have been described for agonist-elicited channel opening, agonist binding and agonist-induced desensitization of the receptor [3, 32]. Noncompetitive inhibitor sites within various depths of the internal channel also have been defined and are the sites of local anesthetic inhibition of receptor function.

Sequence identity among the subunits appears to be greatest in the hydrophobic regions. Various models for the disposition of the peptide chains and subunit assembly have been proposed on the basis of hydropathy plots, electron microscopic reconstruction analysis of *Torpedo* receptor membranes and the AChBP crystal structure [30, 33] (Figs 11-8, 11-9). Homology among the four subunits strongly suggests that the same folding pattern is found in all subunits.

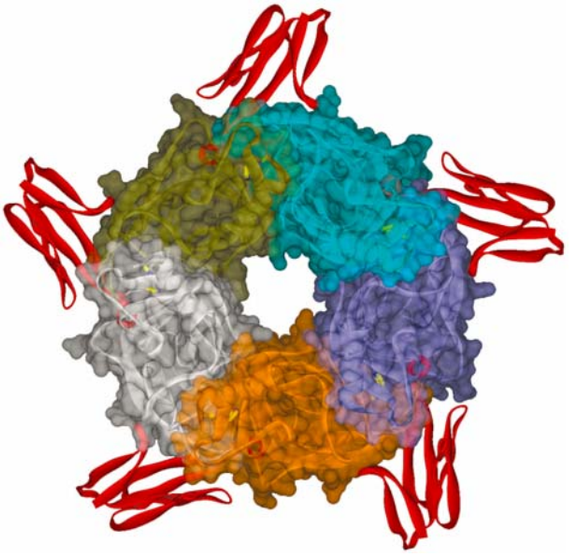
A disulfide loop between Cys 128 and 142 in the α subunit is conserved in the entire receptor channel family (Fig. 11-8). A second disulfide is found in the α subunits between vicinal Cys 192 and 193, and this structural feature is also found in AChBP. Early studies showed that reduction of the Cys 192–193 bond allowed for labeling by the site-directed sulfhydryl-reactive agonist and antagonist, respectively, bromoacetylcholine and *m*-maleimidobenzyl trimethylammonium [32]. Subsequent studies involving photolytic labeling, labeling by the natural coral toxin, lophotoxin, and site-specific mutagenesis identified the region between residues 185 and 200 in the α subunit as being important for forming part of the agonist- and

FIGURE 11-8 Features of the sequence of the acetylcholine receptor. (**A**) Schematic drawing of the sequence showing candidate regions for spanning the membrane. The region M_2 is believed to be an α helical segment and lines the internal pore of the receptor. M_1, M_3 and M_4 contain hydrophobic sequences but it is not certain whether they traverse the membrane as full α helices. The nicotinic β, γ and δ subunits contain homologous M_1 through M_4 hydrophobic domains at similar positions in the linear sequence. Two disulfide loops, 128–142 and 192–193, in the α subunits are shown. While the other subunits contain the larger disulfide loop, they lack cysteines 192 and 193 and tyrosine 190. Sequence features of other homologous subunits in ligand-gated channel (5-hydroxytryptamine [*5-HT*$_3$], GABA and glycine) receptors are shown. The amino-terminal portion is found on the extracellular (synaptic) surface. The soluble ACh binding proteins characterized from *Lymnaea* and *Aplysia* have truncated sequences ending at residue 202–204 and an absence of hydrophobic domains designed to interact with sequences that span the membrane or interact with membrane lipids. (**B**) Structure of the acetylcholine binding protein from *Lymnaea* as a model of the extracellular domain of the nicotinic acetylcholine receptor. Structures are developed from the crystallographic coordinates of Sixma and colleagues [33]. **B-1**. View of the pentameric protein from the apical surface. The protein is a pentamer of five identical subunits surrounding a central cavity, which in the receptor is the vestibule to the ion channel pore. Although the subunits are identical, they are distinguished by color. **B-2**. View of subunit interfaces from the outer perimeter. The C loop, containing the second disulfide bond with vicinal cysteines, overlaps the complementary face on the adjacent subunit. The subunit interfaces (shown in gray, orange and blue and located apical to the C loop) contain binding locations of nicotine and α-neurotoxin (ribbon diagram, shown in red and largely on the membrane side of the C loop) [33]. **B-3**. Interface of the binding protein to the transmembrane spans of the ion channel. Modification of selected residues allowed the acetylcholine binding protein, when linked to the transmembrane spans of the channel, to become a functional ligand-gated ion channel with the expected ligand specificity and channel gating kinetics. **Top** (**a**): Schematic diagram of a chimaeric subunit composed of AChBP and 5-HT_{3A} sequences. The amino terminus contains AChBP (white bars and black font) and 5-HT_{3A} sequences (loop β1-β2, orange; Cys loop , green; β8-β9 loop, magenta). The 5-HT_{3A} pore domain follows, with transmembrane helices M1-M4 (red) and the M2-M3 linker (blue). Red font indicates the start of the M1 domain. **Middle** (**b**): Homology model of the chimaeric receptor with key regions of one subunit colour-coded as in **Top** figure. **Bottom** (**c**): View of the coupling zone from the pore showing the network of binding and pore domain loops. (With permission from reference [34].)

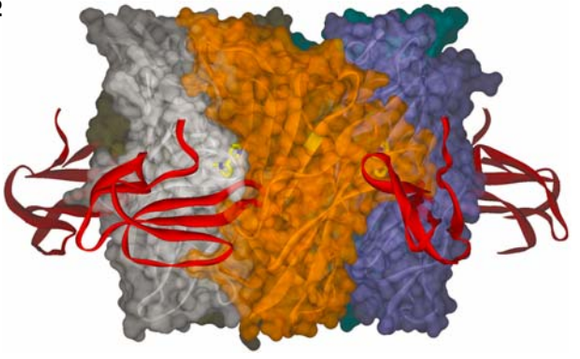
A


Receptor
Nicotinic (α_1-subunit)
Nicotinic (non α_1-subunits)
5-HT_3
$GABA_A$ (α_1-subunit)
Glycine (α_1-subunit)
Acetylcholine binding protein
H_2N
COOH
S-S
M1
M2
M3
M4

B-1

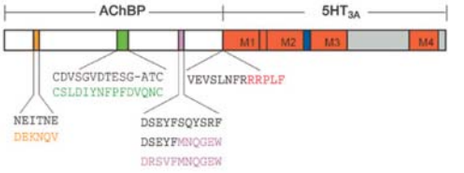


B-2

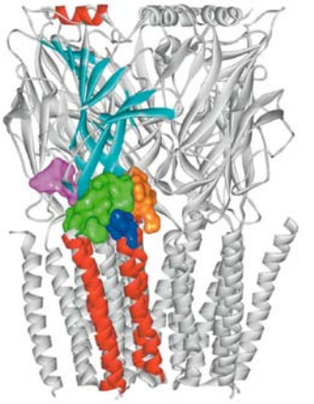


B-3

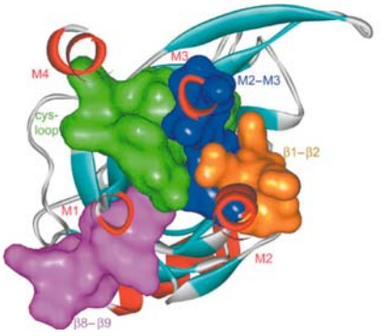
(a)


AChBP
$5HT_{3A}$
M1
M2
M3
M4
CDVSGVDTESG-ATC
CSLDIYNFPFDVQNC
VEVSLNFRRRPLF
NEITNE
DEKNQV
DSEYFSQYSRF
DSEYFMNQGEW
DRSVFMNQGEW

(b)



(c)


M4
M3
M2–M3
Cys-loop
β1–β2
M1
M2
β8–β9

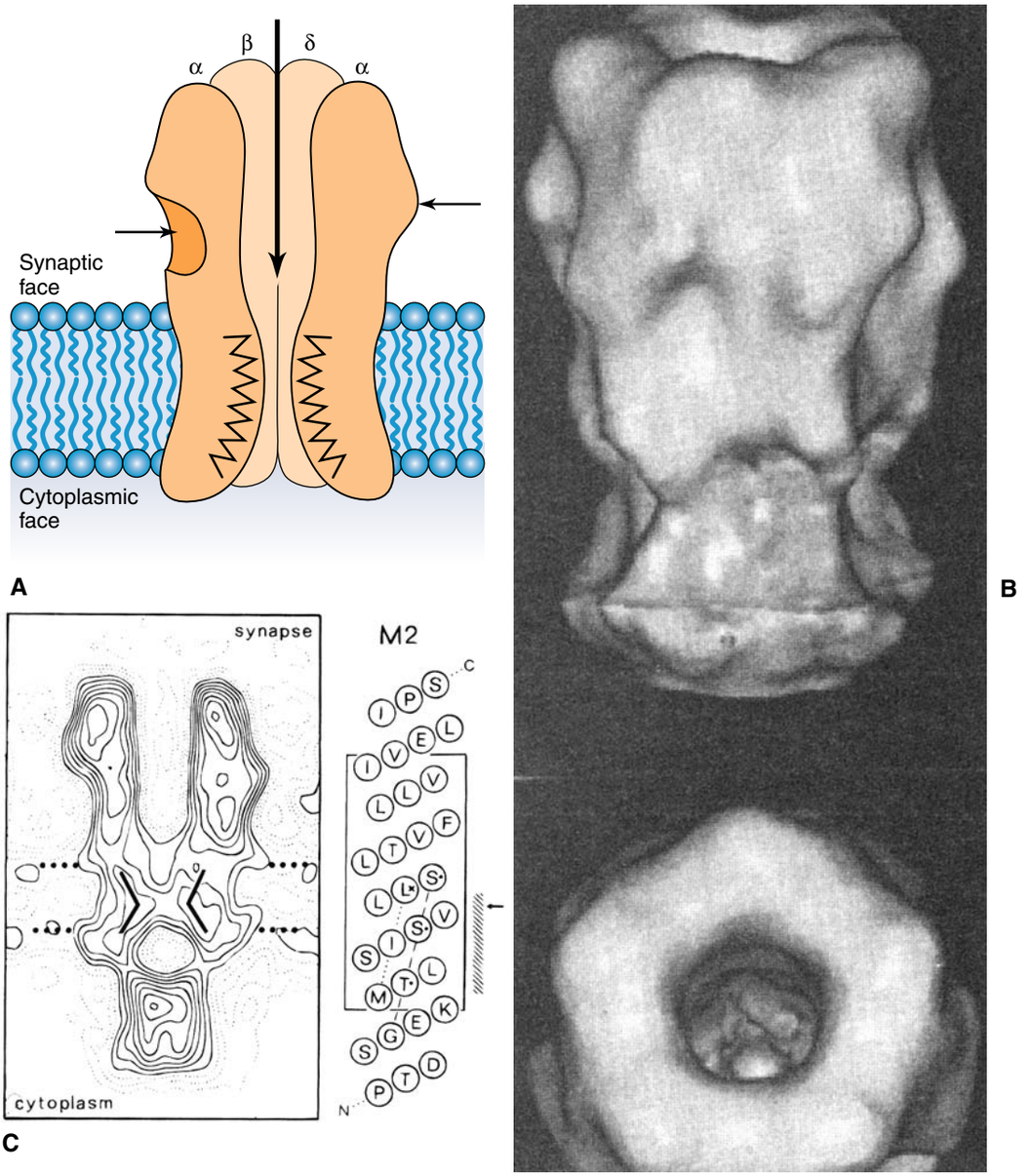


FIGURE 11-9 (**A**) Longitudinal view of the muscle nicotinic acetylcholine receptor with the γ subunit removed. The remaining subunits, two copies of α, one of β and one of δ, surround an internal channel with outer vestibule and its constriction or gating locus deep within the membrane bilayer region. Spans of α helices with bowed structures from the M_2 region of the sequence form the perimeter of the channel (see C). Acetylcholine-binding sites, denoted by *arrows*, are found at the αγ- and αδ (not visible)-subunit interfaces. (**C** shows the data on which this structure is based.) (Adapted with permission from references [25, 59, 60].) (**B**) Image reconstruction of electron micrographs yielding a structure at 9 Å resolution. Shown are side and synaptic views. (Adapted with permission from reference [25, 59, 60].) (**C**) Longitudinal view of the electron density of the receptor. The transmembrane area is shown between the *dots.* The visible transmembrane-spanning helixes are shown by the *V-shaped solid lines.* This helix is believed to be the M_2 region, the sequence of which is shown. The area inside the rectangle is the transmembrane-spanning region. The 'X' denotes the conserved leucine. The additional density in the cytoplasmic region arises from an associated 43 kDa protein, rapsyn. The *shaded area* to the right indicates the zone of narrowest constriction.

antagonist-binding surface. The loop containing the vicinal cysteines overlaps with the neighboring subunit at the outer perimeter of the receptor, and internal to this loop is nested the ligand-binding site. Two other segments of sequence in the α subunit and four discrete segments on the opposing face of the γ and δ subunits also have been identified as forming regions that contribute to the binding surfaces at the αγ and αδ interfaces [34], findings that have now been confirmed from the AChBP–ligand complexes.

Four candidate membrane-spanning regions are found after residue 210 with a large cytoplasmic loop between membrane spans 3 and 4 (Figs 11-8, 11-9). Based on labeling experiments and site-specific mutagenesis, membrane span 2 was found to be proximal to the ion channel. This span, when constructed as an α helix, is amphipathic, with an abundance of serine and threonine residues pointed toward the channel lumen. Positions corresponding to α-Thr 244, α-Leu 251, α-Val 255 and α-Glu 262 in this transmembrane span have been labeled with the noncompetitive, channel-blocking inhibitors chlorpromazine and tetraphenyl phosphonium [3, 32]. Mutation of several of the hydroxyl groups on residues at these positions affects channel kinetics. The channel gate, or constriction, is thought to lie deep within the channel or even close to the cytoplasmic side. The ion selectivity of the channels appears to be controlled in part by rings of charges formed by all five subunits at the extracellular surface of the channel corresponding to α-Glu 262 and at the cytoplasmic surface corresponding to α-Glu241. Exposed amide backbone hydrogens and carbonyl groups and a ring of hydroxylated amino acids corresponding to α-Thr 244 also contribute to ion selectivity and permeation [11, 32, 35].

Analysis of the opening and closing events of individual channels has provided information about ligand binding and activation of the receptor. Electrophysiological studies use high-resistance patch electrodes of 1–2 μm diameter, which form tight seals on the membrane surface [36]. They have the capacity to record conductance changes of individual channels within the lumen of the electrode (see Ch. 10). The patch of membrane affixed to the electrode may be excised, inverted or studied on the intact cell. The individual opening events for ACh achieve a conductance of 25 pS across the membrane and have an opening duration that is distributed exponentially around a value of about 1 ms. The duration of channel opening is dependent on the particular agonist, whereas the conductance of the open-channel state is usually agonist-independent. Analyses of the frequencies of opening events have permitted an estimation of the kinetic constants for channel opening and ligand binding, and these numbers are in reasonable agreement with estimates of ligand binding and activation from rapid kinetic, or stopped-flow, studies. Overall, activation events for the muscle receptor may be described by scheme 1 [3, 11, 36]:

$$2L + R \underset{k\text{-}1}{\overset{2k+1}{\rightleftharpoons}} LR \underset{2k\text{-}1}{\overset{k+1}{\rightleftharpoons}} L_2R \underset{k\text{-}2}{\overset{k\text{-}2}{\rightleftharpoons}} L_2R^*$$

(Closed) (Closed) (Closed) (Open)

Scheme 1

Two ligands (L) associate with the receptor (R) prior to the isomerization step to form the open-channel state L_2R^*. For ACh, the forward rate constant for binding, k_{+1}, is $1–2\times10^8$ mol^{-1} l^{-1} s^{-1}; k_{+2} and k_{-2}, forward and reverse rate constants for isomerization, yield rates of isomerization consistent with opening events in the millisecond time frame. Since k_{+2} and k_{-2} are greater than k_{-1}, the rate constant for ligand dissociation, several opening and closing events with the fully liganded receptor occur prior to dissociation of the first ligand. Binding of the first and second ligands appears not to be identical, even allowing for the statistical differences arising from the two sites. Such a conclusion is consistent with receptor structure since different subunits, such as the γ and δ subunits in muscle, are adjacent to the same face of the α subunits in the muscle receptor pentamer.

Continued exposure of nicotinic receptors to agonist leads to desensitization of the receptors. This diminution of the response occurs even though the concentration of agonist available to the receptor has not changed. Katz and Thesleff examined the kinetics of desensitization with microelectrodes and found that a cyclic scheme in which the receptor existed in two states, R and R′, prior to exposure to the ligand best described the process.

To achieve receptor desensitization and activation by ligand, multiple conformational states of the receptor are required. The binding steps represented in horizontal equilibria are rapid; vertical steps reflect the slow, unimolecular isomerizations involved in desensitization (scheme 2). Rapid isomerization to the open channel state (scheme 1) should be added. To accommodate the additional complexities of the observed fast and slow steps of desensitization, additional states have to be included.

A simplified scheme, in which only one desensitized and one open-channel state of the receptor exist, is represented in scheme 2, where R is the resting (activatable) state, R* the active (open channel) state and R′ the desensitized state of the receptor; M is an allosteric constant defined by R′/R, and K and K' are equilibrium dissociation constants for the ligand.

$$\begin{array}{ccccccc} 2L + R & \underset{}{\overset{k/2}{\rightleftharpoons}} & LR & \underset{}{\overset{2k}{\rightleftharpoons}} & L_2R & \longleftrightarrow & L_2R^* \\ {\scriptstyle M}\uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow & & \\ 2L + R' & \underset{}{\overset{k'/2}{\rightleftharpoons}} & LR' & \underset{}{\overset{2k'}{\rightleftharpoons}} & L_2R' & & \end{array}$$

Scheme 2

In this scheme, $M < 1$ and $K' < K$. Addition of ligand eventually will result in an increased fraction of R′ species because of the values dictated by the equilibrium constants. Direct binding experiments have confirmed the generality of this scheme for nicotinic receptors. Thus, distinct conformational states govern the different temporal responses that ensue upon addition of a ligand to the nicotinic receptor. No direct energy input or covalent modification of the receptor channel is required.

Nicotinic receptor subunits are part of a large superfamily of ligand-gated channels. Nicotinic receptors on neurons, such as those originating in the CNS or neural crest, show ligand specificities distinct from the nicotinic receptor in the neuromuscular junction. One of the most remarkable differences is the resistance of most nicotinic neuronal receptors containing α2–α6 subunits to α-bungarotoxin and related snake α-toxins. This fact and the lack of an abundant source of neuronal CNS receptors limited initial progress in their isolation and characterization. However, low-stringency hybridization with cDNAs encoding the subunits of electric organ and muscle receptors provided a means to clone neuronal nicotinic receptor genes. Isolation of the candidate cDNA clones, their expression in cell systems to yield functional receptors and the discrete regional localizations of the endogenous mRNAs encoding these receptor subunits revealed that the nicotinic receptor subunits are part of a large and widely distributed gene family. They are related in structure and sequence to receptors for inhibitory amino acids (GABA and glycine), to 5-hydroxytryptamine type 3 ($5HT_3$) receptors and, somewhat more distantly, to glutamate receptors (32).

At least 12 distinct genes encoding neuronal nicotinic receptor subunits α2–α10 and β2–β4 have been identified in the central and peripheral nervous systems (Fig. 11-2). The α subunits are similar in sequence to the muscle α1 subunit and contribute to the ligand-binding interface. The β subunits fulfill the role of β1, γ and δ subunits in the muscle receptor. When certain pairs or triplets of cDNAs encoding neuronal α and β subunits are cotransfected into cells or their corresponding mRNAs are injected into oocytes, characteristic ACh-gated channel function can be achieved. The α3 subunit is prevalent in peripheral ganglia, usually with β2, β4, and α5 subunits. The α4β2 subunit combination predominates in the CNS, yet its elimination in knockout studies causes surprisingly limited changes in function. The α5 subunit appears unique in that it will not contribute to function in the absence of other α subunits; its global sequence features are more similar to those of the β subunits. Four permutations of subunits containing α4, α5, α6, β2 and β3 subunits are found in the striatum that mediate dopamine release [36]. The α7 and α8 subunits display function as homologous pentamers. Receptors containing α7 subunits have a high Ca^{2+} permeability, and Ca^{2+} entry may be integral to their function *in vivo*. These receptors also show characteristically rapid rates of desensitization and a high sensitivity to choline as antagonist. α9 and α10 subtypes function in vestibular and cochlear mechanosensory hair cells [37]. While not all combinations of α and β mRNAs lead to the expression of functional receptors on the cell surface, the number of permutations is large [11]. A future challenge is the assignment of pharmacological and biophysical signatures to all of the subunit combinations found *in vivo*.

Substantial evidence points to nicotinic receptors in the CNS functioning at presynaptic locations to regulate release of several CNS transmitters [10]. Electrophysiological and microdialysis studies provide evidence that glutamatergic, dopaminergic, serotonergic, peptidergic and cholinergic pathways are under the control of presynaptic nicotinic receptors. Hence, nicotinic receptors appear to play an important amplification and modulatory role in the CNS. A prime example is the influence of presynaptic nicotinic receptors influencing the release of dopamine that appears to underlie the addictive actions of nicotine [10, 40]. See also Ch. 56.

Both nicotinic receptors and acetylcholinesterase are regulated tightly during differentiation and synapse formation. At present, we understand more about tissue-specific gene expression in muscle than in nerve [41–45]. Both the above proteins show enhanced expression during myogenesis upon differentiating from a mononucleated myoblast to a multinucleated myotube. Curiously, enhanced receptor expression occurs largely by transcriptional activation, while the increase in cholinesterase expression arises to a large extent from stabilization of the mRNA. The receptor appears to cluster spontaneously, which involves a protein on the cytoplasmic side of the membrane, termed 43K or rapsyn (see in Ch. 43) [43, 44]. This protein links the receptor to cytoskeletal elements and restricts its diffusional mobility. Following innervation and synaptic activity, expression of the receptor and AChE persists in endplate, or junctional, regions and disappears in extrajunctional regions. The collagen-tail-containing species of AChE is localized to the basal lamina in the neuromuscular synapse; it links at its distal end to the proteoglycan perlecan [45].

With innervation and the development of electrically excitable synapses, the γ subunit of the receptor is replaced by an ε subunit; small changes in the biophysical properties of the receptor occur concomitantly. Upon denervation, many of the developmental changes associated with innervation are reversed and there is again an increase in expression of extrajunctional receptors containing the γ subunit. In multinucleated muscle cells, particular subsynaptic nuclei drive the expression of these synapse-specific proteins. The factors controlling these regulatory events are incompletely understood, but calcitonin gene-related peptide (CGRP) and the protein ACh receptor-inducing activity (ARIA) may be extracellular mediators of expression. In addition, intracellular Ca^{2+},

membrane depolarization and protein kinase C play distinct roles in maintaining junctional expression of synapse-localized proteins [42–44].

A neurally derived signaling protein, agrin, acts through a receptor tyrosine kinase, MuSK, in the formation of the specialized postsynaptic endplate by interaction with rapsyn. Thus, MuSK–rapsyn interactions are critical in forming the local scaffold for postsynaptic components in the motor endplate [43, 44].

An extensive variety of neuromuscular diseases have been uncovered as congenital conditions involving not only the nAChR and AChE but also proteins that control their expression and synaptic localization. These are discussed in Ch. 43. Study of the underlying genotype or sequence differences has also proved helpful in unraveling the involvement of various amino acid residue determinants in function [41].

MUSCARINIC RECEPTORS

Muscarinic and nicotinic receptors are related more closely to other receptors in their respective families than to one another, both structurally and functionally. The nicotinic receptor is far more similar to other ligand-gated ion channels than to the muscarinic receptor. The muscarinic receptor in turn belongs to a group of seven transmembrane-spanning receptors [46], which transduce their signals across membranes by interacting with GTP-binding proteins (see Ch. 19). Several macromolecular interactions are involved in the responses triggered by activation of the muscarinic receptor. These associations contribute to the 100–250 ms latency characteristic of muscarinic responses, which are slow compared with those mediated by nicotinic receptors.

Muscarinic receptor activation causes inhibition of adenylyl cyclase, stimulation of phospholipase C and regulation of ion channels. Many types of neuron and effector cell respond to muscarinic receptor stimulation. Despite the diversity of responses that ensue, the initial event that follows ligand binding to the muscarinic receptor is, in all cases, the interaction of the receptor with a G protein. Depending on the nature of the G protein and the available effectors, the receptor–G-protein interaction can initiate any of several early biochemical events. Common responses elicited by muscarinic receptor occupation are inhibition of adenylyl cyclase, stimulation of phosphoinositide hydrolysis and regulation of potassium or other ion channels [47] (Fig. 11-10). The particular receptor subtypes eliciting those responses are discussed below. (See also Chs 20 and 21.)

Inhibition of adenylyl cyclase by mAChR activation results in decreased cAMP formation. A decrease in cAMP is most apparent when adenylyl cyclase is stimulated, for example, by activation of adrenergic receptors with catecholamines or by forskolin. Simultaneous addition of cholinergic agonists decreases the amount of cAMP formed in response to the catecholamine, in some tissues almost completely. The result is diminished activation of cAMP-dependent protein kinase (PKA) and decreased substrate phosphorylation catalyzed by this kinase. The mechanism by which the muscarinic receptor inhibits adenylyl cyclase is through activation of an inhibitory GTP-binding protein, G_i. The α subunit of G_i competes with the α subunit of the G protein activated by stimulatory agonists (G_S) for regulation of adenylyl cyclase (see Chs 19, 21). Although muscarinic receptors do not interact with G_s, increases in cAMP formation are seen in response to mAChR stimulation under some circumstances. These may result from stimulatory effects of $\beta\gamma$ subunits released from G_i proteins or from elevated intracellular Ca^{2+} on specific isoforms of adenylyl cyclase or phosphodiesterase.

Activation of phosphoinositide-specific phospholipase C by muscarinic agonists elicits phosphatidylinositol 4,5-bisphosphate stimulates phosphoinositide hydrolysis. The β_1 isoform of phosphoinositide-specific phospholipase C (PI-PLC) is activated by its interaction with the α subunit of a GTP-binding protein, $G_{q/11}$ [48]. This is the primary mechanism by which muscarinic receptors regulate this enzyme. However, some PLC isoforms, most clearly β_2, also are activated by $\beta\gamma$ subunits. This probably accounts for the pertussis-toxin-sensitive, G_i/G_o-mediated activation of PI-PLC seen when high levels of mAChR subtypes that normally couple to G_i (see below) are expressed in heterologous cell lines. The hydrolysis of phosphatidylinositol 4,5-bisphosphate yields two potential second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (see Ch. 20). DAG increases the activity of the Ca^{2+} and phospholipid-dependent protein kinase (PKC). IP_3 mobilizes Ca^{2+} from intracellular stores in the endoplasmic reticulum and thereby elevates cytosolic free Ca^{2+}. Subsequent responses are triggered by direct effects of Ca^{2+} on Ca^{2+}-regulated proteins and by phosphorylation mediated through Ca^{2+}/calmodulin-dependent kinases and PKC. Stimulation of a phospholipase D, which hydrolyzes phosphatidylcholine, also occurs in response to muscarinic receptor activation. This appears to be secondary to activation of PKC and contributes to a secondary rise in DAG.

Regulation of K^+ channels. Muscarinic agonists cause rapid activation of G-protein-coupled, inwardly rectifying potassium channels (GIRKs). This muscarinic effect can be mimicked by GTP analogs in whole-cell clamp experiments, and the response is sensitive to pertussis toxin, which ribosylates and inactivates G_i and a related protein, G_o (see Ch. 19). It is now generally agreed that GIRK1 and GIRK2 are activated directly by binding $\beta\gamma$ subunits released from G_i or G_o. This is a primary mechanism by which muscarinic agonists cause hyperpolarization of

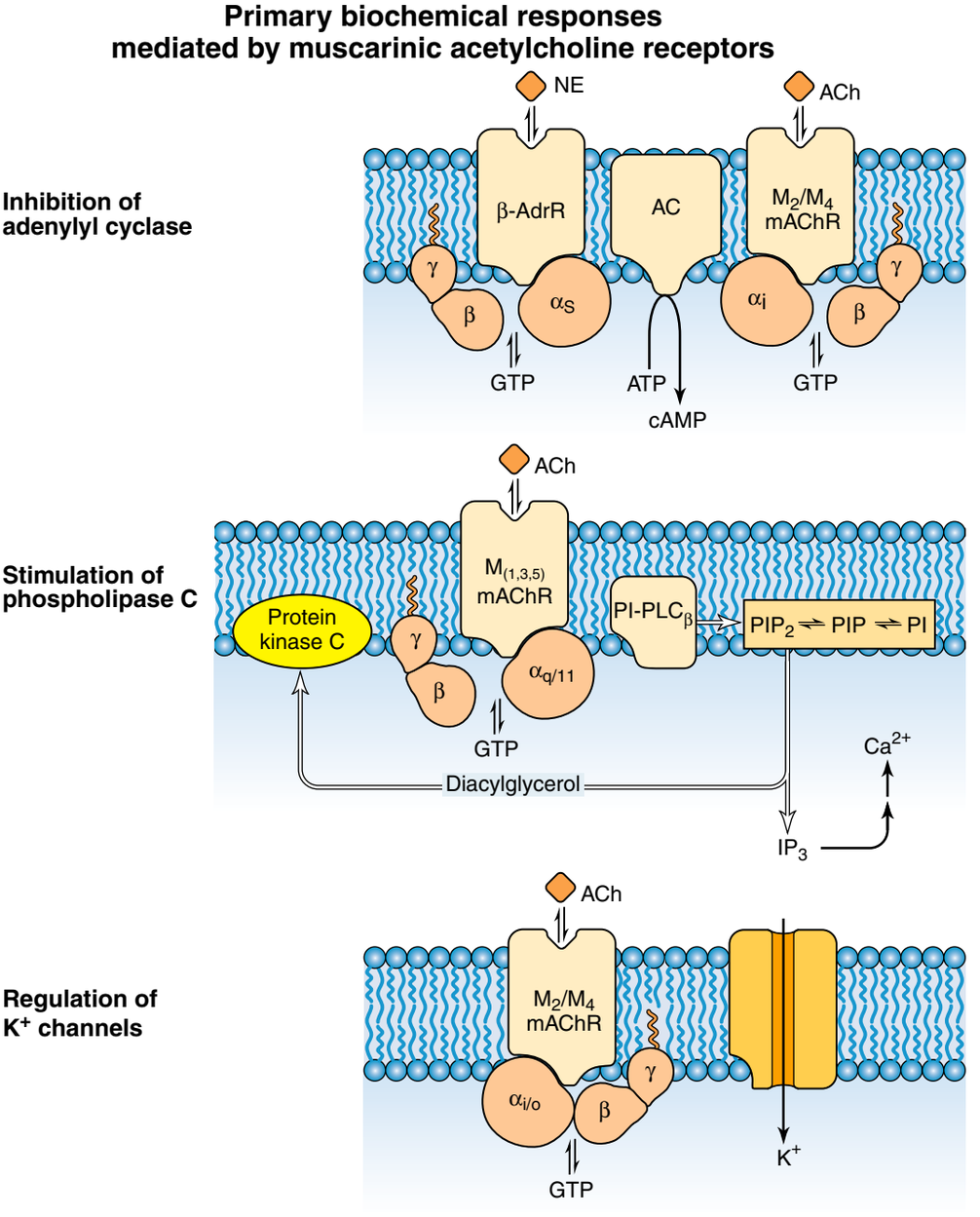


FIGURE 11-10 Acetylcholine (*ACh*) interacts with a muscarinic receptor of the subtypes indicated to induce various responses. The M_2 and M_4 muscarinic acetylcholine receptors (*mAChR*) interact with the α subunit of GTP-binding protein, G_i. When ACh binds, $G\alpha_i$ dissociates from βγ and inhibits adenylyl cyclase (*AC*). The M_1, M_3 and M_5 mAChRs interact with GTP-binding proteins in the G_q and G_{11} family. The $G\alpha_q$ and α_{11} subunits activate phosphoinositide-specific phospholipase C (*PI-PLC*). The M_2 and M_4 mAChRs regulate inwardly rectifying K^+ channels through the βγ subunit of G_i or G_o. Diffusible second messengers formed within the cell include cAMP, inositol trisphosphate (*IP_3*) and diacylglycerol (DAG). IP_3 is generated from phosphatidylinositol bisphosphate (PIP_2). *NE*, norepinephrine; *β-AdrR*, β-adrenergic receptor; *PI*, phosphatidylinositol; *PIP*, phosphatidylinositol-4-phosphate; *PIP_2*, phosphatidylinositol-4,5-bisphosphate.

cardiac atrial cells, as well as of neurons [49]. This pathway contrasts with muscarinic inhibition of the M-current in sympathetic ganglia; suppression of this K^+ channel is mediated indirectly through muscarinic formation of a diffusible second messenger.

Intracellular mediators of muscarinic receptor action. The three events described above, inhibition of adenylyl cyclase, stimulation of PLC and regulation of K^+ channels, occur within the plasma membrane. They can be triggered directly by muscarinic receptor occupation independent of changes in cytosolic mediators. However, these primary events in turn affect the generation of diffusible second messengers such as cAMP, DAG, IP_3 and Ca^{2+}, which generate other metabolic sequelae. For example, an increase in cytosolic free Ca^{2+} probably contributes to activation of phospholipase A_2, generating arachidonic acid, prostaglandins and related eicosanoids (see Ch. 33). These products in turn can stimulate cGMP formation and can regulate ion channel activity. Increased Ca^{2+} also can activate Ca^{2+}-dependent ion channels (K^+, Cl^-), regulate cAMP phosphodiesterase and activate Ca^{2+}/calmodulin kinase-dependent protein phosphorylation. PKC is activated by DAG, generally in concert with Ca^{2+} for conventional PKC isoforms, and has effects on ion channel activity, as well as on cholinergic secretory and contractile responses. Given the obviously complex set of possible interactions between these intracellular mediators, it is easy to explain how diverse cellular responses can be mediated through a single receptor activating relatively few primary responses (see Ch. 10).

Radioligand-binding studies have been used to characterize muscarinic receptors. In membranes or homogenates from heart, brain and other tissues, muscarinic agonists compete for antagonist-binding sites with Hill slopes of less than unity, suggesting that these agonists interact with more than a single population of muscarinic receptors [46]. Direct binding experiments with radiolabeled agonists also show multiple binding sites for agonists. Competition curves are best fitted by a model in which there are sites with low, high and, in some cases, super-high affinity for agonists. Addition of GTP to the binding assay can have a dramatic effect on the agonist competition curve or on direct agonist binding. The effect of GTP is to decrease the apparent affinity of the receptor for agonists. This results from a change in the interaction of the receptor with the GTP-binding protein that transduces its effects.

Agonists vary in their binding properties. Some, like ACh, carbamylcholine and methacholine, bind with high affinity to a large percentage of the total sites and with low affinity to the remainder. In contrast, agonists such as oxotremorine and pilocarpine appear to bind to a single class of sites and may show relatively little high-affinity binding. The capacity of an agonist to induce high-affinity binding correlates with the efficacy of that agonist for eliciting responses such as contraction or phosphoinositide breakdown. It therefore appears that interaction of the receptor and G protein, as reflected in high-affinity agonist binding, is critical to production of the cellular response.

Unlike agonists, most muscarinic antagonists, such as quinuclidinylbenzilate, *N*-methylscopolamine and atropine, bind to the receptor with Hill slopes of unity, as expected for a mass-action interaction with a single receptor type. There is little difference in affinity for these ligands in various tissues. Similar findings with other antagonists initially suggested that all muscarinic receptors were the same. However, a number of functional studies suggested that muscarinic receptors were heterogeneous, and putative subtype-selective antagonists have been described throughout the years.

The binding properties of the antagonist pirenzepine led to the initial classification of muscarinic receptors. Pirenzepine (PZ), in contrast to other known mAChR antagonists, binds to muscarinic receptors in cortex, hippocampus and ganglia with relatively high affinity; these sites have been termed M_1. Heart, gland and smooth muscle mAChR muscarinic receptors, as well as those in brainstem, cerebellum and thalamus, show 30–50-fold lower affinity for PZ [46, 47]. The affinity for classic antagonists like *N*-methylscopolamine is the same in all of these regions, emphasizing the unique selectivity of PZ. Direct binding studies using [^{3}H]-PZ confirmed that only certain tissues and brain regions had receptors with high affinity for this antagonist. Results of pharmacological studies also demonstrated that PZ blocks muscarinic responses in ganglia better than responses in heart. Brain and heart were subsequently used to purify distinct receptors, termed M_1 and M_2 muscarinic receptors, and cDNA clones corresponding to these receptors were isolated from rat brain and heart libraries.

The cDNAs for the muscarinic receptors encode apparent glycoproteins of 55–70 kDa, which contain seven predicted transmembrane-spanning regions, similar to what is seen for the β-adrenergic receptor and other receptors that couple to G proteins (Fig. 11-11). There is only 38% amino acid identity between the proteins cloned from porcine brain and heart. The cDNA encoding the receptor initially cloned from the brain has been termed m_1, whereas that cloned from the heart has been termed m_2, in keeping with the names (M_1 and M_2) applied to the purified recombinant receptors.

The human and rat homologs of these receptor genes, as well as three additional subtypes termed m_3, m_4 and m_5, were subsequently cloned and expressed. Comparison of the amino acid sequences of the five muscarinic receptor subtypes suggests that they are members of a highly conserved gene family. The greatest sequence identity is in the transmembrane-spanning regions, whereas the long cytoplasmic loop (i_3) between transmembrane domains V and VI varies among the receptor subtypes [46, 50]. The

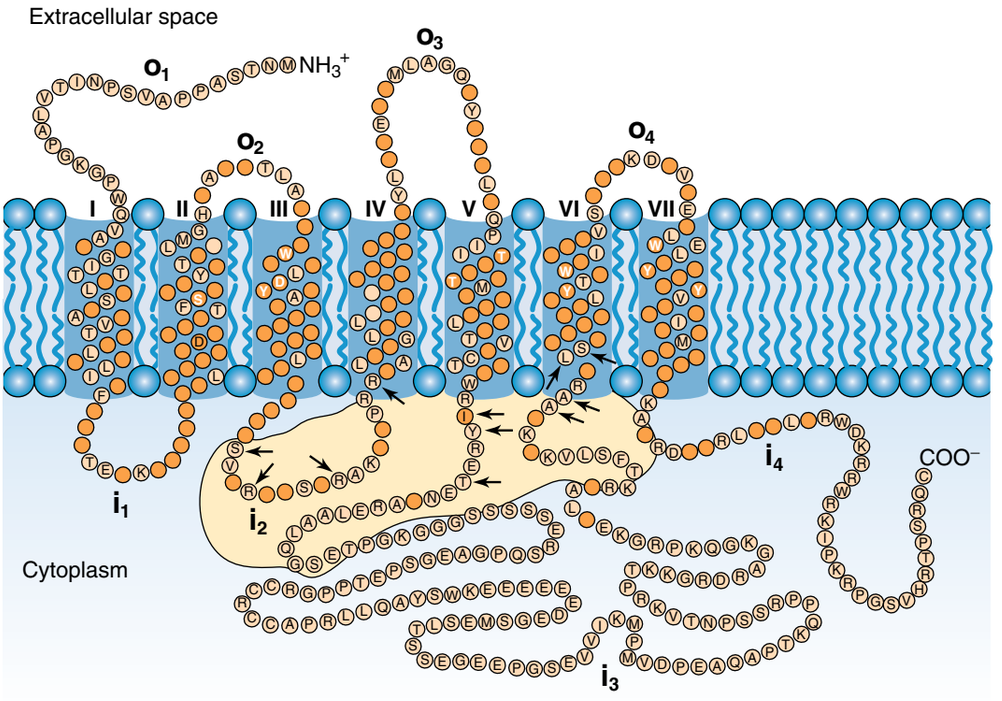


FIGURE 11-11 Predicted amino acid sequence and transmembrane domain structure of the human M_1 muscarinic receptor. Amino acids that are identical among the m_1, m_2, m_3 and m_4 receptors are *dark orange*. The *shaded cloud* represents the approximate region that determines receptor–G-protein coupling. *Arrows* denote amino acids important for specifying G protein coupling. Amino acids predicted to be involved in agonist or antagonist binding are denoted by *white letters* [50].

cloned receptors, expressed in mammalian cells, show differences in antagonist affinity similar to the differences of the pharmacologically defined receptors. Thus, the expressed M_1 receptor is blocked selectively by PZ, the M_2 receptor is blocked by AFDX-116 and methoctramine and the M_3 receptor is blocked by hexahydrosiladifenidol [47]. The regions in the receptor responsible for differences in antagonist affinity have not yet been identified clearly. Ligands are believed to bind to the receptor at sites facing the extracellular space but located in a central cavity deep within the bundle formed by transmembrane domains III–VII. The binding site for the covalent antagonist propylbenzilylcholine mustard has been mapped to a particular aspartic acid residue in the third transmembrane region. This amino acid is conserved in all biogenic amine G-protein-coupled receptors. Mutagenesis of this amino acid profoundly affects both agonist and antagonist binding to muscarinic receptors. It is hypothesized that this residue participates in ionic bonding with the ammonium headgroup of the cholinergic ligand [46].

Expression of the cloned receptors in Chinese hamster ovary cells, other mammalian cells and *Xenopus* oocytes has demonstrated differential coupling of these receptors to cellular responses. In general the M_1, M_3 and M_5 receptors regulate phosphoinositide hydrolysis by stimulating PLC. This occurs through selective coupling of the receptor to the pertussis-toxin-insensitive G protein $G_{q/11}$, which can activate the β isoform of PLC [48]. Calcium-dependent K^+ and Cl^- channels are activated secondarily to the PLC-mediated increase in intracellular Ca^{2+}. In contrast, the M_2 and M_4 receptors couple through a pertussis-toxin-sensitive G protein (G_i) to inhibition of adenylyl cyclase. Regulation of K^+ channels also is mediated through M_2 or M_4 receptor interaction with specific pertussis-toxin-sensitive G proteins.

Chimeric receptors have been used to determine the regions critical for specifying coupling to particular responses. These studies demonstrate that it is the third intracellular (i_3) loop that defines functional specificity [47, 50]. A series of amino acids proximal to the transmembrane domain, that is, at the N- and C- termini of the i_3 loop, carry most of this information. These particular regions are similar in the M_1, M_3 and M_5 receptors and in the M_2 and M_4 receptors but distinguish these two groups from one another. Both site-directed and random mutagenesis studies have identified specific amino acids at the amino-terminus of the i_3 loop which are required for G protein recognition and activation [50, 51]. These critical noncharged amino acids are predicted to reside on the hydrophobic face of an α-helical extension of transmembrane domain V. Conserved amino acids in the carboxy-terminus of the i_3 loop and adjacent transmembrane domain VI also have been demonstrated to specify coupling to G_i- versus G_q-mediated responses. The hydrophobic

regions at the two ends of the i_3 loop thus are suggested to form a surface that binds to and discriminates between different classes of G protein [50]. Other regions, including a portion of the second intracellular loop, also contribute to specifying correct G protein coupling. The early and detailed characterization of distinct regions involved in selective G-protein coupling of mAChR provides a paradigm for analysis of other GPCRs.

The selectivity in muscarinic receptor coupling is not, however, absolute. Overexpression of receptors or of particular G proteins supports interactions that may differ from those described above. For example, M_2 receptors expressed in Chinese hamster ovary cells not only inhibit adenylyl cyclase but also can stimulate phosphoinositide hydrolysis through a pertussis-toxin-sensitive G protein [52]; this is not seen, however, when M_2 receptors are expressed in Y1 cells. These findings indicate that caution must be exercised in interpreting data obtained when receptors are expressed, often at high levels, in cells in which they normally do not function.

Muscarinic receptors of the M_1, M_3 and M_5 subclasses induce transformation or cell proliferation, a feature not shared by M_2 and M_4 receptors [53]. This property has been exploited to develop a high-throughput assay for screening effects of receptor mutations [51]. Mitogen-activated protein kinases (MAP kinases) also are activated by muscarinic receptors but, unlike transformation, this response occurs with receptors of the M_2/M_4 subtype as well as of the M_1/M_3 subtype. Notably, induction of cell growth by muscarinic receptor stimulation is cell-type-specific, however, and is seen only at high levels of receptor expression [53]. Thus, it is questionable whether there is a physiological role for ACh in growth regulation.

Transgenic mice are being generated to assess the functions of receptor subtypes *in vivo*. Knowledge of the anatomical distribution and coupling properties of receptor subtypes can indicate which physiological responses they mediate *in vivo*. However, the lack of good subtype-selective antagonists limits the use of pharmacological approaches to address this question. Generation of transgenic mice in which muscarinic receptors are overexpressed or knockout mice in which receptor genes are disrupted by homologous recombination provides a powerful and necessary approach for evaluation of muscarinic receptor function. The *m_1* gene was the first to have been targeted selectively, eliminating M_1-receptor expression in the forebrain where it is abundantly expressed [54]. Homozygous M_1-receptor-deficient mice are completely resistant to seizures produced by pilocarpine, implicating the M_1 receptor in this model of epilepsy. Furthermore, inhibition of the M-current in sympathetic ganglia, suggested by previous pharmacological experiments to be M_1-receptor-coupled, is ablated in knockout mice. Interestingly, despite the abundance of M_1 receptors in the forebrain, most learning and memory tasks are not impaired in mice lacking M_1 receptors. With regard to the biochemical signaling pathways delineated above, mAChR-stimulated phosphoinositide hydrolysis is attenuated and MAP kinase activation lost in hippocampus and pyramidal neurons. The conclusion that brain M_1 mAChR selectively regulate these responses is supported by the observation that these responses are unaffected in the M_2-, M_3- and M_4-receptor knockout mice described below.

Both the M_2 and M_4 receptors are, as indicated above, coupled to G_i pathways and appear to mediate similar responses. The M_2 receptor is widely expressed in the CNS but also present in heart and smooth muscle, while M_4 is preferentially expressed in the CNS, especially in forebrain. Ablation of the M_2 receptor leads to complete loss of muscarinic-agonist-stimulated bradycardia [55]. In the CNS, deletion of the M_2 receptor abolishes oxotremorine induced akinesia and tremors [56]. Memory and learning tasks including passive avoidance and working memory are impaired in M_2-receptor knockout mice, and there is decreased LTP in hippocampal slices [12].

M_2 receptors are also predominant in the mouse spinal cord and here this receptor subtype appears, from knockout studies, to play a major role in signaling muscarinic-agonist-induced analgesia (although M_4 receptors can also mediate this response) [56]. A major role for M_2 (and M_4) receptors is as presynaptic auto- (or hetero-)receptors. Thus studies using M_2-receptor knockout mice reveal that the M_2 receptor plays a prominent role in inhibiting NE release from sympathetic nerves in atria and other peripheral tissues and that M_2 and M_4 autoreceptors regulate ACh release from atria, urinary bladder and cholinergic neurons in the hippocampus and striatum [12, 57].

Muscarinic and dopaminergic pathways in the CNS interact in control of numerous pathways implicated in diseases, especially those controlling involuntary motor systems. Muscarinic effects on dopamine release are mediated in several ways via different mAChR subtypes. Thus mAChR facilitation of DA release appears to involve M_4 receptors on GABA projection neurons to the striatum, while M_3 receptors on striatal DA neurons are predicted to inhibit striatal DA release [12].

The role of the M_3 mAChR in the CNS remains unclear. The phenotype of the M_3 receptor knockout is hypophagic and lean. This appears to result from altered hypothalamic appetite regulation, as M_3 receptors are relatively highly expressed in this region [12]. The most striking changes in the M_3-receptor knockout animal are peripheral, specifically loss of contractile responses of tracheal, urinary bladder and pupillary smooth muscle to muscarinic agonists. The M_5 receptor also has an uncertain role in the CNS, where it is present at low levels. Recent studies using M_5-receptor knockout mice suggest that these receptors may reside primarily on endothelial cells of brain arteries and arterioles, mediating cholinergic relaxation of these vessels [58].

The information gleaned from studies with mAChR knockout mice is encouraging in demonstrating striking phenotypes that indicate that mAChR agonists and

antagonists have potential for the treatment of Alzheimer's disease, Parkinson's disease, epilepsy and pain. The evidence that the distribution and functions of the various mAChR subtypes are highly specific provides further hope for the design of therapeutic agents without limiting side effects. That mice are not humans means that the general principles learned from these studies are germane, but that much additional work will be needed to define the utility of selective mAChR agents in human disease.

REFERENCES

1. Rama-Sastry, B.V. and Sadavongvivad, C. Non-neuronal acetylcholine. *Pharmacol. Rev.* 30: 65–132, 1979.
2. Dale, H. H. The action of certain esters and ethers of choline and their relation to muscarine. *J. Pharmacol.* 6: 147–190, 1914.
3. Changeux, J. P. Chemical signaling in the brain. *Sci. Am.* 269: 58–62, 1993.
4. Numa, S., Noda, M., Takahashi, H. *et al.* Molecular structure of the acetylcholine receptor. *Cold Spring Harb. Symp. Quant. Biol.* 48: 57–69, 1983.
5. Partington, P., Feeney, J. and Burgen, A. S. V. The conformation of acetylcholine and related compounds in aqueous solutions as studied by nuclear magnetic resonance spectroscopy. *Mol. Pharmacol.* 8: 269–277, 1972.
6. Behling, R. W., Yamane, T., Navon, G. and Jelinski, L. W. Conformation of acetylcholine bound to the nicotinic acetylcholine receptor. *Proc. Natl Acad. Sci. U.S.A.* 85: 6721–6724, 1988.
7. Portoghese, P. S. Relationships between stereostructure and pharmacological activity. *Annu. Rev. Pharmacol.* 10: 51–76, 1970.
8. Cerlie, T. H., Van-Rossum-Fikkert, S. E., Van Dijk, W. J., Brej, K., Smit, A. B. and Sixma, T. Nicotine and carbamylcholine binding to nicotinic receptors as studied in AChBP crystal structures. *Neuron* 41, 907–914, 2004.
9. Hoffman, B. B. and Taylor, P. Neurohumoral transmission: the autonomic and somatic motor nervous systems. In J. G. Hardman and L. E. Limbird (eds.), *Goodman & Gilman's Pharmacological Basics of Therapeutics*, 10th edn. New York: Macmillan, pp.115–154, 2001.
10. Mac Dermott, A. B., Role, L. and Siegelbaum, S. A. Presynaptic ionotropic receptors and the contol of transmitter release. *Annu. Rev. Neurosci.* 22, 443–485, 1999.
11. Clementi, F., Fornasari, D. and Gotti, C. (eds.), *Neuronal Nicotinic Receptors, Handbook of Experimental Pharmacology*. Berlin: Springer, 2000.
12. Wess, J. Muscarinic receptor knock out mice: novel phenotypes and clinical implications. *Ann. Rev. Pharmacol. Toxicol.* 44, 423–500, 2004.
13. Champtiaux, N. and Changeux, J. P. Knock out and knock in mice to investigate the role of nicotinic recepors in the CNS. *Curr. Drug Targets – CNS Neurol. Dis.* 1, 319–330, 2002.
14. Adams, P. R., Brown, D. A. and Constitini, A. Pharmacological inhibition of the M-current. *J. Physiol. (Lond.)* 332: 223–262, 1982.
15. Van der Kloot, W. and Molgo, J. Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiol. Rev.* 74: 898–991, 1994.
16. Wu, D. and Hersh, L. B. Choline acetyltransferase: celebrating its fiftieth year. *J. Neurochem.* 62: 1653–1663, 1994.
17. Okuda, T., Haga, T., Kanai, Y., Endou, H., Ishihara, T. and Katsura, I. Identification and characterization of the high affinity choline transporter. *Nat. Neurosci.* 3, 120–125, 2000.
18. Eiden, L. E. The cholinergic gene locus. *J. Neurochem.* 70, 2227–2240, 1998.
19. Ferguson, S. M. and Blakely, R. D. The choline transporter resurfaces: new roles for synaptic vesicles? *Mol. Interv.* 4, 22–37, 2004
20. Erickson, J. D., Weihe, E., Schafer, M. K. M. *et al.* The VAChT/ChAT 'cholinergic gene locus': new aspects of genetic and vesicular regulation of cholinergic function. *Prog. Brain Res.* 109: 69–82, 1996.
21. Varoqui, H., Mennier, F.-M., Mennier, F. A. *et al.* Expression of the vesicular acetylcholine transporter in mammalian cells. *Prog. Brain Res.* 109: 83–95, 1996.
22. Kuffler, S. W., Nicholls, J. and Martin, R. A. *From Neuron to Brain: A Cellular Approach to the Function of the Nervous System.* Sunderland, MA: Sinauer Associates, 1984.
23. Van der Kloot, W. Loading and recycling of synaptic vesicles in the *Torpedo* electric organ and the vertebrate neuromuscular junction. *Prog. Neurobiol.* 71, 269–303, 2003.
24. Taylor, P. and Radic, Z. The cholinesterases: from genes to proteins. *Annu. Rev. Pharmacol. Toxicol.* 34: 281–320, 1994.
25. Giacobini, E. (ed.) *Cholinesterases and Cholinesterase Inhibitors.* London: Martin Dunitz, 2000.
26. Giacobini, E. (ed.) *Butyrylcholinesterase: its Function and Inhibitors.* London: Martin Dunitz, 2003.
27. Xie, W., Stribley, J., Chatonnet, A. *et al.* Postnatal development delay and supersensitivity to organophosphate in gene targeted mice lacking acetylcholinesterase. *J. Pharmacol. Exp. Ther.* 293: 896–902, 2000.
28. Sussman, J. L., Harel, M., Frolow, F. *et al.* Atomic structure of acetylcholinesterase from *Torpedo californica*. A prototypic acetylcholine-binding protein. *Science* 253: 872–878, 1992.
29. Bourne, Y., Taylor, P. and Marchot, P. Acetylcholinesterase inhibition by fasciculin. Crystal structure of the complex. *Cell* 83: 503–512, 1995.
30. Massoulie, J. The origin and molecular diversity and functional anchoring of cholinesterases. *Neurosignals* 11: 130–143, 2002.
31. Miyazawa, A., Fujiyoshi Y. and Unwin, N. Structure and gating mechanism of the acetylcholine receptor pore. *Nature* 423, 949–955, 2003.
32. Karlin, A. Emerging structures of nicotinic acetylcholine receptors. *Nat. Rev. Neurosci.* 3, 102–114, 2002.
33. Brejc, K., van Dijk, W. J., Klassen, R. V. *et al.* Crystal structure of an Ach binding protein reveals the ligand binding domain of nicotinic receptors. *Nature* 411, 269–276, 2001.
34. Bouzat, C., Gulimar, F., Spitzmaul, G. *et al.* Coupling of agonist binding to channel gating in an Ach-binding protein linked to an ion channel. *Nature* 430, 896–900, 2004.
35. Corringer, P. J., Le Novere, N. and Changeux, J. P. Nicotinic receptors at the amino acid level. *Annu. Rev. Pharmacol. Toxicol.* 40, 431–458, 2000.

36. Sakmann, B. Elementary steps in synaptic transmission revealed by currents through single ion channels. *Science* 256: 503–512, 1992.
37. Salminen, O., Murphy, K. L., M.McIntosh, J. *et al.* Subunit composition of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol. Pharmacol.* 65, 1526-1535, 2004.
38. Lindstrom, J. Nicotinic acetylcholine receptors of muscle and nerves. *Ann. N.Y. Acad. Sci.* 998, 41–42, 2003.
39. Elgoyhen, A., Vetter, D., Katz, E. *et al.* Alpha 10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl Acad. Sci. U.S.A.* 98, 3501–3506, 2001.
40. Dani, J. D. and Zhou, F.-M. Synaptic plasticity and nicotine addiction. *Neuron* 31: 349–352, 2001.
41. Engel, A. G., Ohno, K. and Sine, S. M. Sleuthing molecular targets for neurological diseases at the neuromuscular junction. *Nat. Rev. Neurosci.* 4, 339–352, 2003.
42. Sanes, J. R. The basement membrane/basal lamina of skeletal muscle. *J. Biol. Chem.* 278, 12601–12604, 2003.
43. Yamagata, M., Sanes, J. R. and Weiner, J. A. Synaptic adhesion molecules. *Curr. Opin. Cell Biol.* 15, 621–632, 2003.
44. Burden, S. J. Building the vertebrate neuromuscular synapse. *J. Neurobiol.* 53, 501–511, 2002.
45. Arikawa-Hirasawa, E., Rossi, S. G., Rotundo, R. L. and Yamada, Y. Absence of acetylcholinesterase at the neuromuscular junctions of perlecan-null mice. *Nat. Neurosci.* 5, 119–123, 2002.
46. Hulme, E., Birdsall, N. and Buckley, N. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* 30: 633–673, 1990.
47. Caulfield, M. D. Muscarinic receptors – characterization, coupling and function. *Pharmacol. Ther.* 58: 319–379, 1993.
48. Berstein, G., Blank, J. L., Smrcka, A. V. *et al.* Reconstitution of agonist stimulated phosphatidylinositol 4–5 bisphosphate hydrolysis using purified m_1 muscarinic receptor, $G_{q/11}$, and phospholipase C-β1. *J. Biol. Chem.* 267: 8081–8088, 1992.
49. Kofuji, P., Davidson, N. and Lester, H. Evidence that neuronal G-protein-gated inwardly rectifying K^+ channels are activated by G protein $\beta\gamma$ subunits and function as heteromultimers. *Proc. Natl. Acad. Sci. USA* 92: 6542–6546, 1995.
50. Wess, J. Molecular biology of muscarinic acetylcholine receptors. *Crit. Rev. Neurobiol.* 10: 69–99, 1996.
51. Hill Eubank, D., Burstein, E., Spalding, T., Brauner-Osborne, H. and Brann, M. Structure of a G-protein coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. *J. Biol. Chem.* 271: 3058–3065, 1996.
52. Ashkenazi, A., Winslow, J. W., Peralta, E. G. *et al.* An M_2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science* 238: 672–675, 1987.
53. J. S. Gutkind, E. A. Novotny, M. R. Brann, and K. C. Robbins. Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes. *Proc. Natl Acad. Sci. U.S.A.* 88: 4703–4707, 1991.
54. Hamilton, S. E.. Loose, M. D., Qi, M. *et al.* Disruption of the m_1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. *Proc. Natl Acad. Sci. U.S.A.* 94: 13311-13316, 1997.
55. Stengel, P. W., Gomeza, J., Wess, J. and Cohen, M. L. M2 and M4 receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. *J. Pharmacol. Exp. Ther.* 292:877–85, 2000.
56. Gomeza, J., Shannon, H., Kostenis, E. *et al.* Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. *Proc. Natl Acad. Sci. U.S.A.* 96: 1692–97, 1999.
57. Zhang, W., Basile, A. S., Gomeza, J., Volpicelli, L. A., Levey, A. I. and Wess, J. Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. *J. Neurosci.* 22: 1709–17, 2002.
58. Yamada, M., Lamping, K. G., Duttaroy, A. *et al.* Cholinergic dilation of cerebral blood vessels is abolished in M5 muscarinic acetylcoholine receptor knockout mice. *Proc. Natl Acad. Sci. U.S.A.* 98: 14096–14101, 2001
59. Unwin, N. Nicotinic acetylcholine receptor at 9Å resolution. *J. Mol. Biol.* 229: 1101–1124, 1993.
60. Unwin, N. Refined structure of the nicotinic acetylcholine receptor at 4A resolution. *J. Mol. Biol.* 346: 967–989, 2005.

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CHAPTER

12

Catecholamines

Michael J. Kuhar

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INTRODUCTION

The catecholamines dopamine, norepinephrine and epinephrine are neurotransmitters and/or hormones in the periphery and in the CNS. Norepinephrine is a neurotransmitter in the brain as well as in postganglionic, sympathetic neurons. Dopamine, the precursor of norepinephrine, has biological activity in the periphery, most particularly in the kidney, and serves as a neurotransmitter in several important pathways in the CNS. Epinephrine, formed by the *N*-methylation of norepinephrine, is a hormone released from the adrenal gland, and it stimulates catecholamine receptors in a variety of organs. Small amounts of epinephrine are also found in the CNS, particularly in the brainstem.

BIOSYNTHESIS OF CATECHOLAMINES

The enzymatic processes involved in the formation of catecholamines have been characterized. The component enzymes in the pathway have been purified to homogeneity, which has allowed for detailed analysis of their kinetics, substrate specificity and cofactor requirements and for the development of inhibitors (Fig. 12-1). Their cDNAs have been cloned, and studies with knockout mice clearly indicate the importance of these enzymes since their

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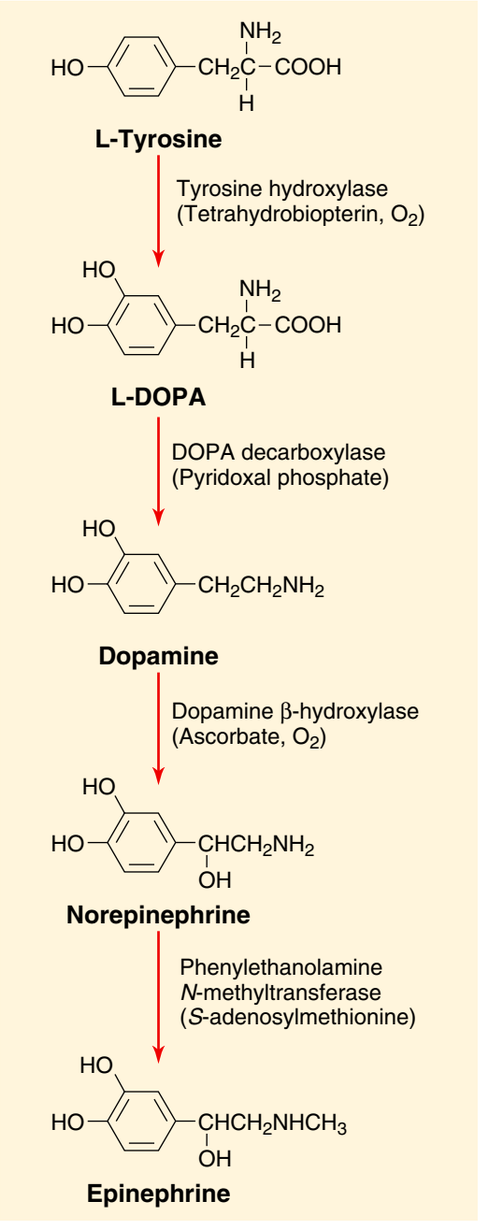

FIGURE 12-1 Biosynthetic pathway for catecholamines.

absence results in a variety of abnormalities, including loss of viability (Table 12-1).

Tyrosine hydroxylase is the rate-limiting enzyme for the biosynthesis of catecholamines. Tyrosine hydroxylase (TH) is found in all cells that synthesize catecholamines and is a mixed-function oxidase that uses molecular oxygen and tyrosine as its substrates and biopterin as its cofactor [1]. TH is a homotetramer, each subunit of which has a molecular weight of approximately 60,000. It catalyzes the addition of a hydroxyl group to the *meta* position of tyrosine, thus forming 3,4-dihydroxy-L-phenylalanine (L-DOPA).

TABLE 12-1 Studies with knockout mice

Tyrosine hydroxylase	Not viable	[54]
Dopamine beta hydroxylase	Hypersensitive to amphetamine	[55]
MAO-A	Aggressive behavior	[54]
COMT	Complex alterations in anxiety and aggression	[16]
VMAT2	Not viable	[54]
DAT	Growth retardation and hyperactivity	[54]
NET	Enhanced opiate-mediated analgesia and responses to psychostimulants	[56]

TH can also hydroxylate phenylalanine to form tyrosine, which is then converted to L-DOPA; this alternative synthetic route may be of significance in patients affected with phenylketonuria, a condition in which phenylalanine hydroxylase activity is depressed. TH has a K_m for tyrosine in the micromolar range. As a result, it is virtually saturated by the high tissue concentrations of endogenous tyrosine. The cofactor, biopterin, may be at subsaturating concentrations within catecholamine-containing neurons and thus may play an important role in regulating catecholamine biosynthesis. TH is primarily a soluble enzyme; however, interactions with membrane constituents, such as phosphatidylserine, or with polyanions, such as heparin sulfate, have been shown to alter its kinetic characteristics. Analogs of tyrosine, such as α-methyl-*p*-tyrosine (AMPT), are competitive inhibitors of TH. Sequence analysis [2] reveals consensus sequences for phosphorylation primarily in the N-terminal portion of the molecule (Fig. 12-2). The gene reveals considerable sequence homology with phenylalanine hydroxylase and tryptophan hydroxylase.

DOPA decarboxylase catalyzes the removal of the carboxyl group from DOPA to form dopamine. DOPA decarboxylase (DDC) is a pyridoxine-dependent enzyme that has a low K_m and a high V_{max} with respect to L-DOPA; thus, endogenous L-DOPA is efficiently converted to dopamine. DDC can also decarboxylate 5-hydroxytryptophan, the precursor of serotonin, as well as other aromatic amino acids; accordingly, it has also been called aromatic amino acid decarboxylase (AADC). DDC is widely distributed throughout the body, where it is found both in catecholamine- and serotonin-containing neurons and in non-neuronal tissues, such as kidney and blood vessels. In dopamine-containing neurons, this enzyme is the final step in the pathway and this is exploited clinically in the treatment of Parkinson's disease by giving patients excess L-DOPA to boost the production of dopamine in the remaining dopaminergic axon terminals. α-Methyldopa inhibits DDC *in vitro* and leads to a reduction in blood pressure after being converted to the false transmitter α-methylnorepinephrine *in vivo*. A false neurotransmitter is a substance stored and released like the authentic transmitter but has lower efficacy.

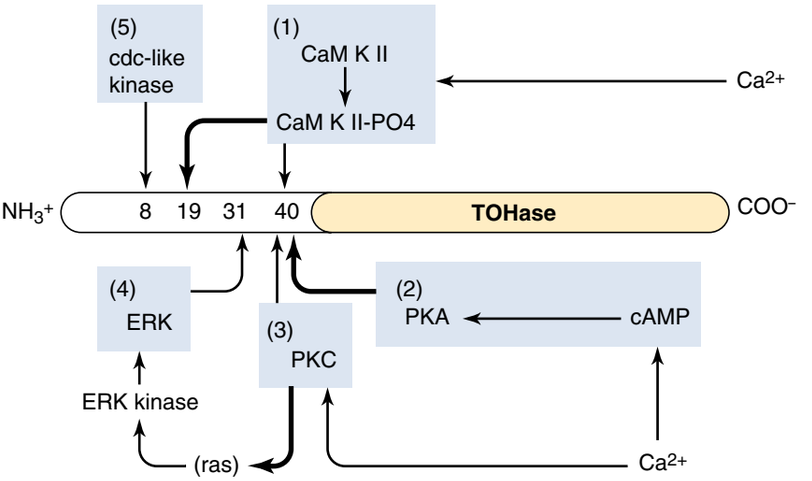

FIGURE 12-2 Schematic diagram of the phosphorylation sites on each of the four 60 kDa subunits of tyrosine hydroxylase (TOHase). Serine residues at the N-terminus of each of the four subunits of TOHase can be phosphorylated by at least five protein kinases. (*1*), Calcium/calmodulin-dependent protein kinase II (CaM KII) phosphorylates serine residue 19 and to a lesser extent serine 40. (*2*), cAMP-dependent protein kinase (PKA) phosphorylates serine residue 40. (*3*), Calcium/phosphatidylserine-activated protein kinase (PKC) phosphorylates serine 40. (*4*), Extracellular receptor-activated protein kinase (ERK) phosphorylates serine 31. (*5*), A cdc-like protein kinase phosphorylates serine 8. Phosphorylation on either serine 19 or 40 increases the activity of TOHase. Serine 19 phosphorylation requires the presence of an 'activator protein', also known as 14-3-3 protein, for the expression of increased activity. Phosphorylation of serines 8 and 31 has little effect on catalytic activity. The model shown includes the activation of ERK by an ERK kinase. The ERK kinase is activated by phosphorylation by PKC. (With permission from reference [72].)

For neurons that synthesize epinephrine or norepinephrine, dopamine β-hydroxylase is the next step in the biosynthetic pathway. Like TH, dopamine β-hydroxylase (DBH) is a mixed-function oxidase that uses molecular oxygen to form the hydroxyl group added to the carbon on the side chain of dopamine [3]. Ascorbate, reduced to dihydroascorbate during the reaction, provides a source of electrons. DBH contains Cu^{2+}, which is involved in electron transfer in the reaction; accordingly, copper chelators, such as diethyldithiocarbamate, are potent inhibitors of the enzyme. DBH is a tetrameric glycoprotein containing subunits of 77 and 73 kDa, as determined by sodium dodecyl sulfate (SDS) gel electrophoresis. A full-length clone encodes a polypeptide chain of 578 amino acids [4]. The enzyme is concentrated within the vesicles that store catecholamines; most of the DBH is bound to the inner vesicular membrane but some is free within the vesicles. DBH is released along with catecholamines from nerves and from the adrenal gland and is found in plasma.

In cells that synthesize epinephrine, the final step in the pathway is catalyzed by the enzyme phenylethanolamine *N*-methyltransferase. This enzyme is found in a small group of neurons in the brainstem that use epinephrine as their neurotransmitter and in the adrenal medullary cells, for which epinephrine is the primary hormone secreted. Phenylethanolamine *N*-methyltransferase (PNMT) transfers a methyl group from *S*-adenosylmethionine to the nitrogen of norepinephrine, forming a secondary amine [5]. The coding sequence of bovine PNMT is contained in a single open reading frame encoding a protein of 284 amino acids [6]. PNMT activity is regulated by corticosteroids in the adrenal gland and superior cervical ganglia [7].

STORAGE AND RELEASE OF CATECHOLAMINES

Catecholamines are concentrated in storage vesicles that are present at high density within nerve terminals. Ordinarily, low concentrations of catecholamines are free in the cytosol, where they may be metabolized by enzymes including monoamine oxidase (MAO). Thus, conversion of tyrosine to L-DOPA and L-DOPA to dopamine occurs in the cytosol; dopamine then is taken up into the storage vesicles. In norepinephrine-containing neurons, the final β-hydroxylation occurs within the vesicles. In the adrenal gland, norepinephrine is *N*-methylated by PNMT in the cytoplasm. Epinephrine is then transported back into chromaffin granules for storage.

cDNA clones encoding vesicular amine transporters have been obtained. The sequences suggest that the proteins have 12 transmembrane domains and are homologous to a family of bacterial drug-resistance transporters (Ch. 5). The expressed protein, referred to as vesicular membrane transporter 2 (VMAT2), has a high affinity for reserpine, which blocks vesicular uptake *in vivo*. The mechanism that concentrates catecholamines within the vesicles is an ATP-dependent process linked to a proton pump. The intravesicular concentration of catecholamines

is approximately 0.5 mol/l, and they exist in a complex with ATP and acidic proteins known as chromogranins. The vesicular uptake process has broad substrate specificity and can transport a variety of biogenic amines, including tryptamine, tyramine and amphetamines; these amines may compete with endogenous catecholamines for vesicular storage sites. Reserpine is a specific, irreversible inhibitor of the vesicular amine pump that blocks the ability of the vesicles to concentrate the amines. Treatment with reserpine causes a profound depletion of endogenous catecholamines in neurons. Knockout mice lacking VMAT2 are not viable (Table 12-1). Altered levels of VMAT2 have been associated with bipolar disorder in humans [8] and lithium, a treatment for this disorder, modulates VMAT2 expression in the brain [9].

The vesicles play a dual role: they maintain a ready supply of catecholamines at the nerve terminal that is available for release, and they mediate the process of release. When an action potential reaches the nerve terminal, Ca^{2+} channels open, allowing an influx of the cation into the terminal; increased intracellular Ca^{2+} promotes the fusion of vesicles with the neuronal membrane (see Ch. 10). The vesicles then discharge their soluble contents–for example, at norepinephrine neurons, norepinephrine, ATP, chromogranin and DBH – into the extraneuronal space [10]. The demonstration that DBH is released concurrently and proportionately with norepinephrine established the fact that release occurs by the process of exocytosis, since proteins would not be expected to diffuse across cell membranes. Exocytotic release from sympathetic neurons may be the source of some of the DBH found in the plasma and cerebrospinal fluid (CSF) of animals and humans. Indirectly acting sympathomimetics, like tyramine and amphetamine, release catecholamines by a mechanism that is neither dependent on Ca^{2+} nor associated with release of DBH. These drugs displace catecholamines from storage vesicles, resulting in leakage of neurotransmitter from the nerve terminals.

The concentration of catecholamines within nerve terminals remains relatively constant. Despite the marked fluctuations in the activity of catecholamine-containing neurons, efficient regulatory mechanisms modulate the rate of synthesis of catecholamines [11]. A long-term process affecting catecholamine synthesis involves alterations in the amounts of TH and DBH present in nerve terminals. When sympathetic neuronal activity is increased for a prolonged period of time, the amounts of mRNA coding for TH and DBH are increased in the neuronal perikarya. DDC does not appear to be modulated by this process. The newly synthesized enzyme molecules are then transported down the axon to the nerve terminals.

In addition, two mechanisms operative at the level of the nerve terminal play important roles in the short-term modulation of catecholamine synthesis and are responsive to momentary changes in neuronal activity [12]. TH, the rate-limiting enzyme in the synthesis pathway, is modulated by end-product inhibition [11]. Thus, free intraneuronal catecholamines inhibit the further activity of TH by competing at the site that binds the pterin cofactor; conversely, neuronal activity results in the release of catecholamines, a decrease in cytoplasmic concentrations and disinhibition of the enzyme. An additional and probably more important effect of depolarization of catecholaminergic terminals is activation of TH, resulting from reversible phosphorylation of the enzyme (Fig. 12-2) [13]. Protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are all capable of inducing phosphorylation of the enzyme, leading to an increase in its activity (see Chs 20 and 21). When phosphorylated, the kinetic characteristics of the enzyme change so that it has a higher affinity for the pterin cofactor and is less sensitive to end-product inhibition.

Monoamine oxidase and catechol-*O*-methyltransferase are primarily responsible for the inactivation of catecholamines. MAO and catechol-*O*-methyltransferase (COMT) are widely distributed throughout the body (Fig. 12-3). MAO is a flavin-containing enzyme located on the outer membrane of the mitochondria [14]. This enzyme oxidatively deaminates catecholamines to their corresponding aldehydes; these can be converted, in turn, by aldehyde dehydrogenase to acids or by aldehyde reductase to form glycols. Because of its intracellular localization, MAO plays a strategic role in inactivating catecholamines that are free within the nerve terminal and not protected by storage vesicles. Accordingly, drugs that interfere with vesicular storage, such as reserpine, or indirectly acting sympathomimetics, such as amphetamines, which displace catecholamines from vesicles, cause a marked increase in deaminated metabolites. Isozymes of MAO with differential substrate specificities have been identified: MAO-A preferentially deaminates norepinephrine and serotonin and is selectively inhibited by clorgyline, whereas MAO-B acts on a broad spectrum of phenylethylamines, including β-phenylethylamine. MAO-B is selectively inhibited by deprenyl. MAO in the gastrointestinal tract and liver plays an important protective role by preventing access to the general circulation of ingested, indirectly acting amines, such as tyramine and phenylethylamine, that are contained in food; however, patients being treated for depression or hypertension with MAO inhibitors are not afforded this protection and can suffer severe hypertensive crises after ingesting foods that contain large amounts of tyramine. Such foods include port wine, Stilton cheese and herring. Drugs that selectively inhibit MAO isozymes are less prone to produce hypertensive crisis and the MAO-A inhibitor moclobemide is an effective antidepressant [15], while the MAO-B inhibitor deprenyl is used in Parkinson's disease.

A methyl substituent on the α carbon of the phenylethylamine side chain protects against deamination by MAO; the prolonged action of amphetamine and related

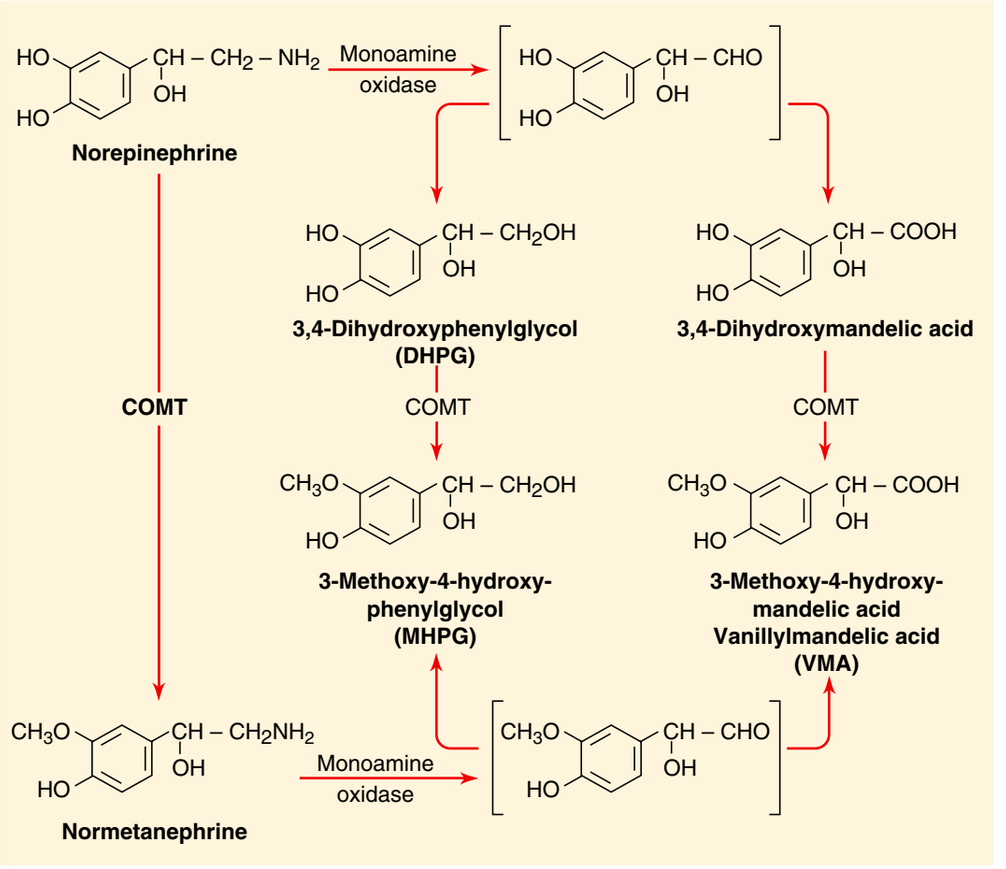

FIGURE 12-3 Pathways of norepinephrine degradation. Unstable glycol aldehydes are shown in brackets. COMT, catechol-*O*-methyltransferase.

indirectly acting stimulants is in part a consequence of the presence of an α-methyl group, which prevents their inactivation by MAO.

COMT is found in nearly all cells, including erythrocytes; thus, this enzyme can also act on extraneuronal catecholamines. Most studies of COMT are carried out with enzyme purified from homogenates of liver. The enzyme, which requires Mg^{2+}, transfers a methyl group from the co-substrate *S*-adenosylmethionine to the 3-hydroxy group on the catecholamine ring. This enzyme has broad substrate specificity, methylating virtually any catechol regardless of the side-chain constituents; for this reason, competitive inhibitors of the enzyme that are of pharmacological significance have not been developed. COMT knockout mice exhibit increased dopamine levels in frontal cortex, but not striatum, and complex, sexually dimorphic behavioral alterations [16]. In humans, a functional polymorphism of the COMT gene has been identified, in which valine (val) is substituted for methionine (met). The met containing enzyme has reduced enzymatic activity compared with the val containing protein. The higher activity, COMT(val) enzyme has been correlated with impaired prefrontal cortical functioning and an increase in risk for schizophrenia [17].

Measurement of catecholamine metabolites can provide insight into the rate of release or turnover of catecholamines in the brain. In clinical studies, metabolites of catecholamines are generally assayed in the CSF because the large quantities derived from the peripheral sympathomedullary system obscure the small contribution from the brain to urinary concentrations. However, acid metabolites are actively excreted from the CSF; more reliable estimates of turnover in the brain are obtained when this transport process is blocked by pretreatment with the drug probenecid.

4-hydroxy-3-methoxy-phenylacetic acid, more commonly known as homovanillic acid (HVA), is a major metabolite of dopamine. Spinal fluid concentrations of HVA provide insight into the turnover of dopamine in the striatum. Concentrations of HVA are decreased, for example, in the CSF of patients with Parkinson's disease (see Ch. 46). A metabolite of norepinephrine formed relatively selectively in the brain is 3-methoxy-4-hydroxyphenylglycol (MHPG). Because this is a minor metabolite of the much larger amounts of norepinephrine metabolized in the periphery, it is estimated that between 30% and 50% of the MHPG excreted in urine is derived from the brain. MHPG has been measured in CSF

and in urine to provide an index of norepinephrine turnover in the brain, and concentrations of MHPG have been shown to be decreased in certain forms of depression (see Ch. 55) and increased in generalized anxiety disorder.

The action of catecholamines released at the synapse is modulated by diffusion and reuptake into presynaptic nerve terminals. Catecholamines diffuse from the site of release, interact with receptors and are transported back into the nerve terminal. Some of the catecholamine molecules may be catabolized by MAO and COMT. The catecholamine-reuptake process was originally described by Axelrod [18]. He observed that, when radioactive norepinephrine was injected intravenously, it accumulated in tissues in direct proportion to the density of the sympathetic innervation in the tissue. The amine taken up into the tissues was protected from catabolic degradation, and studies of the subcellular distribution of catecholamines showed that they were localized to synaptic vesicles. Ablation of the sympathetic input to organs abolished the ability of vesicles to accumulate and store radioactive norepinephrine. Subsequent studies demonstrated that this Na^+- and Cl^--dependent uptake process is a characteristic feature of catecholamine-containing neurons in both the periphery and the brain (Table 12-2).

The uptake process is mediated by a carrier or transporter located on the outer membrane of the catecholaminergic neurons. It is saturable and obeys Michaelis–Menten kinetics. A unique transporter for norepinephrine is found only in noradrenergic neurons, whereas a different transporter is found in dopamine-containing neurons. The genes for transporters responsible for uptake of norepinephrine and dopamine have been cloned, revealing proteins that belong to a larger family of neurotransporters (see Ch. 5) and that display many conserved structural features [19]. The proteins are thought to have 12 transmembrane domains with intracellularly oriented N- and C-termini and a large glycosylated extracellular loop between transmembrane domains 3 and 4. The proteins show the most homology in their transmembrane spanning domains, particularly domains 1, 2, and 4–8, which may be involved in moving the transmitter across the membrane. The transporters are substrates for PKC-dependent phosphorylation, which reduces their activity. The dopamine transporter is phosphorylated on the extreme end of the N-terminal tail, and consensus phosphorylation sites for various other kinases are present in the intracellular loops and domains [20–22] (Fig. 12-4). The dopamine and norepinephrine transporters form functional homo-oligomers, although it is not known if this is required for transport activity, and the transporters also interact with many other membrane proteins that may control their cell-surface expression or other properties.

The uptake process is energy-dependent since it can be inhibited by incubation at a low temperature or by metabolic inhibitors. The energy requirements reflect a coupling of the uptake process with the Na^+ gradient across the

TABLE 12-2 Properties of amine transporters

	NET	DAT	VMAT-2
Mechanism	NaCl-dependent	NaCl-dependent	H^+-dependent
Transmembrane segments	12	12	12
Amino acids	617	620	742
Chromosome	16	5	10
Blockers	Nisoxetine, desipramine	GBR12909, RTI-121	Reserpine, tetrabenazine

The neuronal membrane norepinephrine transporter (NET), the dopamine transporter (DAT) and the vesicular membrane transporter (VMAT-2), which is the same in all catecholamine-containing neurons, have similar numbers of predicted transmembrane segments. They have different numbers of amino acids, pharmacological properties and chromosomal localizations.

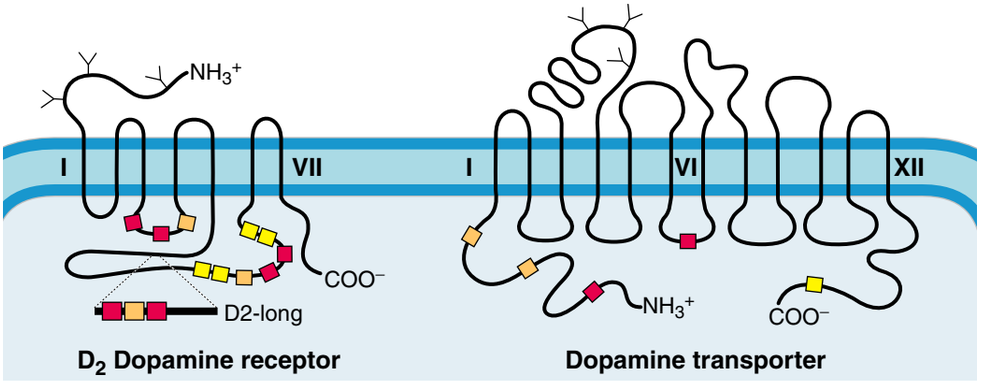

FIGURE 12-4 Schematic of the D_2 receptor and dopamine transporter. The amino acid chain is depicted as a line crossing the membrane. The D_2 receptor has the typical seven transmembrane domains, while the dopamine transporter has approximately 12. The D_2 dopamine receptor has two alternatively spliced mRNA variants that result in a short and a long form of the receptor. The longer variant has the insertion in the third intracellular loop. Putative glycosylation sites are indicated with *Y-shaped symbols* on extracellular sequences. Possible phosphorylation sites are indicated with boxes for various protein kinases: *gray boxes*, protein kinase A; *orange boxes*, protein kinase C; *white boxes*, calcium-calmodulin protein kinase. The dopamine transporter has a large glycosylated extracellular loop between transmembrane regions III and IV.

neuronal membrane. The process is also Cl^--dependent. Drugs such as ouabain, which inhibits Na,K-ATPase, or veratridine, which opens Na^+ channels, inhibit the uptake process.

The linkage of uptake to the Na^+ gradient may be of physiological significance since transport temporarily ceases at the time of depolarization-induced release of catecholamines. The transport of catecholamines can be inhibited selectively by such drugs as tricyclic antidepressants and cocaine. In addition, a variety of phenylethylamines, such as amphetamine, are substrates for carrier; thus, they can be concentrated within catecholamine-containing neurons and can compete with the catecholamines for transport.

Neurotoxins such as mercaptopyrazide pyrimidine (MPP+) and 6-hydroxydopamine are also taken up by transporters, and this is required for their neurotoxic effects. Mice have been prepared with their transporter genes 'knocked out'. Extensive studies with these mice confirm the important role of transporters (Table 12-1). Once an amine has been taken up across the neuronal membrane, it can be taken up by intracellular adrenergic storage vesicles as described above.

The reuptake process does not capture all of the released catecholamine. Diffusion away from the nerve terminal to distant sites can occur and has been termed 'volume transmission' [23]. Volume transmission allows the stimulation of extrasynaptic receptors, which has been described for dopamine [24] and norepinephrine [25]. Brain regions differ in their capacity for catecholamine reuptake; thus, whereas extracellular dopamine concentrations are dominated by release in the cerebral cortex, in the striatum dopamine concentrations are dominated by reuptake [26]. These regional differences in extracellular dopamine kinetics correlate with levels of dopamine transporter [27].

ANATOMY OF CATECHOLAMINERGIC SYSTEMS

The anatomy of catecholaminergic systems has been elucidated. After several decades of research, using a variety of both light and electron microscopic techniques, the anatomical organizations of dopaminergic, noradrenergic and adrenergic neurons have been elucidated [28]. The most important techniques include histofluorescence and immunohistochemistry, which can visualize either the catecholamines themselves or enzymes involved in their synthesis. There appear to be 16 dopamine- and noradrenaline-containing cell groups, and 3 adrenaline-containing cell groups in the CNS. In general, cell bodies containing these transmitters are found in clusters throughout the subcortical brain. In many instances, axons from these cells project to widespread areas and are believed to have broad effects in the CNS, while some of the cell groups have more focused projections and more specific functional effects. Also, other neurotransmitters often colocalize with these catecholaminergic transmitters.

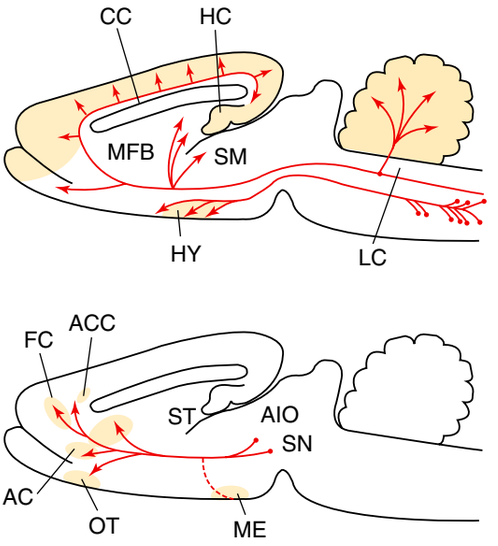

FIGURE 12-5 Some catecholaminergic neuronal pathways in the rat brain. **Upper.** Noradrenergic neuronal pathways. **Lower.** Some dopaminergic neuronal pathways. *A10*, ventral tegmental cell group; *AC*, nucleus accumbens; *ACC*, anterior cingulate cortex; *CC*, corpus callosum; *FC*, frontal cortex; *HC*, hippocampus; *HY*, hypothalamus; *LC*, locus ceruleus; *ME*, median eminence; *MFB*, median forebrain bundle; *OT*, olfactory tubercle; *SM*, stria medullaris; *SN*, substantia nigra; *ST*, striatum. (Courtesy of J. T. Coyle and S. H. Snyder.)

Six groups of cell bodies of noradrenergic neurons are clustered in the medulla oblongata, pons and midbrain. They are considered part of the reticular formation and extend from the spinal cord to the lateral pontine reticular formation. One of the best-studied of these groups is the locus ceruleus (Fig. 12-5), which has many ascending and descending projections from the pons.

Ten groups of dopaminergic neurons are broadly distributed from the retina and olfactory bulb to ventral midbrain and lemniscal area. Some of the most studied of these groups include the tuberoinfundibular neurons involved in endocrine regulation, the nigrostriatal neurons involved in body movement and Parkinson's disease and the mesolimbic and mesocortical ventral tegmental neurons involved in the action of antipsychotic, antihyperactivity and abused drugs (Fig. 12-5). Aside from these brief comments, the functional involvements of these catecholaminergic pathways cannot be summarized here, and the reader is referred to other reviews [29–32] and chapters in Part VIII, Neural Processing and Behavior.

CATECHOLAMINE RECEPTORS

The brain contains many different catecholamine receptors. The effects of dopamine are mediated through interaction with five different receptors; usually referred to as D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4) [33] (Table 12-3).

TABLE 12-3 Properties of human dopamine receptor subtypes

	D_1-like		*D_2-like*		
	D_1	D_5	D_2	D_3	D_4
Amino acids	446	477	415/444	400	387
Chromosome	5	4	11	3	11
Effector pathways	↑cAMP	↑cAMP	↓cAMP ↑K^+ channel ↓ Ca^{2+}channel	↓cAMP ↑K^+ channel ↓Ca^{2+} channel	↓cAMP ↑K^+ channel ↓Ca^{2+} channel
Distribution	Caudate/putamen, nucleus accumbens, olfactory tubercle, cerebral cortex	Hippocampus, hypothalamus, cerebral cortex	Caudate/putamen, nucleus accumbens, midbrain	Olfactory tubercle, hypothalamus,	Frontal cortex, medulla, midbrain, nucleus accumbens

The effects of norepinephrine and epinephrine are mediated through nine distinct receptors grouped into three families (α_1, α_2, β), each containing three subtypes encoded by separate genes [35] (Tables 12-4–12-6). Knockout mice lacking each of these receptors have now been produced and characterized. Knockouts of individual receptors sometimes, but not always, cause overt phenotypical changes (Table 12-7). Because there are so many receptors for each transmitter, it is perhaps not particularly surprising that loss of a single subtype often results in apparently only mild phenotypes.

The postsynaptic receptors on any given neuron receive information from transmitters released from another neuron. Typically, postsynaptic receptors are located on dendrites or cell bodies of neurons, but may also occur on axons or nerve terminals; in the latter case, an axoaxonic synaptic relationship may cause increases or decreases in transmitter release. In contrast, autoreceptors are found on certain neurons and respond to transmitter molecules released from the same neuron. Autoreceptors may be widely distributed on the surface of the neuron. At the nerve terminal, they respond to transmitter molecules released into the synaptic cleft; on the cell body, they may respond to transmitter molecules released by dendrites. Functionally, most autoreceptors appear to decrease further transmitter release in a kind of negative feedback loop. Autoreceptors have been identified for all the catecholamines, as well as for several other neurotransmitters. α_2-adrenergic receptors are often found on noradrenergic nerve terminals of postganglionic sympathetic nerves, as well as on noradrenergic neurons in the CNS [36], and activation of these receptors decreases further norepinephrine release. Dopamine autoreceptors, primarily of the D_2 subtype, are found in the nigrostriatal pathway and also reduce further transmitter release. There is also evidence for other dopamine autoreceptors in the brain and both β and α_1-adrenergic autoreceptors on peripheral sympathetic nerves [37]. However, these are much less common than α_2 and D_2 autoreceptors and can sometimes increase, rather than decrease, further transmitter release.

TABLE 12-4 Properties of human α_1-adrenergic receptor subtypes

	α_{1A}	α_{1B}	α_{1D}
Amino acids	430–476	515	560
Chromosome	8	5	20
Effector pathways	↑Ca^{2+}, protein kinase C	↑Ca^{2+}, protein kinase C	↑Ca^{2+}, protein kinase C
Distribution	Heart, liver, cerebellum, cerebral cortex, blood vessels	Spleen, kidney, fetal brain, blood vessels	Aorta, cerebral cortex

Modified with permission from references [35, 57].

TABLE 12-5 Properties of human α_2-adrenergic receptor subtypes

	α_{2A}	α_{2B}	α_{2C}
Amino acids	450	450	461
Chromosome	10	2	4
Effector pathways	↓cAMP	↓cAMP	↓cAMP
Distribution	Pancreas, small intestine, locus ceruleus, hippocampus	Liver, thalamus	Heart, lung, aorta, hippocampus, olfactory bulb

Modified from [57] with permission.

DOPAMINE RECEPTORS

There are five primary dopamine receptor subtypes. Dopamine receptors are found primarily in brain, although they also exist in kidney [33]. Two subtypes of dopamine receptor were initially identified based primarily on differences in their drug specificities and signaling mechanisms. D_1 receptors were found to stimulate adenylyl cyclase activity, while D_2 receptors inhibited this enzyme (Fig. 12-6). Subsequently, multiple D_1- and D_2-like receptors were identified by molecular cloning (Table 12-3) [33]. All dopamine receptor subtypes are

TABLE 12-6 Properties of human β-adrenergic receptor subtypes

	β_1	β_2	β_3
Amino acids	477	413	402/408
Chromosome	10	5	8
Effector pathways	↑cAMP	↑cAMP	↑cAMP
Distribution	Heart, kidney, cerebral cortex, hypothalamus	Lung, liver, cerebellum, hippocampus, cerebral cortex, smooth muscle, olfactory bulb	Fat, brain (?)

TABLE 12-7 Characteristics of catecholamine receptor knockout mice

Knockout	Phenotype	Reference
α_{1A}-adrenergic receptor	Decreased blood pressure	[58]
α_{1B}-adrenergic receptor	Decreased blood pressure, decreased response to CNS stimulants	[59]
α_{1D}-adrenergic receptor	Decreased blood pressure	[60]
α_{2A}-adrenergic receptor	Increased sympathetic activity, tachycardia, loss of hypotensive response to α_2 agonists	[61]
α_{2B}-adrenergic receptor	Decreased vasoconstrictor response to α_2 agonists	[62]
α_{2C}-adrenergic receptor	No overt phenotype	[63]
β_1-adrenergic receptor	Most die prenatally, survivors have impaired cardiac response	[64]
β_2-adrenergic receptor	Changes in vascular tone and energy metabolism during stress	[65]
β_3-adrenergic receptor	Altered leptin and insulin concentrations after agonist treatment	[66]
D_1-dopamine receptor	Reduced agonist responses, hyperlocomotion	[67]
D_2-dopamine receptor	Parkinsonian-like motor impairment	[68]
D_3-dopamine receptor	Hyperactivity	[69]
D_4-dopamine receptor	Reduced locomotion, hypersensitivity to ethanol and stimulants	[70]
D_5-dopamine receptor	Reduced agonist induced locomotion, startle, and prepulse inhibition	[71]

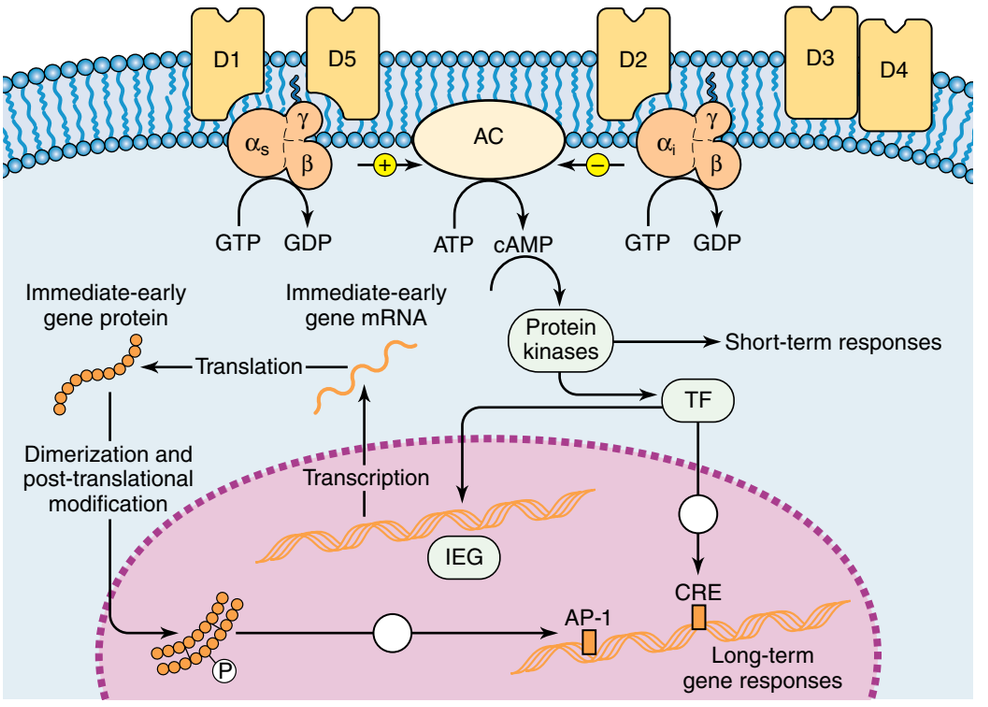

FIGURE 12-6 Effect of dopamine on intracellular signaling pathways. Stimulation of receptors by agonists can change enzyme activities as well as gene expression. Five subtypes of dopamine receptor have been identified. The D_1 and D_5 receptors are coupled to adenylyl cyclase (*AC*) via a stimulatory G protein (G_S). The D_2 receptor inhibits cyclase activity via coupling to an inhibitory G protein (G_i). Activation of D_3 and D_4 receptors also inhibits cAMP production. Adenylyl cyclase catalyzes the conversion of ATP into cAMP, which in turn causes dissociation of the regulatory and catalytic subunits of protein kinase A. The activated catalytic subunit catalyzes conversion of protein substrates into phosphoproteins. For example, PKA phosphorylates and activates DARPP-32 which in turn inhibits protein phosphatase-1 (PP-1). The phosphoproteins activated by the kinase in turn can lead to short-term responses within the cell or activate transcription factors (*TF*) which enter the nucleus and alter gene expression. Long-term gene responses can be initiated by the action of constitutively expressed TFs either directly on DNA or via a mechanism involving transcription of an immediate-early gene (*IEG*) and cytoplasmic production of its protein. This protein can then act on DNA via an adaptor protein 1 (*AP-1*)-binding site. *TF*, constitutively expressed transcription factors, such as a cAMP-response element (CRE), *IEG*, immediate-early genes, such as c-*fos*, c-*jun* and *knox*. For detailed discussion of the regulation of DARRP-32 phosphorylation by D_1 and D_2 receptors see reference [34], and for PP-1 functions, see Ch. 23.

members of the large G-protein-coupled receptor superfamily, which is characterized by seven hydrophobic, transmembrane domains, an extracellular N terminus and an intracellular C terminus (Fig. 12-4). These receptors are subject to post-translational modifications, including glycosylation, palmitoylation and phosphorylation. Consensus sequences for phosphorylation are found in the second (i_2) and third (i_3) intracellular loops and the C-terminal tail. D_1-like receptors have relatively small i_3 loops and long C-tails, while D_2-like receptors have large i_3 loops and short C-tails.

D_1-like receptors include the D_1 and D_5 receptors, have a high affinity for benzazepines like SCH-23390 and a low affinity for benzamides, and are coupled to stimulation of adenylyl cyclase activity through G_S, leading to increases in cyclic AMP and activation of PKA (see Chs 19 and 21). One of the consequences of dopamine receptor activation of PKA is the phosphorylation of DARPP-32, a protein phosphatase inhibitor enriched in dopaminoceptive neurons (see Ch. 23). The most striking pharmacological difference among D_1 and D_5 receptors is the high affinity of D_5 receptors for dopamine. The activity of D_1-like receptors modulates the functioning of the prefrontal cortex [38] and these receptors are increased in the prefrontal cortex of patients with schizophrenia [39].

D_2-like receptors include the D_2, D_3 and D_4 receptors. In addition, molecular cloning has demonstrated the presence of two isoforms of D_2 receptors, designated D_2L and D_2S [40]. These two isoforms differ by a 29-amino-acid insert in the i_3 loop of the receptor (Fig. 12-4), which appears to arise through alternative splicing. Although most (90%) G-protein-coupled receptors contain no introns in their protein coding blocks [41], D_2 receptors are an exception and contain multiple introns, allowing for alternative splicing. Both D_2L and D_2S receptors inhibit adenylyl cyclase activity through G_I, leading to decreased production of cyclic AMP (see Chs 19 and 21). The function of these splice variants has recently been elegantly elucidated by the use of transgenic animals, where D_2L appears to act mainly at postsynaptic sites and D_2S is primarily a presynaptic autoreceptor [42]. Interestingly, the cataleptic effects of the widely used antipsychotic drug haloperidol are absent in D_2L-deficient mice [42], suggesting that this receptor may be one of the main targets for haloperidol. D_2, D_3 and D_4 receptors are found in a variety of brain regions (Table 12-3). Pharmacologically, D_3 receptors have a relatively high affinity for atypical antipsychotics and for dopamine autoreceptor inhibitors, including (+)-UH232 and (+)-AJ76, while D_4 receptors have a particularly high affinity for the atypical antipsychotic drug clozapine. D_3 and D_4 receptors also inhibit adenylyl cyclase through G_i, and D_4 receptors have also been reported to be positively coupled to K^+ channels.

ADRENERGIC RECEPTORS

There are nine primary adrenergic receptor subtypes. Since norepinephrine and epinephrine are important messengers in both the peripheral sympathetic nervous system and the brain, adrenergic receptors are widely distributed in peripheral tissues as well as existing in high concentrations in the brain. Two subtypes of adrenergic receptor were first identified in the 1940s by differences in the relative potencies of catecholamine agonists [43]. α-adrenergic receptors were found to be potently activated by the natural catecholamines norepinephrine and epinephrine but not by the synthetic analog isoproterenol. Conversely, β-adrenergic receptors were potently stimulated by all three agonists. Subtypes of β-adrenergic receptor were first discovered in the 1960s and subtypes of α-adrenergic receptor in the 1970s [44]. Further pharmacological analysis and molecular cloning approaches have now shown that there are nine distinct adrenergic receptors, subclassified into three major families (α_1, α_2, β) based on their pharmacological properties, amino acid sequences and signaling mechanisms [35, 44] (Tables 12-4–12-6). All the adrenergic receptors, like the dopamine receptors, are members of the G-protein coupled receptor superfamily and are thought to have similar seven-transmembrane domain structures.

All subtypes within each adrenergic receptor family appear to be linked to the same primary signaling mechanism, raising the question of whether these receptors are redundant or have distinct functions. α_1-adrenergic receptor subtypes all activate $G_{q/11}$, increasing phospholipase Cβ activity and releasing inositol 1,4,5 trisphosphate and diacylglycerol (see Ch. 20). These actions synergistically increase the activity of protein kinase C, as well as causing many other effects due to increased intracellular calcium. α_2-adrenergic receptor subtypes all activate G_i to inhibit adenylyl cyclase (as do D_2-like receptors), leading to decreases in cyclic AMP and decreased phosphorylation of proteins by PKA. α_2-adrenergic receptor subtypes also appear to be linked to N-type calcium channels, inhibiting their function [45], particularly when they function as autoreceptors. Conversely, β-adrenergic receptor subtypes all activate G_S to increase adenylyl cyclase activity (as do D_1-like receptors), thereby increasing cyclic AMP and subsequent PKA activity.

All three α_1-adrenergic receptor genes contain a conserved intron in their protein coding sequence near the end of the sixth transmembrane domain [41]. Four splice variants have been reported for α_{1A}-adrenergic receptors, which appear to differ only in their final six to 53 amino acids at the distal end of the C-tail. The functional importance of these splice variants are not yet understood, and it is not yet known whether similar splice variants occur for the other two members of this family. Interestingly, the β_3- but not the β_1- or β_2-adrenergic receptor also contains an intron in its protein coding block only a few amino acids from the C-terminus [41]. C-terminal splice variants of this receptor have also been reported, although their function remains unclear.

Adrenergic receptors are also subject to multiple post-translational modifications. Like dopamine receptors, they are glycosylated, palmitoylated and phosphorylated on various residues. There are multiple consensus

phosphorylation sites for serine and threonine residues in the i_3 loops and C termini, and phosphorylation may be important in agonist-induced receptor sequestration and desensitization [46] (see also below). Both cAMP-dependent and non-cAMP-dependent phosphorylations of β-adrenergic receptors have been observed and in many cases the phosphorylated receptor is functionally uncoupled from G_S. Tyrosine phosphorylation of β-adrenergic receptors has also been reported. Other receptors coupled to activation of adenylyl cyclase can also cause what is known as heterologous desensitization. In addition, occupancy of β-adrenergic receptors by agonists results in the activation of β-adrenergic receptor kinase (βARK), which leads to phosphorylation of the receptor. The uncoupling of the receptor from G_S also appears to involve a protein called β-arrestin [46], which is similar to the 48 kDa arrestin protein in the retina (see Ch. 49).

The widespread distribution and the important functional activities of adrenergic receptors have made them attractive therapeutic targets for drug development. All adrenergic receptors are found in brain, although in widely varying patterns. In peripheral tissues, different subtypes often coexist in tissues and cells, but one subtype is often dominant. For example, α_1-adrenergic receptors are most important in smooth muscle contraction, and α_2- receptors in decreasing pancreatic insulin secretion. β_1-receptors are dominant in cardiac muscle, while β_2-receptors are most important in bronchial smooth muscle. Therefore, selective β_1 antagonists such as atenolol are widely used for cardiovascular problems, without the undesirable effect of promoting bronchoconstriction, which is mediated by the β_2-subtype. Conversely, selective β_2-agonists such as albuterol are useful in treating asthma and chronic obstructive pulmonary disease, without undesirable cardiovascular actions. Selective α_1-blockers such as prazosin are used for treating hypertension by blocking sympathetic vasoconstriction and reducing total peripheral resistance. Surprisingly, selective α_2-agonists such as clonidine are also used for treating hypertension by stimulating CNS control centers to reduce total sympathetic outflow. Unfortunately, highly selective agonists and antagonists have not yet been developed for the closely related subtypes within these subfamilies, greatly limiting their therapeutic utility.

The role of particular adrenergic receptor subtypes in brain function is still poorly understood. There is clearly a differential distribution of subtypes within different brain regions, determined primarily by radioligand binding, quantitative autoradiography, and/or *in situ* hybridization with specific mRNA sequences. α_1-adrenergic receptor subtypes are enriched in most brain regions, with the density of α_{1A} and α_{1B} subtypes generally much higher than α_{1D}; α_{2A} and α_{2C} receptors are found in most brain regions while α_{2B} is found primarily in thalamus; β_1 and β_2 subtypes are widely distributed but there is evidence for only low levels of β_3-adrenergic receptor expression in brain [47]. Elucidation of the functional importance of adrenergic receptor subtypes in CNS has been hampered by the lack of subtype-selective agonists and antagonists that cross the blood–brain barrier, but has recently been at least partially clarified by the use of knockout mice. For example, such studies suggest that the α_{2A} subtype is primarily responsible for the central hypotensive effects of α_2-adrenergic receptor agonists [48]. Interestingly, α_{1B} knockout mice show dramatically reduced stimulation of locomotor activity and reward responses to psychostimulants and opiate-type drugs of abuse [49], suggesting an important role for this subtype in vulnerability to addiction.

DYNAMICS OF CATECHOLAMINE RECEPTORS

Chronic exposure to agonists often results in diminished responsiveness. Neurotransmitter receptors are not static entities and, in both the peripheral sympathetic nervous system and the brain, receptor numbers can be modulated by chronic increases or decreases in agonist activation. The phenomenon of desensitization refers to a loss in cellular responsiveness caused by chronic agonist stimulation, often due to uncoupling of the receptor from its immediate effector mechanism. A related phenomenon is termed downregulation, which usually occurs with a slower time course and involves much more extensive cellular adaptations such as receptor degradation. Although occurring with most receptor subtypes, the mechanisms involved in desensitization and downregulation have been most extensively explored for β_2-adrenergic receptors. As mentioned above, within minutes of exposure to agonist, β_2-adrenergic receptors are phosphorylated by a specific kinase, βARK, which phosphorylates only agonist-occupied receptors [46]. Agonist-dependent phosphorylation by βARK promotes binding of β-arrestin to the receptor, which uncouples the receptor from G_S, thereby stopping signaling. This functional uncoupling is followed by a sequestration of cell-surface β_2-adrenergic receptors into intracellular compartments, with partial recycling to the cell surface and partial degradation. Similar, but independent, cAMP-dependent phosphorylation of the receptor has also been observed, and there appears to be a complex interplay between different types of phosphorylation events and the multiple molecular mechanisms involved in desensitization. These events also appear to involve both transcriptional and post-transcriptional regulatory mechanisms, including changes in transcription factor availability and receptor mRNA levels, feedback phosphorylation, changes in mRNA stability and targeting of receptors to particular cellular compartments for recycling or degradation (see also Chs 9 and 21). It now seems likely that similar mechanisms of desensitization occur with many, if not all, of the catecholamine receptors to different extents [50], although this has yet to be definitively proved.

Chronic exposure to antagonists often results in increased responsiveness. It is also well established that chronic treatment with antagonists to block receptor

stimulation, or surgical or chemical denervation, can result in compensatory increases in receptor density and/or responsiveness. This phenomenon, commonly referred to as supersensitivity or sensitization, was first observed after sympathetic denervation of peripheral organs in the early 20th century and has now also been observed extensively in brain [51]. Supersensitivity can result from a rapid loss of release sites for catecholamines following denervation, a more slowly occurring increase in receptor density on postsynaptic cells due to diminished stimulation, or from other compensatory processes resulting in increases in the efficiency of signal transduction from the activated receptor to downstream effectors [51]. Like desensitization, different degrees of supersensitivity have been observed with most of the catecholamine receptors when they are deprived of their normal tonic stimulatory tone.

Changes in receptor density are associated with altered synaptic activity. Destruction of catecholamine-containing nerves can be accomplished by treatment with selective neurotoxins such as 6-hydroxydopamine and usually causes functional supersensitivity of postsynaptic effector cells. Conversely, administration of tricyclic antidepressants or inhibitors of MAO, which would increase the concentrations of catecholamines within the synaptic cleft, often leads to functional subsensitivity. Many of these changes appear to be a compensatory response and involve changes in catecholamine receptor density. Destruction of the dopamine-containing nigrostriatal pathway can be accomplished surgically and has well described behavioral consequences. Unilateral nigrostriatal lesion causes asymmetry in dopamine innervation between the two cerebral hemispheres. Behavioral studies have demonstrated that the dopamine receptors in the denervated corpus striatum are supersensitive. Apomorphine, a dopamine agonist that stimulates dopamine receptors selectively, causes rotational behavior in rats with unilateral lesions in a direction contralateral to the lesion. In contrast, indirect agonists such as cocaine cause rotation in the opposite direction, since they act by releasing or blocking reuptake of dopamine. Thus, these agents will only activate receptors on the side with an intact dopaminergic input (see Ch. 46).

The extent of receptor supersensitivity after unilateral nigrostriatal lesions can be quantified by measuring the extent of rotational behavior. After selective nigrostriatal lesions have been produced in rats by injections of 6-hydroxydopamine into the substantia nigra, the number of dopamine receptors in the ipsilateral corpus striatum increases markedly, and the increase in the number of receptors may correlate with the extent of behavioral supersensitivity as monitored by rotational behavior [52]. Thus, the increase in receptor density appears to play a role in the behavioral supersensitivity of these animals.

The number of D_1 and D_2 receptors can be modulated by antagonists. The density of both D_1 and D_2 receptors can also be increased by chronic administration of antagonists [53]. Different subtypes of dopamine receptor may be coregulated since the D_2 antagonist sulpiride attenuated the ability of SCH-23390 to increase the density of D_1 receptors. The increase in the density of D_2 receptors following chronic administration of antagonists may be responsible for the development of a movement disorder called tardive dyskinesia (see Ch. 46). For example, when either acute or repeated doses of agonists acting at dopamine receptors are administered to rats, the result is an augmentation of the behavioral effects of the drugs. This phenomenon, known as reverse tolerance or sensitization, is characterized by a selective increase in the intensity or duration or a shift to an earlier time of onset of stereotypical behaviors such as locomotion, sniffing, rearing, licking and gnawing. Sensitization to indirect dopamine agonists such as amphetamine or cocaine also occurs. The mechanisms underlying behavioral sensitization are likely to be complex. It is known, for example, that stereotypical behavior and locomotor hyperactivity are critically dependent on activation of both D_1 and D_2 receptors.

Dopamine D_2-like receptors appear to mediate the actions of antipsychotic drugs. A strong correlation exists between the clinical potencies of antipsychotic drugs and their affinity for brain D_2 receptors [53]. This has led to the hypothesis that psychotic disorders result from overstimulation of D_2-like receptors (Chs 46 and 54). Long-term administration of antipsychotic drugs to humans or experimental animals can result in an increase in the density of striatal D_2 receptors and in the appearance of extrapyramidal side effects, including parkinsonian movement disorders and tardive dyskinesia. Certain antipsychotic drugs such as clozapine, olanzapine and quetiapine, referred to as atypical antipsychotics, have been reported to produce fewer extrapyramidal side effects than typical antipsychotics. They have been useful in treatment of patients with schizophrenia who respond poorly to typical antipsychotics such as haloperidol. The relative affinities of D_2, D_3 and D_4 receptors for typical and atypical antipsychotics together with the selective expression of D_3 receptor mRNA in limbic areas of the brain have led to the hypothesis that the clinical utility of antipsychotics in the treatment of psychiatric illness may be due, at least in part, to their ability to antagonize stimulation of D_3 or D_4 receptors, while the motor dysfunction observed following chronic treatment with typical antipsychotics could be due to alterations in the density of D_2 receptors in the striatum.

Changes in dopamine receptor density may be involved in the side effects of antipsychotic drugs. One of the most serious side effects of the antipsychotic drugs is tardive dyskinesia, a disfiguring, excessive motor activity of the tongue, face, arms and legs in patients treated chronically with large doses of the drugs (see Ch. 46). Paradoxically, reduction of the dosage worsens the

symptoms, whereas increasing the dosage alleviates the symptoms. It has been suggested that tardive dyskinesia reflects a supersensitivity of dopamine receptors that have been chronically blocked. This hypothesis gains support from the direct demonstration that chronic treatment with antipsychotics leads to an increase in the number of dopamine receptors in the corpus striatum. Moreover, the ability of antipsychotics to elicit this increase correlates with their ability to block dopamine receptors. The recently introduced antipsychotic aripiprazole is a partial D_2 agonist and has been associated with a very low incidence of extrapyramidal side effects. If this is borne out after more extensive use in the clinic, partial agonists may represent an important and novel avenue for future development of drugs to modulate catecholaminergic neural systems.

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REFERENCES

1. Shiman, R., Akino, M. and Kaufman, S. Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J. Biol. Chem.* 246: 1330–1340, 1971.
2. Grima, B., Lamouroux, A., Blanot, F., Biguet, N. F. and Mallet, J. Complete coding sequence of rat tyrosine hydroxylase mRNA. *Proc. Natl Acad. Sci. U.S.A.* 82: 617–621, 1985.
3. Craine, J. E., Daniels, G. H. and Kaufman, S. Dopamine-beta-hydroxylase. The subunit structure and anion activation of the bovine adrenal enzyme. *J. Biol. Chem.* 248: 7838–7844, 1973.
4. Lamouroux, A., Vigny, A., Faucon Biguet, N. *et al.* The primary structure of human dopamine-beta-hydroxylase: insights into the relationship between the soluble and the membrane-bound forms of the enzyme. *EMBO J.* 6: 3931–3937, 1987.
5. Connett, R. J. and Kirshner, N. Purification and properties of bovine phenylethanolamine *N*-methyltransferase. *J. Biol. Chem.* 245: 329–334, 1970.
6. Baetge, E. E., Suh, Y. H. and Joh, T. H. Complete nucleotide and deduced amino acid sequence of bovine phenylethanolamine *N*-methyltransferase: partial amino acid homology with rat tyrosine hydroxylase. *Proc. Natl Acad. Sci. U.S.A.* 83: 5454–5458, 1986.
7. Bohn, M. C., Bloom, E., Goldstein, M. and Black, I. B. Glucocorticoid regulation of phenylethanolamine N-methyltransferase (PNMT) in organ culture of superior cervical ganglia. *Dev. Biol.* 105: 130–136, 1984.
8. Zubieta, J. K., Huguelet, P., Ohl, L. E. *et al.* High vesicular monoamine transporter binding in asymptomatic bipolar I disorder: sex differences and cognitive correlates. *Am. J. Psychiatry* 157: 1619–1628, 2000.
9. Cordeiro, M. L., Gundersen, C. B. and Umbach, J. A., Lithium ions modulate the expression of VMAT2 in rat brain. *Brain Res.* 953: 189–194, 2002.
10. Weinshilboum, R. M., Thoa, N. B., Johnson, D. G., Kopin, I. J. and Axelrod, J. Proportional release of norepinephrine and dopamine-β-hydroxylase from sympathetic nerves. *Science* 174: 1349–1351, 1971.
11. Alousi, A. and Weiner, N. The regulation of norepinephrine synthesis in sympathetic nerves: effect of nerve stimulation, cocaine, and catecholamine-releasing agents. *Proc. Natl Acad. Sci. U.S.A.* 56: 1491–1496, 1966.
12. Goldstein, M, Long- and short-term regulation of tyrosine hydroxylase. In F. E. Bloom and D. J. Kupfer (eds), *Psychopharmacology: The Fourth Generation of Progress.* New York: Raven Press, 1995, pp. 189–196.
13. Zigmond, R. E., Schwarzschild, M. A. and Rittenhouse, A. R. Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. *Annu. Rev. Neurosci.* 12: 415–461, 1989.
14. Costa, E. and Sandler, M. *Monoamine Oxidase: New Vistas.* New York: Raven Press, 1972.
15. Larsen, J. K., Gjerris, A., Holm, P. *et al.* Moclobemide in depression: a randomized, multicentre trial against isocarboxazide and clomipramine emphasizing atypical depression. *Acta Psychiatr. Scand.* 84: 564–570, 1991.
16. Gogos, J. A., Morgan, M., Luine, V. *et al.* Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. *Proc. Natl Acad. Sci. U.S.A.* 95: 9991–9996, 1998.
17. Egan, M. F., Goldberg, T. E., Kolachana, B. S. *et al.* Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc. Natl Acad. Sci. U.S.A.* 98: 6917–6922, 2001.
18. Axelrod, J. Noradrenaline: fate and control of its biosynthesis. *Science* 173: 598–606, 1971.
19. Amara, S. G. and Kuhar, M. J. Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* 16: 73–93, 1993.
20. Foster, J. D., Pananusorn, B. and Vaughan, R. A. Dopamine transporters are phosphorylated on N-terminal serines in rat striatum. *J. Biol. Chem.* 277: 25178–25186, 2002.
21. Granas, C., Ferrer, J., Loland, C. J., Javitch, J. A. and Gether, U. N-terminal truncation of the dopamine transporter abolishes phorbol ester- and substance P receptor-stimulated phosphorylation without impairing transporter internalization. *J. Biol. Chem.* 278: 4990–5000, 2003.
22. Vaughan, R. Phosphorylation and regulation of psychostimulant-sensitive neurotransmitter transporters. *J. Pharmacol. Exp. Ther.* 310: 1–7, 2004.
23. Agnati, L. F., Zoli, M., Stromberg, I. and Fuxe, K. Intercellular communication in the brain: wiring versus volume transmission. *Neuroscience* 69: 711–726, 1995.
24. Smiley, J. F., Levey, A. I., Ciliax, B. J. and Goldman-Rakic, P. S. D1 dopamine receptor immunoreactivity in human and monkey cerebral cortex: predominant and extrasynaptic localization in dendritic spines. *Proc. Natl Acad. Sci. U.S.A.* 91: 5720–5724, 1994.
25. Aoki, C., Venkatesan, C., Go, C. G., Forman, R. and Kurose, H. Cellular and subcellular sites for noradrenergic action in the monkey dorsolateral prefrontal cortex as revealed by the immunocytochemical localization of noradrenergic receptors and axons. *Cereb. Cortex* 8: 269–277, 1998.
26. Garris, P. A. and Wightman, R. M. Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. *J. Neurosci.* 14: 442–450, 1994.

27. Sesack, S. R.,Hawrylak, V. A., Matus, C., Guido, M. A. and Levey, A. I. Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse immunoreactivity for the dopamine transporter. *J. Neurosci.* 18: 2697–2708, 1998.
28. Bjorklund, A. and Hokfelt, T. (eds) *Handbook of Chemical Neuroanatomy*. New York: Elsevier, 1984.
29. Moore, R. Y. Organization of midbrain dopamine systems and the pathophysiology of Parkinson's disease. *Parkinsonism Relat. Disord.* 9(Suppl 2): S65–S71, 2003.
30. Sesack, S. R. and Carr, D. B. Selective prefrontal cortex inputs to dopamine cells: implications for schizophrenia. *Physiol. Behav.* 77: 513–517, 2002.
31. Charney, D. S. and Deutch, A. A functional neuroanatomy of anxiety and fear: implications for pathophysiology and treatment of anxiety disorders. *Crit. Rev. Neurobiol.* 10: 419–446, 1996.
32. Weiss, J. M. and Simson, P. E. Neurochemical and electrophysiological events underlying stress-induced depression in an animal model. *Adv. Exp. Med. Biol.* 245: 425–440, 1988.
33. Sibley, D. R. New insights into dopaminergic receptor function using antisense and genetically altered animals. *Annu. Rev. Pharmacol. Toxicol.* 39: 313–341, 1999.
34. Svenningsson, P., Nishi, A., Fisone, G., Girault, J.-A., Nairn, A. C. and Greengard, P. DARPP-32: an integrator of neurotransmission. *Annu. Rev. Pharmacol. Toxicol.* 44: 269–296, 2004.
35. Hieble, J. P., Bylund, D. B., Clarke, D. E. *et al.* International Union of Pharmacology. X. Recommendation for nomenclature of alpha 1-adrenoceptors: consensus update. *Pharmacol. Rev.* 47: 267–270, 1995.
36. Starke, K. Presynaptic autoreceptors in the third decade: focus on alpha2-adrenoceptors. *J. Neurochem.* 78: 685–693, 2001.
37. Starke, K. Presynaptic autoreceptors in the third decade: focus on alpha2-adrenoceptors. *J. Neurochem.* 78: 685–693, 2001.
38. Goldman-Rakic, P. S., Muly, E. C. III and Williams, G. V. D(1) receptors in prefrontal cells and circuits. *Brain Res. Brain Res. Rev.* 31: 295–301, 2000.
39. Abi-Dargham, A., Mawlawi, O., Lombardo, I. *et al.* Prefrontal dopamine D1 receptors and working memory in schizophrenia. *J. Neurosci.* 22: 3708–3719, 2002.
40. Giros, B., Sokoloff, P., Martres, M. P., Riou, J. F., Emorine, L. J. and Schwartz, J. C. Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature* 342: 923–926, 1989.
41. Minneman, K. Splice variants of G protein coupled receptors. *Mol. Interv.* 1: 108–116, 2001.
42. Usiello, A., Baik, J. H., Rouge-Pont, F. *et al.* Distinct functions of the two isoforms of dopamine D_2 receptors. *Nature* 408: 199–203, 2000.
43. Ahlquist, R. A study of adrenotropic receptors. *Am. J. Physiol.* 153: 586–600, 1948.
44. Bylund, D. B., Eikenberg, D. C., Hieble, J. P. *et al.* International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* 46: 121–136, 1994.
45. Boehm, S. and Huck, S. Inhibition of N-type calcium channels: the only mechanism by which presynaptic alpha 2-autoreceptors control sympathetic transmitter release. *Eur J. Neurosci.* 8: 1924–1931, 1996.
46. Luttrell, L. M. and Lefkowitz, R. J. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J. Cell Sci.* 115: 455–465, 2002.
47. Pupo, A. and Minneman, K. Adrenergic pharmacology: focus on the central nervous system. *CNS Spectr.* 6: 656–662, 2001.
48. MacMillan, L. B., Hein, L., Smith, M. S., Piascik, M. T. and Limbird, L. E. Central hypotensive effects of the alpha2a-adrenergic receptor subtype. *Science* 273: 801–803, 1996.
49. Drouin, C., Darracq, L., Trovero, F. *et al.* Alpha1b-adrenergic receptors control locomotor and rewarding effects of psychostimulants and opiates. *J. Neurosci.* 22: 2873–2884, 2002.
50. Heck, D. A. and Bylund, D. B. Differential down-regulation of alpha-2 adrenergic receptor subtypes. *Life Sci.* 62: 1467–1472, 1998.
51. Fleming, W. W. Cellular adaptation: journey from smooth muscle cells to neurons. *J. Pharmacol. Exp. Ther.* 291: 925–931, 1999.
52. Pycock, C. J. Turning behaviour in animals. *Neuroscience* 5: 461–514, 1980.
53. Strange, P. G. Antipsychotic drugs: importance of dopamine receptors for mechanisms of therapeutic actions and side effects. *Pharmacol. Rev.* 53: 119–133, 2001.
54. Chen, L. and Zhuang, X. Transgenic mouse models of dopamine deficiency. *Ann. Neurol.* 54(Suppl. 6): S91–S102, 2003.
55. Swoap, S. J., Weinshenker, D., Palmiter, R. D. and Garber, G. Dbh(-/-) mice are hypotensive, have altered circadian rhythms, and have abnormal responses to dieting and stress. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286: R108–R113, 2004.
56. Bohn, L. M., Xu, F., Gainetdinov, R. R. and Caron, M. G. Potentiated opioid analgesia in norepinephrine transporter knock-out mice. *J. Neurosci.* 20: 9040–9045, 2000.
57. Bylund, D. B. Subtypes of alpha 1- and alpha 2-adrenergic receptors. *FASEB J.* 6: 832–839, 1992.
58. Rokosh, D. G. and Simpson, P. C. Knockout of the alpha 1A/C-adrenergic receptor subtype: the alpha 1A/C is expressed in resistance arteries and is required to maintain arterial blood pressure. *Proc. Natl Acad. Sci. U.S.A.* 99: 9474–9479, 2002.
59. Cavalli, A., Lattion, A. L., Hummler, E. *et al.* Decreased blood pressure response in mice deficient of the alpha1b-adrenergic receptor. *Proc. Natl Acad. Sci. U.S.A.* 94: 11589–11594, 1997.
60. Tanoue, A., Nasa, Y., Koshimizu, T. *et al.* The alpha(1D)-adrenergic receptor directly regulates arterial blood pressure via vasoconstriction. *J. Clin. Invest.* 109: 765–775, 2002.
61. Altman, J. D., Trendelenburg, A. U., MacMillan, L. *et al.* Abnormal regulation of the sympathetic nervous system in alpha2A-adrenergic receptor knockout mice. *Mol. Pharmacol.* 56: 154–161, 1999.
62. Link, R. E., Desai, K., Hein, L. *et al.* Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. *Science* 273: 803–805, 1996.
63. Kable, J. W., Murrin, L. C. and Bylund, D. B. In vivo gene modification elucidates subtype-specific functions of alpha(2)-adrenergic receptors. *J. Pharmacol. Exp. Ther.* 293: 1–7, 2000.
64. Rohrer, D. K., *et al.* Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects. *Proc. Natl Acad. Sci. U.S.A.* 93: 7375–7380, 1996.

65. Chruscinski, A. J., Rohrer, D. K., Schauble, E., Desai, K. H., Bernstein, D. and Kobilka, B. K. Targeted disruption of the beta2 adrenergic receptor gene. *J. Biol. Chem.* 274: 16694–16700, 1999.
66. Susulic, V. S., Frederich, R. C., Lawitts, J. *et al.* Targeted disruption of the beta 3-adrenergic receptor gene. *J. Biol. Chem.* 270: 29483–29492, 1995.
67. Xu, M., Moratalla, R., Gold, L. H. *et al.* Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin and in dopamine-mediated behavioral responses. *Cell* 79: 729–742, 1994.
68. Baik, J. H., Picetti, R., Saiardi, A. *et al.* Parkinsonian-like locomotor impairment in mice lacking dopamine D_2 receptors. *Nature* 377: 424–428, 1995.
69. Accili, D., Fishburn, C. S., Drago, J. *et al.* A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. *Proc. Natl Acad. Sci. U.S.A.* 93: 1945–1949, 1996.
70. Rubinstein, M., Phillips, T. J., Bunzow, J. R. *et al.* Mice lacking dopamine D4 receptors are supersensitive to ethanol, cocaine, and methamphetamine. *Cell* 90: 991–1001, 1997.
71. Holmes, A., Hollon, T. R., Gleason, T. C. *et al.* Behavioral characterization of dopamine D5 receptor null mutant mice. *Behav. Neurosci.* 115: 1129–1144, 2001.
72. Waymire, J. C. and Craviso, G. L. Multiple site phosphorylation and activation of tyrosine hydroxylase. *Adv. Protein Phosphatases* 7: 501–513, 1993.

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CHAPTER

13

Serotonin

Julie G. Hensler

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SEROTONIN, THE NEUROTRANSMITTER

The indolealkylamine 5-hydroxytryptamine was initially identified because of its effects on smooth muscle. Since the mid-19th century, scientists have known that after blood clots, a substance in the resulting serum constricts vascular smooth muscle and increases vascular tone. Around the turn of the century, platelets were identified as the source of this substance. It wasn't until 1948 that Rapport, Green and Page isolated and correctly identified the serum vasoconstrictor factor as the substituted indole 5-hydroxytryptamine (5-HT) [1]. Quite appropriately, this serum tonic factor, released into sera from platelets during the clotting of blood, was named 'serotonin'. Independently, Esparmer characterized a substance found in large amounts in the enterochromaffin cells of the gastrointestinal tract, which also constricted smooth muscle. He called this substance 'enteramine'. In 1952, Esparmer and Areso reported that serotonin and enteramine were the same substance. Twarog and Page, in 1953, detected serotonin in extracts of brain using a sensitive bioassay, the mollusk heart. Thus, serotonin was localized to three key systems in the body: platelets, gastrointestinal tract and brain.

The structures of serotonin (5-HT) and related compounds are shown in Figure 13-1. The combination of the hydroxyl group in the 5 position of the indole nucleus and a primary amine nitrogen serving as a proton acceptor at physiological pH makes 5-HT a hydrophilic substance. Therefore, it does not pass the lipophilic blood–brain barrier readily. For this reason, its discovery in brain in 1953 by Twarog and Page was exciting in that it indicated that 5-HT was being synthesized in brain. At about the same time the observation was made that (+)lysergic acid diethylamide (LSD), which was known to have psychedelic or psychotomimetic effects, antagonized a response to 5-HT

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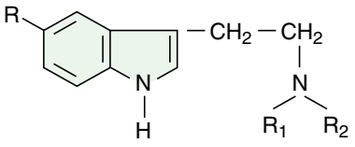

Compound	Position		
	R	R_1	R_2
Tryptamine	H	H	H
Serotonin	OH	H	H
Melatonin	OCH_3	$COCH_3$	H
Diethyltryyptamine (DET)*	H	CH_3CH_2	CH_3CH_2
Dimethyltryptamine (DMT)*	H	CH_3	CH_3
Bufotenine*	OH	CH_3	CH_3

*Psychotropic (modifies mental activity)

FIGURE 13-1 Chemical structure of 5-hydroxytryptamine (5-HT; serotonin) and related indolealkylamines. The indole ring structure consists of the benzene ring and the attached five-member ring containing nitrogen.

(i.e. the contraction of gastrointestinal smooth muscle produced by serotonin) (see detailed discussion in Ch. 56). This further substantiated an important role of 5-HT in brain function. The idea that 5-HT had important behavioral effects stimulated much thought about a central role for 5-HT in mental illness. Subsequently, various theories arose linking abnormalities of 5-HT function in brain to the development of a number of psychiatric disorders, particularly schizophrenia and depression. Psychotherapeutic drugs are now available that are effective in depression, anxiety disorders and schizophrenia, and have potent, and in some cases selective, effects on serotonergic neurons in brain (see Chs 54 and 55).

Understanding the neuroanatomical organization of serotonergic neurons and projections provides insight into this neurotransmitter's functions and possible roles in mental processes. Serotonin-containing neuronal cell bodies are restricted to discrete clusters or groups of cells located along the midline of the brainstem. Their axons, however, innervate nearly every area of the central nervous system (Fig. 13-2). In 1964, Dahlstrom and Fuxe, using the Falck–Hillarp technique of histofluorescence, observed that the majority of serotonergic soma were found in cell body groups that had been previously designated by Taber, Brodal and Walberg as the raphe nuclei. This earlier description of the raphe

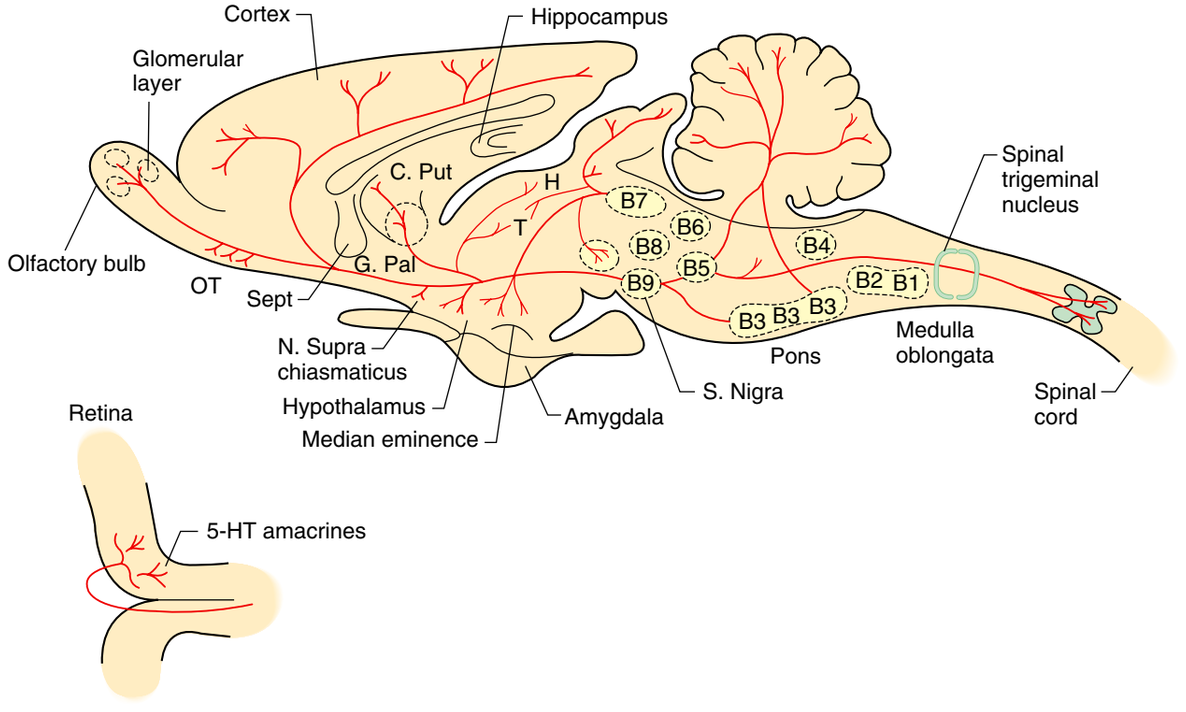

FIGURE 13-2 Schematic drawing depicting the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections. *OT*, olfactory tuberculum; *Sept*, septum; *C. Put*, nucleus caudate-putamen; *G. Pal*, globus pallidus; *T*, thalamus; *H*, habenula. (With permission from Consolazione, A. and Cuello, A. CNS serotonin pathways. In: *The Biology of Serotonergic Transmission.* New York: John Wiley & Sons, 1982, pp 29–61.)

nuclei was based on cytoarchitectural criteria, i.e. on cell body structural characteristics and organization. Dahlstrom and Fuxe described nine groups of serotonin-containing cell bodies, which they designated B_1–B_9 and which correspond for the most part with the raphe nuclei [2] (Table 13-1). Some serotonergic neuronal cell bodies, however, are found outside the raphe nuclei, and not all the cell bodies in the raphe nuclei are serotonergic. In most of the raphe nuclei, the majority of neurons are nonserotonergic. For example, the dorsal raphe contains the largest number of serotonergic neurons; however, only 40–50% of the cell bodies in the dorsal raphe are serotonergic (Fig. 13-3).

Over the course of the last three decades, a variety of techniques have been used to characterize the circuitry of serotonergic neurons in the central nervous system. The density of serotonergic innervation in the forebrain was initially underestimated because the original histofluorescence method was limited in sensitivity and did not permit the detection of many fine axons and terminals.

TABLE 13-1 Classification of serotonergic cell body groups according to Dahlstrom and Fuxe and corresponding anatomical structure

Groups of serotonin-containing cell bodies	Anatomical structure
B_1	Raphe pallidus nucleus
	Caudal ventrolateral medulla
B_2	Raphe obscurus nucleus
B_3	Raphe magnus nucleus
	Rostral ventrolateral medulla
	Lateral paragigantocellular reticular nucleus
B_4	Raphe obscurus nucleus, dorsolateral part
B_5	Median raphe nucleus, caudal part
B_6	Dorsal raphe nucleus, caudal part
B_7	Dorsal raphe nucleus principal, rostral part
B_8	Median raphe nucleus, rostral main part
	Caudal linear nucleus
	Nucleus pontis oralis
B_9	Nucleus pontis oralis
	Supralemniscal region

Source: From Tork [2].

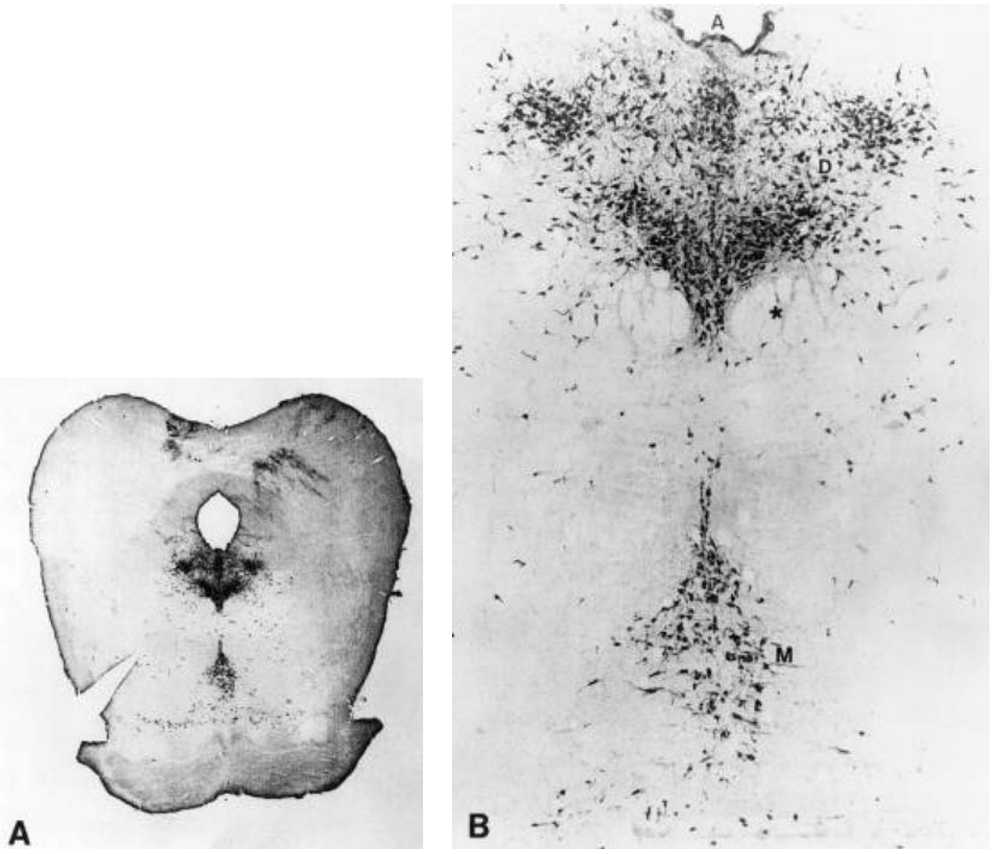

FIGURE 13-3 Serotonergic cell bodies in the midbrain raphe nuclei demonstrated by 5-HT immunocytochemistry. (**A**) Low magnification of transverse section through rat midbrain. The serotonergic cell body groups shown give rise to widespread serotonergic projections to cerebral cortex and forebrain structures. (**B**) Higher magnification micrograph showing serotonergic cell bodies in dorsal and median raphe nuclei. The dorsal raphe nucleus lies in the central gray matter just beneath the cerebral aqueduct. In the transverse plane, the dorsal raphe can be further subdivided into a ventromedial cell cluster between and just above the medial longitudinal fasciculus (MLF), a smaller dorsomedial group just below the aqueduct, and large bilateral cell groups. The median raphe nucleus lies in the central core of the midbrain, below the MLF. *D*, dorsal raphe; *M*, median raphe, *A*, aqueduct. (With permission from Molliver [4].)

Subsequent anatomical techniques (e.g. immunohistochemistry of 5-HT or tryptophan hydroxylase, an enzyme unique to the synthesis of 5-HT; retrograde and anterograde axonal transport studies) have allowed a more complete and accurate characterization of the serotonergic innervation of forebrain areas.

The largest group of serotonergic cells is group B_7 of Dahlstrom and Fuxe. B_7 is continuous with a smaller group of serotonergic cells, B_6. Groups B_6 and B_7 are often considered together as the dorsal raphe nucleus, with B_6 being its caudal extension. Another prominent serotonergic cell body group is B_8, which corresponds to the median raphe nucleus, also termed the nucleus central superior. Group B_9, part of the ventrolateral tegmentum of the pons and midbrain, forms a lateral extension of the median raphe and therefore is not considered one of the midline raphe nuclei. Ascending serotonergic projections innervating the cerebral cortex and other regions of the forebrain arise primarily from the dorsal raphe, median raphe, and B_9 cell group. The serotonergic neurons of the median and dorsal raphe nucleus differ in their electrophysiological characteristics and in their inhibition by somatodendritic autoreceptor activation [3], as well as in the morphology and topographical organization of their axonal projections to the forebrain (see below). These differences may be extremely important in understanding the role of these two distinct serotonergic systems, arising from the dorsal and median raphe nuclei, in normal brain function and in mental illness.

The two main ascending serotonergic pathways from the midbrain raphe nuclei to the forebrain are the dorsal periventricular path and the ventral tegmental radiations. Both pathways converge in the caudal hypothalamus where they join the medial forebrain bundle. Axons of dopaminergic (A8, A9, A10) and noradrenergic (A6) cell body groups also course anteriorly through the medial forebrain bundle.

Ascending projections from the raphe nuclei to forebrain structures are organized in a topographical manner. The dorsal and median raphe nuclei give rise to distinct projections to forebrain regions (Fig. 13-4). The median raphe projects heavily to hippocampus, septum and hypothalamus, whereas the striatum is innervated predominantly by the dorsal raphe. The dorsal and median raphe nuclei send overlapping neuronal projections to the neocortex. Within the dorsal and median raphe, cells are organized in particular zones or groups that send axons to specific areas of brain. For example, the frontal cortex receives heavy innervation from the rostral and lateral subregions of the dorsal raphe nucleus. Moreover, raphe

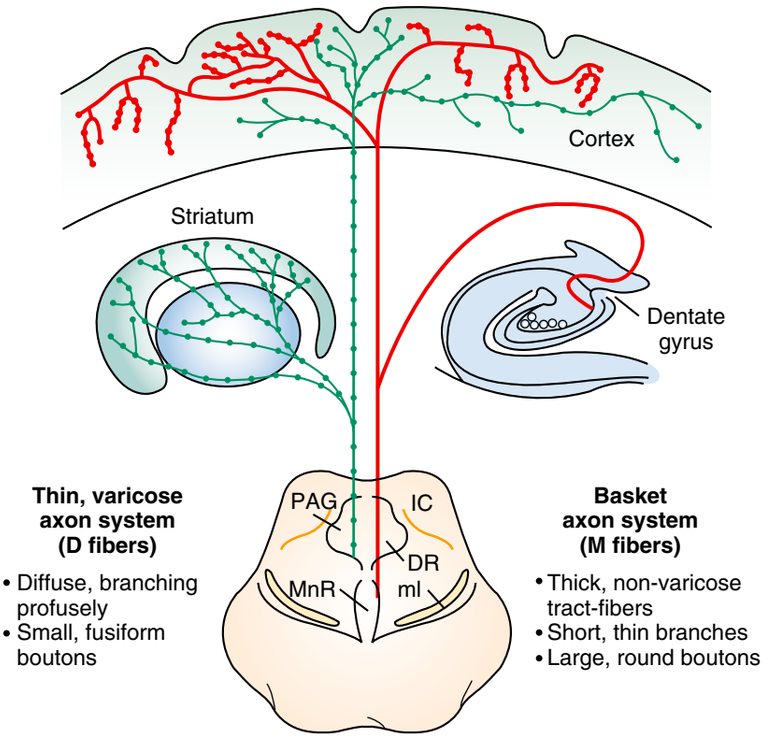

FIGURE 13-4 Simplified diagram of the main features of the dual serotonergic system innervating the forebrain. The fine varicose axon system (*D fibers*) arises from the dorsal raphe (*DR*) nucleus with fibers that branch profusely in their target areas. It is difficult to demonstrate the synaptic connections of these fibers and therefore the incidence of synapses on these fibers is still being debated. The basket axon system (*M fibers*) arises from the median raphe (*MnR*) nucleus with thick, nonvaricose axons, giving rise to branches with characteristic axons that appear beaded, with round or oval varicosities. These large terminals make well-defined synapses with target cells. *PAG*, periaqueductal gray matter; *IC*, inferior colliculus; *ml*, medial lemniscus. (With permission from Tork [2].)

neurons send collateral axons to areas of brain that are related in function such as the amygdala and hippocampus, or substantia nigra and caudate putamen. The highly organized innervation of forebrain structures by serotonergic neurons of the raphe is quite interesting in that it implies independent functions of sets of serotonergic neurons dependent on their origin and terminal projections, as opposed to a nonselective or general role for 5-HT in the central nervous system.

Serotonergic axon terminals appear to exhibit morphological differences related to the raphe nucleus of origin (Fig. 13-4). Serotonergic axons from the median raphe nucleus (type M) look relatively coarse with large spherical varicosities. By contrast, axons from the dorsal raphe (type D) are very fine and typically have small, pleomorphic varicosities. Dorsal raphe axons appear to be more vulnerable to certain neurotoxic amphetamine derivatives, e.g. 3,4-methylenedioxymethamphetamine (MDMA, or 'Ecstasy') and parachloroamphetamine (PCA) (see also Ch. 56). Median raphe axons appear to be more resistant to the neurotoxic effects of these drugs. Blockade of the serotonin transporter (SERT) prevents the neurotoxic effects of these amphetamine derivatives, indicating that activity of this transporter is critical for the neurotoxic effects of these drugs.

The other raphe nuclei, B_1–B_4, are more caudally situated (mid-pons to caudal medulla) and contain a smaller number of serotonergic cells. These cell body groups give rise to serotonergic axons that project within the brainstem and to the spinal cord (Fig. 13-2). The spinal cord receives a strong serotonergic innervation. Three principal descending pathways have been described: (1) from the raphe magnus nucleus (B_3) to laminae I and II of the dorsal horn; (2) from the raphe obscurus nucleus (B_2, B_4) to lamina IX of the ventral horn; (3) from the rostral ventrolateral medulla and the lateral paragigantocellular reticular nucleus (B_3) to the intermediolateral cell column. Projections from the caudal ventrolateral medulla (B_1) are not known at present [2].

Afferent connections to the raphe nuclei include connections between the dorsal and median raphe nuclei, B_9, B_1 and B_3. Connections between the raphe nuclei have been described by retrograde tracing techniques using horseradish peroxidase and wheat germ agglutinin. Such innervation may have considerable physiological and/or pharmacological importance as serotonin released in the vicinity of serotonergic cell bodies regulates the firing of serotonergic neurons through the activation of somatodendritic autoreceptors. The raphe nuclei also receive input from other cell body groups in the brainstem such as the substantia nigra and ventral tegmental area (dopamine), superior vestibular nucleus (acetylcholine), locus ceruleus (norepinephrine), nucleus prepositus hypoglossi and nucleus of the solitary tract (epinephrine). Other afferents include neurons from the hypothalamus, cortex, and limbic forebrain structures such as the amygdala [2, 4].

The amino acid L-tryptophan is the precursor for the synthesis of 5-HT. The synthesis and primary metabolic pathways of 5-HT are shown in Figure 13-5. The initial step in the synthesis of serotonin is the facilitated transport of the amino acid L-tryptophan from blood into brain. The primary source of tryptophan is dietary protein. Other neutral amino acids, such as phenylalanine, leucine and methionine, are transported by the same carrier into the brain. Therefore, the entry of tryptophan into brain is not only related to its concentration in blood but is also a function of its concentration in relation to the concentrations of other neutral amino acids. Consequently, lowering the dietary intake of tryptophan while raising the intake of the amino acids with which it competes for transport into brain lowers the content of 5-HT in brain and changes certain behaviors associated with 5-HT function. This strategy for lowering the brain content of 5-HT has been used clinically to evaluate the importance of brain 5-HT in the mechanism of action of psychotherapeutic drugs.

Serotonergic neurons contain the enzyme L-tryptophan-5-monooxygenase (EC 1.14.16.4), more commonly termed tryptophan hydroxylase, which converts tryptophan to 5-hydroxytryptophan (5-HTP) (Fig. 13-5). Tryptophan hydroxylase contains 444 amino acids, corresponding to a molecular weight of about 51 Da. This enzyme is synthesized in serotonergic cell bodies of the raphe nuclei and is found only in cells that synthesize 5-HT. Therefore its distribution in brain is similar to that of 5-HT itself. The K_m of tryptophan hydroxylase for tryptophan is approximately 30–60 µmol/l, a concentration comparable to that of tryptophan in brain. If the concentration of tryptophan in serotonergic neurons is assumed to be comparable to that in whole brain, the enzyme would not be saturated with substrate, and the formation of 5-HT in brain would be expected to rise as the brain concentration of tryptophan increases. This has been found to occur in response to raising the dietary intake of tryptophan specifically.

The other enzyme involved in the synthesis of 5-HT, aromatic L-amino acid decarboxylase (AADC) (EC 4.1.1.28), is a soluble pyridoxal-5′-phosphate-dependent enzyme, which converts 5-HTP to 5-HT (Fig. 13-5). It has been demonstrated that administration of pyridoxine increases the rate of synthesis of 5-HT in monkey brain, as revealed using position emission tomography (this technique is discussed in Ch. 58). This presumably reflects a regulatory effect of pyridoxine on AADC activity and raises the interesting issue of the use of pyridoxine supplementation in situations associated with 5-HT deficiency.

AADC is present not only in serotonergic neurons but also in catecholaminergic neurons, where it converts 3,4-dihydroxyphenylalanine (DOPA) to dopamine (see Ch. 12). However, different pH optima or concentrations of substrate or cofactor are required for optimum activity of the enzyme in brain homogenates when using either 5-HTP or DOPA as the substrate. cDNAs encoding AADC

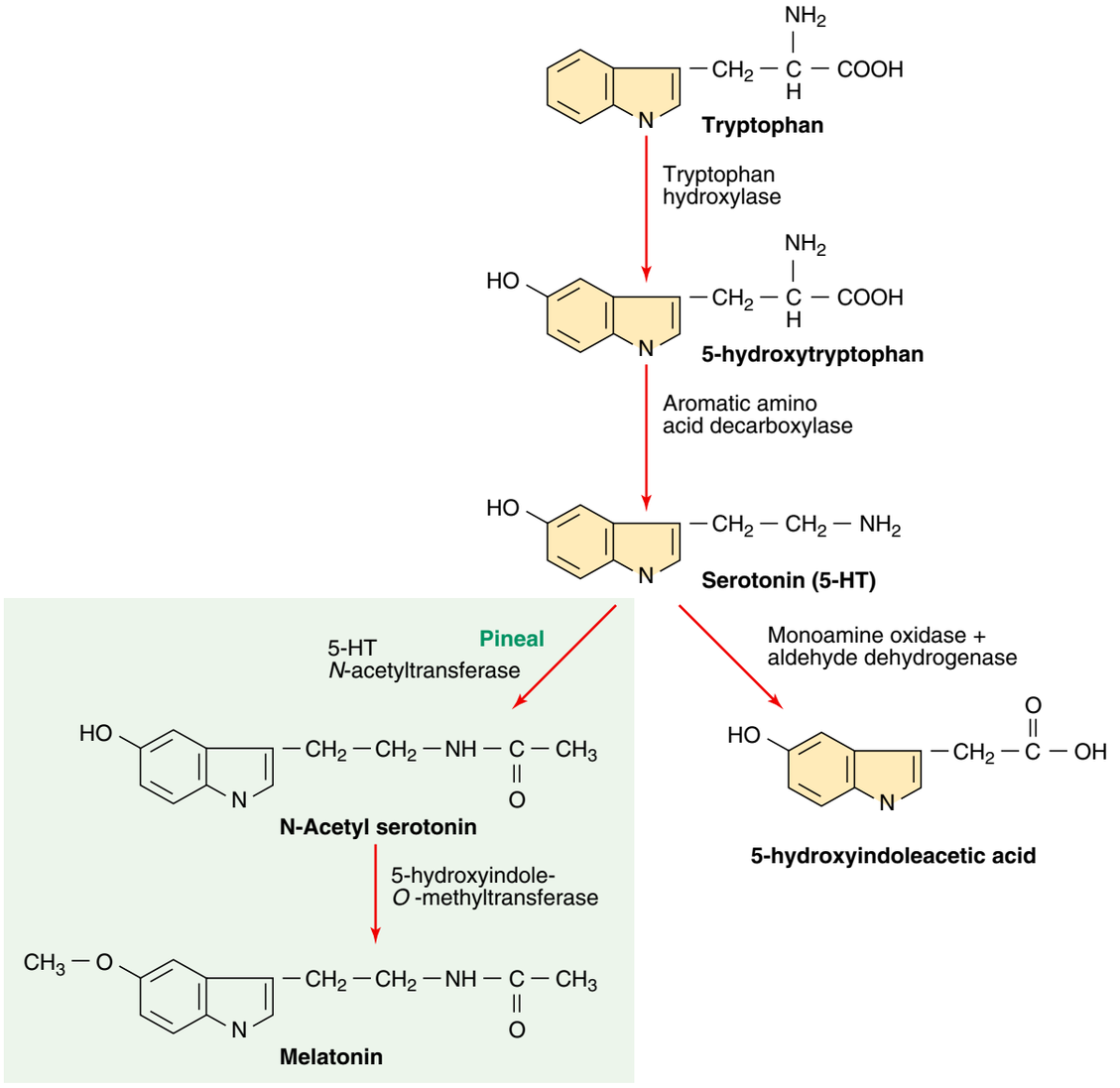

FIGURE 13-5 The biosynthesis and catabolism of serotonin. Note that in the pineal gland, serotonin is converted enzymatically to melatonin.

have been cloned from various species. The encoded protein contains 380 amino acids and has a molecular weight of 54 kDa, but it appears to exist as a dimer. Characterization of the protein expressed in cells transfected with the cDNA shows that it decarboxylates either DOPA or 5-HTP. Also, *in situ* hybridization of the mRNA for the enzyme revealed its presence both in serotonergic cells in the dorsal raphe nucleus and in catecholaminergic cells in brain regions containing catecholaminergic soma [5]. Taken together, these results support the idea that the decarboxylation of both DOPA and 5-HTP are catalyzed by the same enzyme.

Because AADC is not saturated with 5-HTP under physiological conditions, (i.e. the concentration of 5-HTP is much less than the enzyme's K_m of 10 µmol/l), it is possible to raise the content of 5-HT in brain not only by increasing the dietary intake of tryptophan but also by raising the intake of 5-HTP. This procedure, though, results in the formation of 5-HT in cells that would not normally contain it (e.g. catecholaminergic neurons) because of the nonselective nature of AADC.

The initial hydroxylation of tryptophan, rather than the decarboxylation of 5-HTP, appears to be the rate-limiting step in serotonin synthesis. Therefore, the inhibition of this reaction results in a marked depletion of the content of 5-HT in brain. The enzyme inhibitor most widely used in experiments is parachlorophenylalanine (PCPA). *In vivo,* PCPA irreversibly inhibits tryptophan hydroxylase, presumably by incorporating itself into the enzyme to produce an inactive protein. This results in a long-lasting reduction of 5-HT levels. Recovery of enzyme activity, and 5-HT biosynthesis, requires the synthesis of new enzyme. Marked increases in mRNA for tryptophan hydroxylase are found in the raphe nuclei 1–3 days after administration of PCPA [6].

The synthesis of 5-HT can increase markedly under conditions requiring more neurotransmitter. Plasticity is an important concept in neurobiology. In general, this refers to the ability of neuronal systems to conform to either short- or long-term demands placed upon their activity or function (see Plasticity in Ch. 53). One of the processes contributing to neuronal plasticity is the ability to increase the rate of neurotransmitter synthesis and release in response to increased neuronal activity. Serotonergic neurons have this capability; the synthesis of 5-HT from tryptophan is increased in a frequency-dependent manner in response to electrical stimulation of serotonergic soma [7]. The increase in synthesis results from the enhanced conversion of tryptophan to 5-HTP and is dependent on extracellular calcium ion. It is likely that the increased 5-HT synthesis results in part from alterations in the kinetic properties of tryptophan hydroxylase, perhaps due to calcium-dependent phosphorylation of the enzyme by calmodulin-dependent protein kinase II or cAMP-dependent protein kinase (PKA; see Ch. 23).

Short-term requirements for increases in the synthesis of 5-HT can be met by post-translational processes, such as phosphorylation, that change the kinetic properties of tryptophan hydroxylase without necessitating the synthesis of more molecules of tryptophan hydroxylase. By contrast, situations requiring long-term increases in the synthesis and release of 5-HT result in the synthesis of tryptophan hydroxylase protein. For example, partial but substantial destruction (>60%) of central serotonergic neurons results in an increase in the synthesis of 5-HT in residual terminals. Although the increase in synthesis initially results from activation of existing tryptophan hydroxylase molecules, the increased synthesis of 5-HT seen weeks after the lesion results from more tryptophan hydroxylase being present in the residual terminals. An increase in tryptophan hydroxylase mRNA has been reported in residual serotonergic neurons after partial lesion with the neurotoxins 5,7-dihydroxytryptamine or MDMA [8, 9], consistent with the idea of an increase in the synthesis of tryptophan hydroxylase molecules in residual neurons.

5-HT is stored primarily in vesicles and is released into the synaptic cleft by an exocytotic mechanism. Peripheral sources of monoamine-containing cells have been utilized to study the properties of storage vesicles, for example chromaffin cells of the adrenal medulla for catecholamines and parafollicular cells of the thyroid gland for 5-HT [10]. In some respects, the vesicles that store 5-HT resemble those that store catecholamines. For example, drugs such as reserpine and tetrabenazine, which inhibit the activity of the transporter localized to the vesicular membrane, deplete the brain content of 5-HT as well as catecholamines. Storage of 5-HT in vesicles requires its active transport from the cytoplasm. The vesicular 'transporter' uses the electrochemical gradient generated by a vesicular H^+-ATPase to drive transport, such that a cytoplasmic amine is exchanged for a luminal proton – i.e. the uptake of 5-HT is coupled to the efflux of H^+ (Fig. 13-6A) (see also Ch. 5).

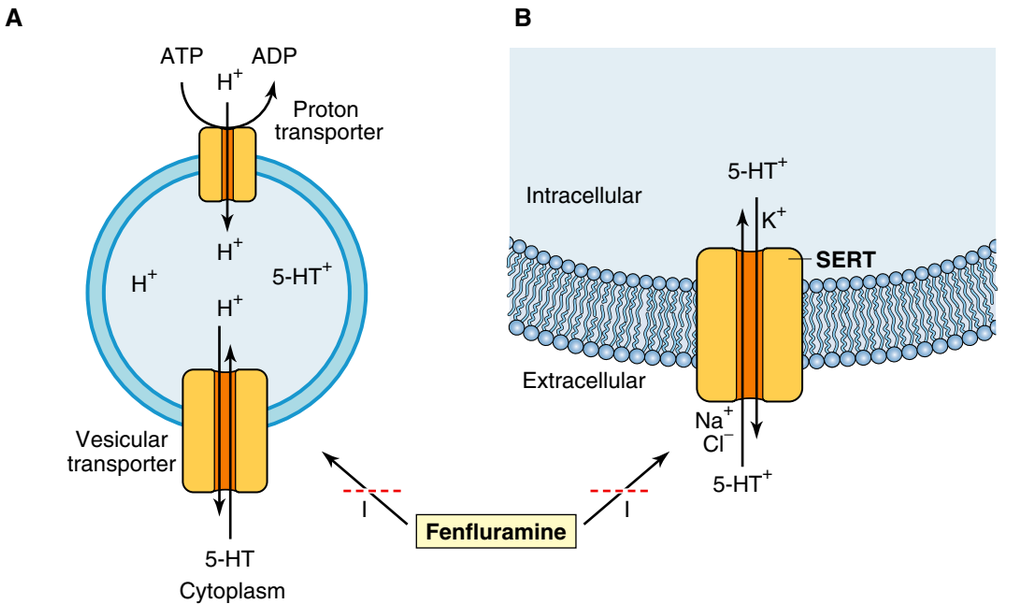

FIGURE 13-6 The substituted amphetamine fenfluramine inhibits the transport of 5-HT by both (**A**) the vesicular transporter, and (**B**) the serotonin transporter (*SERT*). Substituted amphetamines such as fenfluramine and MDMA stimulate the release of 5-HT from serotonergic terminals. These drugs block the vesicular transporter and disrupt the proton gradient across the vesicle membrane. The increase in intracellular 5-HT favors the release of 5-HT by the reverse action of the SERT. These drugs also act as substrates for the SERT so as to inhibit the transport of 5-HT into cells.

Drugs that inhibit the vesicular transporter do not generally block the plasma membrane transporter (serotonin transporter, SERT) and *vice versa*. However, two drugs that have effects at both the vesicular transporter and the SERT are MDMA or 'Ecstasy', and the anorexic agent fenfluramine. Fenfluramine and MDMA inhibit the vesicular transporter directly by competing for its substrate binding site. These agents also dissipate the transmembrane pH gradient to further inhibit 5-HT uptake into the vesicle (Fig. 13-6A). The effects of these drugs on the vesicles storing 5-HT raises cytoplasmic 5-HT and facilitates SERT-mediated efflux of 5-HT. Both fenfluramine and MDMA act as substrates for the SERT and not only inhibit the transport of 5-HT into the cell but also facilitate its outward transport by the SERT (Fig. 13-6B). The consequence of such dual pharmacological actions on both the vesicle pump and the plasmalemma transporter is the stimulation of the release of 5-HT by a calcium ion-independent (i.e. non-exocytotic) process. In contrast to exocytotic release, this release process is not modulated by terminal auto- or heteroreceptors. The release of 5-HT caused by drugs such as fenfluramine is prevented by drugs that block the SERT.

Vesicles storing 5-HT exhibit some differences from those storing catecholamines. In contrast to catecholamine-containing vesicles, there is virtually no ATP in serotonin vesicles. Also, serotonergic synaptic vesicles, but not chromaffin granules, contain a specific protein that binds 5-HT with high affinity in the presence of Fe^{2+}. This serotonin-binding protein (SPB) is packaged in secretory vesicles along with 5-HT, which probably accounts for the observation that newly taken up ^{3}H-5-HT is rapidly complexed with this protein in brain *in situ*. SPB is secreted along with 5-HT by a calcium-dependent process.

There is considerable evidence that the release of 5-HT occurs by exocytosis, i.e. by the discharge from the cell of the entire content of individual storage vesicles. First, 5-HT is sufficiently ionized at physiological pH so that it does not cross plasma membranes by simple diffusion. Second, most intraneuronal 5-HT is contained in storage vesicles and other contents of the vesicle including SPB are released together with serotonin. By contrast, cytosolic proteins do not accompany electrical stimulation-elicited release of 5-HT. Third, the depolarization-induced release of 5-HT occurs by a calcium-dependent process; indeed, it appears that the influx of extracellular calcium ions with or without membrane depolarization can increase the release of 5-HT. Calcium stimulates the fusion of vesicular membranes with the plasma membrane (see Chs 9, 10).

Serotonin release is regulated in part by the firing rate of serotonergic soma in the raphe nuclei. Numerous studies utilizing a variety of techniques have revealed that an increase in raphe cell firing enhances the release of 5-HT in terminal fields. The opposite effect is observed when raphe cell firing decreases. This means that drugs that change the firing rate of serotonergic soma modify the release of 5-HT as well. Important targets for such drugs are somatodendritic autoreceptors, which, as is discussed later, are 5-HT_{1A} receptors (see Fig. 13-8). Activation of these receptors by 5-HT or exogenous agonist slows the rate of firing of serotonergic soma. As discussed below, serotonergic autoreceptors on terminals appear to be either the 5-HT_{1B} or 5-HT_{1D} subtype, depending on the species. Activation of these receptors decreases the synthesis and release of 5-HT. However, in contrast to the activation of somatodendritic autoreceptors, such effects are not due to decreases in the firing rate of serotonergic soma.

The activity of 5-HT in the synapse is terminated primarily by its re-uptake into serotonergic terminals. The synaptic effects of many amino acid and monoaminergic neurotransmitters, including 5-HT, are terminated by the binding of these molecules to specific transporter proteins. The serotonin transporter (SERT) is located on serotonergic neurons. Evidence for this comes from studies showing that the selective lesion of serotonergic neurons in brain markedly reduces both the high-affinity uptake of ^{3}H-5-HT in areas of brain receiving serotonergic innervation and the specific binding of radioligands to the SERT. The mRNA for the SERT has been localized in brain exclusively to the serotonergic cells in the raphe nuclei. Of interest is the fact that mRNA for the SERT has not yet been detected in glia, even though primary cultures of astrocytes *in vitro* can take up 5-HT. Activity of the SERT regulates the concentration of 5-HT in the synapse, thereby influencing synaptic transmission.

The uptake system for 5-HT is saturable and of high affinity, with a K_m value for 5-HT of approximately 0.2–0.5 µmol/l. The uptake of 5-HT is an active process that is temperature-dependent and has an absolute requirement for external Na^+ and Cl^- it is inhibited by metabolic inhibitors as well as by inhibitors of Na^+/K^+-ATPase activity. From these and other data it has been inferred that the energy requirement for 5-HT uptake is not directly used to transport 5-HT but rather is necessary to maintain the gradient of Na^+ across the plasma membrane upon which 5-HT uptake is dependent (see Ch. 5). The current model of transport has one Na^+, one Cl^- and one protonated 5-HT binding to the transporter extracellularly to form a quaternary complex that subsequently undergoes a conformational change to release the neurotransmitter and the ions into the cytoplasm. In the cytoplasm, K^+ associates with the SERT to promote the reorientation of the unloaded carrier for another transport cycle (Fig. 13-6B). This 'alternating access' hypothesis of serotonin transport, however, does not adequately account for electrophysiological data that are better explained by a channel model of coupled transport [11].

The cloning, sequencing and expression of several transporter proteins, including that for 5-HT, has aided considerably in understanding structure/function relationships of transporter proteins [12]. The cDNA for the SERT isolated from rat brain predicts a protein containing

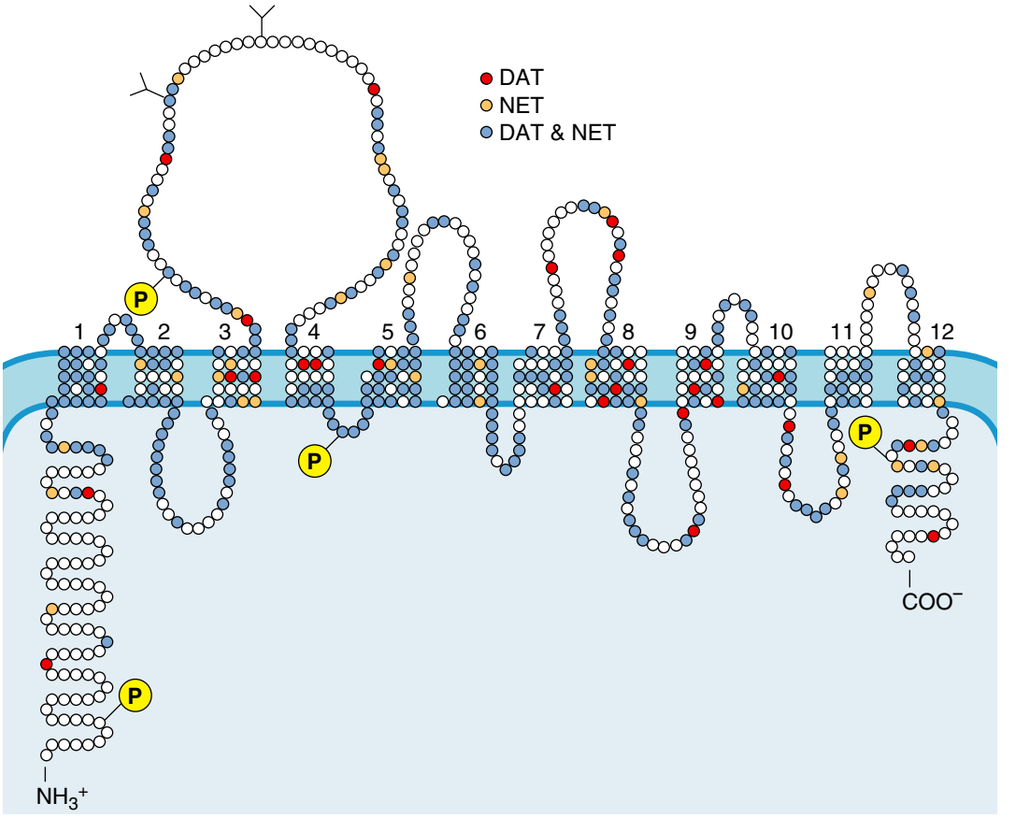

FIGURE 13-7 Putative structure of the rat serotonin transporter (SERT) showing homologous amino acids with the rat dopamine transporter (*DAT*), human norepinephrine transporter (*NET*) or both. Possible phosphorylation (*P*) sites are shown, as are possible glycosylation sites on the large second extracellular loop. Note the considerable degree of homology in the 12 transmembrane-spanning regions. (Courtesy of Dr Beth J. Hoffman, Amgen, Thousand Oaks, CA.)

630 amino acids with a molecular weight of about 68,000 Daltons or 68 kDa. The putative structure of the SERT has 12 transmembrane domains (TMDs), with both the amino- and carboxy-termini being intracellular, and a large extracellular loop containing glycosylation sites connecting TMDs 3 and 4 (Fig. 13-7). Glycosylation seems necessary for optimal stability of the transporter in the membrane but not for 5-HT transport or ligand binding. There are also six potential sites of phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) on the human SERT (see Ch. 23). The predicted structure of the SERT is similar to the predicted structure of other cloned neurotransmitter transporters and quite distinct, for example, from the seven-transmembrane-domain structure of G-protein-linked receptors. These transporters are considered to be members of the Na^+ and Cl^- dependent neurotransporter family, distinct from the vesicular transporter family described earlier. All members of this family are fragmented by multiple introns, raising the possibility of multiple transcripts by alternative RNA processing.

The serotonin transporter exhibits about 50% absolute homology with the transporters for norepinephrine (NET) and dopamine (DAT), with the greatest homology being found in TMDs 1 and 2, and TMDs 4–8. The least conserved regions are the intracellular amino- and carboxy-terminal tails (Fig. 13-7). It has been proposed that the conserved regions may be involved in general transport functions and the least conserved regions in the unique attributes of each carrier, i.e. pharmacological specificity. However, studies in which the terminal regions were exchanged between the SERT and the NET revealed no alteration in substrate or antagonist selectivity, suggesting that the NH_2 and COOH termini may not, in fact, be important for ligand recognition. There is evidence for a lack of strict substrate selectivity among biogenic amine transporters, particularly in situations where the function of one transporter is compromised. For example, studies using *in vivo* chronoamperometry to measure the clearance of 5-HT from the extracellular fluid, indicate that 5-HT can be taken up by the NET [12].

Although there is structural homology among the transporters for 5-HT, norepinephrine and dopamine, some drugs exhibit great selectivity at inhibiting the activity of just one of these proteins. For example, the selective norepinephrine re-uptake inhibitor reboxetine is 150 times more potent at inhibiting norepinephrine transport

than that of 5-HT. By contrast, the selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, sertraline or paroxetine, are 25–100 times more potent in inhibiting the uptake of 5-HT than the uptake of norepinephrine. There are also drugs, such as cocaine, that are nonselective inhibitors of all three transporters.

It has been known for over 25 years that many of the tricyclic antidepressants (TCAs), e.g. imipramine and amitriptyline, are potent inhibitors of both norepinephrine and 5-HT reuptake. Some tricyclic antidepressants, e.g. desipramine, inhibit the uptake of norepinephrine much more potently than the uptake of 5-HT. Thus, it was unclear for some time whether the inhibition of 5-HT uptake played any role in the antidepressant action of those TCAs that possessed this pharmacological property. Recently, however, effective antidepressants such as fluoxetine, paroxetine and sertraline have been marketed and these SSRIs are much more potent inhibitors of the uptake of 5-HT than that of norepinephrine (Fig. 13-8). Thus, selective inhibition of the uptake of either norepinephrine or 5-HT can result in an antidepressant effect (Ch. 55).

The SSRIs, which were initially developed as antidepressants, are now the most widely used agents in the treatment of many additional neuropsychiatric disorders (e.g. eating disorders, anxiety, obsessive–compulsive disorder) (Fig. 13-8). Clomipramine, a TCA that is somewhat selective *in vivo* as an inhibitor of 5-HT uptake, also produces clinically significant amelioration of the symptoms associated with obsessive–compulsive disorder. Thus, the inhibition of 5-HT uptake, not only has an antidepressant effect, but also reduces the symptoms of anxiety disorders, including obsessive–compulsive disorder. This clinical effect is not found with drugs such as desipramine that selectively inhibit the uptake of norepinephrine (see also Ch. 55).

Acute and chronic regulation of serotonin transporter function provides means for altering synaptic 5-HT concentrations and neurotransmission. Long-term exposure to drugs that block the SERT have been shown to downregulate the SERT. A number of studies have shown that chronic administration of SSRIs produces a reduction in SERT activity and expression. In postmortem samples of dorsal raphe nucleus from cocaine users, SERT binding sites are reduced in number, suggesting a downregulation of the SERT with chronic cocaine exposure. In addition to this long-term regulation of SERT function by drugs that block the SERT, there is a

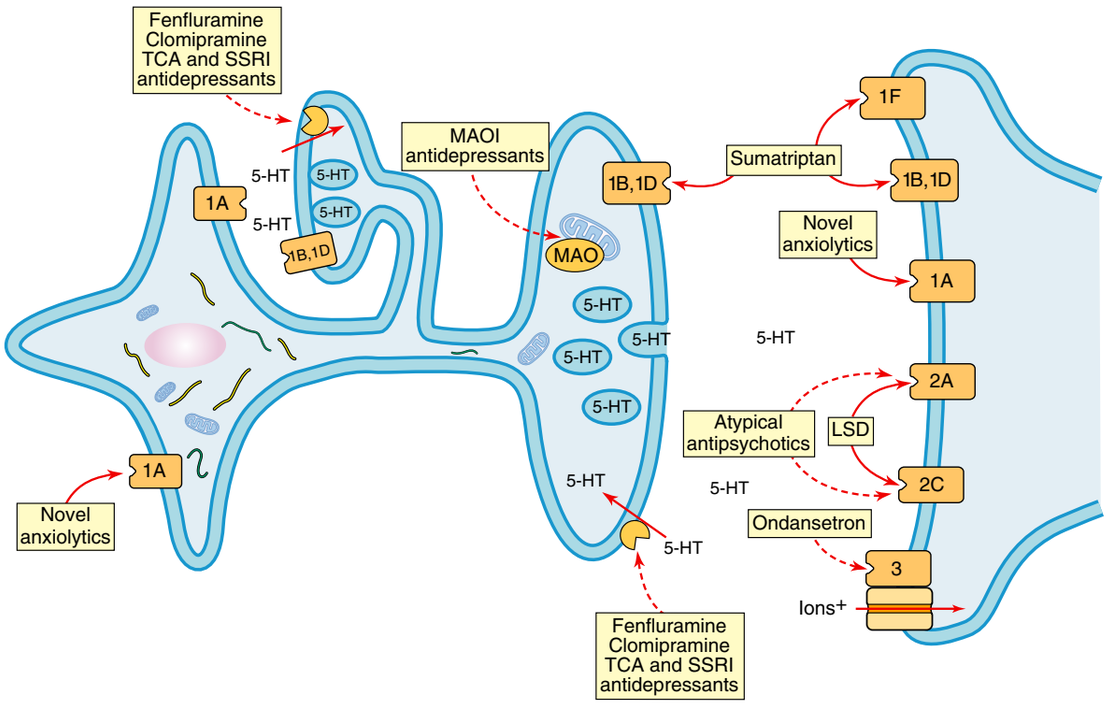

FIGURE 13-8 Serotonin neurons and receptors are targets for a wide variety of therapeutic drugs. Drugs that act as agonists to activate receptors are indicated by *solid-line arrows*, whereas antagonists or inhibitors are shown with *broken-line arrows*. The 5-HT_{1A} receptor acts as both the somatodendritic autoreceptor and a postsynaptic receptor; anxiolytic drugs such as buspirone are agonists at this receptor. In terminal fields, the autoreceptor is either the 5-HT_{1B} or 5-HT_{1D} subtype; these receptors also function as postsynaptic receptors. The antimigraine drug sumatriptan is an agonist at these receptors as well as the 5-ht_{1F} receptor. Hallucinogenic drugs such as LSD are agonists at 5-HT_{2A} and 5-HT_{2C} receptors whereas atypical antipsychotic drugs such as clozapine and olanzapine are antagonists. The 5-HT_3 receptor, a ligand-gated ion channel, is blocked by drugs effective in the treatment of chemotherapy-induced nausea and emesis, such as ondansetron. Another important target for psychotherapeutic drugs is the serotonin transporter, which is blocked by drugs effective in the treatment of depression or obsessive-compulsive disorder (clomipramine). The enzyme responsible for the catabolism of serotonin, MAO, is inhibited by another class of antidepressants.

more dynamic, short-term regulation of SERT function by substrate exposure, membrane potential changes and presynaptic receptor activation/inhibition. Depolarization of the plasma membrane is associated with reduced transport velocity, whereas hyperpolarization results in enhanced transport. The transient inhibition of uptake during neuronal depolarization may allow 5-HT to diffuse away from the presynaptic terminal to interact with postsynaptic receptors. Substrate occupancy and/or transport activity promotes retention of the SERT on the cell surface, thereby maintaining SERT function [12]. Acute presynaptic receptor-mediated regulation of SERT function has been demonstrated in experiments using *in vivo* chronoamperometry; the activation of 5-HT_{1B} terminal autoreceptors increases SERT activity [13].

The regulation of SERT number or function by cyclic changes in estrogen and/or progesterone is a current area of research interest. The number of SERT binding sites is increased in many areas of the brain by estrogen [14]. Because the modulation of SERT function and/or number provides means for altering synaptic 5-HT concentrations and serotonergic neurotransmission, hormonal regulation of the SERT may underlie changes in mood that occur during the reproductive cycle, or increased incidence of major depression in women *versus* men.

The regulation of SERT function can occur at the level of transcription or translation, or by post-translational modifications (e.g. glycosylation or phosphorylation). Research carried out *in vitro* using cells that express the SERT has indicated that second-messenger systems, particularly those activating protein kinases, play a role in the regulation of SERT function. SERT gene expression is influenced by cAMP-dependent pathways and rapidly regulated by changes in intracellular calcium, treatment with calmodulin inhibitors, or activation of protein kinase C (PKC) as well as NOS/cyclic GMP pathways. Most of the changes in enzyme kinetics reflect change in maximal transport capacity (V_{max}) rather than changes in apparent affinity (K_m). Recent work indicates that activation of PKC causes a loss of SERT protein on the cell surface. Thus, occupancy of the SERT by substrate (5-HT) may induce a conformational change of the transporter that either prohibits phosphorylation by PKC (thereby inhibiting PKC-induced internalization of the SERT), or enhances access of phosphatases (thereby reducing phosphorylation and internalization) [12].

The SERT is encoded by a single gene located on the long arm of chromosome 17. The human serotonin transporter gene possesses a functional polymorphism within the promotor region, which affects the transcription and therefore expression of the gene. The long (l/l) variant, compared to the short (s/s) or heterozygous (s/l) forms, is associated with greater SERT expression and function. Alterations in the expression and function of the SERT would be expected to affect 5-HT neurotransmission. The discovery of these polymorphisms has led to much research examining the potentially exciting association of these polymorphisms with different personality traits, a variety of neuropsychiatric disorders, and differing responses to drugs, in particular the SSRIs [15, 16].

The primary catabolic pathway for 5-HT is oxidative deamination by the enzyme monoamine oxidase. Monoamine oxidase (MAO) (E.C.1.4.3.4.) converts serotonin to 5-hydroxy-indoleacetaldehyde, and this product is oxidized by an NAD^+-dependent aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA) (see Fig. 13-5). There are at least two isoenzymes of MAO, referred to as type A and type B. These isoenzymes are integral flavoproteins of outer mitochondrial membranes in neurons, glia and other cells. Evidence for the existence of isoenzymes of MAO was based initially on differing substrate specificities and sensitivities to inhibitors of MAO. For example, 5-HT and norepinephrine are metabolized preferentially by type A MAO. Selective inhibitors of each form of MAO exist, e.g. clorgyline or moclobemide for type A or deprenyl for type B. Definitive proof of the existence of these two forms of MAO comes from the cloning of cDNAs encoding type A and type B MAO from human liver [17]. The deduced amino acid sequences of type A and type B MAOs show about 70% homology and have masses of 59.7 kDa and 58.8 kDa respectively. The activity of the proteins expressed in recombinant cell systems resembles that of the endogenous enzymes from human brain, e.g. the expressed type A MAO prefers 5-HT as a substrate and is preferentially inhibited by clorgyline (see also Chs 12 and 46).

Several different techniques have been used to study the neuroanatomical localization of the two forms of MAO in brain. Originally, both histochemical and immunohistochemical techniques were used. More recently, *in situ* hybridization histochemistry has been used to demonstrate the location of the mRNAs for the two isoenzymes of MAO. The development of radioligands selective for each form of MAO has enabled their distribution to be revealed by quantitative autoradiography. In general, the results from the use of these different techniques are similar. It is of some interest that there is more type A enzyme than type B enzyme throughout rat brain whereas human brain contains more type B MAO than type A. Interestingly, serotonergic cell bodies contain predominantly type B MAO so that serotonergic neurons contain the form of MAO (type B) that does not preferentially metabolize 5-HT. This has led to the hypothesis that type B MAO in serotonergic neurons prevents the cell from accumulating various natural substrates (e.g. dopamine) that could interfere with the storage, release and uptake of 5-HT. Furthermore, treatment of rats with clorgyline, a selective inhibitor of type A MAO, raises the brain content of 5-HT and reduces the conversion of 5-HT to 5-HIAA in brain. Thus, 5-HT may well be oxidized preferentially by type A MAO *in vivo*, as it is *in vitro*, even though serotonergic neurons do not contain much of this form of the enzyme.

Techniques have been developed that permit the selective elimination, or 'knockout' of genes encoding specific proteins in mice. Mice that have either type A or type B MAO knocked out have been developed with this methodology [18]. In the brains of mice deficient in type A MAO, the content of 5-HT remains markedly elevated for about 12 days after birth and then slowly declines, reaching values comparable to those in normal mice after about 7 months. In the type A MAO-deficient mice, the selective inhibitor of type B MAO, deprenyl, had a greater effect on serotonin metabolism than it did in normal mice. Such observations indicate that, in the absence of type A MAO, the type B isoform can metabolize 5-HT *in vivo*. However, mice lacking the MAO B isoenzyme do not have elevated levels of 5-HT in brain. Of interest are the aggressive behaviors exhibited by the mice deficient in type A MAO, consistent with a postulated role of serotonergic neurons in human aggressive behaviors.

Another class of antidepressant drug is the monoamine oxidase inhibitors (MAOIs), e.g. phenelzine or tranylcypromine. These drugs are nonselective in that they irreversibly inhibit the activity of MAO type A and type B (Fig. 13-8). Because MAO metabolizes biogenic amines such as 5-HT, dopamine and norepinephrine, these neurotransmitters have been implicated in the mechanism of action of these drugs. Interestingly, studies have been carried out from which it was inferred that 5-HT is needed for SSRIs or MAOIs to produce a beneficial clinical response in depressed patients. Such data are consistent with the idea that drug-induced enhancement of serotonergic transmission can produce amelioration of depressive symptomatology (see Ch. 55).

In addition to classical synaptic transmission, 5-HT may relay information by volume transmission or paracrine neurotransmission. As expected, serotonergic terminals make the usual specialized synaptic contacts with target neurons and release 5-HT following nerve stimulation. In some areas of the mammalian CNS, however, there are sites where 5-HT is released and there is no evidence for synaptic specializations. For example, it is difficult to demonstrate the synaptic contacts of fine varicose fibers whereas large terminals make well-defined synapses with target cells. The percentage of 5-HT terminals associated with synaptic specializations appears to vary in particular brain regions. This may have important implications for the type of information processing in which 5-HT is involved in these brain areas. The appearance of specialized synaptic contacts suggests relatively stable and strong associations between a presynaptic neuron and its target. Conversely, the lack of synaptic specialization implies a dynamic and perhaps less specific interaction with target neurons. For example, neurotransmitter is released and then diffuses over some distance (as great as several hundred microns); this is termed *volume transmission*. It is also seen in dopamine targets areas (Ch. 12).

Consistent with the idea that a significant mode of transmission for 5-HT is through volume or extra-synaptic transmission is the observation that the SERT is localized perisynaptically and along axons and dendrites, and not confined to the terminals [19]. Thus 5-HT may diffuse away from the site of release before its action is terminated by re-uptake into serotonergic neurons. 5-HT_{1B} receptors, which function as both auto- and heteroreceptors in serotonergic terminal field areas, are also found predominantly at extrasynaptic and nonsynaptic sites [20]. The fact that serotonergic neurons contain predominantly type B MAO, the form of MAO that does not preferentially metabolize 5-HT, is also consistent with the volume or extrasynaptic transmission of 5-HT. As discussed above, 5-HT is oxidized preferentially by type A MAO, even though serotonergic neurons do not contain this form of the enzyme. Thus 5-HT may have a paracrine or neuromodulatory role in brain, adjusting or tuning ongoing activity at synapses some distance from the serotonergic terminal.

5-HT may be involved in a wide variety of behaviors by setting the tone of brain activity in relationship to the state of behavioral arousal/activity. Serotonin has been implicated in practically every type of behavior, e.g. appetitive, emotional, motoric, cognitive, autonomic. However, from a physiologic perspective, it is not clear whether 5-HT affects such behaviors specifically, or more generally by coordinating the activity of the nervous system, particularly to set the tone of activity in conjunction with the organism's level of arousal.

The primary body of data that has contributed to the view that 5-HT has a general effect on behavior by modulating the tone of nervous system activity comes from studies of the firing rate of serotonergic soma in raphe nuclei [21]. Under quiet waking conditions, serotonergic neurons display a slow, clock-like activity of about 1–5 spikes per second, which shows a gradual decline as the animal becomes drowsy and enters slow-wave sleep. A decrease in the regularity of firing accompanies this overall slowing of neuronal activity. During REM (rapid eye movement) sleep, the activity of these neurons becomes silent. In response to certain types of arousing stimulus, the firing rate of these serotonergic neurons increases. Not surprisingly, such data led to the idea that the activity of serotonergic neurons is related to the level of behavioral arousal/activity. These data have contributed also to the idea that the activity of serotonergic neurons is associated with motoric output, since atonia of the major skeletal muscle groups occurs during REM sleep. Also oral–buccal motor activity, such as chewing, biting, licking or grooming, causes a marked increase in the firing rate of a subgroup of serotonergic somata that are also activated by somatosensory stimuli applied to the head, neck and face. However, exposing a cat to environmental stressors such as a loud noise or seeing a dog, although producing strong sympathetic activation and typical

behavioral responses, does not alter the firing rate of serotonergic neurons. Thus, the type of motoric activity that activates serotonergic cell bodies seems to be repetitive, of the type known to be mediated by central pattern generators. Furthermore, activation of serotonergic transmission inhibits information processing in afferent systems. From all such data, it has been suggested that the serotonergic neuronal system functions at the organismic level to integrate functions needed for the animal's behavioral output, i.e. facilitation of motor output with suppression of activity in sensory systems irrelevant to the ongoing behavior.

Perturbation of the 5-HT system can elicit changes in a wide variety of behaviors. Furthermore, drugs that act on serotonergic neurons and their receptors are used to treat diseases such as depression, anxiety disorders and schizophrenia. Thus, 5-HT has been implicated in the regulation of many behaviors and physiological processes. The involvement of 5-HT in three areas – neuroendocrine function, circadian rhythms and feeding behavior – will be highlighted for illustrative purposes.

5-HT modulates neuroendocrine function. The hypothalamus secretes several releasing factors and release-inhibiting factors to control the secretion of hormones from the anterior pituitary gland (see details in Ch. 52). 5-HT is among the many neurotransmitters that participate in the hypothalamic control of pituitary secretion, particularly in the regulation of the secretion of adrenocorticotropin (ACTH), prolactin and growth hormone. A direct synaptic connection between serotonergic terminals and corticotropin-releasing-hormone (CRH)-containing neurons in the paraventricular nucleus of the hypothalamus has been described. Precursors of 5-HT or drugs that enhance the effect of 5-HT increase CRH in portal blood and ACTH in plasma. In addition to effects at the hypothalamus, 5-HT may also have direct effects on the anterior pituitary to stimulate the release of ACTH, and at the level of the adrenal cortex to regulate release of corticosterone or cortisol. However, what role, if any, is played by 5-HT in regulating stress-induced elevations of CRH or the circadian periodicity of the HPA axis is unclear.

Measurement of these endocrine responses after administration of drugs that increase brain serotonin function provides one of the few methods currently available for assessing such function in humans. Precursors of 5-HT, releasing agents, reuptake inhibitors, and receptor agonists and antagonists have all been used to probe serotonergic function. For example, intravenous administration of the serotonin precursor L-tryptophan increases plasma concentrations of prolactin and growth hormone consistently, but not ACTH or cortisol. Fenfluramine (Fig. 13-6) causes a dose-dependent increase in plasma prolactin. When administered to humans, 5-HT receptor agonists that stimulate 5-HT_{1A} or 5-HT_2 receptors also increase plasma ACTH, cortisol, prolactin and perhaps growth hormone levels. The neuroendocrine response in humans to such agents has been used clinically to assess the functioning of the central serotonergic system in patients with psychiatric disorders.

5-HT modulates circadian rhythmicity. Serotonin also appears to be involved in the regulation of *circadian* rhythms. The suprachiasmatic nucleus (SCN) of the hypothalamus generates electrophysiological and metabolic cycles that repeat approximately every 24 hours. When isolated *in vitro*, the SCN continues to produce 24 hour rhythms in metabolism, vasopressin secretion and spontaneous electrical activity, indicating that circadian time-keeping functions or pacemaker activity are endogenous characteristics of the SCN. Ordinarily, this rhythm is synchronized or entrained to the environmental photoperiod, also about 24 hours. A serotonergic contribution to circadian rhythm regulation has been postulated because the SCN receive a very dense serotonergic innervation from the midbrain raphe nuclei. In addition, there is a serotonergic innervation to the intergeniculate leaflet, an area of brain through which photic information indirectly accesses the SCN.

Serotonin appears to function as an inhibitory transmitter that modulates the effects of light on circadian rhythmicity. Direct application of 5-HT or receptor agonists to the SCN block light-induced phase shifts during the subjective night but cause phase advances during the subjective day. Such agents inhibit the excitatory effect of light, measured electrophysiologically, in either the SCN or the lateral geniculate complex. The nonselective 5-HT agonist quipazine has been shown to reset or shift the rhythm of spontaneous electrical activity of single cells recorded extracellularly in SCN isolated in brain slices.

Lesions of serotonergic neurons in laboratory animals have been reported by some, but not all, investigators to disrupt locomotor rhythms or to result in the loss of the daily rhythm of corticosterone. There is evidence that, in the hamster, the median raphe nucleus projects to the SCN whereas the dorsal raphe nucleus innervates the IGL; furthermore, the serotonergic innervation to the SCN and not the IGL is necessary for the photic entrainment of locomotor activity [22]. It appears then, that the SCN circadian pacemaker or clock is modulated by stimulation of serotonergic receptors in the SCN and that serotonergic projections to the SCN may modulate the phase of the SCN in intact animals.

5-HT modulates feeding behavior and food intake. Pharmacological studies primarily have contributed to the idea that 5-HT has an inhibitory effect on feeding behavior. Drugs that either directly or indirectly activate postsynaptic 5-HT receptors decrease food consumption whereas agents that inhibit serotonergic transmission increase food intake. Precisely how this occurs is controversial, with claims that 5-HT governs the selection of macronutrients in the diet, or influences responses to the taste qualities of food, or modulates gastric activity to

reduce feeding. Perhaps the most comprehensive and enduring view is that enhanced serotonergic activity enhances satiety, particularly by increasing the rate of satiation and prolonging the state of satiety [23].

Fenfluramine, originally the racemate and more recently the D-isomer, has been the prototypical drug for studying serotonergic mechanisms in feeding behavior. As mentioned previously, fenfluramine elicits the release of 5-HT and inhibits its reuptake (Fig. 13-7). D-fenfluramine has an active metabolite, D-norfenfluramine, that contributes to the anorectic (appetite suppressant) effects of the parent compound. Fenfluramine decreases meal size, the rate of eating and eating between meals. This is probably related to its ability in humans to decrease the sensation of hunger and to increase the feeling of 'fullness'. Serotonin reuptake inhibitors such as fluoxetine and serotonin precursors mimic these effects of fenfluramine. Fenfluramine's effects on feeding behavior are blocked by the nonselective serotonin receptor antagonist metergoline. Multiple mechanisms in brain appear to be responsible for the effects of serotonergic drugs on satiety; for example, postsynaptic 5-HT_{1B} receptors are involved in regulating the size of meals but 5-HT_{2C} receptors influence the rate of eating.

The sites in brain where these drugs, and presumably 5-HT as well, act to cause such effects remain to be identified. The paraventricular nucleus (PVN) of the hypothalamus may be an important site, although some data indicate that actions on the PVN may be sufficient but not necessary to reduce caloric intake. In addition to brain mechanisms, 5-HT may also act through peripheral mechanisms to produce satiety.

The pharmacological effects produced by drugs such as fenfluramine on feeding behavior in animals has led to its use in the treatment of obesity in humans. In many double-blind placebo-controlled trials, chronic administration of fenfluramine causes greater weight loss than placebo. Although not studied as extensively clinically, fluoxetine produces similar effects. Given all the medical problems associated with obesity, anorectic agents are valuable tools to be used in association with other modalities (diet, exercise) in the treatment of the truly obese individual.

Not only does 5-HT have important physiological effects of its own but it is also the precursor of the hormone melatonin. The pineal gland occupies the depression between the superior colliculi at the posterior border of the corpus callosum and lies 'outside' the blood–brain barrier. Extracts of the pineal gland were reported as early as 1917 to lighten frog skin *in vitro*; in the late 1950s, Lerner and associates isolated the pineal hormone that produced this effect and described its chemical structure, 5-methoxy-*N*-acetyltryptamine (melatonin) (Fig. 13-5). Melatonin is synthesized from serotonin, and the pineal gland contains all the enzymes necessary to synthesize serotonin from tryptophan as well as two additional enzymes required to convert serotonin to melatonin (Fig. 13-5). The rate-limiting enzyme serotonin *N*-acetyl transferase (EC 2.31.87) (arlylalkylamine *N*-acetyltransferase; AANAT) converts serotonin to *N*-acetylserotonin and this product is methylated to form melatonin by the enzyme 5-hydroxyindole-*O*-methyltransferase (HIOMT), which uses *S*-adenosyl methionine as the methyl donor. The human AANAT gene has been cloned and found to have considerable sequence identity to the sheep and rat genes. The human gene is localized on chromosome 17. The gene product is a 23.2 kDa protein that contains putative phosphorylation sites. Such sites are likely to be involved in the cAMP-dependent regulation of enzyme activity.

A unique feature of pineal gland physiology is that the synthesis and secretion of melatonin is markedly influenced by the light–dark cycle, acting through a multisynaptic pathway that relays in the superior cervical ganglia of the sympathetic nervous system. During daylight, the synthesis and secretion of melatonin are reduced, as is impulse flow along the sympathetic nerves innervating the pineal gland. At the onset of darkness, there is activation of these nerves, and the increased release of norepinephrine from them activates β-adrenergic receptors on the pineal to increase the formation of cAMP, with activation of α_1-adrenergic receptors further amplifying the response. This second messenger causes activation of AANAT so as to increase the synthesis of melatonin.

Thus, the pineal gland functions as a neuroendocrine transducer. In mammals, photosensory information impinging on the retina influences the activity of its neuronal projections, which serves ultimately to inhibit or to stimulate the secretion of melatonin. A circadian rhythm of melatonin secretion persists in animals housed in continuous darkness. Thus, melatonin synthesis is turned on by an endogenous 'clock', probably located within the SCN of the hypothalamus, with the daily rhythm normally being entrained to the day–night, light–dark cycle [24]. Melatonin, which provides circadian and seasonal timing cues through activation of G-protein-coupled receptors [25], has been implicated in not only the regulation of biological rhythms but also in sleep and affective disorders.

SEROTONIN RECEPTORS

Pharmacological and physiological studies have contributed to the definition of the many receptor subtypes for serotonin. The initial suggestion that there might be more than one type of receptor for serotonin came from experiments of Gaddum and Picarelli in 1957. Using the isolated guinea pig ileum, they demonstrated that only a portion of the contractile response to 5-HT was blocked by high concentrations of morphine. The remainder of the response to 5-HT was blocked by low concentrations of Dibenzyline (phenoxybenzamine). In the presence of

maximally effective concentrations of Dibenzyline, the remaining contractile response elicited by 5-HT was blocked by low concentrations of morphine. They speculated that there were two different receptors for 5-HT in the ileum, one blocked by morphine (termed the M receptor) and one blocked by Dibenzyline (termed the D receptor). The D receptor was thought to be on the smooth muscle of the ileum whereas the M receptor was considered to be on ganglia or nerves within the muscle.

In the 1970s, the development of radioligand binding assays furthered our understanding of subtypes of receptor for 5-HT. Initially, a number of radioligands, such as ^{3}H-5-HT, ^{3}H-LSD and ^{3}H-spiroperidol, were used to label sites related to 5-HT receptors. In 1972, Farrow and Van Vunakis observed high-affinity, stereospecific binding of ^{3}H-LSD in cortex that was inhibited more potently by serotonin than by other neurotransmitters. Peroutka and Snyder in 1979 demonstrated the presence of two classes of serotonin receptors in brain. Binding sites that were labeled with high affinity by ^{3}H-5-HT were designated the 5-HT$_1$ receptor; binding sites labeled with high affinity by ^{3}H-spiroperidol were termed the 5-HT$_2$ receptor [26].

The binding of ^{3}H-5-HT to 5-HT$_1$ receptors was shown to be displaced by spiperone in a biphasic manner, suggesting that what was termed the 5-HT$_1$ receptor might be a heterogeneous population of receptors. The ^{3}H-5-HT binding site that showed high affinity for spiperone was termed the 5-HT$_{1A}$ subtype, whereas the component of ^{3}H-5-HT binding that showed low affinity for spiperone was called the 5-HT$_{1B}$ subtype. The different neuroanatomical localization of these two 5-HT binding sites, as demonstrated by quantitative autoradiography, was further evidence that these were distinct subtypes of receptor for 5-HT. For example, the 5-HT$_{1A}$ subtype was shown to be present in high density in the hippocampus; the 5-HT$_{1B}$ subtype was shown to be present in high density in the globus pallidus and substantia nigra. A high density of binding sites for ^{3}H-5-HT was also found in the choroid plexus. These ^{3}H-5-HT binding sites were termed the 5-HT$_{1C}$ subtype as they did not show the pharmacological characteristics used to classify the 5-HT$_{1A}$ or 5-HT$_{1B}$ binding site, or the 5-HT$_2$ binding site. Subsequently, a fourth binding site for ^{3}H-5-HT was identified in bovine brain and was called the 5-HT$_{1D}$ receptor. The 5-HT$_{1D}$ receptor was identified by pharmacological criteria only in brains of species devoid of the 5-HT$_{1B}$ receptor such as pig, cow, guinea pig and human.

Bradley and associates in 1986 proposed a classification scheme with three major types of receptors for serotonin, using pharmacological criteria and functional responses primarily in peripheral tissues. The receptors were called '5-HT$_1$-like', 5-HT$_2$ and 5-HT$_3$. The development of potent and selective antagonists of the 5-HT$_2$ receptor, such as ketanserin, facilitated the assignment of certain effects mediated by 5-HT to the 5-HT$_2$ receptor. The D receptor of Gaddum and Picarrelli, originally described in guinea pig ileum, and the 5-HT$_2$ receptor were shown to be pharmacologically indistinguishable. The M receptor of Gaddum and Picarelli, which is pharmacologically distinct from all of the binding sites associated with 5-HT receptors just described, was renamed the 5-HT$_3$ receptor. The development of potent selective antagonists and an agonist, 2-methyl-5-HT, provided useful tools for the pharmacological characterization of 5-HT$_3$ receptors.

Molecular biology techniques led to the discovery of additional 5-HT receptor subtypes and furthered our understanding of their structure and function. The first 5-HT receptor to be cloned and fully sequenced was the 5-HT$_{1A}$ receptor, by Kobilka and co-workers in 1987. Over the course of the next 5 years, the 5-HT$_{1B}$, 5-HT$_{1C}$, 5-HT$_{1D}$, 5-HT$_2$ and 5-HT$_3$ receptors were also cloned. Techniques used in molecular biology also led to the rapid discovery of additional subtypes of receptor for 5-HT, making it necessary to establish an unambiguous system of nomenclature for serotonin receptors. The current classification scheme takes into account not only operational criteria (drug-related characteristics such as selective agonists, selective antagonists, ligand-binding affinities) but also information about intracellular signal transduction mechanisms and molecular structure (amino acid sequence of the receptor protein) [27]. For example, the 5-HT$_{1C}$ receptor was reclassified as a 5-HT$_2$ receptor based on the sequence homology, similar pharmacological characteristics and effector coupling of the 5-HT$_2$ and 5-HT$_{1C}$ receptors. The 5-HT$_2$ receptor was renamed the 5-HT$_{2A}$ receptor and the 5-HT$_{1C}$ was renamed the 5-HT$_{2C}$ receptor. The amino acid sequence of many new 5-HT receptors has been reported. However, the classification of several of these receptors remains tentative because of limited knowledge of their operational and transductional characteristics, which have only been described for these recombinant receptors in transfected cell systems. Because the functions mediated by these 5-HT receptors in intact tissue are unknown, lower case appellations are presently used [27] (Table 13-2).

Three families of serotonin receptor, the 5-HT$_1$ family, the 5-HT$_2$ family and the family that includes the 5-HT$_4$, 5-ht$_6$ and 5-HT$_7$ receptors represent the three major classes of 5-HT receptor that are G-protein-coupled receptors (Ch. 19). The 5-HT$_3$ receptor is a ligand-gated ion channel and is a separate family. Although each serotonin receptor can be potently activated by 5-HT, differences in signal transduction mechanisms, neuroanatomical distribution and affinities for synthetic chemicals create opportunities for drug discovery and make each 5-HT receptor subtype a potential therapeutic target.

The 5-HT$_1$ receptor family comprises the 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-ht$_{1E}$ and 5-ht$_{1F}$ receptors. The five receptor subtypes in the 5-HT$_1$ receptor family share 40–63% overall sequence homology. These 5-HT receptors couple preferentially to the inhibition of adenylyl cyclase via the G$_{i/o}$ family of G proteins (see Ch. 21).

TABLE 13-2 Serotonin receptors present in the central nervous system.

Receptor	Distribution	Effector mechanism
5-HT$_1$ receptor family		
5-HT$_{1A}$	Hippocampus, amygdala, septum entorhinal cortex, hypothalamus, raphe nuclei	Inhibition of adenylyl cyclase; opening of K^+ channels
5-HT$_{1B}$	Substantia nigra, basal ganglia	Inhibition of adenylyl cyclase
(5-HT$_{1D\beta}$)	Frontal cortex, superior colliculus, lateral geniculate, deep nuclei of the cerebellum	
5-HT$_{1D}$	Globus pallidus, substantia nigra, caudate putamen	Inhibition of adenylyl cyclase
(5-HT$_{1D\alpha}$)	Hippocampus and cortex	
5-ht$_{1E}$	?	Inhibition of adenylyl cyclase
5-ht$_{1F}$	Cerebral cortex, striatum, hippocampus,	Inhibition of adenylyl cyclase
5-HT$_2$ receptor family		
5-HT$_{2A}$ 13q14–21	Claustrum, cerebral cortex, olfactory tubercle, striatum, nucleus accumbens	Stimulation of phospholipase C; closing of K^+ channels
5-HT$_{2B}$ 2q36.3–37.1	?	Stimulation of phospholipase C
5-HT$_{2C}$ Xq24	Choroid plexus, globus pallidus, cerebral cortex, hypothalamus septum, substantia nigra, spinal cord	Stimulation of phospholipase C
5-HT$_3$ receptor family		
5-HT$_3$ (5-HT$_{3A}$) (5-HT$_{3B}$)	Hippocampus, entorhinal cortex, amygdala, nucleus, accumbens, solitary tract nerve, trigeminal nerve, motor nucleus of the dorsal vagal nerve, area postrema, spinal cord	Ligand-gated cation channel
5-HT$_4$	Hippocampus, striatum, olfactory tubercle, substantia nigra	Stimulation of adenylyl cyclase
5-ht$_{5A}$	?	Inhibition of adenylyl cyclase
5-ht$_{5B}$	?	?
5-ht$_6$	?	Stimulation of adenylyl cyclase
5-HT$_7$	Cerebral cortex, septum, thalamus hypothalamus, amygdala, hippocampus	Stimulation of adenylyl cyclase

The 5-HT$_{1C}$ receptor appellation is vacant because, as described above, this receptor was renamed the 5-HT$_{2C}$ receptor due to structural, operational and transductional similarities with the 5-HT$_2$ receptor family. A lower case appellation is used for the 5-ht$_{1E}$ and 5-ht$_{1F}$ receptors because a physiological role for these receptors in intact tissue has not been found [28].

The 5-HT$_{1A}$ receptor is present in high density in cortical and limbic structures (i.e. hippocampus, entorhinal cortex, septum, amygdala, frontal cortex). The distribution of the 5-HT$_{1A}$ receptor in brain suggests that this 5-HT receptor subtype may have a role in cognitive or integrative functions as well as in emotional states. The 5-HT$_{1A}$ receptors in terminal field areas of serotonergic innervation are located postsynaptically. 5-HT$_{1A}$ receptors are also present in high density on serotonergic cell body areas, particularly in the dorsal and median raphe nuclei. Here they function as somatodendritic autoreceptors, involved in the negative feedback modulation of serotonergic neuronal activity. Therefore somatodendritic 5-HT$_{1A}$ autoreceptors play a key role in regulating serotonergic neurotransmission. In terminal field areas of serotonergic innervation such as the hippocampus, 5-HT$_{1A}$ receptors are coupled to the inhibition of adenylyl cyclase activity, as well as to the opening of potassium channels, which results in neuronal hyperpolarization (Table 13-2). However, in the dorsal raphe nucleus, 5-HT$_{1A}$ receptors are coupled to the opening of potassium channels but do not appear to be coupled to the inhibition of adenylyl cyclase.

Activation of 5-HT$_{1A}$ receptors in the central nervous system results in a variety of physiological and behavioral responses. 5-HT$_{1A}$ receptors modulate feeding, sexual behavior and body temperature. Activation of 5-HT$_{1A}$ receptors stimulates the release of adrenocorticotrophic hormone (ACTH). The 5-HT$_{1A}$ receptor has been implicated in affective disorders such as anxiety and depression. Newer antianxiety medications, specifically the substituted azapirones (e.g. buspirone), are agonists with high affinity for the 5-HT$_{1A}$ receptor (Fig. 13-8). The sensitivity and function of the 5-HT$_{1A}$ receptor is decreased following chronic administration of a variety of antidepressant drugs as well as 5-HT$_{1A}$ receptor agonists. Because of the critical role of the somatodendritic 5-HT$_{1A}$ autoreceptor in regulating serotonergic neuronal firing, and therefore serotonergic neurotransmission, the desensitization of this receptor by drugs used to treat anxiety and depression is of great interest, particularly in regard to the role of serotonin in these mental disorders (Ch. 55). Drugs with agonist activity at the 5-HT$_{1A}$ receptor are also of interest in the treatment of schizophrenia, and behavioral disorders such as impulsivity and aggression (Chs 54, 55).

The 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes are also linked to inhibition of adenylyl cyclase activity (Table 13-2). Binding sites, pharmacologically defined as 5-HT$_{1B}$ receptors, were originally characterized in certain rodents (rat, mouse, hamster), whereas the 5-HT$_{1D}$ receptor was characterized using pharmacological criteria in species such as guinea pig, dog, pig, cow and human. The 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors were considered to be species variants of the same receptor because the pharmacological profiles of these two receptors are similar, although not identical, and the distribution of these two receptors in brain is very similar.

Although biochemical, pharmacological and functional data suggest that the 5-HT$_{1B}$ receptor found in rats and mice, and the 5-HT$_{1D}$ receptor found in other species, including human, are functionally equivalent species homologues of the same receptor, the story has been somewhat complicated by the discovery of two genes encoding the human 5-HT$_{1D}$ receptor, 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$. The human 5-HT$_{1D\beta}$ receptor was shown to have a high degree of homology with the rodent 5-HT$_{1B}$ receptor. Subsequently, a gene homologous to the human 5-HT$_{1D\alpha}$ was discovered in the rat. This gene encoded a receptor with the pharmacological profile of the 5-HT$_{1D}$. Thus, both rat and human express a 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor. As a result, the nomenclature for the 5-HT$_{1B/1D}$ receptors was reassessed [29]. This new nomenclature scheme recognizes that the human 5-HT$_{1D\beta}$ receptor is a species equivalent of the rodent 5-HT$_{1B}$ receptor, as discussed above, despite differing pharmacology, and realigned the 5-HT$_{1D\beta}$ to the 5-HT$_{1B}$ receptor classification. Prefixes are often used to denote species-specific 5-HT$_{1B}$ receptors because of the significant differences in the pharmacology of the 5-HT$_{1B}$ receptor across species, e.g. r5-HT$_{1B}$ and h5-HT$_{1B}$ denote the rat and human receptors respectively. The 5-HT$_{1D\alpha}$ receptor expressed in both rat and human is reclassified as the 5-HT$_{1D}$ receptor (Table 13-2).

Much of what is known about the function and neuroanatomical distribution of the 5-HT$_{1B}$ receptor is attributed to the receptor designated under the older classification scheme as the 5-HT$_{1B}$ receptor in rodents and the 5-HT$_{1D}$ receptor in bovine, guinea pig and human brain. The 5-HT$_{1B}$ (5-HT$_{1D_JA\beta}$, r5-HT$_{1B}$, h5-HT$_{1B}$) receptor is located in high density in the basal ganglia, particularly in the globus pallidus and the substantia nigra (Table 13-2). The neuroanatomical localization of 5-HT$_{1B}$ receptor raises the interesting possibility that this receptor may be involved in diseases of the brain that involve the basal ganglia, such as Parkinson's disease (see Ch. 46). Functional studies indicate that 5-HT$_{1B}$ receptors are located on presynaptic terminals of serotonergic neurons and modulate the release of serotonin. 5-HT$_{1B}$ receptors are also located postsynaptically where they may modulate the release of other neurotransmitters, such as acetylcholine in the hippocampus and dopamine in the prefrontal cortex. Postsynaptic 5-HT$_{1B}$ receptors are also located on cerebral arteries and other vascular tissues. Agonists at the 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors have been termed 'serenics' because of their selective ability to inhibit aggressive behavior without sedation in rats and mice. Drugs that are agonists at 5-HT$_{1B/1D}$ receptors (e.g. sumatriptan, zolmitriptan) (Fig. 13-8) are currently used clinically in the treatment of migraine. These drugs constrict cranial blood vessels and inhibit the neurogenic inflammation in the dura mater that gives rise to plasma extravasation. Activation of 5-HT$_{1B/1D}$ receptors in the nucleus tractus solitarius may alleviate symptoms of nausea and vomiting in patients having a migraine attack [30].

It has been difficult to determine the distribution of the 5-HT$_{1D}$ (5-HT$_{1D\alpha}$,) receptor in brain because of the lack of selective radioligands. Receptor binding sites attributed to the 5-HT$_{1D}$ receptor are present in basal ganglia (globus pallidus, substantia nigra, caudate putamen) and hippocampus and cortex [31]. With the cloning of the various subtypes of receptors for 5-HT, knowledge of receptor sequences can be used to generate radioactive probes for mRNAs encoding individual serotonin receptor subtypes. Using the technique of *in situ* hybridization histochemistry, the localization of these mRNAs and, thus, the distribution of cells expressing the mRNAs for 5-HT receptors can be established in brain. 5-HT$_{1D}$ receptor mRNA is expressed at low levels in the basal ganglia, dorsal raphe nucleus and locus ceruleus, indicative of the 5-HT$_{1D}$ receptor being located predominantly on axon terminals of both serotonergic and nonserotonergic neurons. The release of 5-HT from dorsal raphe nucleus appears to be under the control of 5-HT$_{1D}$ receptors, presumably located on serotonergic terminals. It has been proposed that neurogenic inflammation and trigeminal nociception may be 5-HT$_{1D}$-receptor-mediated. Therefore, there is much interest in the 5-HT$_{1D}$ receptor as a useful therapeutic target for migraine.

The 5-ht$_{1E}$ receptor was originally identified in homogenates of human frontal cortex by radioligand binding studies using ^{3}H-5-HT in the presence of 5-CT to block 5-HT$_{1A}$ and 5-HT$_{1D}$ receptor sites. Because of the lack of specific radioligands for the 5-ht$_{1E}$ receptor, the overall distribution of this receptor in brain is unknown. 5-ht$_{1E}$ receptor mRNA has been found in the cortex (particularly entorhinal cortex) and caudate putamen. The function of the 5-ht$_{1E}$ receptor in intact tissue is not known due to the lack of selective agonists or antagonists. In transfected cells, the 5-ht$_{1E}$ receptor is coupled to the inhibition of adenylyl cyclase activity [32]. The 5-ht$_{1E}$ receptor displays a higher degree of homology with the 5-HT$_{1D}$ receptor (64%) than any other 5-HT$_{1}$ receptors. Although 5-ht$_{1E}$ receptor mRNA and binding sites have been mapped in the rodent and human brain, confirmation of a true physiological role for 5-ht$_{1E}$ receptors is still lacking; hence, they retain their lower case appellation.

The 5-ht$_{1F}$ receptor was cloned and sequenced in 1993 and shares the greatest sequence homology with the 5-ht$_{1E}$

receptor (61%). 5-ht$_{1F}$ receptor mRNA is found in cortex, hippocampus, dentate gyrus, nucleus of the solitary tract, spinal cord, uterus and mesentery. In transfected cells, the 5-ht$_{1F}$ receptor is coupled to the inhibition of adenylyl cyclase. Because selective agonists or antagonists for the 5-ht$_{1F}$ receptor have not been available until very recently, little is known about the distribution or function of the 5-ht$_{1F}$ receptor in brain. Binding sites attributed to the 5-ht$_{1F}$ receptor have been found in cortex, striatum and hippocampus. Activation of 5-ht$_{1F}$ receptors *in vivo* does not mediate constriction of human vasculature (unlike 5-HT$_{1B/1D}$ receptors), but may be associated with the blockade of dura extravasation. Because 5-ht$_{1F}$ receptor mRNA has been detected in the trigeminal ganglia, 5-ht$_{1F}$ receptor agonists are also of great interest as potential therapeutic drugs in the treatment of migraine.

The 5-HT$_2$ receptor family comprises the 5-HT$_{2A}$, 5-HT$_{2B}$ and 5HT$_{2C}$ receptors. The three receptor subtypes in the 5-HT$_2$ receptor family share 46–50% overall sequence homology. Members of the 5-HT$_2$ receptor family are coupled via the G$_{q/11}$ family of G proteins to the phospholipase C signaling cascade, which involves the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and increases in intracellular calcium ion (see Ch. 20). The 5-HT$_{2A}$ receptor nomenclature refers to the classical D receptor of Gaddum and Picarelli, and 5-HT$_2$ receptor of Peroutka and Snyder [26]. The 5-HT$_{2C}$ receptor was formerly the 5-HT$_{1C}$ receptor. It is important to note that because selective drugs able to differentiate between members of the 5-HT$_2$ receptor family have not been available until very recently, many of the functional and clinical correlates of the 5-HT$_{2A}$ receptor may very well involve or be attributed to the 5-HT$_{2C}$ or 5-HT$_{2B}$ receptor.

5-HT$_{2A}$ receptors are found postsynaptically to serotonergic neurons and are particularly concentrated in the frontal cortex. 5-HT$_{2A}$ receptors are also found in high density in the claustrum, a region that is connected to the visual cortex, in parts of the limbic system (i.e. amygdala and hippocampus) and in the basal ganglia (Table 13-2). In the cortex, the 5-HT$_{2A}$ receptor is located on local GABAergic interneurons, as well as on pyramidal projection neurons, which are known to be glutamatergic. The high level of 5-HT$_{2A}$ receptor expression throughout the cortex suggests that this 5-HT receptor subtype may play a role in higher cognitive or integrative functions. Activation of 5-HT$_{2A}$ receptors in the central nervous system results in an increase in body temperature (hyperthermia) and increased secretion of ACTH. In the periphery, 5-HT$_{2A}$ receptors mediate the contractile responses of vascular smooth muscle, and a component of the contractile response of smooth muscle in the gut, to 5-HT. The 5-HT$_{2A}$ receptor has been implicated in the hallucinogenic effects of 5-HT$_2$ receptor agonists. There is also considerable interest in the role of the 5-HT$_{2A}$ receptor in antipsychotic drug action. The so-called 'atypical' antipsychotic medications (e.g. clozapine, olanzepine) (Fig. 13-8) are antagonists with high affinity for the 5-HT$_{2A}$ and D$_2$ receptors (see Ch. 54).

The cloning of the 5-HT$_{2A}$ receptor has been used to gain insight into a controversy over the nature of agonist binding to the 5-HT$_{2A}$ receptor. The hallucinogenic amphetamine derivative ^{3}H-DOB, an agonist, has been shown to bind to receptor sites with properties very similar to those of the receptor labeled with the antagonist ^{3}H-ketanserin. Some investigators interpreted these and other data as evidence for the existence of a new subtype of 5-HT$_{2A}$ receptor, whereas others interpreted these data as indicative of agonist high-affinity and agonist low-affinity states of the 5-HT$_{2A}$ receptor. In experiments in which the cDNA encoding the 5-HT$_{2A}$ receptor was transfected into clonal cells, binding sites for both the 5-HT$_{2A}$ receptor antagonist ^{3}H-ketanserin and the 5-HT$_2$ receptor agonist ^{3}H-DOB were found. Furthermore, agonists had higher affinities for ^{3}H-DOB binding than for ^{3}H-ketanserin binding. Thus, a single gene produces a protein with both binding sites substantiating the view that agonist and antagonist binding are to different states of the 5-HT$_{2A}$ receptor rather than two different subtypes of the 5-HT$_{2A}$ receptor.

5-HT$_{2B}$ receptor. Although the 5-HT$_{2B}$ receptor is the most recently cloned of the 5-HT$_2$ receptor class, it was among the first of the 5-HT receptors to be characterized using pharmacological criteria. The first report of the sensitivity of rat stomach fundus to 5-HT was published by Vane in 1959. This receptor, whose activation results in the contraction of fundus smooth muscle, was originally placed in the 5-HT$_1$ receptor class because of its sensitivity to serotonin and because responses mediated by this receptor were not blocked by 5-HT$_2$ or 5-HT$_3$ receptor antagonists. It has been reclassified as a 5-HT$_2$ receptor because of its similar pharmacological profile to the 5-HT$_{2C}$ receptor (Table 13-2). The recombinant receptor expressed in clonal cells is coupled to hydrolysis of membrane PIP$_2$. However, in rat stomach fundus, the 5-HT$_{2B}$ receptor appears not to be coupled to PIP$_2$ hydrolysis. 5-HT$_{2B}$-receptor-mediated contraction of rat stomach fundus is dependent upon the influx of calcium ion through voltage-sensitive channels, intracellular calcium release and activation of PKC [33]. The effector system to which this receptor is coupled in the CNS is a subject of research.

5-HT$_{2B}$ receptor mRNA has been detected in the stomach fundus, intestine, kidney, heart and lung, as well as in the brain (cerebellum, cerebral cortex, amygdala, substantia nigra, caudate, thalamus, hypothalamus and retina). The lack of truly selective 5-HT$_{2B}$ receptor agonists and antagonists until recently has limited our knowledge about the functional role of the 5-HT$_{2B}$ receptor in brain and in the periphery. The 5-HT$_{2B}$ receptor is also expressed in a number of blood vessels. Contraction of the renal

artery is mediated in part by the 5-HT_{2B} receptor [34]. Given the vasodilatory role of the 5-HT_{2B} receptor, recently developed 5-HT_{2B} receptor antagonists may be indicated for the treatment of migraine. In addition, the 5-HT_{2B} receptor, which is expressed in heart valves, appears to be responsible for the cardiac valve defects reported in obese patients treated with dex-fenfluramine-containing preparations such as 'fen-phen' [28].

The 5-HT_{2C} receptor is present in high density in the choroid plexus. High-resolution autoradiography has shown that they are enriched on the epithelial cells of the choroid plexus. It has been proposed that 5-HT-induced activation of 5-HT_{2C} receptors could regulate the composition and volume of the cerebrospinal fluid. 5-HT_{2C} receptors are also found throughout the brain, in particular in areas of the limbic system (hypothalamus, hippocampus, septum, neocortex) and those associated with motor behavior (substantia nigra, globus pallidus). 5-HT_{2C} receptors are present in these areas in much lower concentrations than in the choroid plexus (Table 13-2). The lack of truly selective 5-HT_{2C} receptor agonists and antagonists has limited our knowledge about the functional role of the 5-HT_{2C} receptor in brain.

RNA editing is the term applied to the process of altering gene transcripts co- or post-transcriptionally (see Ch. 26). 5-HT_{2C} receptor mRNA undergoes post-transcriptional editing to yield multiple 5-HT_{2C} receptor isoforms with different distributions in brain [35, 36]. Messenger RNA editing alters the binding affinity of the resulting recombinant 5-HT_{2C} receptor isoforms for agonists and therefore alters the functional potency of the agonists. This is potentially of great significance as the isoforms expressed endogenously may have different functional and regulatory properties, and thus provide a novel mechanism for the regulation 5-HT synaptic signaling and plasticity.

5-HT_2 receptors are thought to mediate hallucinogenic properties of some 5-HT agonists, such as LSD (Fig. 13-8) (see Ch. 56). Evidence for this comes from experiments in which animals are trained to discriminate 5-HT_2 receptor agonists and are therefore able to recognize or discriminate other 5-HT_2 receptor agonists but not, for example, 5-HT_1 receptor agonists. Thus, activation of 5-HT_2 receptors is said to lead to a discriminative stimulus. The production of drug discriminative stimulus properties of 5-HT_2 receptor agonists, such as the hallucinogenic amphetamine derivatives, can be blocked by 5-HT_2 receptor antagonists. There is also a close correlation between the human hallucinogenic potency of 5-HT_2 receptor agonists and their affinity for 5-HT_2 (i.e. both 5-HT_{2A} and 5-HT_{2C}) receptor binding sites.

Unlike the other subtypes of receptor for 5-HT, the 5-HT_3 receptor is a ligand-gated ion channel.

The 5-HT_3 receptor refers to the classical M receptor of Gaddum and Picarelli. Based on its overall electrophysiological features and sequence, it is a member of the ligand-gated ion channel superfamily of receptors. This receptor is a cation channel that is gated by 5-HT. Like other members of this receptor superfamily, it possesses additional pharmacologically distinct recognition sites whereby the function of the receptor can be modulated by alcohols and anesthetic agents [37].

5-HT_3 receptors are located postsynaptically to serotonergic neurons in the central and peripheral nervous systems. 5-HT_3 receptors initially appeared to be confined to peripheral neurons, where they mediate depolarizing actions of 5-HT and modulate neurotransmitter release. Serotonin throughout the gastrointestinal tract regulates both motility and intestinal secretion through activation of 5-HT_3 receptors. 5-HT_3 receptors are found in high density in peripheral ganglia and nerves (superior cervical ganglion and vagus nerve) as well as in the substantia gelatinosa of the spinal cord. Their localization in spinal cord and medulla suggest that 5-HT could modulate nociceptive mechanisms via the 5-HT_3 receptor. 5-HT_3 receptors facilitate the release of substance P (see Ch. 18) in the spinal cord. The localization of 5-HT_3 receptor binding sites in cortical and limbic areas of the brain is consistent with behavioral studies in animals that suggest that 5-HT_3 receptor antagonists (Fig. 13-8) may have potential anxiolytic, antidepressant and cognitive effects. In the cortex and hippocampus the majority of neurons expressing 5-HT_3 receptor mRNA are GABAergic. The observation that 5-HT_3 receptors modulate the activity of dopaminergic neurons in the ventral tegmental area has led to the hypothesis that 5-HT_3 receptor antagonists may have potential as antipsychotic drugs and may posses the ability to reduce the rewarding effects of alcohol and certain drugs of abuse (Fig. 13-8). The highest density of 5-HT_3 receptor sites in the brain is in the area postrema, the site of the chemoreceptor trigger zone (Table 13-2).

Antagonists at 5-HT_3 receptors, such as ondansetron or granisetron (Fig. 13-8), are an important class of drugs for the treatment of nausea and vomiting in cancer patients receiving chemotherapy. Chemotherapeutic drugs such as cisplatin and dacarbazine induce the release of 5-HT from enterochromaffin cells of the gastrointestinal tract. The released 5-HT activates 5-HT_3 receptors located on the enteric nerves innervating the smooth muscle of the gut, causing depolarization of visceral afferent nerves and increases in their rate of firing. The enhanced afferent input leads to stimulation of the chemoreceptor trigger zone, which produces nausea and vomiting. Antagonism of 5-HT_3 receptors prevents or attenuates this chain of events. The site of action of these drugs appears to be 5-HT_3 receptors in the gastrointestinal tract, even though the central area regulating emesis, i.e. the chemoreceptor trigger zone, possesses a high density of 5-HT_3 receptors. Unfortunately, there is a more prolonged, often milder, form of emesis caused by chemotherapeutic drugs that is not dependent on the release of 5-HT and is resistant to amelioration with 5-HT_3 receptor antagonists.

The 5-HT_3 receptor is a pentamer made up of five subunits. Julius and co-workers cloned a single subunit of the 5-HT_3 receptor (5-HT_{3A} receptor subunit) in 1991. The cloned receptor subunit exhibits sequence similarity to the alpha subunit of the nicotinic acetylcholine receptor. Later, a second subunit, 5-HT_{3B}, was cloned [38]. Although single subunits of members of the ligand-gated ion channel receptor family can form functional homomeric receptors (receptors composed of subunits of a single type), they generally lack some of the properties of the native receptor. This appears to be the case for the 5-HT_3 receptor. In electrophysiological studies using *Xenopus* oocytes injected with mRNA encoding 5-HT_{3A} and/or 5-HT_{3B} receptor subunits, the heteromeric combination of 5-HT_{3A} and 5-HT_{3B} subunits is necessary to provide the functional characteristics of the native 5-HT_3 receptor. Homomeric receptors composed of either subunit do not display the characteristics of native 5-HT_3 receptors. The exact subunit composition of the native 5-HT_3 receptor is not known. It is entirely possible that the subunit composition of native 5-HT_3 receptors may vary with their location in the body, raising the exciting possibility of differences in the pharmacology and regulation of native 5-HT_3 receptors depending on the tissue or brain region in which they are expressed.

The 5-HT_4, 5-ht_6 and 5-HT_7 receptors are coupled to the stimulation of adenylyl cyclase. 5-HT_4, 5-ht_6 and 5-HT_7 receptors preferentially couple to the stimulation of adenylyl cyclase, increasing cAMP formation, via the Gs family of G proteins (see Chs 19 and 21). These receptors, however, share only >35% overall sequence homology. For this reason, they are classified as distinct receptor groups or classes and not subtypes of a family. The grouping of these receptors together is considered to be somewhat arbitrary and may be modified in the future. A lower-case appellation is used for the 5-ht_6 receptor because a physiological role for these receptors in intact tissue has not been found [28].

The 5-HT_4 receptor was originally described in cultured mouse collicular neurons, and subsequently in mouse and guinea pig brain, by Bockaert and co-workers in the late 1980s. This 5-HT receptor coupled to the stimulation of adenylyl cyclase activity and possessed pharmacological characteristics distinct from that of the 5-HT_1, 5-HT_2 or 5-HT_3 receptors. It had been known for a number of years prior to the pharmacological definition of the 5-HT_4 receptor that 5-HT could stimulate adenylyl cyclase in brain. Studies of the 5-HT_4 receptor were hampered by the absence of a high-affinity radioligand. The synthesis and development of specific radioligands provided the necessary tools for the study and characterization of the 5-HT_4 receptor. 5-HT_4 receptor binding sites are localized with high density in the striatum, substantia nigra and olfactory tubercle, and have been reported in the hippocampus as well (Table 13-2). The 5-HT_4 receptor is located postsynaptically to serotonergic neurons and this receptor modulates the release of several neurotransmitters (including acetylcholine, dopamine and GABA) and, indirectly, 5-HT release as well. 5-HT_4 receptor activation enhances cognitive performance in rats and monkeys. In humans, a role for the 5-HT_4 receptor in memory enhancement remains to be demonstrated in clinical studies [39].

In the periphery, 5-HT_4 receptor mRNA is found in vascular smooth muscle. Newly developed drugs that activate 5-HT_4 receptors are of interest for their potential in treating cardiac arrhythmia. The 5-HT_4 receptor is also located on neurons of the alimentary tract, for example the myenteric plexus of the ileum, and on smooth muscle cells and secretory cells of the gastrointestinal tract, where they evoke secretions and the peristaltic reflex. 5-HT_4 receptor agonists (e.g. cisapride, prucalopride, tegaserod) are used therapeutically in the treatment of constipation-predominant irritable bowel syndrome and in functional motility disorders of the upper gastrointestinal tract.

Multiple isoforms of the 5-HT_4 receptor have been shown to exist, the result of alternative splicing of 5-HT_4 receptor mRNA. Studies indicate specificity in the pattern of expression of these splice variants in different tissues. For example, the human 5-HT_{4a}, 5-HT_{4b} and 5-HT_{4c} receptor isoforms are expressed in the brain, atrium and gut, whereas expression of the 5-HT_{4d} receptor isoform appears to be restricted to the gut. The physiological significance of the differential distribution of many splice variants of the 5-HT_4 receptor in the body remains to be understood. The existence of different isoforms of the 5-HT_4 receptor raises interesting possibilities as to the distinct pharmacological characteristics and regulation of these different receptor isoforms.

The 5-ht_6 receptor, when expressed in transfected cells, shows a pharmacological profile that is unique. Interestingly, this receptor has high affinity for various antipsychotic and antidepressant drugs such as clozapine, amitriptyline, clomipramine, mianserin and ritanserin. The 5-ht_6 receptor stimulates adenylyl cyclase when expressed in recombinant cell systems. The function of the 5-ht_6 receptor in intact tissue has not been characterized because of the lack of selective agonists or antagonists. Expression of 5-ht_6 receptor mRNA has been detected in the striatum, nucleus accumbens, olfactory tubercle, hippocampus and cerebral cortex The recent development of the selective antagonist radioligand [^{125}I] SB 258585 has allowed the visualization of the distribution of 5-ht_6 receptor sites in brain using quantitative autoradiography [40].

The 5-HT_7 receptor is the most recently identified member of the family of G-protein-coupled 5-HT receptors. Although there is no selective agonist or antagonist currently available for the 5-HT_7 receptor, the distinct pharmacological profile of 5-HT_7 receptor sites has been used to delineate the function and distribution of this

receptor *in vivo*. 5-HT_7 receptor binding sites in the rat brain have been described using non-selective radioligands in the presence of drugs to mask 5-HT_{1A} and 5-HT_{1B} receptors. Recently, the distribution of 5-HT_7 receptors has been confirmed in autoradiographic studies using nonselective ligands, and in the brains of mice lacking 5-$HT_{1A/1B}$ receptors. Analysis of the binding of nonselective radioligands in the brains of 5-HT_{1A} and 5-$HT_{1A/1B}$ knockout mice revealed the presence of low densities of 5-HT_7 receptors in the cortex, septum, thalamus, hypothalamus, amygdala and hippocampus [41] (Table 13-2).

A role for the 5-HT_7 receptor in the regulation of circadian rhythms has been implicated. As discussed above, 5-HT has been known for some time to induce phase shifts in behavioral circadian rhythms and modulate neuronal activity in the suprachiasmatic nucleus, the likely site of the mammalian circadian clock. The pharmacological characteristics of the effect of 5-HT on circadian rhythms are consistent with 5-HT_7 receptor. Moreover, mRNA for the 5-HT_7 receptor is found in the suprachiasmatic nucleus. There is also increasing evidence that the 5-HT_7 receptor may play a role in psychiatric disorders. The regional distribution of 5-HT_7 receptors in brain includes limbic areas and cortex. Atypical antipsychotics, such as clozapine and risperidone, and some antidepressants display high affinity for this receptor. In the periphery, 5-HT_7 receptors have been shown to mediate relaxation of vascular smooth muscle.

Alternative mRNA splicing has been reported to generate four 5-HT_7 receptor isoforms. However, these isoforms have not been shown to differ in their respective pharmacology, signal transduction or tissue distribution.

'Orphan' receptors include the 5-ht$_5$ receptor and the 5-HT_{1P} receptor.

5-ht$_5$ receptor. Two subtypes of the 5-ht$_5$ receptor have been cloned (5-ht$_{5A}$ and 5-ht$_{5B}$ receptors). These receptor proteins are 77% identical to each other whereas the homology with other serotonin receptors is low. 5-ht$_{5A}$ receptor mRNA transcripts have been detected by *in situ* hybridization in the cerebral cortex, hippocampus, granule cells of the cerebellum, medial habenula, amygdala, septum, several thalamic nuclei and olfactory bulb of the rat and mouse. 5-ht$_{5B}$ mRNA has been detected by *in situ* hybridization in the hippocampus, habenula and dorsal raphe nucleus of rat and human [27]. Immunohistochemical studies using antibodies to the 5-ht$_{5A}$ receptor have shown this receptor to be expressed predominantly by astrocytes, although some neurons in cortex were labeled as well. When expressed in recombinant cell systems, the 5-ht$_{5A}$ receptor is coupled to the inhibition of adenylyl cyclase. At the present time, the functional correlate and transductional properties of the 5-ht$_{5B}$ receptor are unknown. Because no evidence has been obtained to confirm that the recombinant 5-ht$_5$ receptor is expressed and functions in an endogenous tissue, this receptor retains its lower case appellation [28].

The existence of an atypical 5-HT receptor on the enteric neurons of the gut has been shown in a series of investigations by Gershon and associates. This receptor has high affinity for ^{3}H-5-HT and mediates a slow depolarization of particular myenteric neurons that is not blocked by selective 5-HT_3 antagonists. This receptor has been termed the 5-HT_{1P} receptor as it has a high affinity for 5-HT and is found in the periphery, in high density in the gut. The pharmacology of this receptor is somewhat similar to that of the 5-HT_4 receptor. The available functional and radioligand binding data confirm the orphan status of the 5-HT_{1P} receptor and emphasize the need to establish a rigorous basis for its positive identification [27, 28].

REFERENCES

1. Rapport, M. M., Green, A. A. and Page, I. H. Serum vasoconstrictor (serotonin). IV. Isolation and characterization. *J. Biol. Chem.* 176: 1243–1251, 1948.
2. Tork, I. Anatomy of the serotoninergic system. *Ann. N.Y. Acad. Sci.* 600: 9–34, 1990.
3. Beck, S. G., Pan, Y. Z., Akanwa, A. C. and Kirby, L. G. Median and dorsal raphe neurons are not electrophysiologically identical. *J. Neurophysiol.* 91: 994–1005, 2004.
4. Molliver, M. E. Serotonergic neuronal systems: what their anatomic organization tells us about function. *J. Clin. Psychopharmacol.* 7(6 Suppl.): 3S–23S, 1987.
5. Eaton, M. J., Gudehithlu, K. P., Quach, T., Sivia, C. P., Hadjiconstantinou, M. and Neff, N. H. Distribution of aromatic L-amino acid decarboxylase mRNA in mouse brain by in situ hybridization histology. *J. Comp. Neurol.* 337: 640–654, 1993.
6. Cortes, R., Mengod, G., Celada, P. and Artigas, F. p-Chlorophenylalanine increases tryptophan-5-hydroxylase mRNA levels in the rat dorsal raphe: a time course study using in situ hybridization. *J. Neurochem.* 60: 61–764, 1993.
7. Boadle-Biber, M. C. Regulation of serotonin synthesis. *Prog. Biophys. Mol. Biol.* 60: 1–15, 1993.
8. Bendotti, C., Servadio, A., Forloni, G., Angeretti, N. and Samanin, R. Increased tryptophan hydroxylase mRNA in raphe serotonergic neurons spared by 5,7-dihydroxytryptamine. *Mol. Brain Res.* 8: 342–248, 1990.
9. Garcia-Osta, A., Del Rio, J. and Frechilla, D. Increased CRE-binding activity and tryptophan hydroxylase mRNA expression induced by 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') in the rat frontal cortex but not in the hippocampus. *Mol Brain Res.* 126: 181–187, 2004.
10. Tamir, H., Liu, K. P., Adlersberg, M., Hsiung, S. C. and Gershon, M. D. Acidification of serotonin-containing secretory vesicles induced by a plasma membrane calcium receptor. *J. Biol. Chem.* 271: 6441–6450, 1996.
11. Adams, S. V. and DeFelice, L. J. Ionic currents in the human serotonin transporter reveal inconsistencies in the alternating access hypothesis. *Biophys. J.* 85: 1548–1559, 2003.
12. Zahniser, N. R. and Doolen, S. Chronic and acute regulation of Na^+/Cl^--dependent neurotransmitter transporters: drugs, substrates, presynaptic receptors, and signaling systems. *Pharmacol. Ther.* 92: 21–55, 2001.

13. Daws, L. C., Gould, G. G., Teicher, S. D., Gerhardt, G. A. and Frazer, A. 5-HT1B receptor-mediated regulation of serotonin clearance in rat hippocampus in vivo. *J. Neurochem.* 75: 2113–2122, 2000.
14. Krajnak, K., Rosewell, K. L., Duncan, M. J. and Wise, P. M. Aging, estradiol and time of day differentially affect serotonin transporter binding in the central nervous system of female rats. *Brain Res.* 990: 87–94, 2003.
15. Murphy, D. L., Li, W., Engel, S. *et al.* Genetic perspectives on the serotonin transporter. *Brain Res. Bull.* 56: 487–494, 2001.
16. Lotrich, F. E., Pollock, B. G. and Ferrell, R. E. Polymorphisms of the serotonin transporter: implications for the use of selective serotonin reuptake inhibitors. *Am. J. Pharmacogenomics* 1: 153–164, 2001.
17. Shih, J. C. Molecular basis of human MAO A and B. *Neuropsychopharmacology* 4: 1–3, 1991.
18. Cases, O., Seif, I., Grimsby, J. *et al.* Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. *Science* 268: 1763–1766, 1995.
19. Tao-Cheng, J. H. and Zhou, F. C. Differential Polarization of serotonin transporters in axons versus soma-dendrites: an immunogold electron microscopy study. *Neuroscience* 94: 821–830, 1999.
20. Riad, M., Garcia, S., Watkins, K. C. *et al.* Somatodendritic localization of 5-HT1A and preterminal axonal localization of 5-HT1B serotonin receptors in adult rat brain. *J. Comp. Neurol.* 417: 181–194, 2000.
21. Jacobs, B. L. and Fornal, C. A. 5-HT and motor control: a hypothesis. *Trends Neurosci.* 16: 346–352, 1993.
22. Meyer-Bernstein, E. L. and Morin, L. P. Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. *J. Neurosci.* 16: 2097–2111, 1996.
23. Simansky, K. J. Serotonergic control of the organization of feeding and satiety. *Behav. Brain Res.* 73: 37–42, 1996.
24. Reiter, R. J. Melatonin: the chemical expression of darkness. *Mol. Cell. Endocrinol.* 79: C153–C158, 1991.
25. Dubocovich, M. L., Rivera-Bermudez, M. A., Gerdin, M. J. and Masana, M. I. Molecular pharmacology, regulation and function of mammalian melatonin receptors. *Front. Biol.* 8: d1093–1108, 2003.
26. Peroutka, S. and Snyder, S. H. Multiple serotonin receptors. Differential binding of [^{3}H]-5-hydroxytryptamine, [^{3}H] lysergic acid diethylamide and [^{3}H]-spiroperidol. *Mol. Pharmacol.* 16: 687–699, 1979.
27. Hoyer, D., Clarke, D. E., Fozard, J. R. *et al.* VII. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.* 46: 157–203, 1994.
28. Hoyer, D., Hannon, J. P. and Martin, G. R. Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol. Biochem. Behav.* 71: 533–554, 2002.
29. Hartig, P. R., Hoyer, D., Humphrey, P. P. A. and Martin, G. Alignment of receptor nomenclature with the human genome: classification of 5-HT_{1B} and 5-HT_{1D} receptor subtypes. *Trends Pharmacol. Sci.* 17: 103–105, 1996.
30. Hoskin, K. L., Lambert, G. A., Donaldson, C. and Zagami, A. S. The 5-hydroxytryptamine$_{1B/1D/1F}$ receptor agonists eletriptan and naratriptan inhibit trigeminovascular input to the nucleus tractus solitarius in the cat. *Brain Res.* 998: 91–99, 2004.
31. Bruinvels, A. T., Palacios, J. M. and Hoyer, D. Autoradiographic characterisation and localisation of 5-HT1D compared to 5-HT1B binding sites in rat brain. *Naunyn Schmiedebergs Arch. Pharmacol.* 347: 569–582, 1993.
32. Zgombick, J. M., Schechter, L. E., Macci, M., Hartig, P. R., Branchek, T. A. and Weinshank, R. L. Human gene S31 encodes the pharmacologically defined serotonin 5-hydroxytryptamine$_{1E}$ receptor. *Mol. Pharmacol.* 42: 180–185, 1992.
33. Cox, D. A. and Cohen, M. L. 5-HT_{2B} receptor signaling in the rat stomach fundus: dependence on calcium influx, calcium release and protein kinase C. *Behav. Brain Res.* 73: 289–292, 1996.
34. Watts, S. W. and Thompson, J. M. Characterization of the contractile 5-hydroxytryptamine receptor in the renal artery of the normotensive rat. *J. Pharmacol. Exp. Ther.* 309: 165–172, 2004.
35. Burns, C. M., Chu, H., Rueter, S. M. *et al.* Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature* 387: 303–308, 1997.
36. Fitzgerald, L. W., Iyer, G., Conklin, D. S. *et al.* Messenger RNA editing of the human serotonin 5-HT_{2C} receptor. *Neuropsychopharmacology* 21: 82S–90S, 1999.
37. Parker, R. M. C., Bentley, K. R. and Barnes, N. M. Allosteric modulation of 5-HT_3 receptors: focus on alcohols and anaesthetic agents. *Trends Pharmacol. Sci.* 17: 95–99, 1996.
38. Davies, P. A., Pistis, M., Hanna, M. C. *et al.* The 5-HT_{3B} subunit is a major determinant of serotonin receptor function. *Nature* 397: 359–363, 1999.
39. Barnes, J. M. and Barnes, N. M. Neurochemical consequensces following pharmacological manipulation of central 5-HT_4 receptors. In R. M. Eglen (ed.), *5-HT_4 Receptors Brain Periphery*. Berlin: Springer, 1998, pp 103–126.
40. Woolley, M. L., Marsden, C. A. and Fone, K. C. F. 5-ht_6 receptors. *Curr. Drug Targets C.N.S. Neurol. Disord.* 3: 59–79, 2004.
41. Bonaventure, P., Nepomuceno, D., Kwok, A. *et al.* Reconsideration of 5-hydroxytryptamine (5-HT)$_7$ receptor distribution using [^{3}H]5-carboxamidotryptamine and [^{3}H]8-hydroxy-2-(di-n-propylamino)tatraline: Analysis in brain of 5-HT_{1A} knock-out and 5-$HT_{1A/1B}$ double knock-out mice. *J. Pharmacol. Exp. Ther.* 302: 240–248, 2002.

CHAPTER

14

Histamine

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HISTAMINE: THE MESSENGER AND THE MOLECULE

Histamine is a mediator of several physiological and pathological processes within and outside the nervous system. Although the existence of histamine in the brain was known over 50 years ago, a role for this substance in brain function was not widely appreciated until relatively recently. It is now clear that histamine is formed within and released from central nervous system neurons and is an important regulator of several brain functions [1–3]. Paradoxically, the existence of well-established roles for histamine outside the nervous system is one factor that hampered the acceptance of this amine as a neuronal messenger. As a physiological mediator, histamine is best known as an endogenous stimulant of gastric secretion. Histamine is also released from mast cells and basophils by antigens, certain peptides and small basic drugs. In addition, histamine participates in inflammation and in the regulation of immune responses. Cardiac stores of

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FIGURE 14-1 Chemical structure of histamine, illustrating the two tautomeric forms. The names of the nitrogen atoms are shown on the left tautomer and the numbering scheme for carbon atoms is on the right. For example, *N*$^{\pi}$-methylhistamine, *N*α-methylhistamine and α-methylhistamine are distinct substances and have very different properties (Fig. 14-3 and Table 14-1). This nomenclature system avoids references to the ring nitrogens as 1- and 3-, a designation that becomes confused by the tautomerism.

histamine probably play no physiological role but may be of pathological significance. Endogenous peripheral histamine may also participate in the modulation of sympathetic and/or afferent nerve activity; the storage of this histamine is probably non-neuronal.

The chemical structure of histamine has similarities to the structures of other biogenic amines, but important differences also exist. Chemically, histamine is 2-(4-imidazolyl)ethylamine (Fig. 14-1). The ethylamine 'backbone' is a common feature of many of the amine transmitters (e.g. dopamine, norepinephrine and serotonin). However, the imidazole nucleus, absent from other known transmitters, endows histamine with several distinct chemical properties. Among these is prototypic tautomerism, a property that permits it to exist in two different chemical forms (Fig. 14-1). The tautomeric properties of histamine are thought to be critical in the ability of this molecule to activate some of its receptors. Although histamine has more than one basic center, both tautomers exist predominantly as monocations at physiological pH. This tautomerism has also contributed to confusion in naming histamine congeners (Fig. 14-1).

HISTAMINERGIC CELLS OF THE CENTRAL NERVOUS SYSTEM: ANATOMY AND MORPHOLOGY

The brain stores and releases histamine from more than one type of cell. Mast cells are a family of bone marrow-derived secretory cells that store and release high concentrations of histamine. They are found throughout many connective tissues of the body, but are also present within and surrounding the brain of many species [4, 5]. In many

TABLE 14-1 Interactions between histamine and other transmitters

	Histaminergic modulation of transmitter			*Transmitter's modulation of histamine*		
Transmitter	**Transmitter parameter**	**Receptor**	**Effect**	**Histamine parameter**	**Receptor**	**Effect**
Acetylcholine	Release	H_3	↓*	Release	M_1	↓
Acetylcholine	Release†	H_2	↑	Release†	M_1	↓
Acetylcholine	Turnover	Muscarinic	↓			
Acetylcholine	Turnover	Nicotinic	↓			
CGRP, substance P	Release‡	H_3	↓			
Dopamine	Release	H_3	↓	Release†	D_2	↑
Dopamine	DOPAC levels	H_1	↑§	Release†	D_3	↓
GABA	Release†	$GABA_{A,B}$	↓			
GABA	Turnover	?	↓			
Glutamate	Release†	NMDA	↑			
Norepinephrine	Release	H_3	↓	Release	α_2	↓
Norepinephrine	Release†	H_1	↑			
Norepinephrine	Turnover	H_1	↑			
Opioids	Release	κ	↓			
Opioids	Turnover	κ	↓			
Opioids	Release†	μ	↑			
Opioids	Turnover	μ	↑			
Serotonin	Release	H_3	↓	Release†	$5HT_{2C/2A}$	↑
Serotonin	D. Raphe activity	H_1	↑	Turnover	$5HT_{1A}$	↓
Serotonin	5-HIAA levels	H_1	↑			

Experiments investigating the interactions between brain histamine and other transmitters are summarized. Unless otherwise specified, 'release' experiments were performed *in vitro* with brain slices or synaptosomes. See [71, 89, 90] for references.

*Inhibition by H_3 receptor may not be direct. †Release measured by *in vivo* techniques. ‡Release from isolated perfused heart. §Some effects of histamine on dopaminergic parameters found to depend on noradrenergic activity.

5-HIAA, 5-hydroxyindoleacetic acid; CGRP, calcitonin-gene-related peptide; DOPAC, 3,4-dihydroxyphenylacetic acid; NMDA, *N*-methyl-D-aspartate.

(but not all) species, they are prevalent in thalamus and hypothalamus, as well as in the dura mater, leptomeninges and choroid plexus [4]. The quantitative contribution made by mast cells to brain histamine levels can be substantial in some cases (e.g. rat thalamus). Earlier biochemical studies suggested that histamine in brain mast cells could be distinguished from neuronal histamine by characteristics such as histamine turnover rates, subcellular fractionation and ontogenic pattern [5]. However, activated mast cells may not show a slow histamine turnover rate. Brain and dural mast cells have been characterized histologically and histochemically in detail. Characterization of neuronal and non-neuronal histamine has been facilitated by the study of mast-cell deficient mice and rats.

The functions for brain and dural mast cells are not certain but several interesting hypotheses are being investigated. The close proximity of many of these cells to blood vessels, along with the potent vascular actions of their contents, has led to the suggestion that they regulate blood flow, permeability and/or immunological access to the brain. Dural mast cells are localized in close proximity with sensory nerve fibers and may modulate the release of inflammatory mediators from these cells (Table 14-1). Perhaps most intriguing are recent findings showing that chemosensory cues associated with estrus induction can trigger mast cell infiltration into distinct brain areas [6]. Brain and/or dural mast cells may also participate in neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease or Wernicke's encephalopathy [7, 8]. In addition to mast cells and neurons, other brain cells (e.g. cerebrovascular endothelial cells [5, 9]) may also synthesize and/or store histamine.

Histaminergic fibers originate from the tuberomammillary region of the posterior hypothalamus. The inability to visualize histaminergic neurons greatly limited the understanding and acceptance of this neuronal system. This situation changed in 1984, when antibodies raised against histamine [10] or its biosynthetic enzyme [11] provided the first detailed anatomical studies of these cells and their distribution. In all vertebrates studied, including humans, histaminergic neurons are found in the tuberomammillary (TM)nucleus of the posterior basal hypothalamus (Fig. 14-2A).

Histaminergic neurons have morphological and membrane properties that are similar to those of neurons storing other biogenic amines. Histaminergic perikarya can be of medium size but most are large, bipolar or multipolar cells with diameters of up to 30 µm. Ultrastructural characteristics resemble those of noradrenergic and serotonergic cell bodies [1]. Some of the most ventrally located cells may make direct contact with cerebrospinal fluid.

Electrophysiological properties of histaminergic neurons show spontaneous activity of about 2 Hz. A combination of sodium, calcium and potassium conductances account for their pacemaker properties [1]. Noradrenergic and serotonergic neurons show similar properties. TM histaminergic neuron firing is high during waking or attention, and much lower or absent during slow wave sleep, findings that strongly support a role for histamine in wakefulness.

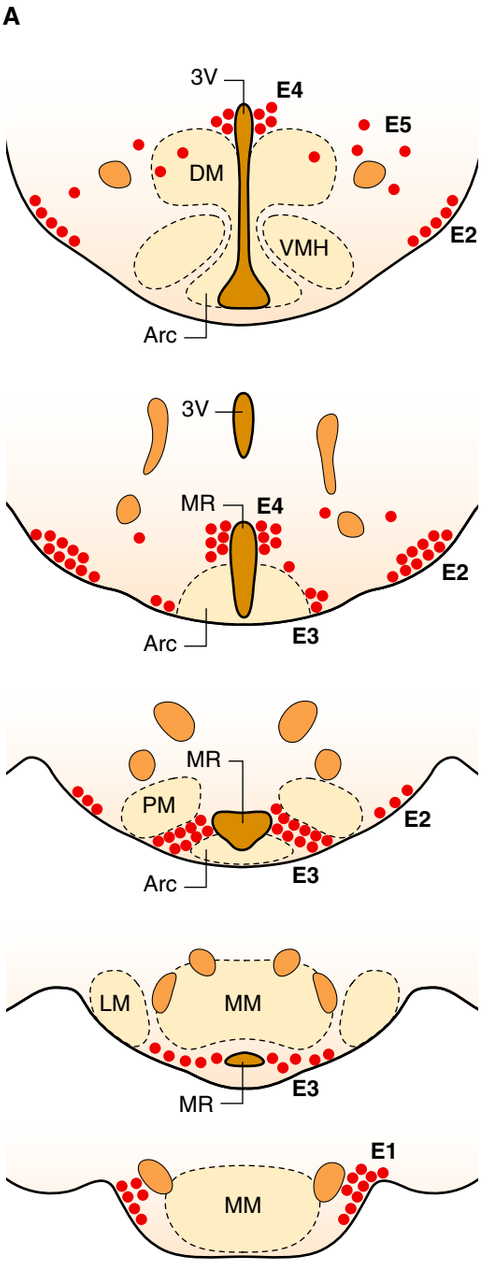

FIGURE 14-2 The histaminergic system of the rat brain. (**A**) Frontal sections through the posterior hypothalamus showing the location of histaminergic neurons. *Arc*, arcuate nucleus; *DM*, dorsomedial nucleus; *LM*, lateral mammillary nucleus; *MM*, medial mammillary nucleus; *MR*, mammillary recess; *PM*, premammillary nucleus; *3V*, third ventricle; *VMH*, ventromedial hypothalamic nucleus. (Modified with permission from reference [5].)

Continued

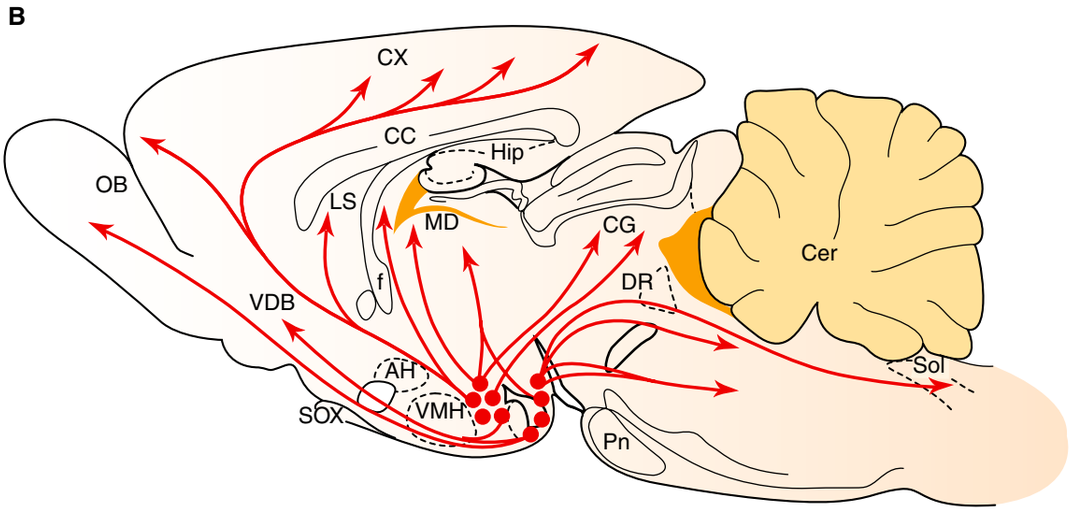

FIGURE 14-2—cont'd (**B**) A sagittal view illustrating the major ascending and descending fiber projections. *AH*, anterior hypothalamus; *CC*, corpus callosum; *Cer*, cerebellum; *CG*, central gray; *CX*, cerebral cortex; *DR*, dorsal raphe; *f*, fornix; *Hip*, hippocampus; *LS*, lateral septum; *MD*, mediodorsal thalamus; *OB*, olfactory bulb; *Pn*, pontine nuclei; *Sol*, nucleus of solitary tract; *SOX*, supraoptic decussation; *VDB*, vertical limb of the diagonal band; *VMH*, ventromedial hypothalamic nucleus. (Modified with permission from reference [2].)

Histaminergic fibers project widely to most regions of the central nervous system. Both ascending and descending efferent pathways account for the histaminergic innervation of the mammalian brain and spinal cord (Fig. 14-2B). The ascending tracts are predominantly ipsilateral. All cell groups appear to contribute to all pathways. Although nearly all CNS areas contain some histaminergic fibers, the density of innervation is heterogeneous [1, 12, 13] and somewhat phylogenetically distinct. The highest densities are found in several hypothalamic nuclei, the medial septum, the nucleus of the diagonal band and the ventral tegmental area. Moderate densities are found in cerebral cortex, amygdala, striatum and substantia nigra. Innervation of the hippocampus and thalamus is substantial is some species. Most areas of the brainstem, as well as retina, cerebellum and spinal cord, contain only small numbers of fibers. These densities follow closely the tissue concentrations of histamine and its biosynthetic enzyme found throughout the brain [14]. In the monkey brain, a homogeneous innervation of several areas of the visual system (including lateral geniculate and superior colliculus) has been documented. Ultrastructural studies in rat show that histaminergic varicosities form only a few synaptic contacts, implying that most neuronal histamine is released by nonsynaptic mechanisms; this seems to be the case for the other amine transmitter systems as well. However, histaminergic synapses have been characterized in some detail, such as in the innervation of the mesencephalic trigeminal nucleus. Histaminergic varicosities also appear to make contact with glia and blood vessels.

A number of substances are colocalized with histamine and its biosynthetic enzyme in hypothalamic tuberomammillary neurons [15]. These include glutamate decarboxylase, GABA, GABA-transaminase, adenosine deaminase, monoamine oxidase-B and the neuropeptide Met-Enk-Arg^6-Phe^7. A subset of cells in rat brain also contain galanin. Thyrotropin-releasing hormone (TRH) is present in some histaminergic neurons of some species. These findings suggest that some of these peptides, as well as GABA and/or adenosine, may function as co-transmitters in this system. Galanin may be a presynaptic inhibitor of neuronal histamine release, similar to its proposed actions on cholinergic and serotonergic fibers. Endogenous GABA appears to regulate neuronal histamine release, but the cellular origin of this GABA is not known (Table 14-1). A recent study found that GABA storage within TM neurons is separate from that of histamine [16].

TM histaminergic cells receive innervation from several brain areas, including the infralimbic prefrontal cortex, several areas within the septum/diagonal band complex and the medial preoptic area of the hypothalamus. These or other afferents seem to contain neuropeptide Y (NPY) or substance P. TM cells receive monoaminergic input from adrenergic (C1–C3), noradrenergic (A1–A2) and serotonergic (B5–B9) cells, but the innervation from dopamine-containing ventral tegmental area is sparse [1]. Since some of these areas seem to have both input and output connections with histaminergic areas, the possibility of reciprocal control has been considered [5]. Orexinergic inputs to the TM cells have also been recently described [1].

Histaminergic neurons are present in many species. Histaminergic neurons have been found in the hypothalamus or diencephalon of a variety of vertebrate brains, including those of fish, snake, turtle and bird. The brain histaminergic system of zebrafish has been studied in

detail, since this species apparently lacks any non-neuronal stores of histamine, such as those found in gastric mucosa or mast cells in mammals [1]. In invertebrates, histamine functions as an important transmitter, for example in arthropod and insect photoreceptors [17, 18]. It is well established that the C2 neuron of *Aplysia* uses histamine as a transmitter [19].

DYNAMICS OF HISTAMINE IN THE BRAIN

Specific enzymes control histamine synthesis and breakdown. Figure 14-3 summarizes the major mechanisms for the synthesis and metabolism of histamine. Biosynthesis is performed in one step by the enzyme L-histidine decarboxylase (HDC, E.C. 4.1.1.22). Histamine metabolism occurs mainly by two pathways. Oxidation is carried out by diamine oxidase (DAO, E.C. 1.4.3.6), leading to imidazole acetic acid (IAA), whereas methylation is effected by histamine *N*-methyltransferase (HMT, E.C. 2.1.1.8), producing *tele*-methylhistamine (t-MH). IAA can exist as a riboside or ribotide conjugate. t-MH is further metabolized by monoamine oxidase (MAO)-B, producing *tele*-methylimidazole acetic acid (t-MIAA). Note that histamine is a substrate for DAO but not for MAO. Aldehyde intermediates, formed by the oxidation of both histamine and t-MH, are thought to be quickly oxidized to acids under normal circumstances. In the vertebrate CNS, histamine is almost exclusively methylated

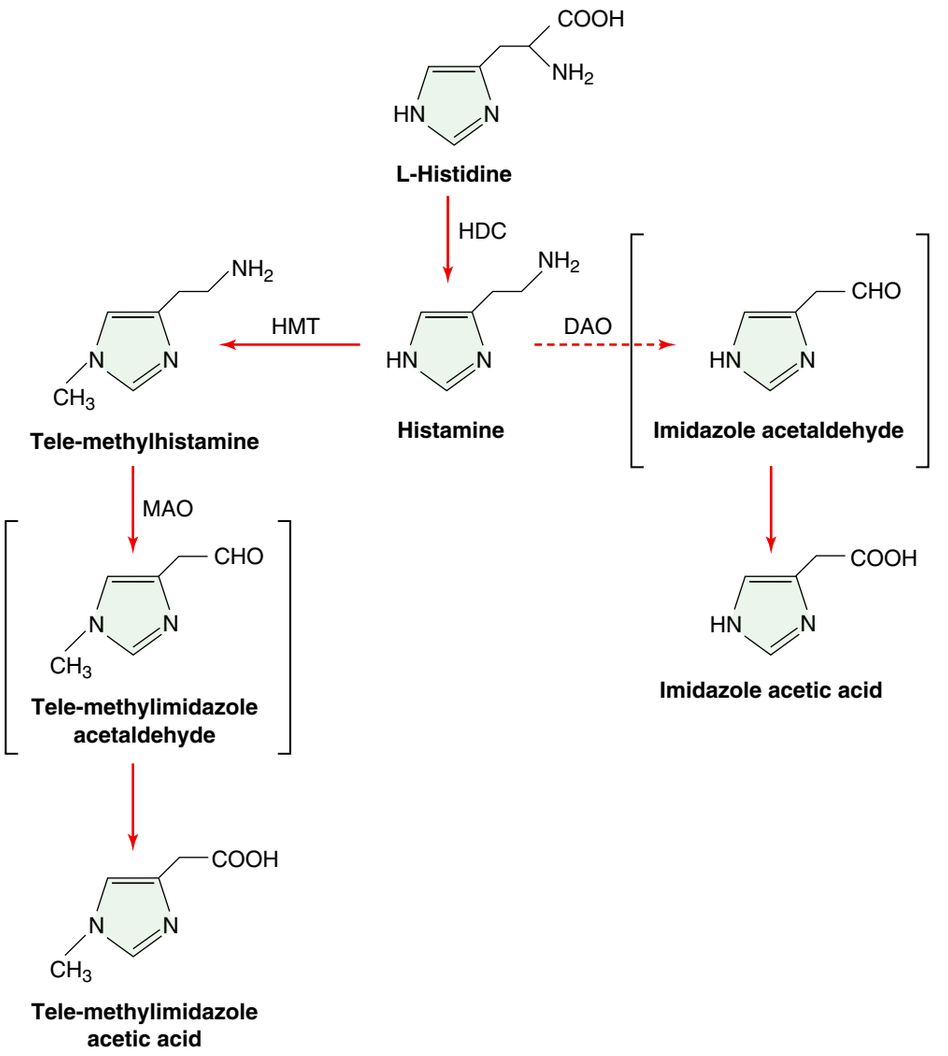

FIGURE 14-3 Synthesis and metabolism of histamine. *Solid lines* indicate the pathways for histamine formation and catabolism in brain. *Dashed lines* show additional pathways that can occur outside the nervous system. *HDC*, histidine decarboxylase; *HMT*, histamine methyltransferase; *DAO*, diamine oxidase; *MAO*, monoamine oxidase. Aldehyde intermediates, shown in brackets, have been hypothesized but not isolated.

and only small amounts of DAO are detectable. However, IAA, a GABA agonist, has been detected and can be formed in the rat brain in small amounts. Although IAA in the brain is probably normally formed by the transamination of histidine, it can also be formed by histamine oxidation under some circumstances. In some invertebrate nervous systems, such as Aplysia, histamine is metabolized to γ-glutamylhistamine.

Several forms of histidine decarboxylase may derive from a single gene. A single cDNA, cloned from rat [20], mouse and human cells, encodes a 74 kDa protein with functional HDC activity. Two species of HDC mRNA have been found in some cells, but the larger one, which contains an additional insert sequence, does not encode a functional enzyme and is not found in brain. Consistent with the immunohistochemical studies discussed above, HDC mRNA is localized to the caudal hypothalamus in rats. The human HDC gene is large, composed of 12 exons with a size of about 2.4 kb. The enzyme, which requires pyridoxal-5′-phosphate as a coenzyme, shares some homology with DOPA decarboxylase (see Ch. 12), another enzyme that requires this cofactor. Protein purification studies have shown HDC to be a dimer composed of two identical 55 kDa subunits. Post-translational modification of the enzyme occurs by an elastase-like enzyme, which converts the 74 kDa form to the smaller protein. Biochemical, biophysical and immunological studies show clear evidence for the existence of HDC isoenzymes [21]. Either post-translational modification of the protein or, possibly, allelic variants may contribute to the existence of these isoforms. The rat HDC gene has two potential *N*-glycosylation sites and two recognition sequences for phosphorylation by cAMP-dependent protein kinase (PKA). These potential modulatory sites could contribute to some of the heterogeneity in HDC.

Histamine synthesis in the brain is controlled by the availability of L-histidine and the activity of histidine decarboxylase. Although histamine is present in plasma, it does not penetrate the blood–brain barrier, such that histamine concentrations in the brain must be maintained by synthesis. With a K_m value of 0.1 mmol/l for L-histidine under physiological conditions, HDC is not saturated by histidine concentrations in the brain, an observation that explains the effectiveness of large systemic doses of this amino acid in raising the concentrations of histamine in the brain. The essential amino acid L-histidine is transported into the brain by a saturable, energy-dependent mechanism [5]. Subcellular fractionation studies show HDC to be localized in cytoplasmic fractions of isolated nerve terminals, i.e. synaptosomes.

HDC activity can be regulated by both hormonal and neuronal factors, most of which are poorly understood. Phosphorylation of the enzyme by PKA may be an important regulatory mechanism. Several regulatory sites have recently been found in the promoter region of the gene.

Unlike catecholamines and indoleamines, histamine itself is not a direct inhibitor of its biosynthetic enzyme, but it exerts feedback control through the H_3 autoreceptor. Perhaps the most powerful tool in the study of the histamine system is *S*-α-fluoromethylhistidine, a highly selective and potent 'suicide' inhibitor of HDC [22]. This compound has been used successfully to study many of the functions of histamine in brain.

Histamine is stored within and released from neurons but a neuronal transporter for histamine has not been found. Newly synthesized neuronal histamine is transported into TM neuronal vesicles by the vesicular monoamine transporter VMAT2 [16]. Both *in vivo* and *in vitro* studies show that depolarization of nerve terminals activates the exocytotic release of histamine by a voltage- and calcium-dependent mechanism. Once released, histamine activates both postsynaptic and presynaptic receptors. Unlike the nerve terminals from other amine transmitters, however, histaminergic nerve terminals do not exhibit a high-affinity uptake system for histamine [5, 9, 23]. Astrocytes may contain a histamine transport system.

In the vertebrate brain, histamine metabolism occurs predominantly by methylation. HMT, the histamine-methylating enzyme, uses the methyl donor *S*-adenosyl-L-methionine. The enzyme has a K_m of about 10 μmol/l for both histamine and the methyl cofactor, and has been localized to the soluble subcellular fraction. Antibodies raised against a highly purified kidney enzyme with a molecular weight of 33,000 co-precipitate the brain enzyme, showing strong similarities between the proteins. A 1.3 kb cDNA has been cloned and expressed in *Escherichia coli*; the encoded protein shows the characteristics of the natural enzyme [24]. Early studies suggested that isoforms of HMT might exist and, recently, it was shown that the HMT gene can be spliced to produce a shorter form (HMT-S, [25]). Although HMT-S mRNA is present in human brain and placenta, the protein does not methylate histamine and its biological significance remains unclear. HMT is subject to inhibition by both its product, t-MH, and, at higher concentrations, its substrate, histamine. Experimental inhibitors of HMT include metoprine and SKF91488. These agents have been used in metabolic studies as well as to probe functions for CNS histamine. As expected, they increase histamine concentrations in the brain and reduce the concentrations of t-MH and t-MIAA. Many other compounds can also inhibit HMT. For example, tacrine, a cholinesterase inhibitor used to treat Alzheimer's disease, also inhibits HMT at therapeutic doses.

Neuronal histamine can be methylated outside of histaminergic nerve terminals. In contrast to the striking regional distribution of histamine and HDC, HMT shows a more even distribution [14], suggesting a widespread HMT localization. In support of this, lesions that destroy

histaminergic fibers cause large reductions in HDC concentrations but with a lesser effect on brain HMT activity. The existence of glial cell lines containing HMT seemed to support an extraneuronal localization for histamine metabolism. However, immunochemical studies of brain HMT found a neuronal and vascular but not glial localization [26]. Because HMT does not follow the characteristic regional distribution of histamine and HDC, yet the HMT product t-MH does show this distribution, it is thought that the formation of t-MH is limited by the rate of histamine release from neurons. The rate at which histamine is formed and methylated (i.e. the histamine turnover rate) also follows this characteristic regional pattern and thus reflects the activity of histaminergic neurons. The hypothesis that histamine is methylated following its neuronal release is supported by recent work showing that extracellular histamine is methylated by isolated nerve terminal fractions to a much greater extent than it is transported [23]. The same study showed the existence of a membrane-bound form of HMT that could methylate newly released histamine in a manner analogous with that of acetylcholinesterase in the cholinergic system [23]. However, attempts to clone a unique membrane-bound form of this enzyme have not succeeded [25].

The product of histamine methylation, t-MH, is a substrate for MAO-B and is ultimately oxidized to t-MIAA, the end product of brain histamine metabolism. Thus, MAO inhibitors increase brain t-MH concentrations and lower t-MIAA concentrations, with little or no effect on histamine concentrations. Both t-MH and t-MIAA have been detected in brain and CSF. In the brain, t-MIAA concentrations are unaffected by probenecid, an inhibitor of the brain transport of other transmitter metabolites.

The activity of histaminergic neurons is regulated by H_3 autoreceptors and by other transmitter receptors. The observation that histamine can inhibit both its own synthesis and release from brain slices and synaptosomes led to the discovery of the histamine H_3 autoreceptor, a hypothesis that was confirmed by the development of unique agonists and antagonists of this receptor [27, 28] (Table 14-2). The prototype H_3 antagonist thioperamide enhances the firing rate of histaminergic neurons and increases neuronal histamine release, brain histamine turnover rates and t-MH concentrations in the brain. H_3 agonists, such as *R*-α-methylhistamine and immepip, produce the opposite effects. H_3 agonists and antagonists are important tools for understanding the brain histamine system, and several new agents are currently being developed for clinical uses. Receptors for several other transmitters have also been shown to regulate histaminergic dynamics (Table 14-1 and see section below).

The stimulatory effects of H_3 antagonists on brain histamine dynamics were initially attributed to the

TABLE 14-2 Characteristics of histamine receptors in the brain

Characteristics	H_1	H_2	H_3	H_4
Cloned?	Yes	Yes	Yes	Yes
Gene localization (mouse)	Chromosome 6	Chromosome 13	Chromosome 2	Chromosome 18
Effectors	PI-PLC: + IP_3 [+Ca^{2+}, +cGMP, +NO] PI-PLC: + DAG [+PKC] PI-PLA_2: + AA, + TXA_2	+ cAMP + PI-PLC: Ca^{2+}, IP_3§	−cAMP −NHE −Ca^{2+} + MAP kinase	−cAMP + MAP kinase + Ca^{2+}
Conductances‖	Excit: ↑Cation Excit: ↓K_{Leak} Inhib: ↑K_S	Excit: ↑ I_H Inhib: ↑ K_{V3} Excit: ↓ I_{kCa}	Reduced cell firing by inhibition of high-voltage-activated Ca channels	Unknown but likely to be similar to H_3
Selective agonists	2-thiazolylethylamine, histaprodifen	Amthamine	R-α-methylhistamine*¶ Immepip*¶ Immethridine	Clobenpropit*¶
Antagonists	Pyrilamine (mepyramine)*	Ranitidine Zolantidine*	Thioperamide*¶ Clobenpropit*¶ Ciproxyfan*	Thioperamide*¶ Iodophenpropit*¶ J&J7777120
Radioligands	^{3}H-pyrilamine ^{125}I-iodobolpyramine	^{125}I-iodoaminopotentidine	^{3}H-N^{α}-methylhistamine ^{125}I-iodophenpropit ^{125}I-iodoproxyfan	[^{3}H]-histamine [^{3}H]-J&J7777120
Distribution†‡	Cerebellum, thalamus, hippocampus	Cerebral cortex, striatum, nucleus accumbens	Striatum, nucleus accumbens, cerebral cortex, substantia nigra	Hippocampus

The characteristics of the four major classes of histamine receptors are summarized. Question marks indicate suggestions from the literature that have not been confirmed. AA, arachidonic acid; DAG, diacylglycerol; $I_{kCa}{}^{2+}$, calcium-activated potassium current; IP_3, inositol 1,4,5-trisphosphate; NHE, sodium–proton exchange, PKC, protein kinase C; NO, nitric oxide; PI-PLC, phosphoinositide-specific phospholipase C; TXA_2, thromboxane A_2.

*Has brain-penetrating characteristics after systemic administration.
†All receptors may exist in non-neuronal brain tissue as well.
‡Distribution in guinea pig (H1 and H2) and rodent (H3, H4) brain. For the H1 receptor, distribution is very different across species.
§Contradictory findings have been reported.
¶Compounds show activities at both H3 and H4 receptors
‖see [1] for details, including abbreviations for conductance changes.

competitive antagonism of continuously-released neuronal histamine, which stimulates the H_3 autoreceptors. However, since there are known to be H_3 antagonists that do not increase brain histamine release, it is now thought that the inverse agonist activity of certain H_3 ligands accounts for their activation of the histaminergic system [29]. This explanation, consistent with *in vitro* studies on recombinant H_3 receptors (see below), would mean that ongoing, *in vivo* H_3 autoreceptor activity is constitutive and therefore does not depend on ongoing histamine-induced H_3 activation. This conclusion does not mean that spontaneous, neuronal histamine release is not occurring, only that it may not be continuously reaching and stimulating its autoreceptors.

MOLECULAR SITES OF HISTAMINE ACTION

Histamine acts on four G-protein-coupled receptors, three of which are clearly important in the brain. Table 14-2 summarizes the characteristics of known histamine receptors in mammals. These histamine receptors subtypes are all linked to G proteins, and all of them have been found inside and outside the brain. Within the brain, the H_1, H_2 and H_3 receptors all have unique regional distributions but none is localized exclusively to neurons. For the recently discovered H_4 receptor, only limited information on its brain localization is currently available. Selective agonists and antagonists are also available for each of the histamine receptors [30]. The judicious use of these compounds for receptor classification and discovery has been reviewed extensively [19, 31, 32].

H_1 receptors are intronless GPCRs linked to Gq and calcium mobilization. The H_1 receptor protein was successfully cloned from a cDNA library of bovine adrenal medulla [33]. This protein is encoded by a single exon and contains 486 (rat), 488 (guinea pig, mouse), 491 (bovine) or 487 (human) amino acids. The homology between the several receptor proteins is quite high in some intracellular domains (≈90%), but is significantly lower in other intracellular and extracellular regions. Features common to many of the G-protein-coupled receptors are present, including seven transmembrane domains, N-terminal glycosylation sites and phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC). The third cytoplasmic loop is large, characteristic of other phospholipase-C-linked receptors (see below).

H_1-linked intracellular messengers. Activated H_1 receptors are known to activate a pertussis-toxin-insensitive G protein, G_q, that stimulates phosphoinositide-specific phospholipase C (PI-PLC), with the subsequent generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These two mediators are known to elevate intracellular Ca^{2+} concentrations and to activate PKC, respectively [34], (see also Ch. 20). Both internal and external Ca^{2+} sources are required to initiate and maintain responses. The distribution of H_1-activated IP_3 concentrations in the brain corresponds well to the distribution of H_1-binding sites in the guinea pig [5].

H_1 receptors also can stimulate the activity of phospholipase A_2 (PLA_2), with the subsequent release of arachidonate and its metabolites. In platelets, this response does not require activation of the phosphoinositide cycle and is inhibited by pertussis toxin, suggesting a second, distinct $G_{i/o}$-protein-mediated transduction mechanism. In cells transfected with the H_1 receptor, PLA_2 activation is partially inhibited by pertussis toxin, also suggesting at least two transduction systems [30, 34].

H_1 receptors in brain slices can also stimulate glycogen metabolism [5] and can positively modulate receptor-linked stimulation of cAMP synthesis. The activation of brain cAMP synthesis by histamine is a well studied phenomenon that reveals a positive interaction between histamine receptors [35]. When studied in cell-free preparations, this response shows characteristics of H_2, but not H_1, receptors. When similar experiments are performed in brain slices, however, both receptors appear to participate in the response. Subsequent work showed that H_1 receptors do not directly stimulate adenylyl cyclase but enhance the H_2 stimulation, probably through the effects of calcium and PKC activation on sensitive adenylyl cyclase isoforms (see Ch. 21).

Activation of brain H_1 receptors also stimulates cGMP synthesis [19]. Outside the brain, histamine is known to relax vascular smooth muscle by activation of endothelial H_1 receptors, thereby increasing endothelial Ca^{2+} concentrations and stimulating the synthesis and release of nitric oxide. The latter, a diffusible agent, then activates the smooth muscle guanylyl cyclase [30]. Although less is known about these mechanisms in the CNS, there is evidence that brain H_1 receptor activation can produce effects that depend on guanylyl cyclase activity [19].

H_1 receptor activation induces depolarizing responses in many brain areas, notably hypothalamus, thalamus and cerebral cortex. In vertebrate brain, many of these effects are mediated by opening cation channels. H_1-induced excitation can also occur by blockade of K_{Leak} conductances [1]. In other cases, however, H_1 receptors can attenuate neuronal excitation by activating certain voltage-gated potassium channels. Most of the H_1 receptor-induced conductance changes are mediated by the IP_3–Ca^{2+} cascade.

H_2 receptors are intronless GPCRs linked to G_s proteins and cyclic AMP synthesis. The H_2 receptor cDNA has been cloned from several species, including dog, rat, guinea pig, mouse and human [28, 30]. Use of the polymerase chain reaction (PCR) with degenerate oligonucleotides and canine gastric parietal cDNA led to the cloning of the first H_2-receptor gene [36]. These intronless genes encode for receptor proteins having 358–359 amino acids

with a molecular weight of approximately 40,000 and show the typical features of G protein-coupled receptors. Like the H_1 receptor, the H_2 protein has sites for phosphorylation by PKC and for glycosylation. Unlike the former, the latter lacks a consensus site for PKA. The H_2 receptor shows only about a 40% homology with the H_1 receptor and shows some features that are often observed for receptors known to be positively coupled to adenylyl cyclase. Features include a short third cytoplasmic loop and a long C-terminal cytoplasmic tail [28]. The human H_2 receptor gene is located on chromosome 5. When expressed in cells, the cloned H_2 receptor shows binding profiles and biochemical characteristics that closely resemble the natural receptor. Both neuronal and non-neuronal cells of brain possess H_2 receptors [5]. H_2 receptors are abundant in the cerebral cortex, corpus striatum and nucleus accumbens of the guinea pig brain (Table 14-2). In the rat, characterization of brain H_2 receptors has been hampered by low concentrations of receptor; however, H_2 receptor mRNA has been observed in this species.

H_2-linked intracellular messengers. H_2 receptors are linked to increases in cAMP but also to other intracellular signals. It is well established that H_2 receptors lead to increases in cAMP concentrations in many tissues by stimulation of adenylyl cyclase [35]. Cells transfected with H_2 receptors demonstrate activation of adenylyl cyclase, confirming that cAMP is an important H_2 messenger. Since this effect is blocked by cholera toxin, a G_s-type protein is implicated in mediating these effects. Elevated cAMP concentrations then result in the activation of PKA, leading to numerous cellular changes (see Ch. 21). The second messenger cAMP is thought to be responsible for most of the effects of H_2 receptor stimulation. However, H_2 receptors activate adenylyl cyclase in homogenates from several regions of guinea pig brain, but the density of H_2-binding sites does not correlate with the magnitude of cyclase activation across regions. These and other experiments suggest that this receptor also uses additional transduction mechanisms, including activation of phospholipase C, increased intracellular Ca^{2+}, increased IP_3 concentrations, increased phospholipid methylation and decreased arachidonate release [28, 30, 35]. Both G_s and G_q proteins have been implicated in these responses, suggesting that the H_2 receptor can activate more than one type of G protein. Similar conclusions have been reached in studies of other G-protein-linked receptors. Further studies are needed to address the importance of the various signal transduction pathways for H_2 receptor function.

H_2 receptor stimulation in mammalian cerebral cortex and hippocampus produces excitation by inhibition of Ca^{2+}-activated K^+ conductances (Table 14-2). This effect resembles that produced by activation of β-adrenergic receptors in these areas and both are mediated by increases in the cAMP-PKA pathway [1]. H_2 receptor activation also facilitates depolarization by enhancing the 'I_H' current, a cation conductance change induced by hyperpolarization [1]. Although most of H_2 receptor signaling is therefore excitatory, H_2 receptors on interneurons of hippocampus also seem to be able to dampen maximum firing rates by inhibiting Kv3 channels [37].

H_3 receptors are a family of GPCRs produced by gene splicing and are linked to $G_{i/o}$. Although the genes encoding the H_1 and H_2 receptors were known since 1991, this information did not help to identify the gene encoding the third HA receptor. This new histamine receptor was already identified by pharmacological means in 1983 by Arrang and colleagues [38] and has for many years been regarded as an interesting drug target for CNS diseases. The molecular identity of the H3 receptor remained unknown until 1999, when, in a search for orphan GPCRs, a GPCR-related expressed sequence tag was identified *in silico* and used to clone a full-length human cDNA [39]. The cDNA contained an open reading frame of 445 amino acids with an aspartate residue in TM3 (Fig. 14-4). Such a residue is highly conserved in the family of biogenic amine receptors and was the first clue that the cDNA might encode for a new histamine receptor. The H_3 receptor protein shows very low homology with the H_1 and H_2 receptor (only to 22% and 20% respectively) or other GPCRs, explaining why the H_3 receptor gene was not cloned by homology screening with H_1- or H_2-receptor-specific probes. Currently, the rat, guinea-pig, mouse and monkey cDNAs have been cloned [40].

H_3 receptor gene splicing. The H_3 receptor gene from several species contains at least 3 introns (Fig. 14-5). Consequently, various isoforms have been identified for the human, rat and guinea pig H_3 receptor as a result of alternative splicing [40]. Alternative splicing of the third intron results in H_3 receptor isoforms, which contain different deletions in the third intracellular I3 loop. The I3 loop is known to be important for the GPCR signaling and indeed significant differences have in this respect been reported for the some of the isoforms (see below). Currently, at least 20 different human H_3 receptor isoform mRNAs have been identified as a result of various alternative splicing events [40]. However, functional characteristics and detailed information on their localization and relative abundance have been reported for only a small number of the isoforms [40].

H_3-linked intracellular messengers. Although the H_3 receptor was initially identified by pharmacological means, detailed information on this receptor's transduction mechanisms was only obtained after the cloning of its cDNA. A -pertussis-toxin-sensitive inhibition of cAMP accumulation in response to H_3 agonists has been observed in a variety of transfected cells, indicating that the H_3 receptor negatively regulates adenylate cyclase activity via $G_{i/o}$ proteins (Fig. 14-6). The H_3-mediated regulation of HDC activity is also controlled via the cAMP-adenylate

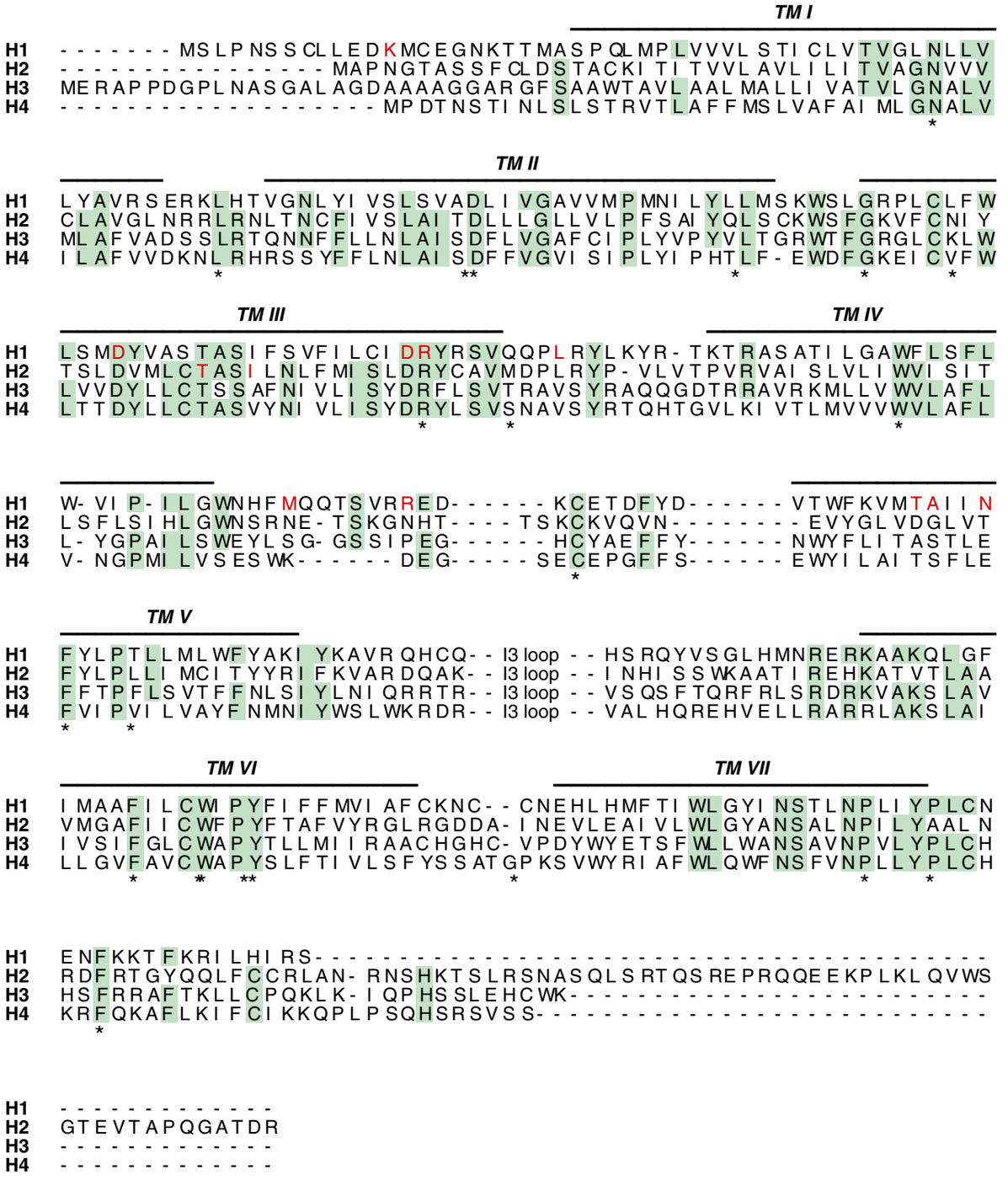

FIGURE 14-4 Amino acid sequence alignment of the four human HA receptors. The figure was produced using ClustalX [87] (see also ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). For ease of presentation, portions of the third intracellular loops (I3) have been omitted. The *green shaded areas* indicated at least 75% conservation between subtypes. The asterisks indicate amino acid conservation with the rhodopsin sequence. Residues in *red* have been mutated and studied for their functional role in receptor function (ligand binding, signaling, glycosylation, phosphorylation), as indicated in the tiny GRAP/GPCRDB database [88] (see also http://tinyGRAP.uit.no/). Above the sequences the putative transmembrane domains (*TM*) are indicated.

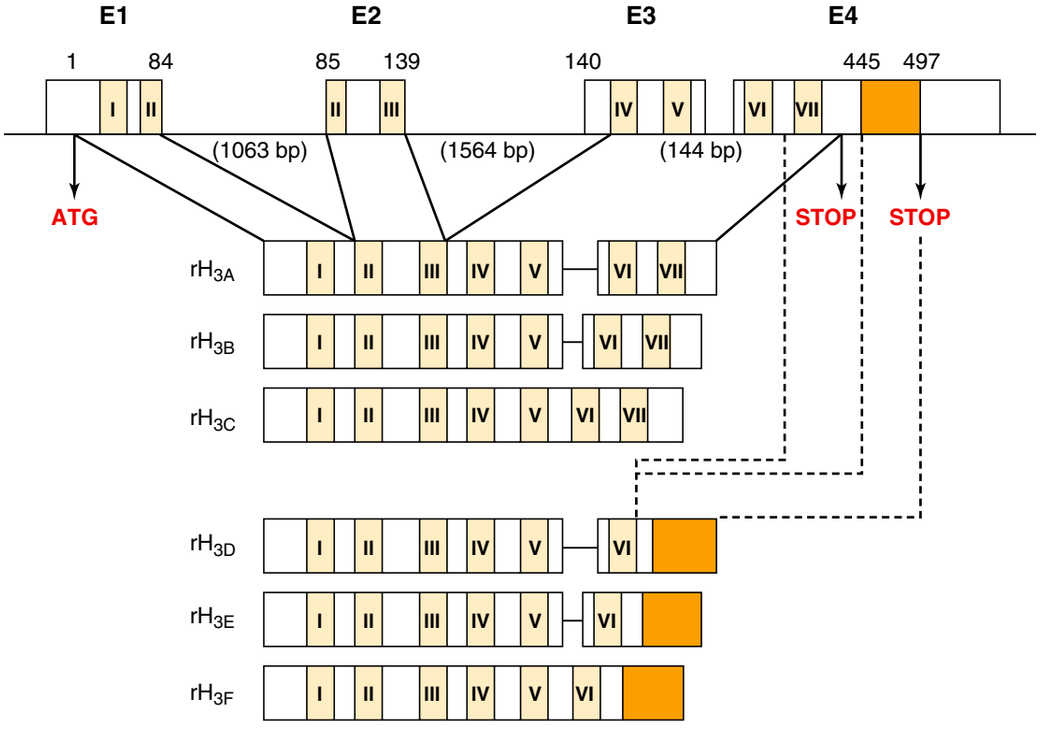

FIGURE 14-5 Isoforms of the rat H_3 receptor. The genomic structure of the receptor (**top**) shows four exons (*boxes*, E1–E4) and three introns (bp sizes are in parentheses). Peptide sequences that correspond to translated TM regions are labeled with roman numerals (I–VII). An alternatively spliced region (*orange box*) is depicted between the stop sequences. Six isoforms of the receptor (labels are on the left) result from alternative splicing. (Modified with permission from reference [2].)

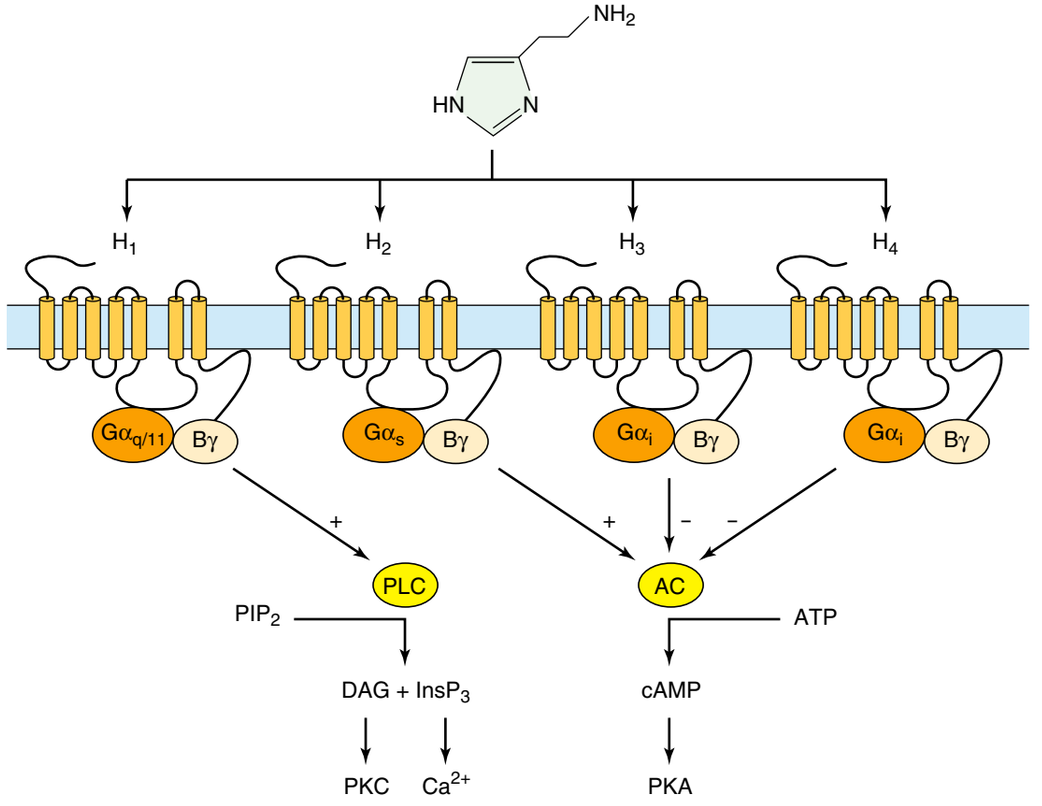

FIGURE 14-6 Main signaling pathways for histamine receptors. Histamine can couple to a variety of G-protein-linked signal transduction pathways via its four different receptors. The H_1 receptor activates the phosphatidylinositol turnover via Gq/11 proteins. The other receptors either positively (H_2 receptor) or negatively (H_3 and H_4 receptor) regulate adenylyl cyclase activity via Gs and $G_{i/o}$ protein activation respectively. Several additional signaling pathways have been described, which are not shown. Abbreviations: *PIP_2*, phosphatidylinositol 4,5-bisphosphate; *PLC*, phospholipase C; *AC*, adenylyl cyclase; *ATP*, adenosine triphosphate; *cAMP*, cyclic AMP; *PKC*, protein kinase C; *PKA*, protein kinase A.

cyclase-PKA pathway [41]. Recently, the H_3 receptor has also been reported to activate the MAPK pathway in both transfected cells and rat brain [42, 43]. Hippocampal administration of a MAPK inhibitor demonstrated that the MAPK activation in CA3 pyramidal cells is important for H_3 receptor-induced memory retention [43].

In transfected cells, H_3 receptor mediated activation of $G_{i/o}$ proteins has also been reported to modulate arachidonic acid release [29] and the Na^+/H^+ exchanger [44], and to inhibit Ca^{2+} influx and exocytosis of [^{3}H] noradrenaline from transfected SH-SY5Y-H_3 cells [45]. The inhibition of Ca^{2+} influx may be particularly relevant in view of the known physiological function of the brain H_3 receptor.

At present it is not known if all H_3 receptor isoforms similarly activate the wide array of signaling pathways. In transfected cells, the rat H_3(413) and H_3(397) isoforms both inhibit adenylate cyclase more efficiently than does the full length rat H_3 receptor isoform (445 amino acids), but the former are less efficient in activating the MAPK pathway. Much less information is available for the human H_3 receptor isoforms but shorter isoforms seem to couple more efficiently to $G_{i/o}$-proteins to inhibit cAMP production in transfected cells [40].

Constitutive H_3 receptor activity. In recent years it has become apparent that G-protein-coupled receptors can signal without the presence of the agonist, a phenomenon referred to as *constitutive activity*. Many GPCR antagonist ligands are able to reduce this agonist-independent signaling, an effect known as *inverse agonism*. All human HA receptors have now been reported to show constitutive activity [29, 46–48]. Moreover, well known therapeutics, such as cetirizine, loratadine, epinastine, cimetidine and ranitidine, act as inverse agonists *in vitro* at constitutively active H_1 or H_2 receptors.

The H_3 receptor is one of the few examples of a GPCR showing a high level of constitutive activity in a physiologically relevant system. Whereas almost all of the findings on constitutively active GPCRs have been obtained with transfected cells that express very high receptor densities, experiments performed *in vivo* have shown that the brain H_3 receptor spontaneously signals in the absence of endogenous histamine. Thus, constitutively active H_3 receptors inhibit histaminergic (and possibly other) neuronal activity and this activity can be inhibited by a structurally diverse set of inverse agonists, thereby increasing neuronal activity [29, 49]. H_3 receptor signaling (both agonist-activated and agonist-independent) occurs through Gi/o –linked inhibition of high voltage-activated calcium conductances (Table 14-2) [1].

H_4 receptors are very similar to H3 receptors in gene structure and signal transduction, but show limited expression in the brain. The H_4 receptor gene was identified in 2000 as a direct consequence of the human genome project. Use of the human H_3 receptor sequence led to the identification of a related orphan GPCR. Heterologous expression of the gene identified the orphan as a new member (H_4) of the histamine receptor family [32, 50]. The human H_4 receptor exhibits an exon/intron organization very similar to that of the H_3 receptor and the occurrence of isoforms can therefore be expected, but none so far have been reported. Within the brain, the H_4 receptor has been shown to be expressed only in the mouse hippocampus but outside the brain eosinophils, T-lymphocytes, neutrophils, mast cells, bone marrow, spleen, heart, lung and kidney show relatively high expression levels. This unique expression profile suggests that this new histamine receptor might be an interesting target for the regulation of immune function.

H_4-linked intracellular messengers. Like the H_3 receptor, the H_4 receptor couples primarily to pertussis toxin-sensitive $G_{i/o}$ proteins. In transfected cells, activation of the H_4 receptor leads to a $G_{i/o}$ protein-mediated inhibition of forskolin-stimulated adenylate cyclase activity, [^{35}S]GTPγS binding, and activation of MAP kinase. Moreover, in eosinophils and mast cells, a pertussis-toxin-sensitive calcium mobilization and chemotaxis has also been reported. The H_4 receptor can also couple to the relatively promiscuous G-proteins $G\alpha_{15}$ and $G\alpha_{16}$, leading to a stimulation of calcium mobilization. In view of the high expression of both the H_4 receptor and $G\alpha_{15}$ and $G\alpha_{16}$ proteins in cells of the immune system, this pathway could represent an important physiological second messenger system. At present no information is available on the signaling of the H_4 receptor in the CNS.

Histamine can modify ionotropic transmission at identified and unidentified sites. Although known receptors for histamine in mammals are all coupled to G proteins, two histamine-operated chloride channels (*hcl*A, *hcl*B, see [51] for discussion of nomenclature) were recently cloned from the fruit fly *D. melanogaster* [52, 53]. Both have the predicted four-transmembrane spanning regions and two cysteine bridges typical of all glycine and glutamate-operated chloride channels. These channels are most closely related in structure to human glycine receptors but also bear resemblance to GABA and glutamate ion channels. They are activated by micromolar concentrations of histamine and are blocked by high concentrations of some H_1 and H_2 antagonists. There is strong evidence that histamine activation of *hcl*A mediates the photoreceptor transmission in insect vision [51]. Although the mammalian homologs of these new histamine channels have not been found, there is substantial recent evidence for their existence in the mammalian brain [54, 55].

Histaminergic activation of ion channels may also occur through NMDA receptors [1]. This action occurs at the polyamine binding site on NR1/NR2B subunits and is sensitive to pH. Endogenous histamine may act at this site to facilitate the induction of long term potentiation, but this has not been established. Additional histamine receptors may still be discovered.

HISTAMINE ACTIONS ON THE NERVOUS SYSTEM

Histamine in the brain may act as both a neuromodulator and a classical transmitter. Histaminergic neurons appear to provide a variety of signaling mechanisms in the brain. A 'neuromodulator' role for histamine has received the most attention. Thus, activation of a small number of TM cells is thought to release histamine, which subsequently increases excitability in target cells distributed widely throughout the brain [56]. As mentioned, most of this histamine release is nonsynaptic, implying wide diffusion of the modulator. Such a system is consistent with the characteristics of known histamine receptors, which function through 'slow' transmission mechanisms requiring the production of intracellular second messengers (Table 14-2, see Ch. 10 for overview). However, as discussed above, neuronal histamine may also be capable of providing fast neurotransmission in the brain. For example, electrical stimulation of the TM cells has been shown to evoke fast excitatory postsynaptic potentials in phasically firing supraoptic neurons, effects which are mimicked by the application of histamine [54]. It was also recently shown that histamine increases chloride conductance in the thalamus by a mechanism that may be related to H_2 receptors, or to the putative mammalian histamine ion channel not yet characterized [55]. These findings imply that, like serotonin (see Ch. 13), histamine can activate both ligand-operated channels and receptors linked to second messengers.

Histaminergic neurons can regulate and be regulated by other neurotransmitter systems. A number of other transmitter systems can interact with histaminergic neurons (Table 14-1). As mentioned, the H_3 receptor is thought to function as an inhibitory heteroreceptor. Thus, activation of brain H_3 receptors decreases the release of acetylcholine, dopamine, norepinephrine, serotonin and certain peptides. However, histamine may also increase the activity of some of these systems through H_1 and/or H_2 receptors. Activation of NMDA, μ opioid, dopamine D_2 and some serotonin receptors can increase the release of neuronal histamine, whereas other transmitter receptors seem to decrease release. Different patterns of interactions may also be found in discrete brain regions.

Histamine in the nervous system may participate in a variety of brain functions. Several of the suspected physiological roles for histamine are related to its ability to increase the neuronal excitability [1, 2, 15]. For example, mutant mice lacking the H_1 receptor show defective locomotor and exploratory behaviors [57]. Neuronal histamine may increase attention and/or arousal by many mechanisms, including by enhancing sensory input [58]. All available evidence from several species shows that histaminergic neurons, when activated, increase wakefulness and induce electrographic arousal. For example, TM cell firing and neuronal histamine release is much higher during wakefulness, and pharmacological inhibition of TM activity induces sedation. H_1 receptors in the ventrolateral hypothalamus are one important site for the waking effect [59], but actions on the thalamus and cerebral cortex may also be significant. Of particular interest may be the role of the cholinergic system in histamine-activated arousal [60, 61]. The onset of sleep has been traced to cells in the ventral preoptic area of the hypothalamus, which, when activated, inhibit the histaminergic TM cells through GABAergic neurons. Thus, histamine is an important regulator of sleep–wake cycles and probably contributes to the diurnal changes in other brain functions as well. Experiments with mice lacking the HDC gene show that histaminergic neurons are essential for the waking needed to explore a novel environment [62].

Histamine also reduces seizure activity, another H_1-receptor-mediated effect. H_1 antagonists increase seizure onset and/or seizure duration in humans and animals, and H_1 receptor numbers are increased in some types of human epileptic foci [15]. H_3 antagonists increase seizure threshold in a variety of models, consistent with an elevation in neuronal histamine release. Centrally administered histamine agonists can also enhance learning and retention in laboratory animals [43, 61] but results with TM lesions suggest the opposite [63]. These apparently contradictory effects do not necessarily weaken the evidence for a histamine's role in learning but rather seem to suggest independent, opposing roles in various paradigms [64].

Histamine is a powerful regulator of many hypothalamic functions. Neuroendocrine responses, especially vasopressin release, are physiologically regulated by histaminergic neurons [54, 65]. Hypothalamic histamine may also participate in the physiological regulation of oxytocin, prolactin, adrenocorticotrophic hormone (ACTH) and β-endorphin release. Regulation of the latter two occurs by changes in release of both corticotropin-releasing hormone and vasopressin [66, 67]. Both H_1 and H_2 receptors seem to function in the histaminergic control of pituitary function.

Neuronal histamine is also an effective modulator of both food and water intake [15, 68]. Histamine and compounds that increase extracellular histamine concentrations are powerful suppressants of food intake. An action on the H_1 receptor in the ventromedial hypothalamus (VMH) seems to account for these effects [69]. Evidence that histamine contributes to the physiological control of appetite includes findings with genetically obese Zucker rats, which have very low concentrations of hypothalamic histamine. Furthermore, several studies suggest that leptin, a powerful physiological suppressant of appetite, signals through histaminergic activation of H_1 receptors. Histamine is also a powerful dipsogen (an agent that induces drinking), whether administered systemically or directly into the hypothalamus. Multiple hormonal and neuronal mechanisms may contribute to these effects [70]. Other suggested

roles for histamine in the regulation of vegetative functions include thermoregulation, regulation of glucose and lipid metabolism, and control of blood pressure [71, 72].

Histamine also induces antinociceptive (i.e. pain-relieving) responses in animals after microinjection into several brain regions [73, 74]. H_1 and H_2 mechanisms are significant and both neuronal and humoral mechanisms may be involved. Brain H_2 receptors appear to mediate some forms of endogenous analgesic responses, especially those elicited by exposure to stressors [75]. Many of the modulatory actions of histamine discussed above appear to be activated as part of stress responses. For reasons that remain unclear, histamine releasers, such as thioperamide, show only mild, biphasic antinociceptive actions, even though histamine is a potent and effective analgesic substance. Outside the brain, both H_1 and H_3 receptors exist on certain types of sensory nerves and activation of these receptors promotes and inhibits, respectively, peripheral nerve transmission related to pain and/or inflammation [76, 77].

Histamine may contribute to nervous system diseases or disorders. There are significant changes in the brain histamine system in several neurological diseases, such as multiple sclerosis, Alzheimer's disease, Down's syndrome and Wernicke's encephalopathy [1, 7, 15]. In some of these cases, there are clear changes in the number or morphology of histaminergic neurons. Whether from neurons or mast cells, histamine may participate in these processes by contributing to changes in vascular function, blood–brain barrier and/or immune activity. The ability of histamine to enhance excitatory transmission at NMDA receptors (discussed above) could explain its neurotoxic actions [7]. However, increases in neuronal histamine do not always enhance brain damage; histamine seems to exert a protective effect in some models of cerebral ischemia. Alterations in brain histamine content or dynamics may also be important for cognitive changes resulting from liver disease or histidinemia (an inborn error of histidine metabolism). In addition, histaminergic neurons are activated by vestibular disturbances, leading to the release of histamine in brainstem emetic centers. Thus, neuronal histamine may be one mediator of motion sickness [78].

Although histamine is not stored in neurons outside of the central nervous system, mast-cell-derived histamine can modify peripheral sensory nerve function. Both acute and chronic pain states can result from inflammation or peripheral nerve cell injury, and there is substantial evidence that mast cell histamine participates in these disorders.

SIGNIFICANCE OF BRAIN HISTAMINE FOR DRUG ACTION

Many clinically available drugs that modify sleep–wake cycles and appetite act through the histaminergic system. Blockade of brain H_1 receptors induces drowsiness and other signs of central nervous system depression in humans. For many years, these side effects limited the use of H_1 antagonists in allergic disorders such as hay fever. Newer, nonsedating, antihistamines (e.g. fexofenadine, loratidine) relieve peripheral allergic symptoms but have a low brain penetration, eliminating these sedative side effects. However, nearly all of the over-the-counter sleep aids are brain-penetrating H_1 antagonists (e.g. chlorpheniramine, diphenhydramine). There are also medications used to produce the opposite effect (i.e. increased wakefulness). One such drug, modafinil, was recently shown to increase the release of neuronal histamine [91].

Blocking H_1 receptors in the human brain has other important consequences. For example, brain-penetrating H_1 antagonists are effective for the treatment of motion sickness and Ménière's disease, a related disorder of the inner ear. Many other centrally acting drugs have a high affinity for H_1 receptors. Among tricyclic antidepressant drugs, those with the most pronounced sedative profile have the highest affinity for blocking H_1 receptors. H_1-receptor blockade also promotes appetite and weight gain. In fact, a recent study concluded that the weight-promoting effects of typical and atypical antipsychotic medications are directly related to the H_1-blocking properties of these drugs [79].

Drugs that modify pain perception act in part through the histaminergic system. Although pain-relieving opioid drugs such as morphine initiate many neurochemical changes, the activation of neuronal histamine release by these agents and the subsequent stimulation of brain H_2 receptors are critical for the mechanism of action of these compounds [80–82]. Stress responses also can contribute to opioid analgesia and histaminergic neurons appear to mediate the stress-induced potentiation of morphine antinociception. Although no pain-relieving drugs have been developed based on H_2 receptors, a family of novel analgesics has been discovered from drugs related to cimetidine (an H_2 antagonist) and burimamide (a drug with both H_2 and H_3 properties). Thus far, these drugs have been used only as research tools. Outside the brain, H_1 receptors on sensory nerve fibers are activated during some kinds of pain and inflammation, and H_1 antagonists are used for their anti-inflammatory and analgesic profiles [76]. By an opposing mechanism, H_3 agonists reduce pain transmission evoked by chemical and mechanical stimuli [77, 83]

Drugs that act on the H_3 receptor are being developed for the treatment of obesity, sleep disturbances, epilepsy and cognitive disorders. The ability of histamine to promote arousal, suppress appetite, elevate seizure threshold and stimulate cognitive processes implies that compounds able to enhance the release of neuronal histamine should mimic these effects. Several H_3 antagonists currently in development demonstrate such activity and show promise as effective and novel therapeutic agents [40, 84–86]. Because H_3 agonists suppress the release of

neuronal histamine, compounds that can activate brain H_3 receptors may become clinically useful sleep-promoting drugs.

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REFERENCES

1. Haas, H. and Panula, P. The role of histamine and the tuberomamillary nucleus in the nervous system. *Nat. Rev. Neurosci.* 4: 121–130, 2003.
2. Hough, L. B. and Leurs, R. Histamine receptors. In C. H. Davis and M. N. Pangalos (eds), *Understanding G Protein-Coupled Receptors and Their Role in the CNS*. Oxford: Oxford University Press, 2002.
3. Brown, R. E., Stevens, D. R. and Haas, H. L. The physiology of brain histamine. *Prog. Neurobiol.* 63: 637–672, 2001.
4. Hough, L.B., Goldschmidt, R. C., Glick, S. D. and Padawer, J. Mast cells in rat brain: characterization, localization, and histamine content. In C. R. Ganellin and J. C. Schwartz (eds), *Frontiers in Histamine Research: A Tribute to Heinz Schild*. Advances in the biosciences. New York: Pergamon Press, 1985, pp. 131–140.
5. Schwartz, J. C., Arrang, J. M., Garbarg, M., Pollard, H. and Ruat, M. Histaminergic transmission in the mammalian brain. *Physiol. Rev.* 71: 1–51, 1991.
6. Kriegsfeld, L. J., Hotchkiss, A. K., Demas, G. E., Silverman, A. J., Silver, R. and Nelson, R. J. Brain mast cells are influenced by chemosensory cues associated with estrus induction in female prairie voles (*Microtus ochrogaster*). *Horm. Behav.* 44: 377–384, 2003.
7. Langlais, P. J., McRee, R. C., Nalwalk, J. A. and Hough, L. B. Depletion of brain histamine produces regionally selective protection against thiamine deficiency-induced lesions in the rat. *Metab. Brain Dis.* 17: 199–210, 2002.
8. Rouleau, A., Dimitriadou, V., Trung Tuong, M. D. *et al.* Mast cell specific proteases in rat brain: changes in rats with experimental allergic encephalomyelitis. *J. Neural Transm.* 104: 399–417, 1997.
9. Hough, L. B. Cellular localization and possible functions for brain histamine: recent progress. In G. A. Kerkut and J. W. Phillis (eds), *Progress in Neurobiology 30*. Oxford: Pergamon Press, pp. 469–505, 1988.
10. Panula, P., Yang, Y. H.T. and Costa, E. Histamine-containing neurons in the rat hypothalamus. *Proc. Natl Acad. Sci. U.S.A.* 81: 2572–2576, 1984.
11. Watanabe, T., Taguchi, Y., Shiosaka, S. *et al.* Distribution of the histaminergic neuron system in the central nervous system of rats: a fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. *Brain Res.* 295: 13–25, 1984.
12. Inagaki, N., Yamatodani, A., Ando-Yamamoto, M., Tohyama, M., Watanabe, T. and Wada, H. Organization of histaminergic fibers in the rat brain. *J. Comp. Neurol.* 273: 283–300, 1988.
13. Panula, P., Pirvola, U., Auvinen, S. and Airaksinen, M. S. Histamine-immunoreactive nerve fibers in the rat brain. *Neuroscience* 28: 585–610, 1989.
14. Hough, L. B. and Green, J. P. Histamine and its receptors in the nervous system. In A. Lajtha (ed.), *Handbook of Neurochemistry*, 2nd edn. New York: Plenum Press, pp. 148–211, 1984.
15. Onoder, K., Yamatodani, A., Watanabe, T. and Wada, H. Neuropharmacology of the histaminergic neuron system in the brain and its relationship with behavioral disorders. *Prog. Neurobiol.* 42: 685–702, 1994.
16. Kukko-Lukjanov, T. K. and Panula, P. Subcellular distribution of histamine, GABA and galanin in tuberomamillary neurons in vitro. *J. Chem. Neuroanat.* 25: 279–292, 2003.
17. Hardie, R. C. A histamine-activated chloride channel involved in neurotransmission at a photoreceptor synapse. *Nature* 339: 704–706, 1989.
18. Melzig, J., Buchner, S., Wiebel, F. *et al.* Genetic depletion of histamine from the nervous system of *Drosophila* eliminates specific visual and mechanosensory behavior. *J. Comp. Physiol.* [A] 179: 763–773, 1996.
19. Prell, G. D. and Green, J. P. Histamine as a neuroregulator. *Annu. Rev. Neurosci.* 9: 209–254, 1986.
20. Joseph, D. R., Sullivan, P. M., Wang, Y. M. *et al.* Characterization and expression of the complementary DNA encoding rat histidine decarboxylase. *Proc. Natl Acad. Sci. U.S.A.* 87: 733–737, 1990.
21. Fleming, J. V. and Wang, T. C. The production of 53–55-kDa isoforms is not required for rat L-histidine decarboxylase activity. *J. Biol. Chem.* 278: 686–694, 2003.
22. Watanabe, T., Yamatodani, A., Maeyama, K. and Wada, H. Pharmacology of α-fluoromethylhistidine, a specific inhibitor of histidine decarboxylase. *Trends Pharmacol. Sci.* 11: 363–367, 1990.
23. Barnes, W. G. and Hough, L. B. Membrane-bound histamine *N*-methyltransferase in mouse brain: possible role in the synaptic inactivation of neuronal histamine. *J. Neurochem.* 82: 1262–1271, 2002.
24. Takemura, M., Tanaka, T., Taguchi, Y. *et al.* Histamine *N*-methyltransferase from rat kidney. *J. Biol. Chem.* 267: 15687–15691, 1992.
25. Barnes, W. G., Grinde, E., Crawford, D. R., Herrick-Davis, K. and Hough, L. B. Characterization of a new mRNA species from the human histamine *N*-methyltransferase gene. *Genomics* 83: 168–171, 2004.
26. Nishibori, M., Tahara, A., Sawada, K., Sakiyama, J., Nakaya, N. and Saeki, K. Neuronal and vascular localization of histamine *N*-methyltransferase in the bovine central nervous system. *Eur. J. Neurosci.* 12: 415–424, 2000.
27. Arrang, J. M., Garbarg, M., Lancelot, J. C. *et al.* Highly potent and selective ligands for histamine H3-receptors. *Nature* 327: 117–123, 1987.
28. Arrang, J.-M., Drutel, G., Garbarg, M., Ruat, M., Traiffort, E. and Schwartz, J.-C. Molecular and functional diversity of histamine receptor subtypes. *Ann. N.Y. Acad. Sci.* 757: 314–323, 1995.
29. Morisset, S., Rouleau, A., Ligneau, X. *et al.* High constitutive activity of native H3 receptors regulates histamine neurons in brain. *Nature* 408: 860–864, 2000.

30. Leurs, R., Smit, M. J. and Timmerman, H. Molecular pharmacological aspects of histamine receptors. *Pharmacol. Ther.* 66: 413–463, 1995.
31. Hill, S. J., Ganellin, C. R., Timmerman, H. *et al.* International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol. Rev.* 49: 253–278, 1997.
32. Hough, L. B. Genomics meets histamine receptors: new subtypes, new receptors. *Mol. Pharmacol.* 59: 1–5, 2001.
33. Yamashita, M., Fukui, H., Sugama, K. *et al.* Expression cloning of a cDNA encoding the bovine histamine H_1 receptor. *Proc. Natl Acad. Sci. U.S.A.* 88: 11515–11519, 1991.
34. Leurs, R., Traiffort, E., Arrang, J. M., Tardivel-Lacombe, J., Ruat, M. and Schwartz, J.-C. Guinea pig histamine H_1 receptor. II. Stable expression in Chinese hamster ovary cells reveals the interaction with three major signal transduction pathways. *J. Neurochem.* 62: 519–527, 1994.
35. Hill, S. J. Distribution, properties, and functional characteristics of three classes of histamine receptor. *Pharmacol. Rev.* 42: 45–83, 1990.
36. Gantz, I., Schaffer, M., DelValle, J. *et al.* Molecular cloning of a gene encoding the histamine H_2 receptor. *Proc. Natl Acad. Sci. U.S.A.* 88: 429–433, 1991.
37. Rudy, B. and McBain, C. J. Kv3 channels: voltage-gated K^+ channels designed for high-frequency repetitive firing. *Trends Neurosci.* 24: 517–526, 2001.
38. Arrang, J. M., Garbarg, M. and Schwartz, J. C. Autoinhibition of brain histamine release mediated by a novel class (H_3) of histamine receptors. *Nature* 302 832–837, 1983.
39. Lovenberg, T. W., Roland, B. L., Wilson, S. J. *et al.* Cloning and functional expression of the human histamine H_3 receptor. *Mol. Pharmacol.* 55: 1101–1107, 1999.
40. Hancock, A. A., Esbenshade, T. A., Krueger, K. M. and Yao, B. B. Genetic and pharmacological aspects of histamine H3 receptor heterogeneity. *Life Sci.* 73: 3043–3072, 2003.
41. Gomez-Ramirez, J., Ortiz, J. and Blanco, I. Presynaptic H_3 autoreceptors modulate histamine synthesis through cAMP pathway. *Mol. Pharmacol.* 61: 239–245, 2002.
42. Drutel, G., Peitsaro, N., Karlstedt, K. *et al.* Identification of rat H_3 receptor isoforms with different brain expression and signaling properties. *Mol. Pharmacol.* 59: 1–8, 2001.
43. Giovannini, M. G., Efoudebe, M., Passani, M. B. *et al.* Improvement in fear memory by histamine-elicited ERK2 activation in hippocampal CA3 cells. *J. Neurosci.* 23: 9016–9023, 2003.
44. Silver, R. B., Mackins, C. J., Smith, N. C. *et al.* Coupling of histamine H_3 receptors to neuronal Na^+/H^+ exchange: a novel protective mechanism in myocardial ischemia. *Proc. Natl Acad. Sci. U.S.A.* 98: 2855–2859, 2001.
45. Silver, R. B., Poonwasi, K. S., Seyedi, N., Wilson, S. J., Lovenberg, T. W. and Levi, R.. Decreased intracellular calcium mediates the histamine H_3-receptor-induced attenuation of norepinephrine exocytosis from cardiac sympathetic nerve endings. *Proc. Natl Acad. Sci. U.S.A.* 99: 501–506, 2002.
46. Alewijnse, A. E., Smit, M. J., Hoffmann, M., Verzijl, D., Timmerman, H. and Leurs, R. Constitutive activity and structural instability of the wild-type human H_2 receptor. *J. Neurochem.* 71: 799–807, 1998.
47. Bakker, R. A., Wieland, K., Timmerman, H. and Leurs, R. Constitutive activity of the histamine H_1 receptor reveals inverse agonism of histamine H_1 receptor antagonists. *Eur. J. Pharmacol.* 387: R5-R7, 2000.
48. Morse, K. L., Behan, J., Laz, T. M. *et al.* Cloning and characterization of a novel human histamine receptor. *J. Pharmacol. Exp. Ther.* 296: 1058–1066, 2001.
49. Wieland, K., Bongers, G., Yamamoto, Y. *et al.* Constitutive activity of histamine H_3 receptors stably expressed in SK-N-MC cells: display of agonism and inverse agonism by H_3 antagonists. *J. Pharmacol. Exp. Ther.* 299: 908–914, 2001.
50. Oda, T., Morikawa, N., Saito, Y., Masuho, Y. and Matsumoto, S. Molecular cloning and characterization of novel type of histamine receptor preferentially expressed in leukocytes. *J. Biol. Chem.* 275: 36781–36786, 2000.
51. Gengs, C., Leung, H. T., Skingsley, D. R. *et al.* The target of *Drosophila* photoreceptor synaptic transmission is a histamine-gated chloride channel encoded by ort (hclA). *J. Biol. Chem.* 277: 42113–42120, 2002.
52. Gisselmann, G., Pusch, H., Hovemann, B. T. and Hatt, H. Two cDNAs coding for histamine-gated ion channels in D. melanogaster. *Nat. Neurosci.* 5: 11–12, 2002.
53. Zheng, Y., Hirschberg, B., Yuan, J. *et al.* Identification of two novel *Drosophila* melanogaster histamine-gated chloride channel subunits expressed in the eye. *J. Biol. Chem.* 277: 2000–2005, 2002.
54. Hatton, G. I. and Yang, Q. Z. Synaptically released histamine increases dye coupling among vasopressinergic neurons of the supraoptic nucleus: mediation by H_1 receptors and cyclic nucleotides. *J. Neurosci.* 16: 123–129, 1996.
55. Lee, K. H., Broberger, C., Kim, U. and McCormick, D. A. Histamine modulates thalamocortical activity by activating a chloride conductance in ferret perigeniculate neurons. *Proc. Natl Acad. Sci. U.S.A.* 101: 6716–6721, 2004.
56. Wada, H., Inagaki, N., Yamatodani, A. and Watanabe, T. Is the histaminergic neuron system a regulatory center for whole- brain activity. *Trends Neurosci.* 14: 415–418, 1991.
57. Inoue, I., Yanai, K., Kitamura, D. *et al.* Impaired locomotor activity and exploratory behavior in mice lacking histamine H_1 receptors. *Proc. Natl Acad. Sci. U.S.A.* 93: 13316–13320, 1996.
58. Uhlrich, D. J., Manning, K. A. and Xue, J. T. Effects of activation of the histaminergic tuberomammillary nucleus on visual responses of neurons in the dorsal lateral geniculate nucleus. *J. Neurosci.* 22: 1098–1107, 2002.
59. Lin, J. S., Sakai, K., Vanni-Mercier, G. *et al.* Involvement of histaminergic neurons in arousal mechanisms demonstrated with H_3-receptor ligands in the cat. *Brain Res.* 523: 325–330, 1990.
60. Cecchi, M., Passani, M. B., Bacciottini, L., Mannaioni, P. F. and Blandina, P. Cortical acetylcholine release elicited by stimulation of histamine H1 receptors in the nucleus basalis magnocellularis: a dual-probe microdialysis study in the freely moving rat. *Eur. J. Neurosci.* 13: 68–78, 2001.
61. Passani, M. B., Bacciottini, L., Mannaioni, P. F. and Blandina, P. Central histaminergic system and cognition. *Neurosci. Biobehav. Rev.* 24: 107–113, 2000.
62. Parmentier, R., Ohtsu, H., Djebbara-Hannas, Z., Valatx, J. L., Watanabe, T. and Lin, J. S. Anatomical, physiological, and pharmacological characteristics of histidine decarboxylase knock-out mice: evidence for the role of brain histamine in behavioral and sleep-wake control. *J. Neurosci.* 22: 7695–7711, 2002.
63. Huston, J. P., Wagner, U. and Hasenöhrl, R. U. The tuberomammillary nucleus projections in the control of learning, memory and reinforcement processes: evidence for an inhibitory role. *Behav. Brain Res.* 83: 97–105, 1997.

64. Dere, E., Souza-Silva, M. A., Topic, B., Spieler, R. E., Haas, H. L. and Huston, J. P. Histidine-decarboxylase knockout mice show deficient nonreinforced episodic object memory, improved negatively reinforced water-maze performance, and increased neo- and ventro-striatal dopamine turnover. *Learn. Mem.* 10: 510–519, 2003.
65. Kjaer, A., Knigge, U., Rouleau, A., Garbarg, M. and Warberg, J. Dehydration-induced release of vasopressin involves activation of hypothalamic histaminergic neurons. *Endocrinology* 135: 675–681, 1994.
66. Knigge, U., Matzen, S., Bach, F. W., Bang, P. and Warberg, J. Involvement of histaminergic neurons in the stress-induced release of pro-opiomelanocortin-derived peptides in rats. *Acta Endocrinol. (Copenh.)* 120: 533–539, 1989.
67. Knigge, U. and Warberg, J. The role of histamine in the neuroendocrine regulation of pituitary hormone secretion. *Acta Endocrinol. (Copenh.)* 124: 609–619, 1991.
68. Sakata, T. and Yoshimatsu, H. Homeostatic maintenance regulated by hypothalamic neuronal histamine. *Methods Find. Exp. Clin. Pharmacol.* 17(Suppl. C): 51–56, 1995.
69. Ookuma, K., Sakata, T., Fukagawa, K. *et al.* Neuronal histamine in the hypothalamus suppresses food intake in rats. *Brain Res.* 628: 235–242, 1993.
70. Kraly, F. S., Keefe, M. E., Tribuzio, R. A., Kim, Y. M., Finkell, J. and Braun, C. J. H_1, H_2, and H_3 receptors contribute to drinking elicited by exogenous histamine and eating in rats. *Pharmacol. Biochem. Behav.* 53: 347–354, 1996.
71. Philippu, A. Interactions with other neuron systems. In T. Watanabe and H. Wada (eds), *Histaminergic Neurons: Morphology and Function.* Boca Raton, FL: CRC Press, pp. 323–344, 1991.
72. Sakata, T., Kurokawa, M., Oohara, A. and Yoshimatsu, H. A physiological role of brain histamine during energy deficiency. *Brain Res.* Bull 35: 135–139, 1994.
73. Thoburn, K. K., Hough, L. B., Nalwalk, J. W. and Mischler, S. A. Histamine-induced modulation of nociceptive responses. *Pain* 58: 29–37, 1994.
74. Lamberti, C., Bartolini, A., Ghelardini, C. and Malmberg-Aiello, P. Investigation into the role of histamine receptors in rodent antinociception. *Pharmacol. Biochem. Behav.* 53: 567–574, 1996.
75. Gogas, K. R. and Hough, L. B. Inhibition of naloxone–resistant antinociception by centrally-administered H_2 antagonists. *J. Pharmacol. Exp. Ther.* 248: 262–267, 1989.
76. Raffa, R. B. Antihistamines as analgesics. *J. Clin. Pharm. Ther.* 26: 81–85, 2001.
77. Cannon, K. E., Nalwalk, J. W., Stadel, R. *et al.* Activation of spinal histamine H_3 receptors inhibits mechanical nociception. *Eur. J. Pharmacol.* 470: 139–147, 2003.
78. Takeda, N., Morita, M., Hasegawa, S., Horii, A., Kubo, T. and Matsunaga, T. Neuropharmacology of motion sickness and emesis. A review. *Acta Otolaryngol. (Stockh.) Suppl.* 501: 10–15, 1993.
79. Kroeze, W. K., Hufeisen, S. J., Popadak, B. A. *et al.* H1-histamine receptor affinity predicts short-term weight gain for typical and atypical antipsychotic drugs. *Neuropsychopharmacology* 28: 519–526, 2003.
80. Gogas, K. R., Hough, L. B., Eberle, N. B. *et al.* A role for histamine and H_2 receptors in opioid antinociception. *J. Pharmacol. Exp. Ther.* 250: 476–484, 1989.
81. Eriksson, K. S., Stevens, D. R. and Haas, H. L. Opposite modulation of histaminergic neurons by nociceptin and morphine. *Neuropharmacology* 39: 2492–2498, 2000.
82. Nishibori, M., Oishi, R., Itoh, Y. and Saeki, K. Morphine-induced changes in histamine dynamics in mouse brain. *J. Neurochem.* 45: 719–724, 1985.
83. Rouleau, A., Stark, H., Schunack, W. and Schwartz, J. C. Anti-inflammatory and antinociceptive properties of BP 2–94, a histamine H-3-receptor agonist prodrug. *J. Pharmacol. Exp. Ther.* 295: 219–225, 2000.
84. Fox, G. B., Pan, J. B., Radek, R. J. *et al.* Two novel and selective nonimidazole H_3 receptor antagonists A-304121 and A-317920: II. In vivo behavioral and neurophysiological characterization. *J. Pharmacol. Exp. Ther.* 305: 897–908, 2003.
85. Peschke, B., Bak, S., Hohlweg, R. *et al.* Cinnamic amides of (S)-2-(aminomethyl)pyrrolidines are potent H_3 antagonists. *Bioorg. Med. Chem.* 12: 2603–2616, 2004.
86. Yates, S. L., Phillips, J. G., Gregory, R. *et al.* Identification and pharmacological characterization of a series of new 1H-4-substituted-imidazoyl histamine H3 receptor ligands. *J. Pharmacol. Exp. Ther.* 289: 1151–1159, 1999.
87. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876–4882, 1997.
88. Kristiansen, K., Dahl, S. G. and Edvardsen, O. A database of mutants and effects of site-directed mutagenesis experiments on G protein-coupled receptors. *Proteins* 26: 81–94, 1996.
89. Schwartz, J.C., Arrang, J. M., Garbarg, M., Gulat-Marnay, C. and Pollard, H. Modulation of histamine synthesis and release in brain via presynaptic autoreceptors and heteroreceptors. *Ann. N.Y. Acad. Sci.* 604: 40–54, 1990.
90. Schlicker, E., Malinowska, B., Kathmann, M. and Göthert, M. Modulation of neurotransmitter release *via* histamine H_3 heteroreceptors. *Fundam. Clin. Pharmacol.* 8: 128–137, 1994.
91. Ishizuka, T., Sakamoto, Y., Sakurai, T. and Yamatodani, A. Modafinil increases histamine release in the anterior hypothalamus of rats. *Neurosci. Lett.* 339: 143–146, 2003.

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CHAPTER

15

Glutamate

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THE AMINO ACID GLUTAMATE IS THE MAJOR EXCITATORY NEUROTRANSMITTER IN THE BRAIN

Glutamate mediates most of the fast excitatory neurotransmission in the CNS, and it excites virtually every neuron. Glutamate is the principal mediator of sensory information, motor coordination, emotions and cognition, including memory formation and memory retrieval. As many as 90% of the neurons of the brain use glutamate as their neurotransmitter, and approximately 80–90% of the synapses in the brain are glutamatergic [1]. Repolarization of membranes that are depolarized during glutamatergic activity may account for as much as 80% of the energy expenditure of the brain [2]. The very high consumption of glucose and oxygen by the brain (see Ch. 31) therefore largely fuels glutamatergic activity. The concentration of glutamate in brain gray matter structures varies between 10 and 15 µmol per gram of tissue, higher than in virtually all other tissues of the body. In white matter the glutamate concentration is 4–6 µmol/g. Glutamate takes part in many reactions in the brain (Fig. 15-1). Formation of glutamate is a step in the metabolism of glucose and amino acids; glutamate is a precursor for γ-aminobutyric acid (GABA) in GABAergic neurons and for glutamine in glial cells; it is a constituent of proteins and peptides, e.g. glutathione (γ-glutamyl-cysteinyl-glycine), which is a major defense against oxidative stress in cells. Therefore, all cells of the brain, neuronal and glial, contain glutamate, which is found in both the cytosol and mitochondria, in both the cell bodies and their processes. In glutamatergic neurons, glutamate is concentrated in synaptic vesicles in which compartment it represents 'transmitter glutamate'.

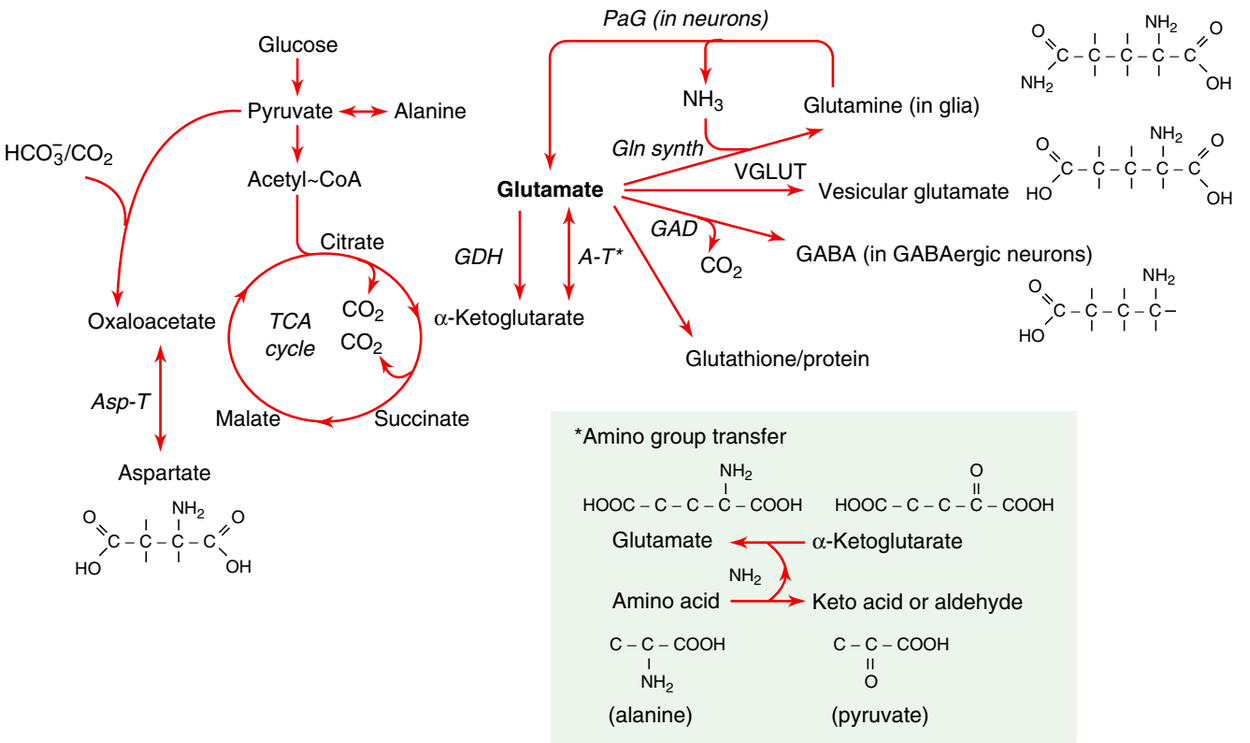


FIGURE 15-1 The formation of glutamate from α-ketoglutarate, a TCA cycle intermediate. α-Ketoglutarate, which is formed from glucose, constitutes the carbon backbone of glutamate. The amino group derives from another amino acid, which after donation of its amino group becomes a keto acid or an aldehyde (insert). This amino donor may be aspartate, GABA, alanine (shown in the insert) or some other amino acid. Glutamate takes part in many biochemical reactions: in glial cells it is converted to glutamine, in GABAergic neurons it is converted into GABA. Transmitter glutamate is defined by its accumulation into synaptic vesicles. In the **upper right corner** the glutamine cycle is shown, with conversion of glutamate into glutamine in glia and conversion of glutamine into glutamate in neurons. To **upper left** is shown pyruvate carboxylation (anaplerosis), which replenishes the TCA cycle (glial or neuronal) with malate or oxaloacetate to compensate for the loss of glutamate, glutamine, or (in GABAergic neurons) GABA. *Asp-T*, aspartate aminotransferase; *A-T*, aminotransferase reaction (Asp-T or other); *GABA*, γ-aminobutyric acid; *GAD*, glutamic acid decarboxylase; *GDH*, glutamate dehydrogenase; *Gln synth*, glutamine synthetase; *PaG*, phosphate-activated glutaminase; *TCA* cycle, tricarboxylic acid cycle; *VGLUT*, vesicular glutamate transporter. A more comprehensive scheme of glutamate metabolism can be found at http://www.genome.ad.jp/kegg/pathway/map/map00251.html.

BRAIN GLUTAMATE IS DERIVED FROM BLOOD-BORNE GLUCOSE AND AMINO ACIDS THAT CROSS THE BLOOD–BRAIN BARRIER

From a metabolic point of view, glutamate may be thought of as a carbon backbone (with hydrogens and hydroxyl groups attached) on the one hand and an amino group on the other. The carbon backbone derives from serum glucose, which, through glycolysis and the tricarboxylic acid (TCA) cycle (see Ch. 31), is converted to α-ketoglutarate. α-Ketoglutarate then receives an amino group from another amino acid through transamination and becomes converted into glutamate (Fig. 15-1). Glutamate is in equilibrium with – and continuously reconverted to – α-ketoglutarate and metabolized through the TCA cycle. The cerebral metabolic rate for glucose in human brain is approximately 0.4 μmol/min/g tissue, and the turnover of glutamate is approximately 0.8 μmol/min/g tissue [3]. This means that virtually all the glucose that enters the brain is metabolized through glutamate, since one molecule of glucose gives rise to two molecules of acetyl-CoA, which enter the TCA cycle to become α-ketoglutarate and hence glutamate (Fig. 15-1). In the rat brain the turnover of glutamate is approximately twice that of the human brain.

The amino group of glutamate derives from serum amino acids that cross the blood–brain barrier. Current evidence points to blood-borne branched-chain amino acids (leucine, isoleucine and valine) as important amino donors for glutamate synthesis. Because the uptake of amino acids into the brain is much less than the uptake of glucose, the amino group of glutamate must be recycled in the brain when the carbon backbone is broken down to CO_2 and water. Aspartate is one important amino group reservoir for glutamate synthesis. The highly active aspartate aminotransferase shuttles the amino group between α-ketoglutarate and another TCA cycle intermediate, oxaloacetate. These two intermediates are converted into glutamate and aspartate respectively when they accept an amino group. Other amino acids, such as alanine and GABA (in GABAergic neurons) are also important amino group reservoirs (Fig. 15-1). Theoretically, glutamate could be formed by reversal of the glutamate dehydrogenase (GDH) reaction: Glutamate + NAD(P) → α-ketoglutarate + NH_3 + NAD(P)H + H^+. This is unlikely, however, since the K_m for NH_3 and α-ketoglutarate in the reverse reaction, >12 mmol/l and 1 mmol/l, respectively, are far above normal brain concentrations (<300 μmol/l and 150 μmol/l, respectively). The specific role of glutamate dehydrogenase in the brain is not clear but it is an important enzyme in protein degradation: The metabolism of protein-derived amino acids involves transamination of the various amino acids into ketoacids; α-ketoglutarate accepts the NH_2 group in these transaminations and becomes converted into glutamate. Glutamate dehydrogenase removes the NH_2 group from glutamate as free NH_3 and shunts glutamate back into the TCA cycle as α-ketoglutarate.

GLUTAMINE IS AN IMPORTANT IMMEDIATE PRECURSOR FOR GLUTAMATE: THE GLUTAMINE CYCLE

Much of the glutamate that is released from nerve terminals is taken up from the extracellular fluid into astrocytic processes that surround synapses (Fig. 15-2). The mechanism of uptake is described later in this chapter. In astrocytes glutamate reacts with ammonia to form glutamine through the activity of glutamine synthetase, a cytosolic, ATP-dependent enzyme that astrocytes and oligodendrocytes express but neurons do not. This reaction is important even for the detoxification of free ammonia; accumulation of ammonia would interfere with synaptic function (see Ch. 34). Glutamine, which does not have neurotransmitter properties, is exported to the extracellular fluid and taken up by neurons (see below). In neurons glutamine is converted back to glutamate by phosphate-activated glutaminase, a mitochondrial and possibly neuron-specific enzyme in the brain. The trafficking of glutamate and glutamine between neurons and astrocytes is called the 'the glutamine cycle'. This cycle seems to be very active, its flux has been estimated at approximately 40% of the glutamate turnover rate [4], meaning that almost half the glutamate formed in neurons is transferred to glial cells. Whether all the neuronal glutamate taken up by glia is vesicular in origin is not known; reversal of glutamate transporters (see below) or exchange of intracellular glutamate for extracellular cystine may both be important mechanisms by which glutamate can leave neurons [5].

Several findings support the concept that glutamine is an important precursor for transmitter glutamate. When brain slices are incubated with radiolabeled glutamine, radiolabeled glutamate is formed that is releasable through a calcium-ion-dependent mechanism. The calcium dependence identifies the released glutamate as vesicular in origin (see Ch. 10). A way to study the fate of glutamine in the intact brain is to use isotopically labeled acetate to label glutamine. Carbon-13-labeled acetate does not enter neurons but it readily enters astrocytes, where it becomes metabolized to glutamine. After intravenous injection of ^{13}C-labeled acetate, magnetic resonance spectroscopy of the brain is consistent with the formation of ^{13}C-glutamine in astrocytes and the formation of ^{13}C-glutamate from that glutamine in neurons (magnetic resonance spectroscopy is discussed in Ch. 58).

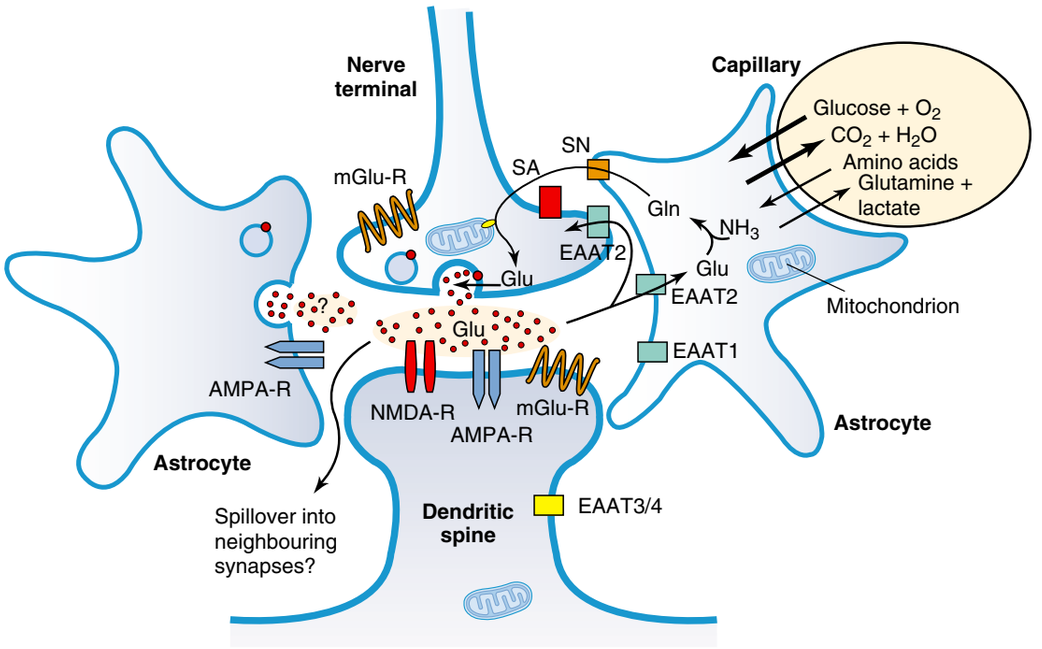


FIGURE 15-2 A glutamatergic, axodendritic synapse consisting of a presynaptic nerve terminal and a postsynaptic dendritic spine. The nerve terminal contains synaptic vesicles with glutamate transporters (*red dot*), mitochondria (*blue*) with glutaminase in nerve terminal (*yellow dot*), metabotropic glutamate receptors, and transporters for glutamate (EAAT2) and glutamine (SA transporters). The postsynaptic dendritic spine contains glutamate receptors, both ionotropic (AMPA and NMDA type) and metabotropic, and glutamate transporters (EAAT3 and EAAT4, the latter in cerebellar Purkinje cells). Surrounding the synapse are astrocytic processes with glutamate (EAAT1 and EAAT2) and glutamine transporters (SN transporters), glutamate receptors and even glutamate-filled vesicles. Green rectangles in plasma membrane of the axon terminal represent EAAT2 and of the astrocyte represent EAAT1/EAAT2 glu transporters. Glutamate that escapes out of the synapse without being cleared by transporters may spill over into neighboring synapses. *AMPA-R*, AMPA receptors; *EAAT1–4*, excitatory amino acid transporters 1–4; *Gln*, glutamine; *Glu*, glutamate; *mGlu-R*, metabotropic glutamate receptors; *SA* and *SN*, system A and system N transporters respectively (for glutamine).

Release of glutamate from nerve endings leads to loss of α-ketoglutarate from the tricarboxylic acid cycle. The glutamine cycle counteracts an important metabolic challenge inherent in glutamatergic neurotransmission: it helps prevent loss of α-ketoglutarate from the neuronal TCA cycle. When neurons release glutamate and astrocytes take it up, the neuronal TCA cycle loses α-ketoglutarate. If this loss of α-ketoglutarate from the TCA cycle is not countered, the downstream product oxaloacetate will not be available for the formation of citrate (Fig. 15-1; see Ch. 31), and the TCA cycle will eventually grind to a halt, with the consequence that energy metabolism stops. The return of glutamine for transmitter glutamate reduces the loss of neuronal α-ketoglutarate but probably does not prevent it completely, partly because astrocytes metabolize some transmitter glutamate through their own TCA cycle as an energy substrate. Two additional mechanisms exist that maintain the level of glutamate and TCA cycle intermediates in the nerve terminal: one is reuptake of glutamate from the extracellular fluid back into the nerve terminal, the other is the reaction of pyruvate (derived from glycolysis) with CO_2 to form TCA cycle intermediate malate, the precursor of oxaloacetate. This process is called pyruvate carboxylation. Pyruvate carboxylation in the nerve terminal may be catalyzed by malic enzyme, a reversible enzyme that utilizes CO_2 and NADPH to form malate from pyruvate [4]. Pyruvate carboxylation also takes place in astrocytes where it may be catalyzed by pyruvate carboxylase, an ATP-dependent, unidirectional enzyme that utilizes bicarbonate for the carboxylation of pyruvate to oxaloacetate. This reaction may support a net production and export of glutamine from astrocytes [4].

Because the carbon backbone of glutamate derives from serum glucose, the formation and further metabolism of glutamate (e.g. to glutamine or GABA) may be followed in the awake human brain or in animals with magnetic resonance spectroscopy after intravenous injection of ^{13}C-labeled glucose [3] (see MRS in Ch. 58). Glutamate and GABA become labeled with ^{13}C within a few minutes after injection of ^{13}C-labeled glucose. The labeling of glutamine lags somewhat behind that of glutamate, probably reflecting the time involved in release of glutamate from nerve endings, uptake by astrocytes, and conversion to glutamine (Fig. 15-2).

SYNAPTIC VESICLES ACCUMULATE TRANSMITTER GLUTAMATE BY VESICULAR GLUTAMATE TRANSPORTERS

One defining characteristic of transmitter glutamate is its accumulation in synaptic vesicles. The concentration of

glutamate in synaptic vesicles has been estimated at 60–250 mmol/l. The concentration of glutamate in the cytosol is only a few mmol/l, so glutamate is concentrated inside the vesicles. The inner radius of a glutamate-containing vesicle is on average about 17 nm, which gives a volume of 2×10^{-20} liters; a glutamate concentration of 100 mmol/l (or 6×10^{22} molecules/l) would therefore yield 1200 molecules of glutamate in each vesicle. Specialized proteins in the vesicular membrane, the vesicular glutamate transporters (VGLUTs), cause the accumulation of glutamate in synaptic vesicles. VGLUTs are multimeric protein complexes that are proton/glutamate antiporters (see Ch. 5). Vacuolar (V-type) H^+-dependent ATPase establishes a proton-based electric gradient across the vesicular membrane at the expense of ATP: protons accumulate in the lumen of the vesicle, which becomes positive relative to the cytosol. This gradient is the main driving force for the accumulation of glutamate, but the low pH of the vesicular lumen also contributes. However, if we consider the small volume of a vesicle, a vesicular pH in excess of 5 (a measured value) would imply less than 0.1 proton per vesicle. This probably means that a free proton is present in the vesicle less than 10% of the time, and that this is enough to attract glutamate. The VGLUTs have a K_m for glutamate of 1–2 mmol/l, are stimulated by chloride at a concentration of 4–10 mmol/l (which is the normal cytosolic concentration) and are sodium-independent. Three VGLUTs have been cloned. VGLUT 1 and 2 are localized to different populations of glutamatergic neurons. VGLUT3, on the other hand, is found in GABAergic, cholinergic and monoaminergic neurons, raising the possibility of novel roles for transmitter glutamate [6]. Even some astrocytes have glutamate-containing vesicles with VGLUT 1 and 2, and astrocytes have been shown to release glutamate in a calcium-ion-dependent manner that involves the participation of SNARE proteins (see Ch. 10) (Fig. 15-2).

The fraction of glutamate contained in vesicles in gray matter may be estimated from the density of glutamatergic synapses ($\approx1/\mu m^3$ or $1/10^{-15}$ liters), the number of vesicles per terminal (100–500), the volume of each vesicle ($\approx2\times10^{-20}$ liters) and the concentration of glutamate in the vesicles (60–250 mmol/l). With a total concentration of glutamate of 12 mmol/kg tissue (or 12 mmol/l), the above values mean that vesicular glutamate may account for 1–20% of the total level of glutamate, depending on the number of vesicles per synapse and the intravesicular concentration of glutamate. This value may seem small considering the high level of glutamate in the brain, but the high level of glutamate is mainly caused by the high metabolic activity of the brain rather than by vesicular glutamate. The higher the metabolic flux, the higher the level of metabolic intermediates, among them glutamate.

Zinc is present together with glutamate in some glutamatergic vesicles. Zinc colocalizes with glutamate in subpopulations of synaptic vesicles throughout the brain. Approximately 10% of the synaptic vesicles of glutamatergic terminals in the hippocampus contain a substantial amount of zinc, which is released together with glutamate. Vesicles accumulate zinc through the ZnT3 zinc transporter, which is located in the vesicular membrane. The importance of this colocalization lies in zinc's ability to modulate the activation of glutamate receptors, as described below.

IS ASPARTATE A NEUROTRANSMITTER?

Aspartate, like glutamate, can be released from brain slices in a calcium-dependent manner by depolarizing stimuli, and intriguingly the ratio of released aspartate to glutamate is not fixed. Aspartate, which is closely related to glutamate and which has excitatory properties, does not appear to be concentrated in synaptic vesicles and for this reason may not be a transmitter in the classic sense. Aspartate might, however, be released directly from the cytosol of nerve endings. Aspartate exclusively activates NMDA receptors, having no effect (agonist or antagonist) at AMPA receptors [7]. The role of aspartate in excitatory neurotransmission remains to be determined.

LONG-TERM POTENTIATION OR DEPRESSION OF GLUTAMATERGIC SYNAPSES MAY UNDERLIE LEARNING

Learning is a crucial aspect of our behavior. This is true for the motor, sensory, emotional and cognitive components of behavior (see also Ch. 53). Complex learning processes lie behind our ability to move our body in a purposeful manner, our ability to discern and understand the sensory information that we receive, the perception of stimuli as harmful or good, as well as memory formation, language acquisition and thought processes. Learning must have an anatomical substrate: the CNS must undergo some form of change that allows the storage of what is to be learnt. Our current understanding is that the changes that underlie such information storage occur at the synaptic level through processes collectively referred to as 'synaptic plasticity'. There is no general agreement as to whether these changes occur predominantly on the presynaptic or on the postsynaptic side of the synaptic cleft, but several molecular mechanisms that could be important for learning have been identified on both sides. Electrophysiologically, two phenomena can be seen that may be components of learning at the synaptic level. These are long-term potentiation (LTP) and long-term depression (LTD) of synaptic efficacy. A glutamatergic synapse that is briefly but strongly activated by high-frequency stimulation (e.g. 100 Hz) may relay impulses more efficiently thereafter and will do so for a long time,

for months or longer. This phenomenon, which was first described by Lømo in 1966, is termed LTP; the synapse has been 'potentiated' or strengthened (see also Hebb's postulate of learning, Ch. 53). However, prolonged low-frequency stimulation (1 Hz) of a glutamatergic synapse may cause it to relay impulses less efficiently on a long-term basis, a phenomenon termed LTD, reflecting that the synapse has been 'weakened'. Presynaptic mechanisms that may contribute to LTP include a greater success rate for the release of glutamate when an action potential arrives at the terminal and release of greater amounts of glutamate per action potential [8]. Postsynaptic mechanisms that mediate LTP and LTD are related to activation and trafficking of glutamate receptors, which is described below.

THE NEURONAL PATHWAYS OF THE HIPPOCAMPUS ARE ESSENTIAL STRUCTURES FOR MEMORY FORMATION

LTP has been shown in many parts of the brain but it has been most extensively studied in the hippocampus, a phylogenetically old part of the cerebral cortex that in humans is embedded in the temporal horn and in rats and rabbits lies beneath the parietal and temporal neocortex (Fig. 15-3A). The hippocampus is essential for (declarative) memory formation; in rats the role of hippocampus in acquisition of spatial information has been studied in

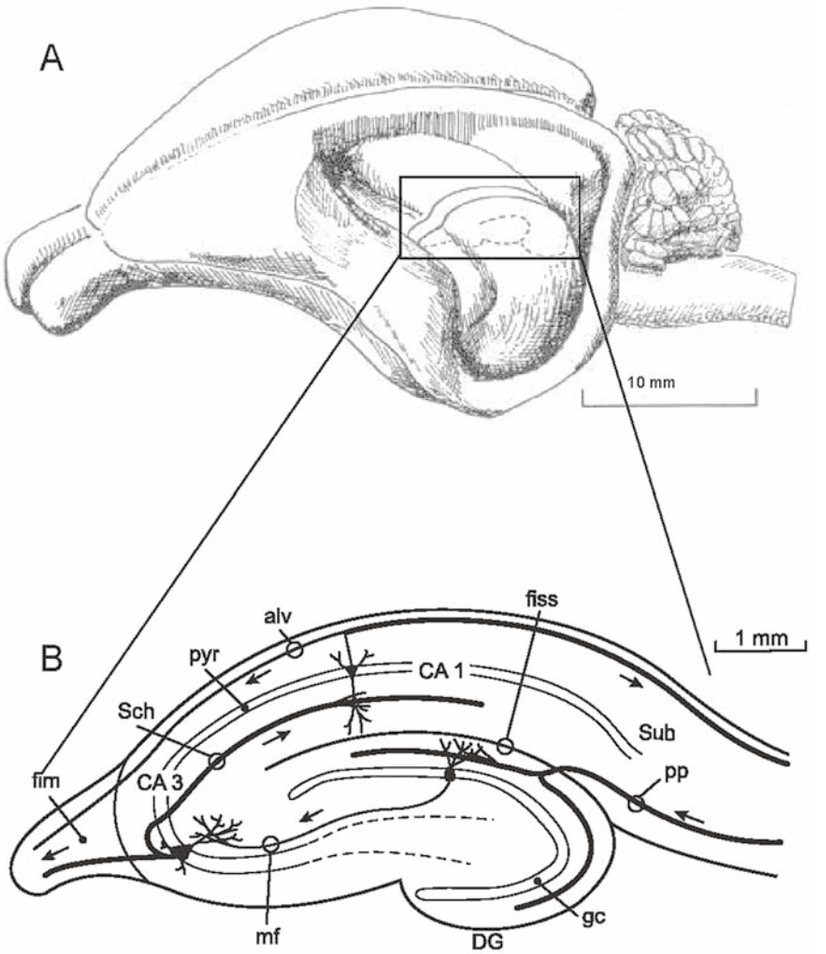


FIGURE 15-3 Hippocampus. (**A**) Its position beneath the parietal cortex in the rabbit brain (With permission from Andersen, P., Bliss, T. and Skrede, K. *Exp. Brain Res.* 13: 222–238, 1971; copyright Springer Verlag). (**B**) The main pathways of the hippocampus as seen in a hippocampal slice (With permission from Skrede, K. and Westgaard, R. H. *Brain Res.* 35: 589–593, 1971.). The main input to the hippocampus is the perforant path (*pp*), which originates in the entorhinal cortex and traverses the hippocampal fissure (*fiss*) to the granule cell layer (*gc*) of the dentate gyrus (*DG*). The perforant path axons traverse the middle part of the dendritic trees of the granule cells, making synapses with several granule cells as they pass through (synapses '*en passage*'). The axons of the granule cells make up the mossy fibres (*mf*) that make large synapses with pyramidal cells (*pyr*) of the CA3 region, also of the *en passage* type. The axons of the CA3 pyramidal cells have two branches: one exits the hippocampus and enters fimbria (*fim*) to reach septum; the other branch, the Schaffer collateral (*Sch*), makes synapses *en passage* with CA1 pyramidal cells. The CA1 pyramidal cells exit the hippocampus as the alveus and project mainly to the subiculum (*Sub*) and more sparsely through fimbria to the septum.

great detail. In the hippocampus the neuronal connections are highly ordered, so it is easy to identify specific populations of neurons and synapses. One may cut the hippocampus into transverse slices (Fig 15-3B) in which many of the neuronal connections are maintained. Such slices are excellent for electrophysiological recordings, a technique pioneered by Skrede and colleagues in the early 1970s. The anatomy of the hippocampus was described around 1900 by Santiago Ramón y Cajal. Axons from neurons in the entorhinal cortex enter the dentate gyrus as the perforant path (so called because it traverses the hippocampal fissure and 'perforates' the dentate gyrus). These axons form glutamatergic synapses with granule cells of the dentate gyrus. Axons of granule cells, the mossy fibers, form glutamatergic synapses with pyramidal cells of the CA3 region (CA stands for cornu ammonis–Ammon's horn). These neurons send axons to the septum through fimbria as well as collaterals (the Schaffer collaterals) to the CA1 region where they form glutamatergic synapses with pyramidal cells. In addition to this multisynaptic pathway from the entorhinal cortex to the CA1 region, there is a direct pathway from neurons in layer III of the entorhinal cortex to pyramidal neurons of the CA1 region. The axons of the CA1 pyramidal neurons exit the hippocampus as the alveus and project mainly to the subiculum, but some project to the septum through fimbria (Fig 15-3B). LTP can be induced at all these glutamatergic synapses.

IONOTROPIC AND METABOTROPIC GLUTAMATE RECEPTORS ARE PRINCIPAL PROTEINS AT THE POSTSYNAPTIC DENSITY

The classical glutamatergic synapse is a point of communication between a presynaptic nerve terminal and a postsynaptic dendritic spine (axo-dendritic synapses) or another nerve terminal (axo-axonal synapses). However, even astrocytes, oligodendrocytes and microglia express some types of glutamate receptor that may be stimulated by glutamate released from nerve terminals. Whereas glutamatergic terminals typically contact dendritic spines, GABAergic receptors are often located directly on dendritic shafts or on the soma. Glutamatergic synapses are easily recognized at high magnification as 'asymmetric': the postsynaptic membrane appears thicker than the presynaptic membrane (Fig. 15-4A). This postsynaptic density, or PSD, is on average 50 nm thick, and may contain as many as 100 different proteins, among them the glutamate receptors (Fig. 15-4B).

Glutamate receptors belong to one of two main categories: Ionotropic receptors are cation channels whose opening is enhanced when glutamate binds to the receptor. Metabotropic receptors do not conduct ion fluxes; instead they activate intracellular enzymes through G proteins when they bind glutamate (G proteins are discussed in Ch. 19).

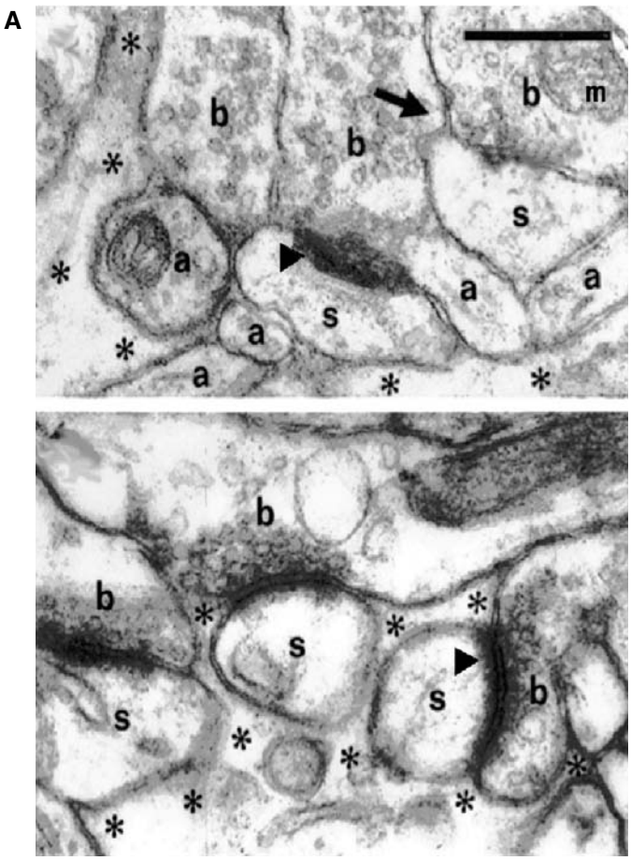
THREE CLASSES OF IONOTROPIC GLUTAMATE RECEPTORS ARE IDENTIFIED

For ionotropic glutamate receptors, the agonist binding sites and associated ion channel are incorporated into the same macromolecular complex. Agonist binding forces a conformational change in the receptor that increases the probability of channel opening. The three classes of ionotropic receptor were originally named after reasonably selective agonists – *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA). The affinity for glutamate is different for the different glutamate receptors. The EC50 for glutamate at NMDA receptors is approximately 1 μmol/l while at AMPA receptors it is approximately 400 μmol/l [7]. Several endogenous molecules may activate NMDA receptors, some of them are, just like glutamate itself, acidic amino acids, e.g. aspartate and homocysteate.

Six functional families of ionotropic glutamate receptor subunit can be defined by structural homologies. Since the cloning of the first glutamate receptor, GluR1, in 1989 [9], more than 16 mammalian genes that encode structurally related proteins have been identified. Currently, six families of ionotropic glutamate receptor subunit have been described. The subunits assemble into functional receptors shown in Figure 15-5. Within a given family, members show at least 80% identity at the amino acid level over the ≈400 amino acid stretch of membrane-spanning regions. Between families, however, a lower degree of identity exists (55% or less).

The ligand-gated ion channel receptors appear to be tetrameric assemblies of the individual subunits. A significant feature of the glutamate receptors is that different subunit combinations produce functionally different receptors as described below. *In situ* hybridization and immunohistochemistry have highlighted regional differences in expression of subunits encoding glutamate receptors. These differences illustrate the heterogeneity of glutamate receptors throughout the CNS. Together with differences in electrophysiological properties of different subunit combinations expressed in cell lines or oocytes, diverse patterns of subunit expression throughout the CNS support the existence of multiple subtypes of AMPA, kainate and NMDA receptors.

AMPA and kainate receptors are both blocked by quinoxalinediones but have different desensitization pharmacologies. AMPA receptors are widespread throughout the CNS; they serve as receptors for fast excitatory synaptic transmission mediated by glutamate. AMPA receptor subunits are GluR1-GluR4, kainate receptor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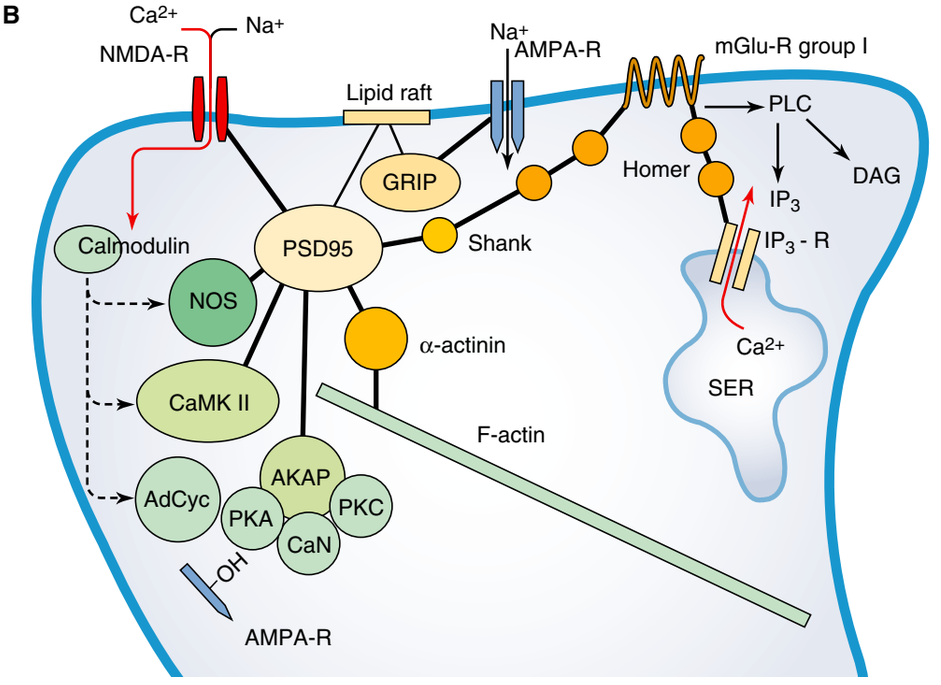
B
Ca2+
Na+
NMDA-R
Lipid raft
Na+
AMPA-R
mGlu-R group I
PLC
DAG
Homer
IP3
IP3 - R
GRIP
Calmodulin
PSD95
Shank
NOS
α-actinin
Ca2+
SER
CaMK II
F-actin
AKAP
AdCyc
PKA
PKC
CaN
OH
AMPA-R

FIGURE 15-4 Organization of the postsynaptic membrane. (**A**) Electron micrographs of glutamatergic axodendritic synapses in hippocampus CA1 (**upper panel**; stratum radiatum) and cerebellum (**lower panel**; molecular layer). The terminals (or boutons: *b*) are recognizable by their vesicles, and the postsynaptic spines (*s*) have the characteristic postsynaptic density (*arrowhead*). Note that glial processes (*) surround synapses and axons (*a*), but that one of the terminals (*arrow*) is in close contact with a neighboring synaptic cleft, which could allow for spillover of glutamate from one synapse to another. *m:* a mitochondrion in a nerve terminal. Scale bar, 400 nm. (With permission from Lehre, K. P. and Danbolt, N. C. *J. Neurosci.* 18: 8751–8757, 1998; copyright Society for Neuroscience). (**B**) Schematic representation of a dendritic spine of a glutamatergic synapse with some proteins of the postsynaptic density (*PSD*). Lipid rafts, scaffolding proteins (such as PSD95, GRIP, shank and homer) and cytoskeletal proteins (F-actin) help to concentrate and stabilize effector proteins at the spine. Effector proteins are glutamate receptors (AMPA-R, NMDA-R, mGlu-R), protein kinases and protein phosphatases, enzymes for the production of second messengers (nitric oxide, cAMP). *AdCyc,* adenylate cyclase; AKAP, (protein kinase) A-kinase anchoring protein; AMPA-R, AMPA receptor; CaMK II, Ca^{2+}/calmodulin-dependent kinase II; *CaN,* calcineurin (protein phosphatase 2B); *DAG,* diacylglycerol; *F-actin,* filamentous actin; *GRIP,* glutamate receptor-interacting protein; IP_3, inositol-1,4,5-triphosphate; *mGluR,* metabotropic glutamate receptor; *NMDA-R,* NMDA-receptor; *NOS,* nitric oxide synthase; *PKA* and *PKC,* protein kinase A and C respectively (activated by cAMP and Ca^{2+}/DAG respectively); *PLC,* phospholipase C; *PSD95,* postsynaptic density protein with a molecular weight of 95 kDa; *SER,* smooth endoplasmatic reticulum.

subunits are GluR5-GluR7 plus KA1 and KA2. GluRs are approximately 900 amino acids long, compared to about 480 amino acids for nicotinic, $GABA_A$ or glycine receptor subunits. The extra length of AMPA receptors is due to an unusually large N-terminal extracellular domain. GluR1–GluR4 subunits (also named GluRA–GluRD or, in mouse α1–α4) co-assemble with one another to form tetramers with the pharmacologic profile of AMPA receptors (Fig. 15-6). Thus, when *Xenopus* oocytes are injected with mixtures of GluR1–GluR4 mRNAs, receptors are formed that can be blocked specifically by certain quinoxalinediones, notably 6-nitro 7-sulphamobenzo[f] quinoxaline-2,3-dione (NBQX). NBQX is a potent and selective competitive antagonist of AMPA receptors but has a weak or no effect on other receptors.

Subunits of the GluR5–GluR7 family, on the other hand, coassemble with KA1 or KA2 into functional kainate receptors when studied in heterologous expression systems. Results of experiments with radioligands demonstrate that homomeric GluR5 and GluR7 receptors expressed in mammalian cell lines bind [^{3}H]kainate with low affinity (K_d 80–100 nmol/l). Such homomeric receptors may correspond to the 'low-affinity' kainate binding sites identified earlier in studies of plasma membranes from brain. Homomeric GluR6, KA1 and KA2 receptors, on the other hand, bind kainate with an affinity between 4 and 15 nmol/l respectively and may contribute to the 'high-affinity' kainate binding sites in brain. KA1 and KA2 are virtually inactive as ion channels when expressed alone and might therefore be thought of as modulatory subunits. Although none of the homomeric receptors is sensitive to AMPA, heteromeric complexes of GluR6 and KA1 do respond to AMPA with a desensitizing current.

AMPA receptors desensitize within milliseconds upon exposure to AMPA, and kainate receptors likewise upon exposure to kainate. AMPA and kainate receptors can be securely distinguished from one another by their response to two drugs, cyclothiazide and the lectin concanavalin A [10]. Cyclothiazide relieves AMPA receptor desensitization without affecting kainate receptors.

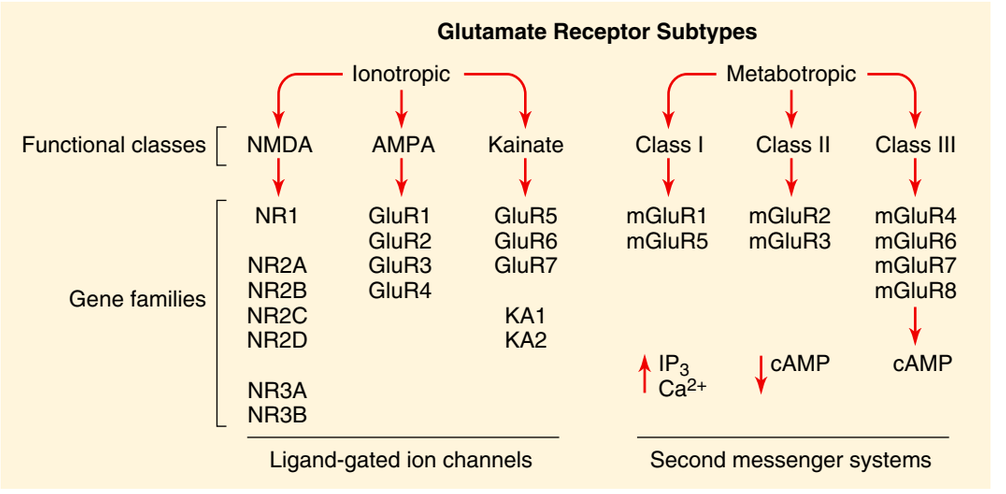

FIGURE 15-5 Molecular families of glutamate receptors. Each of the two main glutamate receptor divisions comprises three functionally defined groups (classes) of receptor. These are made up of numerous individual subunits, each encoded by a different gene.

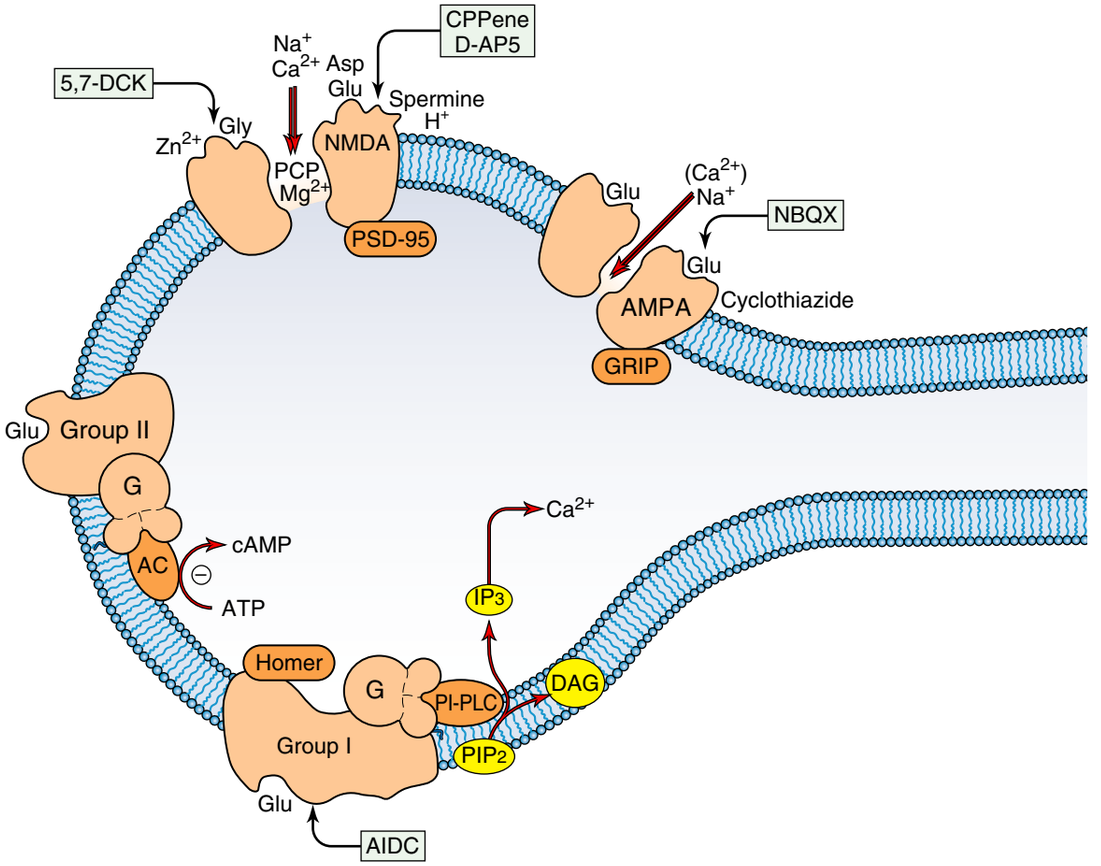

FIGURE 15-6 Schematic views of four types of glutamate receptor. Two heteromeric ionotropic receptors are shown, the NMDA and AMPA receptors, as well as group I and group II metabotropic receptors. Competitive antagonists of each receptor are boxed. The NMDA receptor channel is additionally blocked by Mg^{2+} and phencyclidine (*PCP*). Zn^{2+} is both a negative and a positive modulator. Protons suppress NMDA receptor activation, and polyamines, such as spermine, relieve the proton block. Cyclothiazide removes desensitization of AMPA receptors. Both classes of metabotropic receptor are coupled via G proteins (*G*) to intracellular enzymes, phosphoinositide-specific phospholipase C (*PI-PLC*) for group I receptors and adenylate cyclase (*AC*) for group II receptors. PI-PLC catalyzes the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (*DAG*) from phosphatidylinositol-4,5-bisphosphate (*PIP2*). The resulting increase in cytoplasmic IP_3 triggers release of Ca^{2+} from intracellular stores. Activation of group II metabotropic glutamate receptors typically results in inhibition of AC. The cytoplasmic proteins PSD-95, GRIP and Homer anchor the receptors to the PSD complex. *AIDC*, 1-aminoindan-1,5-dicarboxylate; *5,7-DCK*, 5,7-dichlorokynurenic acid; *D-AP5*, D-2-amino-5-phosphonopentanoic acid; *CPPene*, 3-(2-carboxypiperazin-4-yl)1-propenyl-1-phosphoric acid; *NBQX*, 6-nitro 7-sulfamobenzo[f] quinoxaline-2,3-dione.

Conversely, concanavalin A relieves desensitization of kainate receptors, presumably via interaction with surface sugar chains, but has insignificant effects on AMPA receptors.

An important class of noncompetitive antagonists selective for AMPA receptors is represented by the 2,3-benzodiazepines such as GYKI 53655. These compounds act at sites different from those acted on by cyclothiazide and are useful tools for isolating synaptic responses mediated by kainate receptors. These compounds also show some promise as neuroprotective drugs for treating ischemic neuronal injury.

_N_-methyl-D-aspartate (NMDA) receptors have multiple regulatory sites. To date, three NMDA receptor subunit families have been identified, one represented by a single gene (NR1, encoding a protein of ≈900 amino acids), the second by four genes (NR2A–NR2D, ≈1450 amino acids) and the third by two known genes (NR3A–NR3B, about 1000 amino acids) (Fig. 15-5). Most if not all NMDA receptors in the brain are heteromeric receptors, as is likely to be the case with AMPA and kainate receptors. The mRNAs encoding most NMDA receptor subunits are differentially distributed, as are those of other glutamate receptors. Expression of NR1 mRNA is nearly ubiquitous in the CNS. In contrast, the four NR2 genes show differential patterns of expression (Fig. 15-7). NR2A is present throughout the forebrain and the cerebellum. NR2B and NR2C, however, have a more limited distribution. NR2B is expressed in highest levels in the forebrain and NR2C in the cerebellum where NR2B mRNA is not detected. NR2D expression seems virtually complementary to that of NR2A in being high in the midbrain and hindbrain but

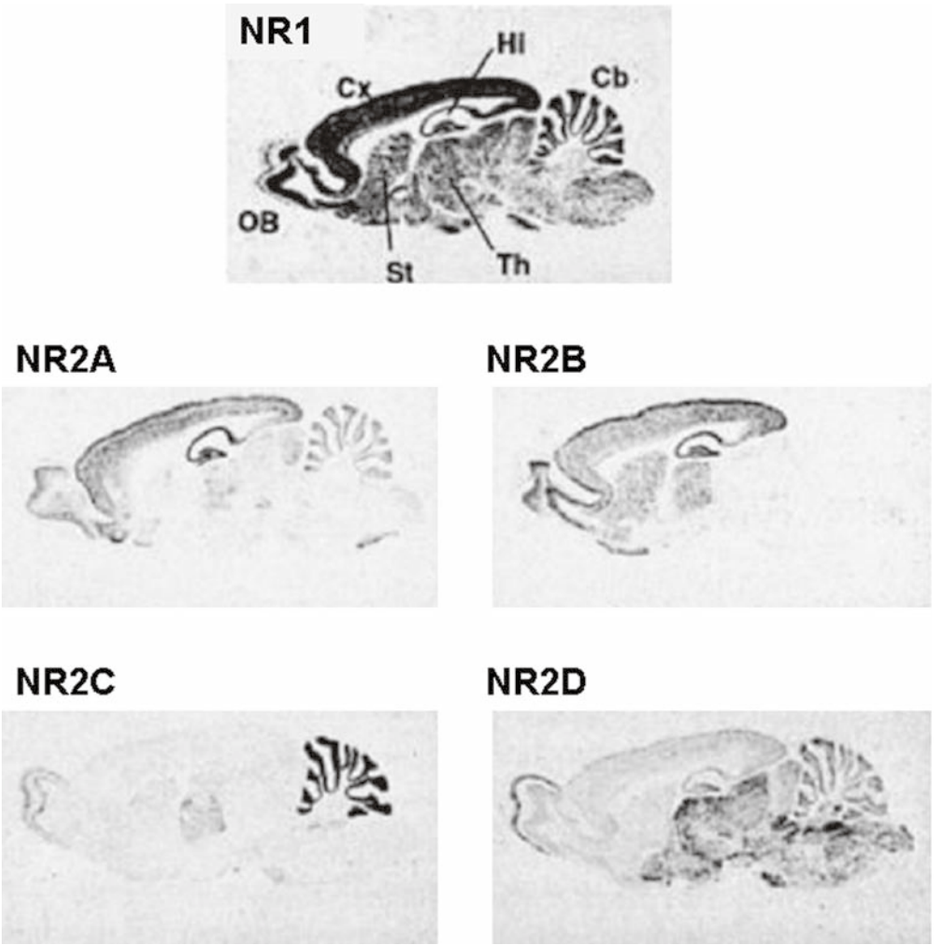

FIGURE 15-7 Regional distribution of mRNAs encoding the five NMDA receptor genes in adult rat brain, by *in situ* hybridization. *OB*, olfactory bulb; *Cx*, cortex; *Hi*, hippocampus; *Cb*, cerebellum; *Th*, thalamus; *St*, striatum. (With permission from Nakanishi, S. *Science* 258, 597–603, 1992.)

low in the forebrain. NR3A is expressed in the spinal cord and cortex, whereas NR3B is found mainly in motor neurons in the spinal cord, pons and medulla.

NMDA receptors are some of the most tightly regulated neurotransmitter receptors. There are no fewer than six distinct binding sites for endogenous ligands that influence the probability of ion channel opening (Fig. 15-6). These consist of recognition sites for two different agonists (glutamate and glycine) and a polyamine regulatory site, all of which promote receptor activation, and separate recognition sites for Mg^{2+}, Zn^{2+} and H^{+} that act to inhibit ion flux through receptors that have bound agonists. The redox state of the receptor also affects NMDA-receptor-mediated responses. One of the three pairs of cysteine residues may either be reduced (which enhances NMDA receptor-mediated currents) or oxidized and form disulfide bridges (which reduces currents).

NMDA receptor agonists are typically short-chain dicarboxylic amino acids such as glutamate, aspartate and NMDA. Acting at a site on the NR2 subunit, glutamate is the most potent endogenous agonist in the mammalian brain. NMDA itself, although a very selective agonist at these receptors, is 30 times less potent than glutamate in electrophysiological assays. Competitive antagonists of the glutamate recognition site are formed from the corresponding agonists by extending the carbon chain, sometimes in a ring structure and often including replacement of the ω-carboxyl group with a phosphonic acid group. Numerous competitive antagonists of this recognition site are available, notably D-2-amino-5-phosphonopentanoic acid (D-AP5) and 3-(2-carboxypiperazin-4-yl)1-propeny-1-phosphonic acid (2R-CPPene), the latter having a K_d of approximately 40 nmol/l in binding and functional studies. These compounds are polar and penetrate the blood–brain barrier only poorly, although recently several NMDA receptor blockers have been developed that have better access to the brain from the blood.

The NMDA receptor is unique among all known neurotransmitter receptors in its requirement for the simultaneous binding of two different agonists for activation. In addition to the conventional agonist-binding site typically occupied by glutamate, the binding of glycine to a

site on the NR1 subunit is required for receptor activation [7, 11]. Because neither glycine nor glutamate acting alone can open this ion channel, they are referred to as 'coagonists' of the NMDA receptor [11]. The glycine site on the NMDA receptor is pharmacologically distinct from the classical inhibitory glycine receptor (see Ch. 16) in that it is not blocked by strychnine and is not activated by β-alanine. Several small analogs of glycine, including serine and alanine, also act as agonists at this site. In both cases the D-isomer is 20–30-fold more potent than the L-isomer. D-serine, formed by serine racemase, is a potent endogenous agonist at the glycine site [12]. Bicyclic compounds and many derivatives of either kynurenic acid or quinoxalinedicarboxylic acid are competitive antagonists at the glycine site. Interestingly, most glycine site antagonists in these two series also block competitively the glutamate recognition site of AMPA receptors, suggesting possible structural similarities in the two ligand recognition sites. Halogenation of both ring structures typically induces a large increase in potency, with 5,7-dichlorokynurenic acid (5,7-DCK) being a potent (K_d = 60 nmol/l) and highly selective glycine-site antagonist.

Thus, glutamate and glycine act in concert to open NMDA receptor ion channels. In contrast, a very important brake on NMDA receptor activation is provided by extracellular Mg^{2+}, which exerts a voltage-dependent block of the open ion channel [13]. Other voltage-dependent blockers of NMDA receptor channels include MK-801, the anesthetic ketamine and the recreational drug of abuse phencyclidine (PCP). These blockers (and Mg^{2+}) exhibit varying degrees of voltage dependence and therefore probably recognize somewhat different domains in the channel of the NMDA receptor. Interestingly, the NR3A/NR3B subunits also possess a glycine binding site. Incorporation of an NR3 subunit into NMDA receptors reduces Ca^{2+} permeability of the receptor channel.

Another important endogenous allosteric inhibitor of NMDA receptor activation is H^+. Protons reduce the frequency of channel opening at NR2B-containing receptors over the physiological pH range, with a midpoint at pH 7.4. At pH 6.0, receptor activation is nearly completely suppressed [14]. This suggests that an ionizable histidine or cysteine may play a key role in receptor activation. One or more modulatory sites that bind polyamines such as spermine and spermidine are also found on NMDA receptors. Occupancy of one of the polyamine sites relieves tonic proton block and thus potentiates NMDA receptor activation in a pH-dependent manner [14]. At higher concentrations, however, polyamines produce a voltage-dependent block of the ion channel and thus inhibit ion flux through activated receptors.

In addition to the regulatory mechanisms discussed above, an interesting form of Ca^{2+}-dependent inactivation of NMDA receptors is brought about by calmodulin. Activated by Ca^{2+} entry, calmodulin interacts with the C-terminal domain of the NR1 subunit (Fig 15-4B); this interaction causes inactivation of the receptor manifested by reduced channel opening frequency and reduced channel open time [15]. The Ca^{2+}/calmodulin-dependent phosphatase calcineurin was also shown to inactivate NMDA receptors [16], suggesting a two-step process for modulation involving dephosphorylation of the NMDA receptor followed by binding of Ca^{2+}-calmodulin.

The transmembrane topology of glutamate receptors differs from that of nicotinic receptors. Knowledge about which segments of a receptor are intracellular, extracellular and transmembrane is necessary for identifying the ligand recognition sites, for understanding the mechanism by which ligand binding leads to channel opening, for interpreting mutagenesis data and for identifying potential targets for drug development. A variety of experimental approaches indicate that nicotinic receptor subunits have four transmembrane domains, with both N- and C-termini facing the extracellular fluid (Ch. 11). However, analysis of the AMPA receptor subunit GluR3 indicated only three transmembrane domains [7, 17]. Most interestingly, the channel-lining M2 domain, which harbors the Q/R site (see below), was found to be a re-entrant pore loop, with both ends facing the cytoplasm (Fig. 15-8A). Subsequent studies of NMDA receptor subunits provided evidence for a similar transmembrane topology [7,18], suggesting that the glutamate receptor family has a different topology from that of other ligand-gated ion channels typified by the nicotinic acetylcholine receptors.

Structure of the agonist binding site has been analyzed. Homology mapping of the glutamate receptors on to the crystal structure of bacterial amino-acid-binding proteins and functional evaluation of GluR3–GluR6 chimeras suggested that agonist binding requires portions of both the large N-terminus and a short region between M3 and M4. An important step forward was provided by Gouaux and collaborators, who crystallized a soluble fusion protein consisting of these two binding domains of the GluR2 subunit connected by a short amino acid linker [19]. The agonist binding pocket lies at the hinge region of a clamshell-like gorge (Fig 15-8B). Much has been learned about the mechanism of receptor activation by studying the structure of these two binding domains crystallized with different agonists and antagonists. Agonists contact residues on both upper and lower lobes of the clamshell (Figs 15-8C,D) and binding causes partial closure of the clamshell. The resulting torque on the receptor is thought to be transmitted mechanically to the pore region, flipping the channel into the open conformation (reviewed in [20]). Interestingly, partial agonists appear to cause less domain closure than full agonists and competitive antagonists work by binding in the pocket and preventing domain closure by a foot-in-the-door mechanism. The structure of the glycine binding site of the NR1 subunit has also been solved and, like the GluR2 binding pocket, the glycine binding site lies at the base of

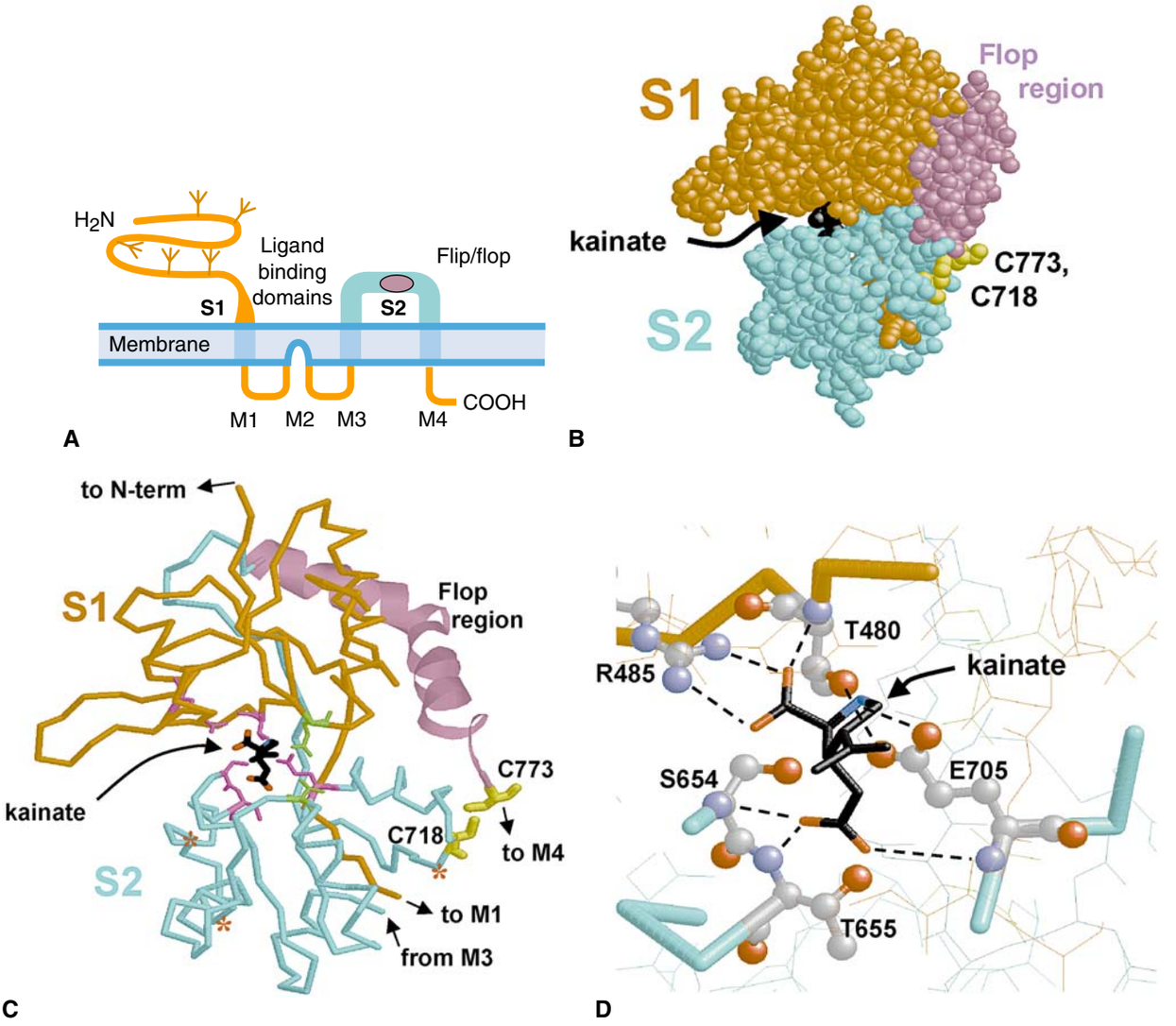

FIGURE 15-8 Transmembrane topology and binding pocket of glutamate receptors. (**A**) Schematic of a glutamate receptor subunit with the two domains that contain agonist-binding residues colored in *orange* (*S1*) and *turquoise* (*S2*). The flip/flop region is indicated in *violet*. (**B**) Space-filled representation of the kainate-bound S1 and S2 domains of GluR2 joined by an 11-residue linker peptide, with coloration the same as in (**A**) The flop domain is helical and located on a solvent-exposed face of the protein. The position of a single-kainate agonist molecule (*black*) within a deep gorge of the protein is indicated; the two disulfide-bonded cysteines (C718 and C773) are shown in *yellow*. (**C**) Backbone representation of the subunit, with kainate (*black*) docked into its binding site. The kainate-binding residues are shown as stick figures in *magenta*, the two cysteines in *yellow* and the flop helix structure in *violet*. The two *green* residues (E402 and T686) do not directly bind to kainate but instead interact with each other, helping to hold the clamshell in the closed conformation.(**D**) Close-up view of the ligand-binding pocket. The binding residues are in space-filled representation, with atoms colored conventionally (*gray* carbon, *light blue* nitrogen, *red* oxygen), (Courtesy of E. Gouaux; from reference [7]).

a cleft between the two large extracellular lobes. A comparison of the GluR2 and NR1 binding pockets explains clearly the basis of agonist and antagonist selectivity.

Genetic regulation via splice variants and RNA editing further increases receptor heterogeneity: the flip/flop versions and the Q/R site. Splice variants that impart functional differences and/or different cellular expression patterns have been found for most of the glutamate receptor subunits. The first splice variants to be described were the so-called 'flip' and 'flop' versions of the AMPA receptor subunits GluR1–GluR4 [21]. The messenger RNA (mRNA) encoding each of these subunits exists in two versions differing by a 115 bp segment. Within this encoded cassette of 38 amino acids that lie in the extracellular loop between the M3 and M4 segments (Fig. 15-8), the two alternative versions differ by only 9–11 amino acids, mostly conservative substitutions. The flip and flop splice variants give rise to receptors that differ in desensitization rate and in their regional distribution in the brain. C-terminal splice isoforms of many glutamate receptor subunits also exist and control the

linkage to cytoskeletal proteins and their anchoring in the subsynaptic membrane. Alternative exon selection within the large N-terminal extracellular domain of NR1 influences many modulatory sites, including those sensitive to pH, Zn^{2+} and polyamines (e.g. spermine). Thus, alternative exon selection is used by neurons to fine-tune the properties of all three classes of ionotropic glutamate receptor.

An important form of regulation is achieved by editing of the primary RNA transcripts of the AMPA and kainate receptor subunits. AMPA receptors that contain the GluR2 subunit are much less permeable to Ca^{2+} than those assembled without GluR2. This important feature of GluR2 was traced by site-directed mutagenesis techniques to a single amino acid within the second membrane-associated domain [22]. A glutamine (Q) resides in this position in GluR1, GluR3 and GluR4, but an arginine (R) is present in GluR2 (Fig. 15-9); this site has thus been named the 'Q/R site'. In addition to Ca^{2+} permeability, the Q/R site influences single channel conductance and the sensitivity of the activated receptor to block by polyamine spider toxins and cytoplasmic polyamines. Voltage-dependent block by cytosolic polyamines gives rise to inward rectification of AMPA receptors with low GluR2 abundance. Interestingly, the genomic DNA sequence has a glutamine codon in the Q/R position, even for the GluR2 subunit in which the mature mRNA has an arginine codon in this site. A small family of double-stranded RNA adenosine deaminases are employed as 'editors' to control the amino acid encoded by this critical codon [23]. RNA editing of the Q/R and other sites, with associated changes in ionic permeability, have also been demonstrated for some kainate receptor subunits. The conditions under which neurons utilize RNA editing to regulate the permeability properties of their glutamate receptors remain to be demonstrated.

Given this combination of internal and C-terminus splice variants and site-specific RNA editing, it appears that four to eight or more mature RNAs can be made from each of the 16 known genes encoding mammalian ionotropic receptor subunits. Thus, neurons have a massive degree of flexibility in constructing a potentially huge number of receptors. The actual degree of glutamate receptor heterogeneity utilized by neurons remains a major unanswered question.

The permeation pathways of all ionotropic glutamate receptors are similar, but *vive la difference*. AMPA, kainate and NMDA receptors contain ion channels that conduct fluxes of Na^+ and Ca^{2+}, but ion selectivity depends on the subunit composition of the receptor. Current flow through AMPA receptors containing GluR2 is normally largely carried by the movement of Na^+ from the extracellular face to the intracellular compartment; these receptors have very low Ca^{2+} permeability. However, receptors that lack GluR2 are three to five times more permeable to Ca^{2+} than to Na^+, because of the absence of an arginine in the Q/R site, as mentioned above. An asparagine residing in the homologous site of all NMDA receptor subunits confers high Ca^{2+} permeability on these receptors [24]. Replacement by site-directed mutagenesis of this asparagine with an arginine produces NMDA receptors with very low Ca^{2+} permeability, similar to that of GluR2-containing AMPA receptors. An arginine or glutamine residing in the Q/R site of all known kainate receptor subunits also reduces the degree of Ca^{2+} permeability. Thus it is likely that the critical portion of the pore that forms the cation selectivity filter is similar among all glutamate receptors.

The permeation pathway of NMDA receptors has a property that sets them apart from other conventional ligand-gated receptors. At membrane potentials more negative (hyperpolarized) than about −50 mV, the concentration of Mg^{2+} in the extracellular fluid of the brain is sufficient to virtually abolish ion flux through NMDA receptor channels even in the presence of the coagonists glutamate and glycine [13] (Fig. 15-10C). Thus, although glutamate and glycine are bound to their receptive sites and the channel is 'activated', the entry of Mg^{2+} into the channel pore blocks the movement of ions through

AMPA Receptors: Role of Q/R Site in Permeation

M2

GluR1	Q
GluR2	NEFGIFNSLWFSLGAFMRQGCDISPRSLS
GluR3	Q
GluR4	Q

Receptors	Internal polyamines	Pca	External polyamine spider toxins	γ (pS)
Lacking GluR2	Block	High	Block	7/16/27
+ GluR2 (R)	No block	Low	No effect	4/8
+ GluR2 (Q)	Block	High	Block	7/16/24

FIGURE 15-9 Location and role of Q/R site in ion permeation through AMPA receptors. The amino acid sequences of GluR1–GluR4 are identical within the M2 pore loop (Fig. 15-8A) except for the Q/R site, as shown (*Q*, glutamine; *R*, arginine). The phenotypes of AMPA receptors containing edited or unedited GluR2 or lacking GluR2 are summarized. *Pca*, calcium permeability; *γ(pS)*, single channel conductance in picosiemens.

the channel. In the presence of Mg^{2+} ions NMDA receptor channels exhibit a characteristic J-shaped current–voltage relationship. As the membrane potential is made less negative or even positive, the affinity of Mg^{2+} for its binding site decreases and the block becomes ineffective. Depolarization-induced relief from voltage-dependent channel block by Mg^{2+} is at the root of several of the most interesting aspects of NMDA receptor function (see below).

GLUTAMATE PRODUCES EXCITATORY POSTSYNAPTIC POTENTIALS

Both NMDA and AMPA receptor components of excitatory postsynaptic potentials (EPSPs) are produced by the brief (1 ms) appearance of free transmitter in the synaptic cleft (Fig 15-10A). Synaptically released glutamate thus

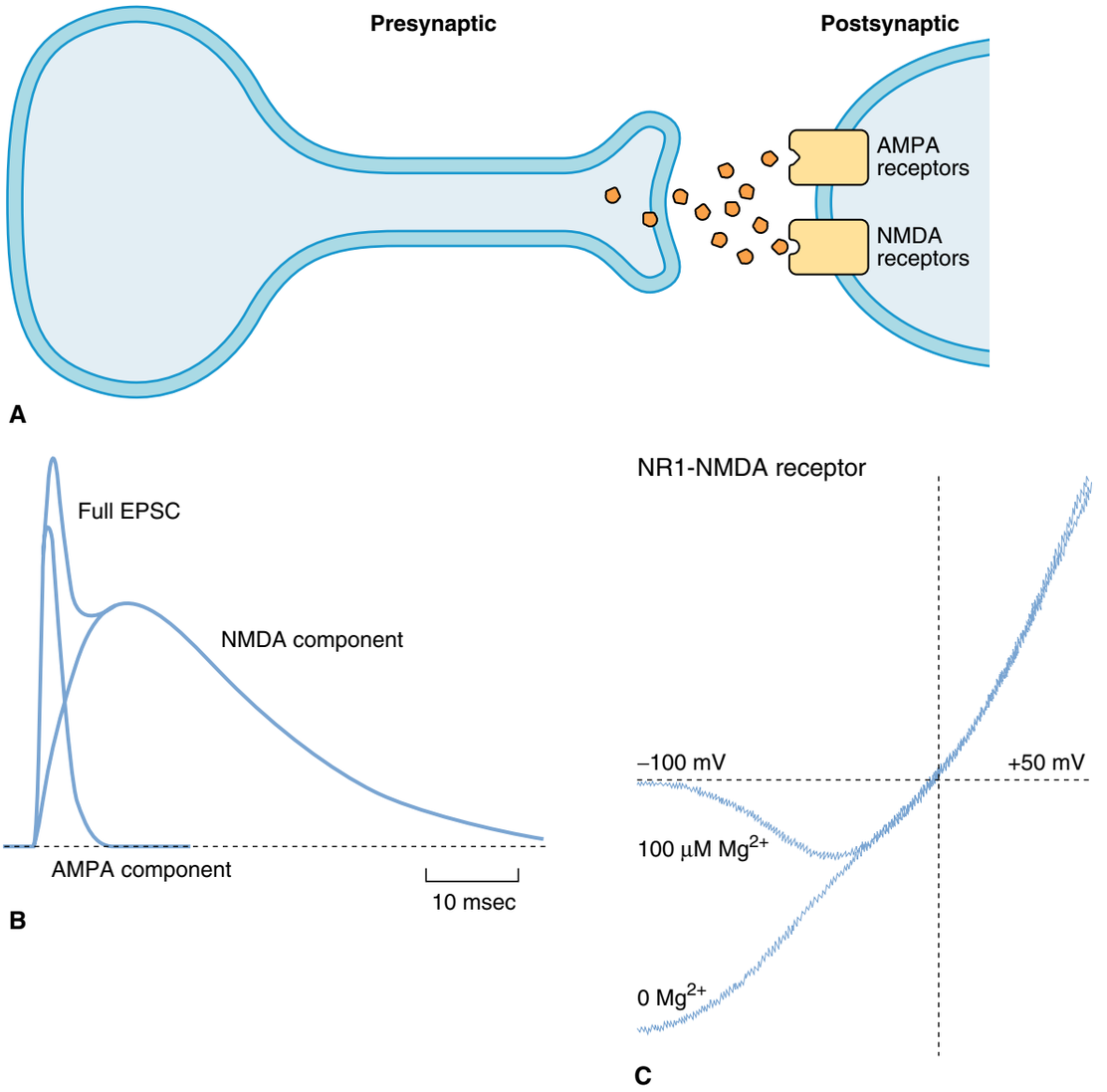

FIGURE 15-10 Biophysical properties of synaptic AMPA and NMDA receptors. (**A**) Diagram showing a postsynaptic cluster of NMDA and AMPA receptors and release of glutamate into the synaptic cleft. (**B**) Excitatory postsynaptic currents (EPSCs) of hippocampal neurons comprise both AMPA- and NMDA-receptor components. EPSCs are upward-going at positive membrane potentials. Treatment with the NMDA receptor blocker D-2-amino-5-phosphonopentanoic acid (D-AP5) or the AMPA receptor antagonist 6-nitro 7-sulfamobenzo[f] quinoxaline-2,3-dione (NBQX) reveals the other component in isolation, as shown. The EPSC is composed of a brief AMPA receptor component and a more prolonged NMDA component. (**C**) Current voltage relationships for NMDA receptors. Receptors can be expressed in *Xenopus* oocytes by microinjection of *in vitro* transcribed RNA encoding various glutamate-receptor subunits. Oocytes were voltage-clamped to permit measurement of ionic currents flowing across the plasma membrane following exposure to agonists. In the presence of an agonist, the membrane potential was slowly ramped from –100 to 50 mV and the membrane current recorded. The *horizontal dashed line* shows zero membrane current. Receptors formed from homomeric NRIA subunits exhibit a roughly linear current–voltage relationship in the absence of Mg^{2+} but, in the presence of Mg^{2+}, current flux through NMDA receptor channels becomes progressively smaller as the membrane potential is made more negative. At –100 mV, the NMDA-induced current is virtually abolished by Mg^{2+}.

results in a two-component excitatory postsynaptic current (EPSC) upon binding to AMPA and NMDA receptors at most central synapses (Fig. 15-10B). Activation of AMPA receptors mediates a component that has rapid onset and decay, whereas the component mediated by NMDA receptor activation has a slower rise time and a decay lasting up to several hundred milliseconds. Rapid desensitization of AMPA receptors may control the time course of EPSPs at many synapses. The long time course of NMDA receptor activation, by contrast, provides more opportunity for temporal and spatial summation of multiple inputs. The resulting summed depolarization may allow other synaptic inputs or nonsynaptic membrane channels to initiate action potential firing. The decay time of the NMDA receptor component is approximately 100 times longer than the mean open time of the channel, so before the bound ligands dissociate, the receptor cycles through the open and closed states many times. The more prolonged activation of NMDA receptors is thought to be due to the higher affinity of glutamate for NMDA than AMPA receptors; high affinity often results from a slow dissociation of the agonist from its receptor.

Genetic knockouts provide clues to ionotropic receptor functions. In most neurons, full editing of the GluR2 subunit mRNA ensures that synaptic AMPA receptors will permit only insignificant Ca^{2+} influx. However, mice engineered to harbor a GluR2 gene that cannot be edited at the mRNA level expressed AMPA receptors with increased calcium permeability [25]. These mice develop seizures and die by 3 weeks of age, demonstrating that GluR2 editing is essential for normal brain development. Moreover, deletion of the GluR2 editing enzyme, RAD1, by gene targeting is also lethal, and this phenotype can be rescued by expression of a GluR2 subunit that has arginine hard-coded into the Q/R site. These results indicate that GluR2 mRNA itself is the most important target of the RAD1 editor. Surprisingly, complete deletion of the GluR2 allele, which also increases Ca^{2+} permeability of AMPA receptors in the targeted mouse neurons, neither induced seizures nor proved lethal in homozygous mice. Rather, Ca^{2+} entry through GluR2-lacking synaptic AMPA receptors could produce a form of LTP [26]. These two genetic manipulations might have had such different outcomes because of different genetic backgrounds of the mice in the two studies. Alternatively, it is possible that a complete absence of GluR2 impairs AMPA receptor assembly, because the density of synaptic AMPA receptors appeared to be low in the latter study.

Conventional gene targeting of the NMDA receptor NR1 subunit interferes with breathing and is lethal within a few hours of birth. However, mouse strains in which the NR1 gene knockout is restricted to the CA1 region of the hippocampus survive and grow normally [27]. Remarkably, LTP is impaired in the CA1 but not in other hippocampal regions, and these mice exhibit impaired spatial memory in a water maze task. These findings demonstrate the essential role of NMDA receptors in LTP in the CA1 region, and also strongly suggest that LTP in the CA1 region is necessary for the acquisition of spatial memory.

METABOTROPIC GLUTAMATE RECEPTORS MODULATE SYNAPTIC TRANSMISSION

Eight metabotropic glutamate receptors (mGluRs) have been identified that embody three functional classes. Metabotropic glutamate receptors are so named because they are linked by trimeric G proteins to cytoplasmic enzymes [28]. To date eight mGluRs have been cloned and named mGluR1–mGluR8. The corresponding genes encode proteins that are thought to span the plasma membrane seven times and, like the ionotropic receptors, they possess an unusually large N-terminal extracellular domain preceding the membrane-spanning segments. mGluRs have been grouped into three functional classes based on amino-acid sequence homology, agonist pharmacology and the signal transduction pathways to which they are coupled (Fig 15-5). Members of each class share ≈70% sequence homology, with about 45% homology between classes. Alternatively spliced variants have been described for mGluR1, mGluR4, mGluR5 and mGluR7.

Glutamate itself activates all of the recombinant mGluR, but with widely varying potencies ranging from 2 nmol/l (mGluR8) to 1 mmol/l (mGluR7). Highly selective agonists for each of the three groups have been identified. 3,5-dihydroxyphenylglycine (DHPG) appears to be a selective group I agonist, 2R,4R-4-aminopyrrolidine-2–4-dicarboxylate (APDC) is highly selective and reasonably potent (400 nmol/l) agonist for group II, whereas L-amino-4-phosphonobutyrate (L-AP4) is a selective agonist of group III mGluR. Some phenylglycine derivatives are mGluR antagonists but highly group-selective antagonists remain to be identified.

mGluRs are linked to diverse cytoplasmic signaling enzymes. The classification of mGluR into three groups is further supported by a consideration of their signal transduction mechanisms. Group I mGluRs stimulate phospholipase C activity and the release of Ca^{2+} from cytoplasmic stores (Fig. 15-6). The ability to increase intracellular Ca^{2+} levels differs between the members of this class and their splice variants, a property probably attributable to the different affinities each receptor has for the G protein. Activation of phospholipase C leads not only to the formation of IP_3 but also diacylglycerol, which in turn activates protein kinase C (see Ch. 20). Activation of group II and group III mGluRs results in the inhibition of adenylate cyclase (Fig. 15-6). This response is blocked by pertussis toxin, which suggests that a G protein of the G_i family is involved (Chs 19 and 21).

Postsynaptic mGluR activation modulates ion channel activity. mGluR located on the postsynaptic membrane modulate a wide variety of ligand- and voltage-gated ion channels expressed on central neurons, as would be expected if receptor activation is coupled to multiple effector enzymes (ion channels are discussed in Ch. 6). Activation of all three classes of mGluR has been shown to inhibit L-type voltage-dependent Ca^{2+} channels, and both group I and II mGluR inhibit N-type Ca^{2+} channels. mGluR activation also decreases a high-threshold Ca^{2+} current in spiking neurons of *Xenopus* retina. Activation of mGluR closes voltage-dependent, Ca^{2+}-dependent K^+ channels in hippocampal and other cortical neurons, leading to slow depolarization and consequent neuronal excitation. The exact mechanism of mGluR modulation of K^+ currents is at present unclear but could occur through activation of CaMKII. In cerebellar granule cells, mGluR activation increases the activity of Ca^{2+}-dependent and inwardly rectifying K^+ channels, leading to a reduction in excitability.

A large number of ligand-gated channels are also modulated by mGluR activation including NMDA and kainate receptors as well as dopamine, $GABA_A$ and norepinephrine receptors. Whether activation of mGluR acts to inhibit or potentiate an ionotropic receptor depends on what component of the signal transduction mechanism is affected and this is often tissue-specific. For example, in hippocampal pyramidal cells group I, mGluR activation potentiates currents through NMDA receptors. This effect is reduced by inhibitors of either protein kinase C or src kinase, and may proceed through dual signaling pathways. In contrast, in cerebellar granule cells, mGluR activation inhibits NMDA-receptor-induced elevations of intracellular calcium also by a mechanism thought to involve PKC.

Presynaptic mGluR activation can lead to presynaptic inhibition. Immunohistochemical studies at both the light and electron microscopic level have firmly placed a number of mGluRs on the presynaptic terminals of central neurons. Activation of presynaptic mGluR blocks both excitatory glutamatergic and inhibitory GABAergic synaptic transmission in a variety of central structures. For example, activation of mGluR2 located on presynaptic granule cell terminals blocks EPSPs evoked in CA3 hippocampal pyramidal neurons by mossy fiber stimulation. In contrast, transmission at synapses between Schaffer collaterals and CA1 pyramidal cells is resistant to presynaptic mGluR2 activation but is blocked by activation of mGluR4 or 7, as suggested by the effect of L-AP4, an agonist of all group III mGluRs. The actual mechanism of how mGluR activation regulates synaptic transmission is unclear. However, because all three mGluR groups are known to inhibit voltage-dependent Ca^{2+} channels it seems probable that presynaptic Ca^{2+} channels are targets for mGluR modulation. A direct demonstration of this has been shown in presynaptic terminals of the calyx of Held in the brain stem, where mGluR agonists suppress a high-voltage-activated P/Q-type Ca^{2+} conductance, thereby inhibiting transmitter release at this glutamatergic synapse [29].

Genetic knockouts provide clues to mGluR functions. The ability to ascertain the precise physiological functions of mGluR has been hampered, in part, by the lack of sufficiently potent and selective pharmacological agents. The use of alternative strategies for the study of mGluR is therefore clearly warranted. To address this problem, several research groups have used gene targeting to produce 'knockout' mice that are devoid of the mGluR subtype of interest. These experiments point directly to a physiological role for a number of mGluRs. Establishment of specific neuronal connections in the mature nervous system occurs by a process in which redundant connections formed during development are eliminated. In the adult cerebellum, each Purkinje cell (PC) is innervated by a single climbing fiber (CF) that originates from the inferior olive of the medulla. This one-to-one relationship is preceded by a developmental stage in which each PC is innervated by multiple CFs. Massive elimination of synapses formed by CFs occurs postnatally, and a monosynaptic relationship is established at around postnatal day 20 in the mouse. Mice that lack the *mGluR1* gene show symptoms of cerebellar dysfunction such as ataxic gait, intention tremor and dysmetria, and they are impaired in motor coordination and motor learning [30]. These mutant mice are deficient in long-term depression (LTD) at CF-PC synapses; LTD in the cerebellum is thought to be a cellular basis for motor learning. In these *mGluR1* mutant mice, innervation of multiple CFs onto a PC persists into adulthood, suggesting that the precise sculpting of these synaptic connections requires activation of mGluR1 during development.

The strong expression of mGluR2 in dentate gyrus granule cells has suggested a role for this receptor in presynaptic regulation of transmission at the mossy fiber–CA3 pyramidal neuron synapses as described above. In mice lacking the *mGluR2* gene, basal synaptic transmission was indistinguishable from wild-type responses. In contrast, presynaptic inhibition at mossy fiber synapses induced by the mGluR2 agonist DCG-IV was markedly reduced in the mutant mice, confirming a role for mGluR2 receptors in the presynaptic regulation of neurotransmission. Interestingly, this mGluR2 deficiency was without effect on the magnitude of LTP induced at these synapses, but LTD was significantly impaired [31]. In a variety of CNS structures including the hippocampus, olfactory tract, spinal cord and thalamus, activation of presynaptically located Group III mGluRs by L-AP4 inhibits synaptic transmission. One member of this group, mGluR4, is preferentially expressed in the cerebellum and has a role in the presynaptic regulation of synaptic transmission. Targeted elimination of the *mGluR4* gene reveals that mGluR4 is essential in providing a presynaptic mechanism for maintaining synaptic efficacy during repetitive

activation, and suggests that the presence of mGluR4 at the parallel fiber–PC synapse is required for maintaining normal motor function [32]. Likewise, mGluR8 seems to be the exclusive mediator of presynaptic inhibition at the lateral perforant path in the mouse.

GLUTAMATE RECEPTORS DIFFER IN THEIR POSTSYNAPTIC DISTRIBUTION

NMDA and AMPA receptors are spread across the postsynaptic density (PSD), whereas metabotropic glutamate receptors (except mGluR7) are located along the periphery of the PSD (Fig. 15-2). NMDA receptors appear to be present at most or all glutamatergic synapses whereas the content of AMPA receptors is variable – from zero to about 50 receptors per PSD [33]. Some synapses are 'silent', meaning that activation of them does not elicit AMPA receptor currents when the plasma membrane is hyperpolarized and Mg^{2+} blocks NMDA receptors. Such silent synapses contain only NMDA receptors. However, AMPA receptors are recruited from the cytosol to the PSD to activate such silent synapses in LTP.

PROTEINS OF THE POSTSYNAPTIC DENSITY MEDIATE INTRACELLULAR EFFECTS OF GLUTAMATE RECEPTOR ACTIVATION

The PSD is a multiprotein complex with many functions (Fig. 15-4B). From the perspective of glutamatergic neurotransmission it may be viewed as a signal transduction machine that converts the extracellular glutamate signal into various intracellular signals. Apart from glutamate receptors, the PSD contains ion channels, glycolytic enzymes, transporters (e.g. a lactate transporter), proteins of intracellular signaling pathways, cell adhesion molecules and scaffolding proteins that link the proteins of the PSD together. Some of the proteins of the PSD are made lipophilic by the binding of two palmitic acid residues. Therefore they have an affinity for so-called lipid rafts, small areas of the plasma membrane that are rich in cholesterol and sphingomyelin [34]. Such rafts are present in dendritic spines and help to concentrate proteins of the PSD at the spines (Fig. 15-4B).

A major scaffolding protein of the PSD is PSD95. Two N-terminal cysteines of this protein bind palmitic acid residues, which anchor PSD95 to lipid rafts [30]. PSD95 contains several domains that bind other proteins: three so-called PDZ domains (short for PSD95/disc large/zona occludens-1), a src homology (SH3) domain, and a guanylate kinase (GK) domain. This family of proteins are called MAGUKs (membrane-associated guanylate kinases, although the guanylate kinase domain is catalytically inactive). PDZ domains are stretches of about 90 amino acids that bind C-termini of other proteins.

Neuroligin is a protein that binds to the PDZs of PSD95. Neuroligin reaches into the synaptic cleft and interacts with β-neurexin, a protein that reaches into the synaptic cleft from the presynaptic side; β-neurexin is anchored presynaptically to the PDZ domain of another MAGUK, CASK. This arrangement connects the pre- and postsynaptic elements and stabilizes the synapse mechanically.

The NR2 subunit of the NMDA receptor binds to PDZ domains of PSD95 with its cytoplasmic C-terminus. PSD95 also binds α-actinin, which again binds to filamentous actin (F-actin), a main cytoskeletal protein in dendritic spines. In this manner PSD95 anchors the NMDA receptor to the cytoskeleton of the dendritic spine.

PSD95 also couples the NMDA receptor to intracellular signaling systems that mediate intracellular effects of NMDA receptor activation. Calcium ions that enter the postsynaptic cell through the NMDA receptor bind to calmodulin probably at all glutamatergic synapses. Calmodulin with bound Ca^{2+} activates a protein kinase, Ca^{2+}/calmodulin-dependent kinase II (CaM kinase II). CaM kinase II is one of the most abundant proteins at the PSD; it connects to α-actinin via the scaffolding protein densin. Activated CaM kinase II can phosphorylate a host of other proteins. One such protein is the GluR1 subunit of the AMPA receptor, which is phosphorylated on a serine residue (ser 831). Phosphorylation of GluR1 by CaM kinase II increases the conductance of the AMPA receptor, which is one mechanism that contributes to potentiation of glutamatergic synapses.

The Ca^{2+}–calmodulin complex may also activate nitric oxide synthase (NOS), which binds to a PDZ domain of PSD-95. Activated NOS produces NO from arginine; NO, in turn, activates guanylate cyclase, the enzyme that catalyzes the conversion of GTP to the intracellular messenger cGMP, which activates protein kinase G (PKG).

AKAP (A-kinase anchor protein) is another scaffolding protein of the PSD. AKAP binds PKA (protein kinase A), PKC (protein kinase C), and the protein phosphatase calcineurin (PP2B) as well as the SH3 and GK domains of PSD95. This arrangement ensures a close proximity of NMDA receptors to PKA. Calcium influx through the NMDA receptor causes Ca^{2+}/calmodulin activation of adenylate cyclase and formation of cAMP. cAMP activates PKA, which, among other things, phosphorylates GluR1 on serine 845; this phosphorylation is necessary for recruitment of AMPA receptors to the plasma membrane of the dendritic spine. In fact, AMPA receptors are to a variable degree located intracellularly, whence they may be recruited to the postsynaptic membrane to strengthen the synapse during induction of LTP. Long-term depression of glutamatergic synapses, which is also mediated by NMDA

receptor activation, involves Ca^{2+}-dependent activation of the phosphatase calcineurin, which causes the dephosphorylation of GluR1 on serine 845 [35]. The result of this dephosphorylation is the relocation of AMPA receptors from the plasma membrane to the cytosol.

AMPA receptors do not appear to bind directly to PSD95, but may do so indirectly through other proteins, such as TARPs (*trans*-membrane AMPA-R regulatory proteins), and this interaction appears necessary for the recruitment of AMPA receptors from the intracellular pool to the PSD. The C termini of AMPA receptors bind directly to other PSD proteins with PDZ domains: GRIP (glutamate receptor-interacting protein), PICK1 (protein interacting with C kinase 1), ABP (AMPA receptor-binding protein) and SAP97 (synapse-associated protein of 97 kDa). GRIP is another protein that is palmitoylated and therefore has an affinity for lipid rafts [34].

GRIP, PICK1 and PSD95 also interact with kainate receptor subunits GluR5 and 6. PKC, which binds to PICK1, may phosphorylate these subunits, thereby causing them to anchor more stably at the synapse, a mechanism that could strengthen kainate receptor-mediated transmission.

Metabotropic glutamate receptors are anchored to the periphery of the PSD by the scaffolding proteins homer and shank (Fig. 15-4). Homer is a cytoplasmic protein that may multimerize and link proteins that have homer-binding motifs, such as mGluRs and shank. Shank binds to the GK domain of PSD95 and thus links mGluRs to PSD95. Through homer, mGluRs also connect to the IP_3 receptor (1,4,5-trisphosphate receptors) [36]; the activation of the IP_3 receptor by IP_3 causes the release of Ca^{2+} from the smooth endoplasmic reticulum (Ch. 20). The proximity of mGluRs and IP_3 receptors ensures that the IP_3 that is formed upon stimulation of type I mGluRs reaches the IP_3 receptor. Similar arrangements occur presynaptically, where mGluR7a binds to the PDZ domain of PICK1.

SMALL GTP-BINDING PROTEINS (GTPases) MEDIATE CHANGES IN GENE EXPRESSION UPON NMDA RECEPTOR ACTIVATION

Synaptic plasticity manifesting as LTP or LTD depends in part on formation of new proteins, which requires transcription of specific genes. Activation of NMDA receptors during LTP may cause more than a hundred genes to be transcribed, resulting in the formation of mRNAs. Some genes are turned on immediately upon NMDA receptor activation, others are activated later. The genes encode transcription factors, AMPA receptor subunits, cytoskeletal proteins, intracellular signaling molecules, ion pumps, and enzymes of energy metabolism [37] (see also Ch. 26).

The intracellular mechanisms that relay glutamate receptor activation to transcription constitute a much-studied field. Small, monomeric GTPases are important activators or silencers of signaling pathways that lead to changes in gene transcription. A final step in these pathways is the activation of transcription factors that cause initiation of transcription.

Ras is a small GTPase that is central in mediating alteration of gene transcription upon NMDA receptor activation. Ras stimulates several intracellular signaling pathways, including the Raf–MEK–ERK pathway and the PI3 kinase (phosphoinositide 3-OH kinase) pathway, the latter leading to activation of protein kinase B (Akt/PKB) as well as the MEKK–JNKK–JNK pathway. Phosphorylated (activated) ERK translocates from the cytosol to the nucleus, where it activates the transcription factors Elk and CREB. Akt/PKB activation leads to the activation and nuclear translocation of transcription factors NFκB and CREB. Activation and nuclear translocation of JNK lead to the activation of the transcription factors c-Jun, c-Fos and ATF2.

Small GTPases, such as Ras, cycle between an active GTP-bound state and an inactive GDP-bound state (Ch. 19). They are regulated by activating GEFs (guanyl exchange factors) and inhibitory GAPs (GTPase activating proteins). GEFs stimulate the exchange of GDP for GTP, which enables the GTPases to bind to their effector proteins. GAPs stimulate the GTPases to hydrolyze their bound GTP to GDP, which causes them to lose signaling activity. GEFs and GAPs may become activated through binding of calcium ions, Ca^{2+}/calmodulin and/or diacylglycerol. A Ras-specific GAP, synGAP (synaptic GAP), binds to PSD95. A Ras GEF has been identified that associates with NMDA receptor subunit NR2B [38]. Thus, glutamate receptor activation may activate both GAPs and GEFs to modulate the activity of Ras and its downstream effectors. Recent evidence suggests that NMDA receptor-triggered activation of Ras and Raf kinases mediates LTP and LTD, respectively, by facilitating the insertion or removal of AMPA receptors in the postsynaptic membrane ([39] for review).

An enigma of LTP research has been how the neuron comes to strengthen just the activated synapse and not all its synapses, since the mRNA made in the cell body will tend to become distributed throughout the cell. An answer seems to be that the LTP-inducing stimulus, in addition to activating genes in the cell body, marks the synapse where the mRNAs are to be translated into protein. The 'mark' may be the activation of cytoplasmic polyadenylation element binding protein (CPEB) by phosphorylation [40]. CPEB, a component of the PSD that binds to the 3′ end of translationally dormant mRNAs, is phosphorylated upon NMDA receptor activation and initiates a process that causes elongation of the mRNA poly (A) tail. This polyadenylation of the mRNAs triggers their translation into protein only at the potentiated synapse.

DENDRITIC SPINES ARE MOTILE, CHANGING THEIR SHAPE AND SIZE IN RESPONSE TO SYNAPTIC ACTIVITY WITHIN MINUTES

Intense synaptic activity leads to enlargement of the dendritic spine and the PSD. Although the pathways that regulate spine morphology in response to synaptic activity are not fully known, influx of calcium ions through the NMDA receptor is an important first signal and modulation of actin is a final result. Interestingly, overexpression of the GluR2 AMPA receptor subunit increases the number and size of spines in cortical neurons, whereas GluR2 depletion causes spine retraction [41]. This effect was traced to a region in the GluR2 N-terminus, which probably interacts with another extracellular protein. The spines are highly motile structures. Most of the actin of the dendritic spine is broken down and rebuilt within a minute. Small GTPases of the Rho family regulate actin morphology [42]. Among them, Rac and Cdc42 promote outgrowth of the actin cytoskeleton to create or enlarge spines, whereas activation of Rho itself has been shown to cause a loss of spines. The balance between Rac and Cdc42 on the one hand and Rho on the other is therefore important in controlling spine morphology in response to NMDA receptor activation. Rho GEFs have been identified that bind to PSD-95. Citron, a downstream effector of Rho, also binds to PSD95. In addition, both Rac and Cdc42 may be activated through Ras-dependent pathways.

SODIUM-DEPENDENT SYMPORTERS IN THE PLASMA MEMBRANES CLEAR GLUTAMATE FROM THE EXTRACELLULAR SPACE

The area of the synaptic cleft is approximately $0.1\,\mu m^2$ and the width is ≈20 nm, which gives a volume of approximately 2×10^{-18} liter, 100 times greater than that of a synaptic vesicle. Therefore, the concentration of glutamate in the cleft when a vesicle has emptied its content will be in the low millimolar range. Rapid removal of glutamate from the extracellular space is important for the synapse to function properly on a millisecond time scale and to avoid overexcitation of the postsynaptic cell. This is accomplished by diffusion of glutamate out of the synaptic cleft; however, to maintain a gradient for diffusion glutamate must be actively removed from the extracellular fluid.

Removal of glutamate occurs by both high-affinity transporters (with K_ms in the low micromolar range) and low-affinity transporters (with K_ms around 0.5 mmol/l). Only the former have been characterized on a molecular level. Glutamate removal has two components: binding of glutamate to the transporters and translocation to the interior of the cell. Binding is an important component, as the following calculation will show: The density of glutamatergic synapses in gray matter is roughly $1/\mu m^3$. A bouton may release the content of about 2–5 vesicles per second. With a content of ≈1,200 molecules of glutamate per vesicle, the amount of glutamate released per second would be 2,500–6,000. In the same volume 15,000–20,000 glutamate transporters may be present [43]. Therefore, binding of glutamate to transporters is a rapid form of inactivation. The translocation of glutamate to the interior of the cell may take only a few additional milliseconds.

The high density of glutamate transporters and the tendency for astrocytic processes to cover the synaptic cleft (Fig. 15-2) controls the degree of spillover of glutamate into neighboring synapses. Spillover of glutamate from one synapse to another does occur, however, and has been shown to modulate transmission at nearby synapses, both glutamatergic, GABAergic and monoaminergic. Modulation of GABAergic and monoaminergic neurotransmission through spillover of glutamate occurs through activation of presynaptic metabotropic glutamate receptors on nerve terminals.

Five high-affinity glutamate transporters have been identified. They are termed EAAT 1–5 (for excitatory amino acid transporter). EAAT2 is the principal transporter in the forebrain; it is present in both astrocytes and nerve terminals (Fig. 15-2). EAAT1 is only present in astrocytes; it is abundant in cerebellum but is also expressed in the forebrain. EAAT3 is expressed in neurons in the whole brain and is found presynaptically on GABAergic nerve terminals, whereas EAAT4 is largely expressed on dendrites of cerebellar Purkinje cells. EAAT5 is expressed in retina. In the rat EAAT1–3 are termed GLAST, GLT and EAAC1, respectively; EAAT4 and 5 have the same names in man and rat.

The high density of glutamate transporters means that they may compete with glutamate receptors for binding of transmitter glutamate and thus modulate the glutamate signal. The density of glutamate transporters at the cell membrane as well as their affinity for glutamate are probably subject to regulation. Activation of PKC leads to a loss of EAAT2 from the cell membrane but causes an increase of EAAT3 in the cell membrane. Glutamate transporter-associated proteins (GTRAPs) have been identified that bind to and regulate the affinity of transporters for glutamate.

Electron microscopic studies with immunocytochemical detection of glutamate transporters show that the majority of the transporters are localized in astrocytic cell membranes, and it is generally assumed that most transmitter glutamate gets taken up by astrocytes as a part of the glutamine cycle (see above). However, when brain slices are exposed to the glutamate analog D-aspartate, this compound is found in nerve terminals, not primarily in astrocytes. Therefore, there is some uncertainty regarding

the quantitative importance of astrocytes versus neurons for uptake of transmitter glutamate.

The uptake of glutamate occurs against its concentration gradient and so needs a driving force, which is provided primarily by the sodium electrochemical gradient across the plasma membrane. Glutamate enters cells together with three sodium ions and one proton; one potassium ion is counter-transported [43]. Uptake of glutamate is therefore electrogenic, resulting in a net movement of positive charge across the plasma membrane. The glutamate transporters also act as chloride channels, but the chloride conductance is not coupled to the transport of glutamate.

Because glutamate uptake is driven by cation gradients it is a secondarily active process that relies on the activity of the sodium-potassium ATPase (Ch. 5). For each molecule of glutamate that is taken up, one molecule of ATP is spent on the extrusion of the three sodium ions in exchange for two potassium ions. In addition, one molecule of ATP is spent if glutamate is converted into glutamine in astrocytes by the ATP-dependent glutamine synthetase. Several studies suggest that this energy expenditure is covered by increased glycolysis. Glycolysis gives two molecules of ATP per molecule of glucose that is converted to lactate. Excess lactate exits the astrocytes to the extracellular fluid and enters neurons where it is used as an energy source [44] (see also Ch. 31).

SODIUM-DEPENDENT GLUTAMINE TRANSPORTERS IN PLASMA MEMBRANES MEDIATE THE TRANSFER OF GLUTAMINE FROM ASTROCYTES TO NEURONS

Astrocytes convert much of transmitter glutamate into glutamine, and glutamine is exported to the extracellular space from which neurons accumulate it. Both astrocytes and neurons have transporters that are sodium-driven and that concentrate glutamine inside the cells. The astrocytic and neuronal transporters are called system N transporters (SN) and system A transporters (SA), respectively (Fig. 15-2). They are closely related, but the astrocytic SN transporter exchanges a proton for sodium and does therefore not carry a net charge into the cell. It therefore depends entirely on the chemical component of the sodium gradient. This feature allows for transporter reversal when the sodium gradient becomes reduced, as would occur when astrocytes depolarize, so that some glutamine may leak out of the astrocytes into the extracellular fluid. An increase in intracellular glutamine or pH (reduction in $[H^+]$) would contribute in the same direction. System A transporters do not exchange a proton for sodium and therefore carry a net charge into the neuron. Therefore system A transporters are driven by both the sodium gradient and the membrane potential, which makes them less likely to leak glutamine to the extracellular fluid [45]. The net effect of SN being more leaky than SA is a flux of glutamine from astrocytes to neurons.

EXCESSIVE GLUTAMATE RECEPTOR ACTIVATION MAY MEDIATE CERTAIN NEUROLOGICAL DISORDERS

Glutamate and its analogs can be neurotoxins and cause excitotoxicity. Glutamate and structurally related ligands, such as NMDA and kainate, in addition to their powerful excitatory effects at glutamate receptors, are potent neurotoxins (see also Ch. 32). The toxic effect of glutamate and its analogs is related to their excitatory properties at glutamate receptors, and this type of toxicity is therefore called excitotoxicity. That glutamate and other amino acids act as neurotoxins was first realized in the 1970s when these agents were given orally to immature animals. Acute neurodegeneration was observed in those areas not well protected by the blood–brain barrier, notably the arcuate nucleus of the hypothalamus. The mechanisms of neurodegeneration are divergent, and activation of all classes of ionotropic glutamate receptor has been implicated.

Intracerebroventricular injection of kainic acid has been shown to result in a well-characterized pattern of neuronal cell damage. In the hippocampus, kainic acid causes a selective lesion of the CA3 pyramidal neurons, an area rich in KA1 and GluR6 receptors. The lesion does not compromise passing axons, which is why this type of (excitotoxic) lesion is often referred to as 'axon-sparing'. Kainic acid injection into the hippocampus also leads to epileptiform discharges in cells normally innervated by the damaged pyramidal neurons.

Some dietary neurotoxins may cause excessive glutamate receptor activation and cell death. Some rare food contaminants are glutamate analogs and cause excessive glutamate receptor activation leading to neuronal cell death. Domoic acid is a shellfish poison that potently activates kainate receptors and produces seizures and death or, if the patient survives, chronic memory impairment because of damage to the hippocampus. Amyotrophic lateral sclerosis (ALS) is a disorder that entails degeneration of upper and lower motor neurons (in the motor cortex and spinal cord/medulla oblongata respectively). While 90% of cases are sporadic, 10% are hereditary. The high incidence of ALS in the Pacific island of Guam was determined to be due to the dietary ingestion of the cycad *Cycas circinalis*. This seed contains an amino acid, β-*N*-methylamino-L-alanine (BMAA). In the presence of bicarbonate, BMAA becomes excitotoxic through a mechanism that involves the activation of AMPA and NMDA receptors. The action of BMAA can be blocked

by the NMDA receptor antagonist D-AP5. Another disorder that also primarily affects upper and lower motor neurons is neurolathyrism, a disorder occurring in East Africa and India. It is associated with the dietary consumption of the chick pea *Lathyrus sativus*. The glutamate-like excitant β-*N*-oxalylamino-L-alanine (BOAA) has been identified as the toxin in this plant. The action of BOAA at AMPA receptors may be responsible for the observed degeneration of motor neurons (see Motor Neuron Diseases, Ch. 44).

A mold that has been reported to contaminate sugar cane in China produces nitropropionic acid, a toxin that causes destruction mainly of the basal ganglia if ingested. Nitropropionic acid is not a glutamate analog, but an inhibitor of succinate dehydrogenase of the TCA cycle. The toxin causes ATP depletion and secondary loss of ion gradients that drive the glutamate transporters; the result is extracellular accumulation of glutamate and excessive stimulation of glutamate receptors, which leads to neuronal death. Such over-stimulation of glutamate receptors caused by energy failure is typical even in hypoxia and ischemia, as discussed below.

Glutamate-mediated excitotoxicity contributes to ischemic neuronal death. Glutamate-mediated toxicity is a central event in several acute brain disorders. In stroke, cardiac arrest, drowning and pre- or perinatal hypoxia/ischemia the delivery of oxygen and glucose to the brain is impaired. As a result, ATP production and energy-dependent processes are inhibited and, most importantly, the maintenance of ion gradients across plasma, vesicular, mitochondrial and endoplasmatic membranes are dissipated. With respect to glutamate this situation means that the transporters of the plasma membrane, which are driven by the sodium gradient, reverse and let glutamate out into the extracellular space (Fig. 15-11; see Ch. 32). Transporter reversal occurs rapidly after inhibition of ATP formation, since the half-life of ATP in the brain is only a few seconds. Because of the lack of ATP, vesicular release of glutamate (an ATP-dependent process) probably does not contribute much to the high extracellular levels of glutamate during energy deficiency. Massive activation of glutamate receptors with influx of sodium and calcium ions follows from the high extracellular levels of glutamate. The influx of sodium, which secondarily causes influx of chloride and water, leads to intracellular edema, which may cause rupture of the cell membrane and lysis of the cell.

The influx of calcium causes a multitude of effects, although the many phosphorylation-dependent processes that are normally triggered by calcium influx must be inhibited during energy failure because of the rapid depletion of ATP and GTP. Calcium influx may cause formation of reactive oxygen species, such as NO through the activation of NO synthase and superoxide through the activation of xanthine oxidase or through the derangement of the electron transport chain of the mitochondria.

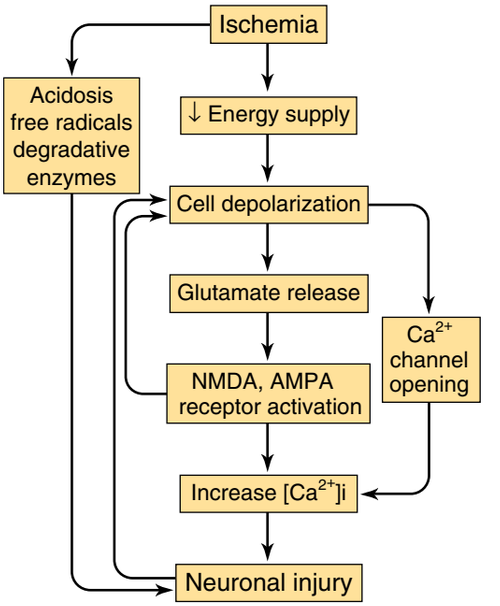

FIGURE 15-11 Potential paths leading to neuronal injury resulting from an episode of ischemic insult. An ischemic episode initiates a complex pathway involving the depletion of cellular energy stores and the release of free radicals. The energy depletion permits sustained activation of glutamate receptors and the consequent entry of Ca^{2+} via NMDA receptors, (GluR2-lacking) AMPA receptors and voltage-gated Ca^{2+} channels. Elevation of intracellular Ca^{2+} causes excessive activation of a variety of Ca^{2+}-dependent enzymes. Excessive neuronal depolarization results in neuronal injury and often cell death.

NO may react with superoxide to yield the highly reactive peroxynitrite, $ONOO^-$. Superoxide may also be converted into H_2O_2 and the reactive hydroxyl radical, •OH. In this way excessive activation of glutamate receptors leads to oxidative damage. The calcium influx has a major effect on mitochondria and causes them to depolarize and swell. This leads to a pore being formed in the outer mitochondrial membrane, which allows the escape of cytochrome c and procaspases from the mitochondria into the cytosol. Cytochrome c activates the caspase cascade, which leads to apoptotic cell death (Ch. 35).

Inhibition of glutamate receptors may ameliorate excitotoxicity. The important role of glutamate in ischemic and hypoxic brain damage is illustrated by the protective effect that antagonists of glutamate receptors have. In animal models of stroke antagonists of NMDA receptors or AMPA receptors reduce the volume of the infarct by approximately 50% and may be effective even when given several hours after the initial insult. Unfortunately, such antagonists have not yet proved useful in humans, partly because the infarct volumes tend to be greater and more variable in humans and the antagonists do not penetrate readily into the ischemic tissue, partly because some glutamate antagonists cause side effects, such as hypotension, ataxia and cognitive disturbances, that have sidelined their clinical development.

Ischemic tissue is acidotic because of accumulation of lactate and other acidic metabolites. A low pH is generally detrimental for brain tissue but should shut down certain splice variants of NMDA receptors, as described above. Interestingly, neuroprotective drugs in the ifenprodil class inhibit NMDA receptors by shifting the pK for the proton-accepting group in an alkaline direction, such that at physiological pH protons occupy a larger fraction of the proton sites and thus render a larger fraction of NMDA receptors unavailable for activation.

Epileptiform activity involves glutamate receptor activation. Involvement of excitatory amino acids in epilepsy is well documented. A large number of animal models of epilepsy have clearly implicated a central role for the glutamate receptor family (see Ch. 37). Excessive stimulation of glutamatergic pathways, block of glutamate transporters or pharmacological manipulation resulting in glutamate receptor activation can precipitate seizures. Epileptiform activity usually begins with excessive AMPA receptor activation; as seizure activity intensifies, an increased involvement of NMDA receptors is observed. Results of studies with a variety of animal models have shown that NMDA receptor antagonists can reduce the intensity and duration of seizure activity. Antagonism of AMPA receptor activation usually prevents initiation of the seizure. This suggests that epileptiform activity depends on the interplay between synaptic AMPA and NMDA receptors. Evidence from human tissue supports the role of glutamate receptors in epilepsy. For example, in patients with refractory complex partial seizure, epileptic hippocampal tissue shows an upregulation of AMPA and NMDA receptors. Moreover, prolonged opening of GluRs by an anti-Glu receptor antibody in Rasmussen's encephalitis produces uncontrollable seizures (see Ch. 37).

REFERENCES

1. Braitenberg, V. and Schüz, A. *Cortex: Statistics and Geometry of Neuronal Connectivity*, 2nd edn. Berlin, Springer, 1998.
2. Attwell, D. and Laughlin, S. B. An energy budget for signaling in the grey matter of the brain. *J. Cereb. Blood Flow Metab.* 21: 1133–1145, 2001.
3. Shen, J., Petersen, K. F., Behar, K. L. *et al.* Determination of the rate of the glutamate/glutamine cycle in the human brain by in vivo ^{13}C NMR. *Proc. Natl Acad. Sci. U.S.A.* 96: 8235–8240, 1999.
4. Broman, J., Hassel, B., Rinvik, E. and Ottersen, O. P. Biochemistry and anatomy of transmitter glutamate. In O. P. Ottersen and J. Storm-Mathisen (eds.) *Handbook of Chemical Neuroanatomy 18.* Elsevier, 2000, pp.1–44.
5. Baker, D. A., McFarland, K., Lake, R. W. *et al.* Neuroadaptations in cystine–glutamate exchange underlie cocaine relapse. *Nat. Neurosci.* 6: 743–749, 2003.
6. Fremeau, R. T. Jr, Voglmaier, S, Seal, R. P. and Edwards, R. H. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci.* 27: 98–103, 2004.
7. Dingledine, R., Borges, K., Bowie, D. and Traynelis, S, F. The glutamate receptor ion channels. *Pharmacol. Rev.* 51: 7–61, 1999
8. Skrede, K. K. and Malthe-Sørenssen, D. Increased resting and evoked release of transmitter following repetitive electrical tetanization in hippocampus: a biochemical correlate to long-lasting synaptic potentiation. *Brain Res.* 208: 436–441, 1981.
9. Hollmann, M., O'Shea-Greenfield, A., Rogers, S. W. and Heinemann, S. Cloning by functional expression of a member of the glutamate receptor family. *Nature* 342: 643–648, 1989.
10. Fletcher, E. J. and Lodge, D. New developments in the molecular pharmacology of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors. *Pharmacol. Ther.* 70: 65–89, 1996.
11. Kleckner, N. W. and Dingledine, R. Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. *Science* 241: 835–837, 1988.
12. Wolosker, H., Blackshaw, S. and Snyder, S. H. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-*N*-methyl-D-aspartate neurotransmission. *Proc. Natl Acad. Sci. U.S.A.* 96: 13409–13414, 1999.
13. Nowak, L., Bregestovski, P., Ascher, P. *et al.* Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* 307: 462–465, 1984.
14. Traynelis, S. F., Hartley, M. and Heinemann, S. F. Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science* 268: 873–876, 1995.
15. Ehlers, M.D., Zhang, S., Bernhardt, J. P. and Huganir, R. L. Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* 84: 745–755, 1996.
16. Tong, G., Shepard, D. and Jahr, C. E. Synaptic desensitization of NMDA receptors by calcineurin. *Science* 267: 1510–1512, 1995.
17. Bennett, J. A. and Dingledine, R. Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron* 14: 373–384, 1995.
18. Kuner, T., Wollmuth, L. P., Karlin, A., Seeburg, P. H. and Sakmann, B. Structure of the NMDA receptor channel M2 segment inferred from the accessibility of substituted cysteines. *Neuron* 17: 343–352, 1996.
19. Armstrong, N., Sun, Y., Chen, G. Q. and Gouaux, E. Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature* 395: 913–917, 1998.
20. Mayer, M. L. and Armstrong, N. Structure and function of glutamate receptor ion channels. *Ann. Rev. Physiol.* 66: 161–181, 2004.
21. Sommer, B., Keinanen, K., Verdoorn, T. A. *et al.* Flip and flop: a cell specific functional switch in glutamate-operated channels of the CNS. *Science* 249: 1580–1585, 1990.
22. Hume, R. I., Dingledine, R. and Heinemann, S. F. Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* 253: 1028–1032, 1991.
23. Higuchi, M., Single, F. N., Kohler, M. *et al.* RNA editing of AMPA receptor subunit GluRB: a base-paired intron-exon structure determines position and efficiency. *Cell* 75: 1361–1370, 1993.
24. Wollmuth, L. P., Kuner, T., Seeburg, P. H. and Sakmann, B. Differential contribution of the NR1- and NR2A-subunits

to the selectivity filter of recombinant NMDA receptor channels. *J. Physiol.* 491: 779–797, 1996.
25. Brusa, R.. Zimmermann, F., Koh, D. S. *et al.* Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. *Science.* 270: 1677–1680, 1995.
26. Jia, Z., Agopyan, N., Miu, P. *et al.* Enhanced LTP in mice deficient in the AMPA receptor GluR2. *Neuron* 17: 945–956, 1996.
27. Tsien, J. Z., Huerta, P. T. and Tonegawa, S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* 87: 1327–1338, 1996.
28. Conn, P. J. Physiological roles and therapeutic potential of metabotropic glutamate receptors. *Ann. N.Y. Acad. Sci.* 1003: 12–21, 2003.
29. Takahashi, T., Forsythe, I. D., Tsujimoto, T., Barnes-Davies, M. and Onodera, K. Presynaptic calcium current modulation by a metabotropic glutamate receptor. *Science* 274: 594–597, 1996.
30. Kano, M., Hashimoto, K., Kurihara, H. *et al.* Persistent multiple climbing fiber innervation of cerebellar Purkinje cells in mice lacking mGluR1. *Neuron* 18: 71–79, 1997.
31. Yokoi, M., Kobayashi, K., Manabe, T. *et al.* Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science* 273: 645–647, 1996.
32. Pekhletski, R., Gerlai, R., Overstreet, L. S. *et al.* Impaired cerebellar synaptic plasticity and motor performance in mice lacking the mGluR4 subtype of metabotropic glutamate receptor. *J. Neurosci.* 16: 6364–6373, 1996.
33. Kennedy, M. B. Signal-processing machines at the postsynaptic density. *Science* 290: 750–754, 2000.
34. Hering, H., Lin, C. C. and Sheng, M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *J. Neurosci.* 23: 3262–3271, 2003.
35. Kameyama, K., Lee, H. K., Bear, M. F. and Huganir, R. L. Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression. *Neuron* 21: 1163–1175, 1998.
36. Sala, C., Piech, V., Wilson, N. R. *et al.* Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31: 115–130, 2001.
37. Hong, S. J., Li, H., Becker, K. G., Dawson, V. L. and Dawson, T. M. Identification and analysis of plasticity-induced late-response genes. *Proc. Natl Acad. Sci. U.S.A.* 101: 2145–2150, 2004.
38. Krapivinsky, G., Krapivinsky, L., Manasian, Y. *et al.* The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron* 40: 775–784, 2003.
39. Esteban, J. A. AMPA receptor trafficking: a road map for synaptic plasticity. *Mol. Interv.* 3: 375–385, 2003
40. Si, K., Giustetto, M., Etkin, A. *et al.* A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. *Cell* 115: 893–904, 2003.
41. Passafaro, M., Nakagawa, T., Sala, C. and Sheng, M. Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. *Nature* 424: 677–681, 2003.
42. Kennedy, M. B., Beale, H. C., Carlisle, H. J., Washburn, L. R. Integration of biochemical signalling in spines. *Nat. Rev. Neurosci.* 6: 423–434, 2005.
43. Danbolt, N. C. Glutamate uptake. *Prog. Neurobiol.* 65: 1–105, 2001.
44. Voutsinos-Porche, B., Bonvento, G., Tanaka, K. *et al.* Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron* 37: 275–286, 2003.
45. Chaudhry, F. A., Schmitz, D., Reimer, R. J. *et al.* Glutamine uptake by neurons: interaction of protons with system a transporters. *J. Neurosci.* 22: 62–72, 2002.

CHAPTER

16

GABA and Glycine

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GABA AND GLYCINE ARE THE MAJOR RAPIDLY ACTING INHIBITORY NEUROTRANSMITTERS IN BRAIN 300

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS. It was discovered in 1950 by Roberts and Awapara. Electrophysiological studies between 1950 and 1965 suggested a role for GABA as a neurotransmitter in the mammalian CNS. Since then, GABA has met the five classical criteria for assignment as a neurotransmitter: it is present in the nerve terminal; it is released from electrically stimulated neurons; there is a mechanism for terminating the action of the released neurotransmitter; its application to target neurons mimics the action of inhibitory nerve stimulation; and specific receptors exist [1, 2].

In view of the ubiquitous nature of GABA in the CNS, it is perhaps not too surprising that its functional significance should be far-reaching. A growing body of evidence suggests a role for altered GABAergic function in neurological and psychiatric disorders of humans, primarily related to hyperexcitability. These include developmental malfunctions, mental retardation and epilepsy; sleep disorders; drug dependence, especially alcoholism; sensorimotor processing, including various types of learning, as well as schizophrenia; and motor coordination disorders such as tardive dyskinesia, Huntington's disease and Parkinson's disease. Pharmacological manipulation of GABAergic transmission is an effective approach for the treatment of anxiety [3–6]. In addition, it has been demonstrated that the nervous-system-depressant actions of the major clinical general anesthetics result from an enhancement of inhibitory synaptic transmission mediated by $GABA_A$ receptors [7, 8].

GABA SYNTHESIS, UPTAKE AND RELEASE

GABA is formed *in vivo* by a metabolic pathway referred to as the GABA shunt. The GABA shunt is a closed-loop process with the dual purpose of producing and conserving the supply of GABA. GABA is present in high

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concentrations (millimolar) in many brain regions. These concentrations are about 1,000 times higher than concentrations of the classical monoamine neurotransmitters in the same regions. This is in accord with the powerful, abundant, and specific actions of GABAergic neurons in these regions. Glucose is the principal precursor for GABA production *in vivo*, although pyruvate and other amino acids also can act as precursors. The first step in the GABA shunt is the transamination of α-ketoglutarate, formed from glucose metabolism in the Krebs cycle, by GABA: α-ketoglutarate transaminase (GABA-T, also known as. α-oxoglutarate-T) into L-glutamic acid [1, 2] (Fig. 16-1). Glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of glutamic acid to form GABA. GAD appears to be expressed only in cells that use GABA as a neurotransmitter. GAD, localized with antibodies or mRNA hybridization probes, serves as an excellent marker for GABAergic neurons in the CNS. Two related but different genes for GAD have been cloned, suggesting independent regulation and properties for the two forms of GAD: GAD_{65} and GAD_{67} [2]. GAD_{65} is localized to nerve endings where it synthesizes GABA for secretory synaptic vesicles; mouse knockouts for this isozyme show seizure susceptibility and impaired experience-modulated plasticity [9]. GAD_{67} appears to be associated with synthesis of nonvesicular GABA; GABA in this pool can be released from cells by reversal of the plasma membrane transport process [2]. Furthermore, expression of GAD and some GABA receptor subunits has been demonstrated in some non-neural tissues, indicating the likely function of GABA outside of the CNS. A mouse knockout of GAD_{67} shows normal brain activity but cleft palate [2].

GABA is metabolized by GABA-T to form succinic semialdehyde. To conserve the available supply of GABA, this transamination generally occurs when the initial parent compound, α-ketoglutarate, is present to accept the amino group removed from GABA, reforming glutamic acid (see also Ch. 15). Therefore, a molecule of GABA can be metabolized only if a molecule of precursor is formed. Succinic semialdehyde can be oxidized by succinic semialdehyde dehydrogenase (SSADH) into succinic acid and can then re-enter the Krebs cycle, completing the loop [1] (Fig. 16-1).

GABA release into the synaptic cleft is stimulated by depolarization of presynaptic neurons. GABA diffuses across the cleft to the target receptors on the postsynaptic surface. The action of GABA at the synapse is terminated by reuptake into both presynaptic nerve terminals and surrounding glial cells. The plasmalemma transport system mediating reuptake of GABA is both temperature- and ion-dependent. These transporter molecules are capable of bidirectional neurotransmitter transport. They have an absolute requirement for extracellular Na^+ ions with an additional dependence on Cl^- ions (see also Ch. 5). The ability of the reuptake system to transport GABA against a concentration gradient has been demonstrated using synaptosomes. Under normal physiological conditions, the ratio of internal to external GABA is about 200. The driving force for this reuptake process is supplied by the movement of Na^+ down its concentration gradient [2, 10] (see Ch. 5). GABA taken back up into nerve terminals is available for reutilization, but GABA in glia is metabolized to succinic semialdehyde by GABA:T and cannot be resynthesized from glutamate in this compartment, since glia lack GAD. Ultimately, GABA can be recovered from this source by a circuitous route involving the Krebs cycle [1]; GABA in glia is converted to glutamine, which is transferred back to the neuron, where

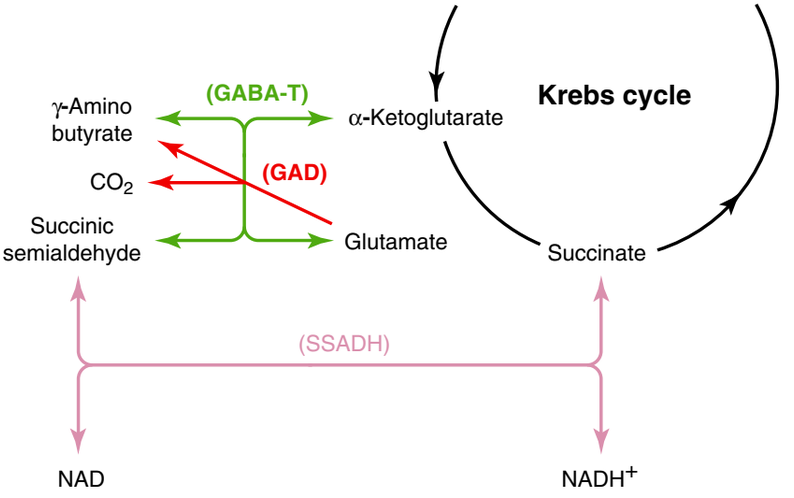

FIGURE 16-1 GABA shunt. These reactions, which participate in the synthesis and metabolism of GABA, are called the 'GABA shunt' because the amount of α-ketoglutarate that is metabolized via these reactions to succinate is shunted around the succinic dehydrogenase step of the TCA cycle and thus decreases the formation of succinyl-CoA. GAD produces GABA and CO_2 from glutamate (*red arrows*). GABA-T removes GABA by converting it to succinic semialdehyde (*SSA*) via transamination with α-ketoglutarate which re-forms glutamate (*green arrows*). SSA is oxidized by NAD and SSA dehydrogenase (*lavender arrows*) to $NADH^+$ and succinate, which re-enters the tricarboxylic cycle.

glutamine is converted by glutaminase to glutamate. The latter re-enters the GABA shunt (see further details in Chs 15 and 31).

The family of GABA plasmalemma transporters is a set of 80 kDa glycoproteins with 12 transmembrane regions; they have no sequence homology with GABA receptors but helped to establish the superfamily of sodium-dependent neurotransmitter transporters. Pharmacological and kinetic studies have suggested a variety of subtypes, and four separate but related entities have been demonstrated by molecular cloning [10]. This has led to rapid developments in understanding the localization, pharmacological specificity, structure-function and mechanism of GABA transport across the cell membrane [10, 11]. Separate proteins with 10 membrane-spanning regions are involved in active transport of GABA from the cytosol into secretory vesicles inside the cell, a mechanism energized by an ATP-dependent proton electrochemical gradient, independent of Na^+ [2, 12] (see also Ch. 5).

GABA RECEPTOR PHYSIOLOGY AND PHARMACOLOGY

GABA receptors have been identified electrophysiologically and pharmacologically in all regions of the brain. Because GABA is widely distributed and utilized throughout the CNS, early GABAergic drugs had very generalized effects on CNS function. The development of more selective agents has led to the identification of at least two distinct classes of GABA receptor, $GABA_A$ and $GABA_B$. They differ in their pharmacological, electrophysiological and biochemical properties. Like the receptors for acetylcholine and glutamate, receptors for GABA employ both ligand-gated ion channels and G-protein-coupled receptors (discussed in Ch. 19). Electrophysiological studies of the $GABA_A$-receptor complex indicate that it mediates an increase in membrane conductance with an equilibrium potential near the resting level of −70 mV. This conductance increase often is accompanied by a membrane hyperpolarization, resulting in an increase in the firing threshold and, consequently, a reduction in the probability of action potential initiation, causing neuronal inhibition. This reduction in membrane resistance is accomplished by the GABA-dependent facilitation of Cl^- ion influx through a receptor-associated channel. On the other hand, increased Cl^- permeability can depolarize the target cell under some conditions of high intracellular Cl^-. This in turn potentially can excite the cell to fire or to activate Ca^{2+} entry via voltage-gated channels and has been proposed as a physiologically relevant event, especially in embryonic neurons [3] (see also Ch. 6).

Electrophysiological data suggest that there are two GABA-recognition sites per $GABA_A$-receptor complex [13]. An increase in the concentration of GABA results in an increase in the mean channel open time because of opening of doubly liganded receptor forms, which exhibit open states of long duration. It has been demonstrated that the increase in the ionic permeability of the $GABA_A$ receptor complex is transient in the continuing presence of agonist [13]. This phenomenon is known as desensitization and is rapidly reversible. The molecular mechanism of desensitization is not understood, and various hypotheses remain under investigation.

STRUCTURE AND FUNCTION OF GABA RECEPTORS

$GABA_B$ receptors are coupled to G proteins and a variety of effectors. $GABA_B$ receptors were identified by their insensitivity to the $GABA_A$ antagonist bicuculline and certain $GABA_A$-specific agonists [2–4, 13]. The GABA analog (−) baclofen (β-(4-chloro-phenyl)-γ-aminobutyric acid) was found to be a potent and selective $GABA_B$ agonist. $GABA_B$ receptors [14] are members of the group III G-protein-coupled receptors (Ch. 19), with structural similarity to the metabotropic glutamate receptors.

$GABA_B$ receptors can mediate both postsynaptic and presynaptic inhibition, through coupling to a variety of effector systems [14]. Some $GABA_B$ receptors are coupled to activation of certain K^+ channels, producing slow inhibitory synaptic currents. Other $GABA_B$ receptors can decrease Ca^{2+} conductance, regulate inositol trisphosphate production (Ch. 20) and/or inhibit cAMP production (Ch. 21). Presynaptic inhibition may occur as a result of $GABA_B$ receptors on nerve terminals causing a decrease in the influx of Ca^{2+}, and thereby reducing the release of neurotransmitters (Chs10 and 22). These responses may have a net excitatory effect when inhibiting GABA nerve ending release (autoreceptors) or may inhibit the release of other neurotransmitters including glutamate, having a net inhibitory effect.

$GABA_B$ receptors are heterodimers. Two $GABA_B$ receptor subunits have been cloned, R1 and R2. Neither of these appears to express functional receptors on their own, but they are active when coexpressed, suggesting that a dimer is trafficked to the cell surface and forms an active complex. Evidence shows that the R1 subunit contains the GABA binding site while the R2 subunit interacts with the G protein [14].

$GABA_A$ receptors are chloride channels and members of a superfamily of ligand-gated ion channel receptors. The $GABA_A$ receptor is a pseudo-symmetric pentamer spanning the membrane and forming a chloride ion channel (Figs 16-2, 16-3). The binding of GABA or of structural analogs with agonist activity opens the chloride ion channel [3, 4, 13]. $GABA_A$ receptors have structural and functional homology with certain other

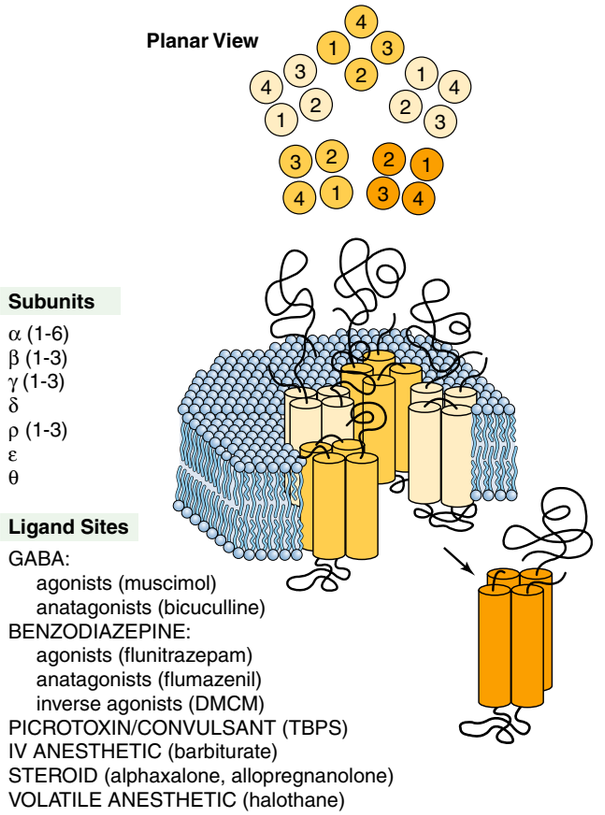

FIGURE 16-2 Schematic of $GABA_A$ receptors. (**Lower panel**) The protein is shown as a pseudosymmetrical membrane-spanning ion channel protein made of five homologous subunits, each of which has four membrane-spanning regions (MSRs, numbered 1–4) as shown in one of the subunits that is pulled out. (**Upper panel**) The view from outside the cell (plan view) shows the arrangement of the five subunits (each with four MSRs) around the central core, the chloride ion channel. Also listed are the subunit families that can be utilized in composing each receptor, and the ligands that have binding sites on the receptor (see also Fig. 16-3).

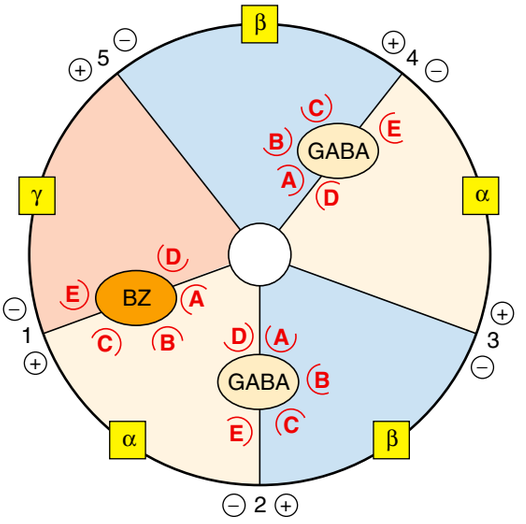

FIGURE 16-3 Donut model of $GABA_A$ receptor heteropentamer showing ligand binding and subunit interaction domains. The five subunits (*–β–γ–α–β–α–*) and five subunit interfaces (*1–5*) are shown, indicating the two different subunit interfaces for each subunit ((–) and (+)), the ligand binding pockets for GABA and benzodiazepines (*BZ*), and the various peptide loops (*A–E*) involved in ligand binding and subunit interfaces. These loops are homologous in other members of the ligand-gated ion channel superfamily, including the glycine receptor. The pore in the center represents the chloride channel.

Cys-loop-containing ligand-gated ion channel receptors, and their subunits have sequence homology, thus defining a gene superfamily. This family includes the nicotinic acetylcholine receptor (Ch. 11), the strychnine-sensitive glycine receptor (see below), and the serotonergic 5-HT_3 receptor (Ch. 13). The superfamily is also sometimes called the Cys-loop family, to distinguish it from the ionotropic excitatory amino acid receptors [4].

Each $GABA_A$ receptor subunit contains four putative α-helical membrane-spanning domains (M1–M4) with a predominantly hydrophobic character. One or more membrane-spanning regions from each subunit, principally M2, form the walls of the channel pore (see also Ch. 11). The sequences of these transmembrane segments are highly conserved between the subunits of the $GABA_A$ receptor as well as between members of the gene superfamily. The region between M3 and M4 contains a long, variable, intracellular domain. This contributes to the subtype specificity and may participate in intracellular regulatory mechanisms such as phosphorylation and interaction with other cellular constituents [2–4].

A family of pentameric $GABA_A$-receptor protein subtypes exists. The $GABA_A$ receptor was first cloned using partial protein sequence, and verification of these cDNAs as GABA-receptor subunits was made by expression in *Xenopus* oocytes of GABA-activated channels [2–4, 13]. According to current understanding of the molecular structure of the $GABA_A$ receptor-ionophore complex, it is a heteropentameric glycoprotein of about 275 kDa composed of combinations of at least 19 different but closely related polypeptides. The subunits are 50–60 kDa and have 20–30% sequence identity between classes and about 70% identity within a class: α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3 (Fig. 16-2). The mammalian ε and θ subunits probably correspond to γ4 and β4 identified in birds. This suggests that the genes probably evolved from a common ancestral sequence; the evolutionary relationships are shown in the dendrogram of Figure 16-4 [4, 5, 15]. In addition, splice variants exist for several of the subunits.

Differential distribution of $GABA_A$-receptor-subunit mRNAs and polypeptides in brain is consistent with data indicating regional variations in physiological function, pharmacology and biochemistry. It seems clear that different combinations with differing pharmacologic properties and conductances are expressed in different neuronal populations, and even different membrane locations in a given cell. The subunit composition of native isoforms

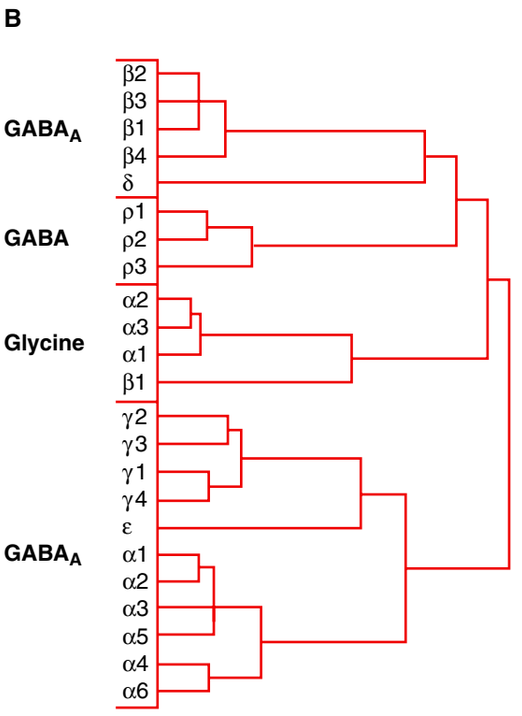

FIGURE 16-4 Evolutionary relationships between DNA sequences of $GABA_A$ receptors and glycine receptors. The dendrogram is based on literature data for human cDNAs, provided by Tim Hales and Ewen Kirkness, analyzed by Ziwei Chen, Kui Yang and Richard Olsen.

has been deduced by a combination of techniques: determining which polypeptides are present in a given cell, finding which ones can be isolated together as an oligomer by using subunit-specific antibodies, and analyzing which pharmacological properties can be reconstituted from recombinant subunits of known combinations. Table 16-1 summarizes data on the most abundant isoforms identified, their localization and their unique pharmacological properties [2–5, 13, 15]. The ρ subunits are expressed primarily, if not exclusively, in the retina, where they appear to form Cl^- channels, possibly homomers, with novel pharmacology. Pharmacological responses to GABA that are insensitive to both bicuculline and baclofen (non-A, non-B) are attributed by some authors to receptors termed $GABA_C$ [16]. Some, but not all, of these responses are produced by the ρ subunit, which is also insensitive to the usual $GABA_A$ modulatory drugs such as benzodiazepines and anesthetics [2, 4, 16].

Proteins associated with the intracellular loop of $GABA_A$ receptor subunits participate in the trafficking of receptors from Golgi membranes to the plasmalemma, the concentrating of receptors at synaptic sites, and the production of changes in subcellular localization and density associated with plasticity. Such changes in plasticity include endocytotic removal of receptors from the plasmalemma following long-term ligand exposure [17–19] (see details of relevant topics of trafficking, endocytosis and plasticity in Chs 9, 30 and 53). Proper direction of receptors to specific cell surface loci and specific synapses requires the γ2 subunit and the microtubule linker-protein, GABARAP, which is, however, not a synaptic anchoring protein. The protein gephyrin, isolated with glycine receptors (see below), colocalizes with synaptic $GABA_A$ receptors and appears to have an anchoring function. The construction of synapses and remodeling during experience is currently an intensive area of research. Other subunits may substitute for γ2 in pentamers; for example, the δ subunit forms receptors that are excluded from synapses. Extrasynaptic receptors appear to play a functional role in mediating tonic inhibition, responding to spillover of synaptically released transmitter or even to ambient extracellular levels, depending on the location. They also appear to be the major target of action of certain modulatory drugs (see below).

Phosphorylation sites for one or more kinases are present on virtually all of the subunits. Phosphorylation of the β subunits by cAMP-dependent protein kinase (PKA) and phosphorylation of β and γ subunits by protein kinase C and tyrosine kinase have been reported [2, 17]. Current studies are directed toward an understanding of the functional consequences of phosphorylation of

TABLE 16-1 Summary of native $GABA_A$ receptor subtypes

Subunit composition	Location	Function	Comments
α1β2γ2	Widespread, esp. GABA neurons	Sedation, anticonvulsant	Adult, BZ-sensitive Reduced in drug tolerance?
α2β3γ2	Forebrain, spinal cord	Anxiety, muscle relaxant	Axon hillock in some cells, BZ-sensitive Embryonic and adult, BZ-sensitive
α2β1γ1	Glia		
α3β3γ2	Cortex	Anticonvulsant	Embryonic and adult, BZ-sensitive
α4β2γ2	Thalamus		Insensitive to agonist BZ
α4β2/3γ2	Dentate gyrus		Elevated in drug withdrawal?
α4β2δ	Thalamus	Tonic inhibition	Extrasynaptic, BZ- insensitive, adult
α4β2/3δ	Dentate gyrus		
α5β3γ2	Hippocampus CA1 Sensory ganglia	Tonic inhibition	Extrasynaptic, BZ-sensitive
α6β2/3γ2	Cerebellar granule cells		Insensitive to agonist BZ
α6β2/3δ	Cerebellar granule cells	Tonic inhibition	Extrasynaptic, BZ-insensitive, adult
γ3, θ, ε	Little information		

$GABA_A$ receptors for both acute and more prolonged time frames (see also Chs 21 and 24).

The $GABA_A$ receptor is the major molecular target for the action of many drugs in the brain. The $GABA_A$ receptor-chloride ion channel complex includes five major types of ligand-binding domain. Two copies of the GABA-binding site are present at α/β subunit interfaces (Fig. 16–3). One of the most useful agonists is the compound muscimol, a naturally occurring GABA analog isolated from the psychoactive mushroom *Amanita muscaria*. It is a potent and specific agonist at $GABA_A$ receptors and has been a valuable tool for pharmacological and radioligand-binding studies (Fig. 16–2). Other GABA agonists include isoguvacine, 4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridin-3-ol (THIP), 3-aminopropane-sulfonate and imidazoleacetic acid. The classical $GABA_A$-receptor antagonist is the convulsant bicuculline, which reduces current by decreasing the opening frequency and mean open time of the channel [2, 13]. It is likely that bicuculline produces its antagonistic effects on $GABA_A$ receptor currents by competing with GABA for its binding sites. On the other hand, the convulsant picrotoxin binds to separate sites that block the chloride channel.

The GABA-gated chloride ion channel is modulated by several classes of drugs that bind to allosteric sites on the receptor complex: the benzodiazepines, barbiturates and related intravenous general anesthetics such as etomidate and propofol, as well as anesthetic steroids and endogenous neurosteroids. It appears that some types of $GABA_A$ receptor are directly enhanced by ethanol and volatile general anesthetics (Fig. 16-2) [7, 8, 20].

Benzodiazepine receptor-binding sites copurify with the GABA-binding sites, including co-immunoprecipitation with antibodies that were developed to recognize the protein containing the GABA-binding site [2, 3, 13, 15]. This indicates that the benzodiazepine receptor is an integral part of the $GABA_A$ receptor–Cl^- channel complex (Fig. 16-3). Benzodiazepine agonists represent the currently most useful group of agents in the general class of depressant drugs, which also includes barbiturates, that show anticonvulsant, anxiolytic and sedative-hypnotic activity (Chs 37 and 55). Well-known examples include diazepam and chlordiazepoxide, which often are prescribed for their antianxiety effects [3–5]. The mechanism of action of benzodiazepine agonists is to enhance GABAergic transmission. From electrophysiological studies, it is known that these benzodiazepines increase the frequency of channel opening in response to GABA, thus accounting for their pharmacological and therapeutic actions [2, 6, 13]. In addition, the benzodiazepine site is coupled allosterically to the binding sites for the other modulatory ligands, such as barbiturate, steroid, anesthetic and picrotoxin [2]. $GABA_A$ receptors show heterogeneity with respect to certain benzodiazepine-site ligands [4–6]. A wide variety of nonbenzodiazepines, such as the β-carbolines, cyclopyrrolones and imidazopyridines, also bind to the benzodiazepine site.

Barbiturates comprise another class of drugs commonly used therapeutically for anesthesia and control of epilepsy (Ch. 37). Phenobarbital and pentobarbital are two of the most commonly used barbiturates. Phenobarbital has been used to treat patients with epilepsy since 1912. Pentobarbital is also an anticonvulsant but is sedative at the effective concentration. Barbiturates at pharmacological concentrations allosterically increase binding of benzodiazepines and GABA to their respective binding sites [2]. Measurements of mean channel open times show that barbiturates act by increasing the proportion of channels opening to the longest open state (9 ms) while reducing the proportion opening to the shorter open states (1 and 3 ms), resulting in an overall increase in mean channel open time and Cl^- flux [13].

Channel blockers, such as the convulsant compound picrotoxin, cause a decrease in mean channel open time. Picrotoxin works by preferentially shifting opening channels to the briefest open state (1 ms). Thus, both picrotoxin and barbiturates appear to act on the gating process of the $GABA_A$ receptor channel, but their effects on the open states are opposite to each other. Experimental convulsants such as pentylenetetrazol and the cage convulsant t-butyl bicyclophosphorothionate (TBPS) act in a manner similar to picrotoxin, preventing Cl^- channel permeability. The antibiotic penicillin is a channel blocker with a net negative charge. It blocks the channel by interacting with the positively charged amino acid residues within the channel pore, consequently occluding Cl^- passage through the channel [2, 13].

There have been numerous studies on the role of $GABA_A$ receptors in anesthesia. A considerable amount of evidence has been compiled to suggest that general anesthetics, including barbiturates, volatile gases, steroids and alcohols, enhance GABA-mediated Cl^- conductance. A proper assessment of this phenomenon requires not only a behavioral assay of anesthesia but also *in vitro* models for the study of receptor function. In this regard, not only electrophysiological methods but also neurochemical measurements of Cl^- flux and ligand binding have been useful. For example, a strong positive correlation exists between anesthetic potencies and the stimulation of GABA-mediated Cl^- uptake. This is seen with barbiturates and anesthetics in other chemical classes [21].

Comparison of ligand-gated ion channels that vary in sensitivity to anesthetic modulation, using the chimera and site-directed mutagenesis approach, has identified amino acids in the membrane-spanning domains that are critical for anesthetic sensitivity [8]. Direct evidence of ethanol augmentation of $GABA_A$ receptor function, measured either by electrophysiological techniques or agonist-mediated Cl^- flux, has been reported [8, 20]. The similarity between the actions of ethanol and sedative drugs such as benzodiazepines and barbiturates that enhance GABA action suggests that ethanol may exert some of its effects by enhancing the function of $GABA_A$ receptors. Ethanol potentiation of $GABA_A$ receptor function appears to be dependent upon the cell type tested and

the method of assay. This suggests that the ethanol interaction may be an indirect action, or specific for certain receptor subtypes [2, 8, 20].

Neurosteroids, which may be physiological modulators of brain activity, enhance $GABA_A$ receptor function. This enhancement by steroids involves direct action on the membrane receptor protein rather than through the classical genomic mechanism mediated by soluble high-affinity cytoplasmic steroid hormone receptors (see Ch. 52). Chemically reduced analogs of the hormones progesterone and corticosterone derivatives administered to animals and humans exert sedative–hypnotic and anti-anxiety effects. This led to the development of a synthetic steroid anesthetic, alphaxalone. These neuroactive steroids are potent modulators of $GABA_A$-receptor function *in vitro* [8, 13, 22]. Neurosteroids can be produced in the brain endogenously and may influence CNS function under certain physiological or pathological conditions. Numerous observations suggest that neurosteroids physiologically affect the CNS: these include the rapid behavioral effects of administered steroids; diurnal and estrous cycle effects on behavior; sex-specific pharmacology, especially of GABAergic drugs; and the development of withdrawal symptoms following cessation of chronically administered steroids. Neuroactive steroids have effects similar to those of barbiturates in that they enhance agonist binding to the GABA site and allosterically modulate benzodiazepine and TBPS binding [2, 3, 13, 22]. Also, like barbiturates, high concentrations of neurosteroids directly activate the $GABA_A$ receptor Cl^- channel. These observations led to the hypothesis that the neurosteroid-binding site may be similar to the barbiturate site but the sites of action for the two classes of drugs are clearly not identical [2].

The three-dimensional structures of ligand-gated ion channel receptors are being modeled successfully. Recent remarkable progress in structural definition of the nicotinic acetylcholine receptor, both the extracellular domain and the membrane-spanning domains (see Ch. 11), are beginning, in turn, to allow modeling of the $GABA_A$ receptor. This will be helpful in suggesting mechanisms whereby GABA binding can gate the chloride ion channel, and the action of modulatory ligands. Photoaffinity-labeling and site-directed mutagenesis of the $GABA_A$ receptors suggest that the binding sites for benzodiazepines are localized at the interface of the α and γ subunits and that those for GABA ligands are located at the interface between the α and β subunits [13, 15]. The binding pockets for each class of ligand appear to be formed from several loops of amino acids (Fig. 16-3). These models are consistent with studies on recombinant $GABA_A$ receptors expressed in heterologous cells. Such studies show that the nature of the α and β subunits determines the pharmacological specificity at the GABA and benzodiazepine sites and that the γ subunits are necessary for sensitivity to benzodiazepines and insensitivity to Zn^{2+} inhibition [3–5, 13, 15]. $GABA_A$ receptors come in five flavors with respect to benzodiazepines: combinations including the α1 subunit have a high affinity for certain 'type 1-selective' benzodiazepine site ligands, while those with the α2, α3 and α5 subunits have moderate affinity. The α5 subunit has a unique specificity to bind most benzodiazepine ligands but not the sedative drug zolpidem [4–6]. Some $GABA_A$ receptors apparently lack benzodiazepine-binding sites altogether or have a novel pharmacological profile at this site. Subunit combinations containing the α4 or α6 subunit with a γ subunit bind benzodiazepine inverse agonists but not agonists and are moderately sensitive to Zn^{2+} and neurosteroids. Combinations containing α4 or α6 with a δ subunit instead of a γ subunit do not bind benzodiazepine-site ligands, are highly sensitive to Zn^{2+} and are relatively more sensitive to neurosteroids and possibly other modulators such as ethanol [5, 6, 8, 20, 22]. This implicates the extrasynaptic $GABA_A$ receptors in the action of endogenous neurosteroids, general anesthetics and alcohol, and suggests a role for these receptors in tonic inhibition.

Mouse genetics reveal important functions for $GABA_A$ receptor subtypes. The use of mouse genetics has given tremendous insights into the functions of various subtypes of $GABA_A$ receptors. Gene targeting (knockouts) for several subunits reveal important phenotypic traits resulting from the loss of the receptor isoform. Most striking has been the relatively benign nature of phenotypes in mice lacking major $GABA_A$ receptor subunits, including α1 and β2, as well as α6. The γ2 and β3 knockouts are neonatally lethal, indicating important functions. The α5 and δ knockout phenotypes are more subtle. Mice lacking δ become insensitive to neurosteroids, while mice lacking α5 show improved performance in hippocampus-dependent spatial memory acquisition [2, 5, 6] (see also Ch. 53).

Further, the removal of benzodiazepine sensitivity in a selective α subunit in a mouse using the gene knockin technique has established that the α1 subunit plays a major role in the sedative and amnesiac effects of benzodiazepines, part of the anticonvulsant effect and little of the anxiolytic effect; the latter effects are more importantly mediated by the α2 subunit [5, 6]. The β subunit selectivity for the drugs loreclezole (an anxiolytic) and etomidate (an anesthetic) allowed determination that a single residue in the M2 domain could account for this selectivity (β2 = β3 >β1). When a mouse knockin selectively removed the etomidate sensitivity of the β2 subunit, the animals showed reduced sensitivity to sedative effects of etomidate but no reduction of the true anesthetic effects. In contrast, mutation of the β3 subunit to negate etomidate sensitivity of that subunit alone resulted in a mouse with no sensitivity to the anesthesia produced by etomidate. This proved that the GABA receptor is the target of at least this one anesthetic (etomidate) and, furthermore, that the specific locations in the brain of β3 subunits are important for anesthetic action, while the

corresponding β2 subunits, even when insensitive to this drug, did not produce a loss of anesthetic action in the organism [5–7].

GLYCINE AS A CENTRAL NEUROTRANSMITTER: GLYCINE SYNTHESIS, UPTAKE AND DEGRADATION

It was first proposed in 1965 that glycine acts as a neurotransmitter in mammalian spinal cord, and since then glycine has been demonstrated to meet all of the criteria for that designation. Glycine is widely recognized as a major inhibitory neurotransmitter in the vertebrate CNS, especially the spinal cord and brainstem, where it is crucial for the regulation of motoneuron activity [1]. In addition, glycinergic interneurons are found in the retina, the auditory system and other areas involved in the processing of sensory information. Like GABA, glycine inhibits neuronal firing by gating Cl^- channels but with a characteristically different pharmacology.

The immediate precursor of glycine is serine, which is converted to glycine by the activity of the enzyme serine hydroxymethyltransferase (SHMT) (see Fig. 40-3). Glycine is packaged into synaptic vesicles by the H^+-dependent vesicular inhibitory amino acid transporter (VIAAT or vGAT), which also transports GABA [12] (see Ch. 5). As for GABA, Ca^{2+}-dependent release of glycine and specific postsynaptic glycine receptors have been rigorously demonstrated. The postsynaptic action of glycine is terminated by its reuptake via high-affinity plasmalemma transporter systems located in glycinergic nerve terminals and glial cells. Molecular cloning has identified two glycine transporter genes, *glyT1* and *glyT2*, which are members of the Na^+/Cl^--dependent transporter superfamily [10, 23]. GlyT1 is abundantly expressed in astrocytes throughout the CNS, whereas GlyT2 is highly localized in glycine-releasing nerve terminals of spinal cord and brain stem. Both glycine transporters differ in their transport stoichiometries and substrate affinities [24] and appear to have different roles at glycinergic synapses. GlyT1 catalyzes the removal of glycine from postsynaptic glycine receptors, whereas GlyT2 is essential for replenishing the presynaptic pool of glycine from which synaptic vesicles are reloaded with neurotransmitter [23]. GlyT1 may in addition regulate glycine levels at excitatory NMDA receptors (see below), and selective GlyT1 inhibitors may therefore prove useful in the treatment of diseases associated with impaired glutamatergic transmission, such as schizophrenia [23]. Glycine is degraded intracellularly by the glycine cleavage system (see Fig. 40-3), a multienzyme complex composed of four different proteins, which, in the CNS, appears to be primarily localized in astrocytes [25]. Mutations in the glycine cleavage system cause nonketotic hyperglycinemia, a disease characterized by severe mental retardation ('glycine encephalopathy') (discussed in Ch. 40).

GLYCINE RECEPTOR PHYSIOLOGY AND PHARMACOLOGY

A number of amino acids can activate, to varying degrees, the inhibitory glycine receptor. The amino acids that can activate the glycine receptor include β-alanine, taurine, L-alanine, l-serine and proline. GABA is largely inactive at this receptor [1, 26]. There are only few known high-affinity antagonists of the inhibitory glycine receptors. They include the plant alkaloid strychnine, which is highly selective for the glycine receptor, and the amidine steroid RU 5135, which is less selective. Both compounds bind to the glycine receptor with nanomolar affinities. In addition, different low-affinity antagonists exist [27]. The binding sites for glycine and competitive antagonists like strychnine are overlapping but not identical. Current findings indicate that up to three molecules of glycine are required to activate the glycine receptor. This high cooperativity may reflect the low binding energy of a ligand as small as glycine.

As for the $GABA_A$ receptor, positive modulators that enhance glycine receptor activity have been identified. These include alcohols, neurosteroids, tropeines and the divalent metal ion Zn^{2+}, which is highly enriched in some types of excitatory neuron [27]. Zn^{2+} release from such neurons may potentiate glycine receptors at neighboring inhibitory synapses and thus facilitate inhibition following strong excitation.

Glycine is inhibitory on ligand-gated, strychnine-sensitive Cl^- channel receptors but excitatory on *N*-methyl-D-aspartate receptors. Although acting as a classical neurotransmitter at inhibitory ion channel receptors, glycine is also an activating ligand at a class of excitatory ion channel receptors, the *N*-methyl-D-aspartate (NMDA) receptors (see Ch. 15). Different investigators have shown that glycine is an essential coagonist of the NMDA receptor that is required for channel activation in addition to glutamate, and that glycine is normally present in the extracellular space at the required concentrations. Regulation of NMDA receptor activity via the release and/or reuptake of glycine may be important for allowing cross-talk between inhibitory and excitatory synapses in different regions of the CNS [28]. It appears curious that common amino acids like glycine and glutamate, which have other roles in metabolism, are employed as signaling molecules in the nervous system and that glycine is utilized as both an inhibitory and an excitatory neurotransmitter. However, sequence analysis indicates that receptors for signaling molecules evolved from prokaryotic proteins utilized for binding and recognition of nutrients in the environment, including glycine, glutamate and GABA. GABA, a carbon- and nitrogen-storage

molecule in plants and algae, appears to act as a colony-stimulating factor for abalone, an invertebrate mollusk. Thus, amino acids may have been used as informational molecules already early in evolution.

GLYCINE RECEPTORS: STRUCTURE, PATHOLOGY AND LOCALIZATION

Glycine receptors belong to the same gene superfamily as the $GABA_A$ receptor. The native glycine receptor is a macromolecular complex of about 250 kDa composed of a combination of homologous polypeptides identified as α (48 kDa) and β (58 kDa), which form a quasisymmetrical pentameric arrangement around a central ion pore [26, 27]. There is approximately 50% amino acid sequence identity between the α and β subunits; a comparatively high homology to $GABA_A$ receptor proteins is also evident (Fig. 16-4). In addition, a 93 kDa polypeptide named gephyrin copurifies with the glycine receptor. Photoaffinity labeling of the glycine receptor has indicated that binding sites for glycine and strychnine are found on the α subunit. The α and β subunits span the postsynaptic membrane and are glycosylated. Like the $GABA_A$ receptor and nicotinic acetylcholine receptor subunits, glycine receptor subunits have four hydrophobic segments, M1–M4, which may span the lipid bilayer as α-helices (Fig. 16-2) [27].

Unlike the α and β subunits, the glycine receptor associated protein gephyrin is a highly hydrophilic polypeptide that is cytoplasmically localized at postsynaptic membranes. Gephyrin anchors the glycine receptor in the postsynaptic membrane by attaching it to cytoskeletal elements [18] (Fig. 16-5). Such a role appears analogous to that of the ankyrin family of proteins, which restrict lateral mobility of many membrane proteins, including transporters and channels. The 43 kDa protein rapsyn appears to play this cytoskeleton-linking role with acetylcholine receptors (see Ch. 11); gephyrin or related proteins are also associated with $GABA_A$ receptors. Antisense treatment and gene knockout indicate that gephyrin is essential for the clustering of glycine receptors and certain $GABA_A$ receptor subtypes at developing inhibitory synapses. In addition, in peripheral organs, gephyrin has an enzymatic function in the biosynthesis of the molybdenum cofactor, a coenzyme of oxidoreductases.

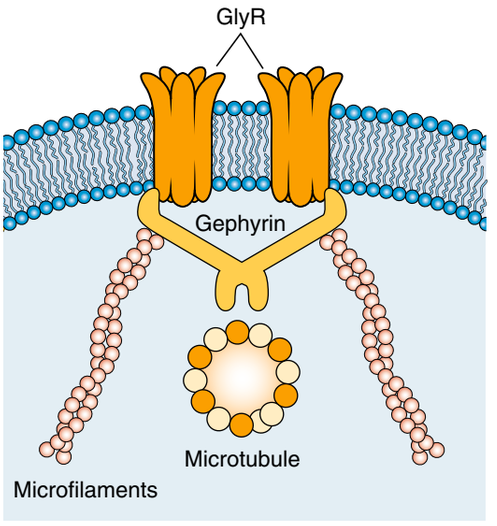

FIGURE 16-5 Model of gephyrin-dependent glycine receptor (GlyR). Trafficking/anchoring. Gephyrin (*light orange*) links the GlyR in the membrane to the intracellular cytoskeleton (microtubules and microfilaments) for trafficking to the surface, clustering at synapses and dispersion of clusters during plasticity.

Site-directed mutagenesis studies of glycine receptor subunits have led to a greater understanding of domains within the ligand-gated ion channel polypeptides that participate in pentamer assembly and interaction with the cytoskeleton, as well as the agonist-binding pocket and ion channel domains [26, 27]. For example, α subunits can produce functional homopentameric channels in recombinant expression systems, while β subunits cannot. The α homomeric glycine receptors are sensitive to the $GABA_A$ antagonist picrotoxin and may represent extrasynaptically located receptors. Heteromeric αβ GlyRs are insensitive to picrotoxin and are clustered at synaptic sites. Mutagenesis of certain amino acid residues in the extracellular domain of β subunits, making them similar to α subunits, confers the ability to assemble into homomeric channels. Mutagenesis of other amino acid residues that differ between α and β subunits has identified domains involved in binding glycine and Zn^{2+}, a metal ion that modulates glycine receptor function [27].

Glycine receptors are mutated in hyperekplexia and may co-localize with $GABA_A$ receptors. Currently, four α subunit genes, but only one β gene, have been identified in vertebrates [27]. The α2 (and α4) subunits are primarily expressed neonatally, whereas α1 and α3 are found primarily postnatally. Functional expression of the α_1–α_4 transcripts in frog oocytes generates glycine-gated Cl^- channels that are blocked by nanomolar concentrations of strychnine. Mutations of the α_1 and β subunits, in particular, substitutions by leucine or glutamine of an arginine residue located at position 271 of the α1 polypeptide, have been associated with hyperekplexia, a rare neurological disease characterized by an exaggerated startle response. Mutations in both α and β subunits have also been implicated in single-gene mutations in mice that show neurologically distinct phenotypes [26, 27, 29].

Immunocytochemical mapping with monoclonal antibodies specific for the α subunits reveals glycine receptor immunoreactivity in regions that also bind [^{3}H]strychnine in autoradiographic studies. Although most glycine receptors are found in spinal cord and brainstem, a small but significant population is located in more rostral brain regions. Interestingly, β subunit mRNA is abundant in many brain structures and even non-neural tissues, where neither [^{3}H]strychnine binding nor known α subunit

mRNAs are found. Thus, glycine receptor proteins may have yet unknown functions.

Ultrastructural and electrophysiological studies indicate that glycine and GABA may be co-utilized as neurotransmitters at distinct subsets of inhibitory synapses. Immunoelectron microscopy revealed glycine receptor antigen apposed to nerve terminals that contain the GABA synthesizing enzyme GAD, which constitutes a highly reliable marker for GABAergic neurons [21]. Furthermore, co-release of GABA and glycine at single postsynaptic sites has been demonstrated by electrophysiological recording in slice preparations from spinal cord and has been shown to result in the simultaneous activation of both $GABA_A$ and glycine receptors [30]. As glycine and $GABA_A$ receptors differ in their elementary channel conductances and kinetic response properties, glycine and GABA co-release may allow the formation of postsynaptic response properties that are distinct from those of purely glycinergic or GABAergic synapses, respectively.

GABA AND GLYCINE ARE THE MAJOR RAPIDLY ACTING INHIBITORY NEUROTRANSMITTERS IN BRAIN

The roles of these two neurotransmitters are very similar: both use ionotropic ligand-gated chloride channels with structural homology, although the receptors are clearly distinct entities and with totally different regional localization. Diversity of subunit isoforms has been exhibited in both $GABA_A$ and glycine receptors. The data suggest a varied pharmacology and physiology associated with differing isoform combinations. An understanding of the nature of these combinations should assist in the development of a new series of therapeutic agents that interact with $GABA_A$ or glycine receptors in a more specific manner than currently available drugs. Furthermore, a detailed understanding of the functional domains of the proteins may aid in rational drug design. Gene-targeting studies and analyses of existing mutant mice have revealed important roles for GABA and glycine receptors in nervous system function and development. Finally, plastic changes in subunit composition have been documented as a result of environmental experiences, giving new clues to understanding mechanisms of learning, disease states and drug dependence [2, 17, 19, 26, 27].

REFERENCES

1. Olsen, R. W. and DeLorey, T. M. GABA and glycine. In G. J. Siegel, B. W. Agranoff, R. W. Albers, S. K. Fisher and M. D. Uhler (eds), *Basic Neurochemistry*, 6th edn. New York: Lippincott-Raven, pp.335–346, 1999.
2. Martin, D. L. and Olsen, R. W. (eds) *GABA in the Nervous System: The View at 50 Years.* Philadelphia: Lippincott Williams & Wilkins, 2000.
3. Olsen, R. W. GABA., In K. L. Davis, D. Charney, J. T. Coyle and C. Nemeroff (eds), *Neuropsychopharmacology: Fifth Generation of Progress.* American College of Neuropsychopharmacology. Philadelphia: Lippincott Williams & Wilkins, pp. 159–168, 2001.
4. Barnard, E. A., Skolnick, P., Olsen, R. W. *et al.* Subtypes of $GABA_A$ receptors: classification on the basis of subunit structure and receptor function. *Int. Union Pharmacol. XV. Pharmacol. Rev*. 50: 291–313, 1998.
5. Whiting, P. J., Bonnert, T. P., McKernan R.M. *et al.* Molecular and functional diversity of the expanding $GABA_A$ receptor family. *Ann. N.Y. Acad. Sci.* 868: 645–653, 1999.
6. Rudolph, U., Crestani, F. and Mohler, H. $GABA_A$ receptor subtypes: Dissecting their pharmacological functions. *Trends Pharmacol. Sci.* 22: 188–194, 2001.
7. Jurd, R., Arras, M., Lambert, S. *et al.* General anesthetic actions in vivo strongly attenuated by a point mutation in the $GABA_A$ receptor β3 subunit. *FASEB J.* 17: 250–252, 2003.
8. Yamakura, Y., Bertaccini, E., Trudell, J. and Harris, R. A. Anesthetics and ion channels: molecular models and sites of action. *Ann. Rev. Pharmacol. Toxicol.* 41: 23–51, 2001.
9. Hensch, T. K., Fagiolini, M., Mataga, N. *et al.* Local GABA circuit control of experience-dependent plasticity in developing visual cortex. *Science* 282: 1504–1508, 1998.
10. Chen, N.-H., Reith, M. E. A. and Quick, M. W. Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6. *Pflugers Arch. Eur. J. Physiol.* 447: 519–531, 2004.
11. Schousboe, A., Sarup, A., Larsson, O. M. and White, H. S. GABA transporters as drug targets for modulation of GABAergic activity. *Biochem. Pharmacol.* 68: 1557–1563.
12. Gasnier, B. The loading of neurotransmitters into synaptic vesicles. *Biochimie* 82: 327–337, 2000.
13. Olsen, R. W. and Macdonald, R. L. $GABA_A$ receptor complex: structure and function. In J. Ejeberg, A. Schousboe and P. Krogsgaard-Larsen (eds), *Glutamate and GABA Receptors and Transporters: Structure, Function, and Pharmacology*. London: Routledge, pp. 202–235, 2002.
14. Bowery, N. G., Bettler, B., Froestl, W. *et al.* Mammalian γ-aminobutyric $acid_B$ receptors: structure and function. *Int. Union Pharmacol. XXXIII. Pharmacol Rev*. 54: 247–264, 2002.
15. Sieghart, W. and Sperk, G. Subunit composition, distribution, and function of $GABA_A$ receptor subtypes. *Curr. Top. Med. Chem.* 2: 795–816, 2002.
16. Chebib, M. and Johnston, G. A. R. GABA-activated ligand-gated ion channels: medicinal chemistry and molecular biology. *J. Med. Chem.* 43: 1427–1447, 2000.
17. Moss, S. J. and Smart, T. G. Constructing inhibitory synapses. *Nat. Rev. Neurosci.* 2: 240–250, 2001.
18. Kneussel, M. and Betz, H. Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: the membrane activation model. *Trends Neurosci.* 23: 429–435, 2000.
19. Fritschy, J.-M. and Brunig, I. Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. *Pharmacol. Ther.* 98: 299–323, 2003.
20. Wallner, M., Hanchar, H. J. and Olsen, R. W. Ethanol enhances α4β3δ and α6β3δ $GABA_A$ receptors at low concentrations known to have effects in humans. *Proc. Natl. Acad. Sci. U.S.A.* 100: 15218–15223, 2003.

21. Triller, A., Cluzeaud, F. and Korn, H. γ-aminobutyric acid-containing terminals can be apposed to glycine receptors at central synapses. *J. Cell Biol.* 104: 947–956, 1987.
22. Smith, S. S. (ed.) *Neurosteroid Effects in the Central Nervous System: The Role of the $GABA_A$ Receptor.* Boca Raton, FL: CRC Press, 2003.
23. Gomeza, J., Ohno, K., Armsen, W. *et al.* Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. *Neuron* 40: 797–806, 2003.
24. Roux, M. J. and Supplisson, S. Neuronal and glial glycine transporters have different stoichiometries. *Neuron* 25: 373–383, 2000.
25. Sakata, Y., Owada, Y., Sato, K. *et al.* Structure and expression of the glycine cleavage system in rat central nervous system. *Brain Res. Mol. Brain Res.* 94: 119–130, 2001.
26. Schofield, P. R. The role of glycine and glycine receptors in myoclonus and startle syndromes. *Adv. Neurol.* 89: 263–274, 2002.
27. Laube, B., Maksay, G., Schemm, R. and Betz, H. Modulation of glycine receptor function: a novel approach for therapeutic intervention at inhibitory synapses? *Trends Pharmacol. Sci.* 23: 519–527, 2002.
28. Ahmadi, S., Muth-Selbach, U., Lauterbach, A. *et al.* Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. *Science* 300: 2094–2097, 2003.
29. Shiang, R., Ryan, S. G., Zhu, Y.-Z. *et al.* Mutations in the α_1 subunit of the inhibitory glycine receptor cause the dominant neurologic disorder hyperekplexia. *Nat. Genet.* 5: 351–358, 1993.
30. Jonas, P., Bischofberger J. and Sandkuhler, J. Co-release of two fast neurotransmitters at a central synapse. *Science* 281: 360–361, 1998.

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CHAPTER

17

Purinergic Systems

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NOMENCLATURE OF PURINES AND PYRIMIDINES

Purines such as ATP and adenosine play a central role in the energy metabolism of all life forms. This fact probably delayed recognition of other roles for purines as autocrine and paracrine substances and neurotransmitters. Today it is recognized that purines are released from neurons and other cells and that they produce widespread effects on multiple organ systems by binding to purinergic receptors located on the cell surface. The principal ligands for purinergic receptors are adenosine, ATP and UTP. Primordial voltage-gated ion channels may have evolved at an early stage to respond to ATP, an early chemical signal. Such a possibility is suggested by the high homology between cloned P_{2X} receptors and epithelial Na^+ channels.

A nucleoside consists of a purine or pyrimidine base linked to a pentose, either D-ribose to form a ribonucleoside or 2-deoxy-D-ribose to form a deoxyribonucleoside. Three major purine bases and their corresponding ribonucleosides are adenine/adenosine, guanine/guanosine and hypoxanthine/inosine. The three major pyrimidines and their corresponding ribonucleosides are cytosine/cytodine, uracil/uradine and thymine/thymidine. A nucleotide such as ATP (Fig. 17-1) is a phosphate or polyphosphate ester of a nucleoside.

PURINE RELEASE AND METABOLISM

Interstitial nucleotides are derived from intracellular sources. In addition to its central role in cellular energy metabolism, ATP is a classical neurotransmitter that is packaged into secretory granules of neurons and adrenal chromafin cells and released in quanta in response to

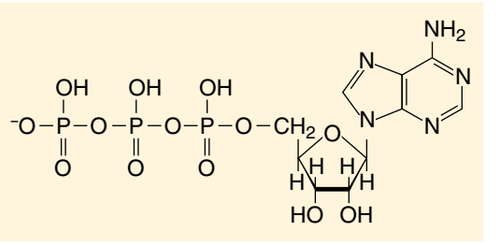

FIGURE 17-1 Adenosine 5′-triphosphate. A purine nucleotide consisting of adenine, ribose and triphosphate.

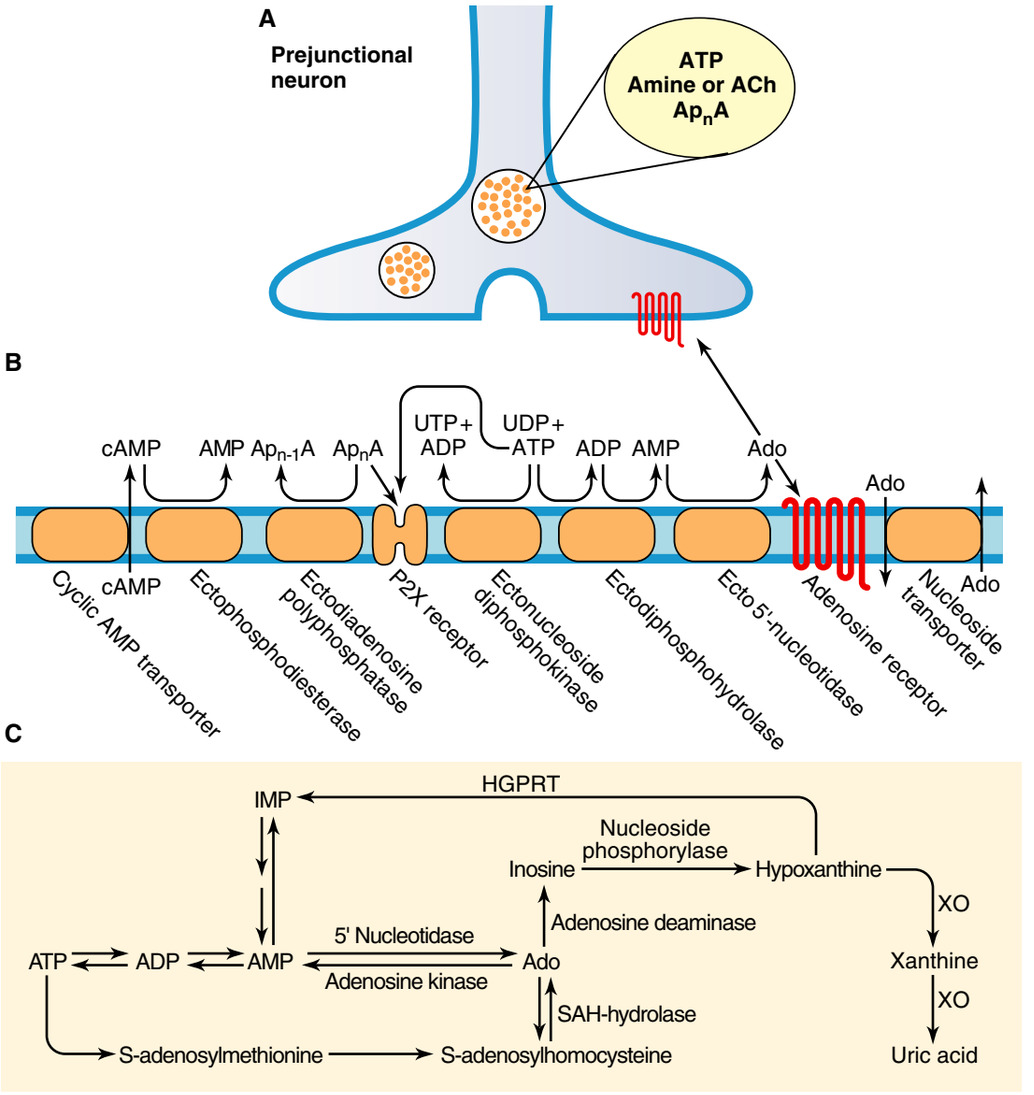

FIGURE 17-2 Purine release and metabolism. (**A**) Prejunctional neuron. Adenine nucleotides are schematically depicted as being stored as cotransmitters in synaptic granules (*see enlargement*). *Amine*, aminergic neurotransmitter; *Ach*, acetylcholine; Ap_nA, diadenosine polyphosphate. (**B**) Postjunctional membrane. Adenine nucleotides (ANs) and uridine nucleotides, diadenosine polyphosphates and cyclic AMP are degraded by ectoenzymes. Adenosine (*Ado*) and inosine accumulate in hypoxic, ischemic or metabolically active cells. Cyclic AMP accumulates as a second messenger in response to various neurotransmitters (see Ch. 21). These purines can be transported to the interstitial space by membrane-associated transport proteins. Both prejunctional (**A**) and postjunctional (**B**) adenosine receptors are depicted as traversing the plasma membrane seven times, as is typical of receptors that interact with GTP-binding proteins, including P_1 and P_{2Y} (not shown) receptors. A P_{2X} postjunctional ATP receptor is depicted as a ligand-gated channel. (**C**) Cytosol. The major pathways of intracellular adenosine metabolism are shown. *HGPRT*, hypoxanthine-guanine phosphoribosyl transferase; *IMP*, inosine monophosphate; *SAH*, *S*-adenosylhomocysteine; *XO*, xanthine oxidase.

action potentials, as illustrated in Figure 17-2A. Thus, ATP is released from synaptosomal preparations of cortex, hypothalamus and medulla. In cortical synaptosomes, a portion of the ATP that is released is co-released with acetylcholine or norepinephrine, but the majority is released from neurons that are neither adrenergic nor cholinergic. In affinity-purified cholinergic nerve terminals, ATP and acetylcholine are co-released in a ratio of 1:10. ATP is also released through channels or transporters from endothelial, epithelial and other cell types in response to hypoxia, mechanical stress or other stimuli to become available to cell-surface receptors and enzymes [1].

Extracellular nucleotides are regulated by ectoenzymes. Ectoenzymes are involved in the rapid metabolism of ATP and other nucleotides in the extracellular space [2]. ATP applied to rat brain hippocampal slices is mostly converted to adenosine in less than a second. Some of the enzymes involved in ATP, UTP and nucleoside metabolism are depicted in Figure 17-2B. Inhibitors of these

TABLE 17-1 Substrates and inhibitors of enzymes that are involved in nucleotide and nucleoside metabolism

Enzyme	Substrate	Inhibitor
ATP diphosphohydrolase	ATP, ADP	ARL67156
Diadenosine polyphosphatase	APnA	Suramin*
5′ nucleotidase	AMP	AOPCP
Nucleoside transporter	Adenosine	Dipyridamole, NBTI, mioflazine
Adenosine deaminase	Adenosine	EHNA, 2-deoxycoformycin
Adenosine kinase	Adenosine	5′-iodotubercidin, 5′-deoxy-5′-amino-adenosine
Xanthine oxidase	Hypoxanthine, xanthine	Allopurinol, CMTA
Nucleoside phosphorylase	Inosine	8-aminoguanosine

* Suramin also is an inhibitor of P_2 receptors.

AOPCP, α, β-methylene-adenosine diphosphate; AP_nA, diadenosine polyphosphate (n=3–6); ARL 67156, 6-*N*,*N*-diethyl-D-β,γ-dibromomethylene ATP; CMTA, 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NBTI, nitrobenzylthioinosine.

enzymes that are useful as experimental tools are listed in Table 17-1.

Ecto-ATP diphosphohydrolase (ADPase or apyrase) is a plasmalemma-bound enzyme that dephosphorylates extracellular ATP and ADP to produce AMP. This enzyme is identical to CD39, an activation marker found on B lymphocytes. A selective inhibitor of ecto-ATPase is ARL67156 (Table 17-1). This compound potentiates the effect of endogenously released as well as endogenously added ATP. Extracellular AMP is converted to adenosine by ecto-5′-nucleotidase, an enzyme that is attached to the cell surface by a glycosyl phosphatidylinositol linker. 5′-Nucleotidases catalyze the conversion of purine and pyrimidine nucleoside monophosphates to the corresponding nucleosides. CD73 is a 5′-nucleotidase that is found on T and B lymphocytes [3]. Ecto-5′-nucleotidase can be blocked by α,β-methylene-adenosine diphosphate (AOPCP). In histochemical studies, ecto-5′-nucleotidase has been found to be associated with plasma membranes of glial cells and astrocytes, particularly near synaptic terminals. Soluble cytosolic 5′-nucleotidases also exist and are involved in the formation of adenosine during increased metabolic activity. Even a small decrease in ATP concentration within cells can lead to a large fractional increase in AMP, because under normal conditions the concentration of ATP inside cells is about 50 times higher than that of AMP. The differentiation of neural cells is dependent on 5′-nucleotidase activity, suggesting that adenosine formation from continuously released nucleotides is essential for neuron survival. 5′-Nucleotidase is phosphorylated and activated by protein kinase C. In rat brain, ischemia results in an upregulation of 5′-nucleotidase on activated astrocytes. This is thought to increase the capacity of damaged tissue to form neuroprotective adenosine. Extracellular adenosine also can be derived from the metabolism of extracellular cyclic AMP by an ectocyclic AMP phosphodiesterase. Stimulation of receptors that stimulate cyclic AMP accumulation (β-adrenergic or VIP receptors) in cultured rat cortical neurons causes the accumulation of extracellular adenosine, which can be blocked by inhibition of cyclic AMP transport, cyclic nucleotide phosphodiesterase, or 5′-nucleotidase, indicating that extracellular cyclic AMP is a source of the adenosine.

Diadenosine polyphosphates are found in the synaptic granules of some nerves, can activate some P_2 receptors and are degraded in the extracellular space. In neural tissues the activity of ectodiadenosine polyphosphatases is lower than ecto-ATP-diphosphohydrolase. Hence, the diadenosine polyphosphates have a longer half-life in the extracellular space than does ATP.

UDP and UTP are selective agonists of certain of the P_{2Y} receptors. It is not yet clear what factors control the release of uridine nucleotides into the extracellular space. UTP can be formed from UDP in the extracellular space by the action of the enzyme nucleoside diphosphokinase, which catalyzes the transfer of the γ-phosphate of nucleoside triphosphates to nucleoside diphosphates, e.g., ATP + UDP → ADP + UTP.

There are several sources of extracellular adenosine. Adenosine is not a classical neurotransmitter because it is not stored in neuronal synaptic granules or released in quanta. It is generally thought of as a neuromodulator that gains access to the extracellular space in part from the breakdown of extracellular adenine nucleotides and in part by translocation from the cytoplasm of cells by nucleoside transport proteins, particularly in stressed or ischemic tissues (Fig. 17-2C). Extracellular adenosine is rapidly removed in part by reuptake into cells and conversion to AMP by adenosine kinase and in part by degradation to inosine by adenosine deaminases. Adenosine deaminase is mainly cytosolic but it also occurs as a cell surface ectoenzyme.

Adenosine and homocysteine are formed from the hydrolysis of *S*-adenosylhomocysteine (SAH) by the enzyme SAH hydrolase (Fig. 17-2C). Attempts to measure intracellular adenosine are complicated by the fact that

over 90% of intracellular adenosine may be weakly bound to this enzyme. SAH is formed from *S*-adenosylmethionine (SAM), which is a cofactor in transmethylation reactions. SAH is the precursor of a sizable fraction of adenosine under resting conditions, but most adenosine is derived from the 5′-nucleotidase pathway during conditions of hypoxia, ischemia or metabolic stress. The accumulation of high concentrations of adenosine under these conditions also leads to a large increase in inosine resulting from adenosine deamination. Intracellular adenosine can be reincorporated into the nucleotide pool upon phosphorylation by the cytosolic enzyme adenosine kinase. In normoxic resting tissues, most adenosine is rephosphorylated since the K_m of adenosine kinase is 10–100 times lower than the K_m of adenosine deaminase. Deamination, which leads to a large accumulation of inosine, becomes the major pathway of adenosine metabolism when adenosine levels are elevated, because maximal adenosine kinase activity is much less than maximal adenosine deaminase activity. Inhibitors of adenosine kinase or adenosine deaminase, by elevating adenosine, have been found to produce adenosine-like actions in laboratory animals. The concentrations of adenosine and inosine in the interstitial fluid of brain and other tissues are increased when oxygen demand exceeds oxygen supply. The effect of adenosine is to increase oxygen delivery by dilating most vascular beds and generally to decrease oxygen demand by reducing cellular energy utilization. In the brain this is usually manifested as a decrease in neuronal firing and decreased release of excitatory neurotransmitters.

Adenosine and inosine can be transported across cell membranes in either direction, facilitated by a membrane-associated nucleoside transport protein. Concentrative transporters have also been identified. Messenger RNA for a pyrimidine-selective Na^+-nucleoside cotransporter (rCNT1) and a purine-selective Na^+-nucleoside cotransporter (rCNT2) are found throughout the rat brain. Most degradation of adenosine is intracellular, as evidenced by the fact that inhibitors of adenosine transport, such as dipyridamole, increase interstitial levels of adenosine. Dipyridamole is used clinically to elevate adenosine in coronary arteries and produce coronary vasodilation. In high doses, dipyridamole can accentuate adenosine-receptor-mediated actions in the CNS, resulting in sedation and sleep, anticonvulsant effects, decreased locomotor activity and decreased neuronal activity.

Hypoxanthine is derived from inosine by the enzyme nucleoside phosphorylase. Hypoxanthine can be converted to IMP by hypoxanthine-guanine phosphoribosyl transferase (HGPRT), one of the enzymes of the purine salvage pathway. Lesch–Nyhan syndrome is a severe neurological disorder caused by a deficiency of HGPRT (Box 17-1). AMP can be formed from IMP by insertion of an amino group at C-6 in place of the carbonyl oxygen. This is a two-step reaction involving the formation of adenylosuccinate as an intermediate. Unsalvaged hypoxanthine is oxidized to xanthine, which is further oxidized to uric acid by xanthine oxidase (Fig. 17-2C, 17-3). Molecular oxygen, the oxidant in both reactions, is reduced to H_2O_2 and other reactive oxygen species. In man, uric acid is the final product of purine degradation and is excreted in the urine.

PURINERGIC RECEPTORS

Receptors for both ATP and adenosine are widely distributed in the nervous system, as well as in other tissues. The notion that there are purinergic receptors, i.e. proteins on the surface of cells that bind and respond to purines, was slow to evolve. The first evidence was the observation of cardiovascular effects of purines. Drury and Szent-Gyorgyi [4] first noted effects of adenine nucleotides on cardiac and vascular tissues in 1929. Then, 34 years later, Berne identified a physiological role for adenosine as a mediator of coronary vasodilation in response to myocardial hypoxia [5]. In the 1970s adenosine was found to stimulate cyclic AMP formation in brain slices. Since then, physiological effects of adenosine on almost all tissues have been described. Based on the responses of various tissues to purines, Burnstock proposed that there are distinct receptors that bind adenosine or ATP, designated P_1 and P_2 receptors respectively [6]. The existence of adenosine receptors was not widely accepted until the 1980s, when saturable binding sites for radioactive adenosine analogs were demonstrated in brain. The existence of adenosine receptors was proved unequivocally when the first adenosine receptors were cloned in 1990 [7]. Originally, the 'P' in P_1 and P_2 was meant to designate 'purinergic' receptors. However, it has now been discovered that some of the P_2 receptors bind pyrimidines (UTP or UDP) preferentially over the purine, ATP. Hence, the 'P' in P_2 is now used to designate purine or pyrimidine. Despite these exceptions, P_1 and P_2 receptors collectively are still generally referred to as purinergic receptors.

In addition to adenosine, various synthetic adenosine analogs activate P_1 but not P_2 receptors and synthetic ATP analogs or UTP analogs activate P_2 receptors but not adenosine receptors. However, not all purines activate P_1 or P_2 receptors. For example, adenine, guanosine and uric acid do not activate P_1 receptors. Inosine, the purine nucleoside product of adenosine deamination, generally has weak activity at P_1 receptors, but inosine binds to adenosine A_3 receptors, interferes with adenosine metabolism, modulates immune function and has neuroprotective effects. Its biological potency at A_3 adenosine receptors is greater in rodent species than in human. These actions of inosine may be physiologically significant because inosine accumulates to very high levels (>1 mmol/l) in ischemic tissues. Inosine stimulates mast-cell degranulation by binding to A_3 receptors but also produces anti-inflammatory effects by blocking the activation of macrophages, lymphocytes and neutrophils. In brain, inosine decreases

BOX 17-1

Inherited diseases of purine metabolism

George J. Siegel

Lesch–Nyhan syndrome (LNS) is an X-linked recessive inherited disorder usually evident at 6–10 months of age with choreiform movements, compulsive self-mutilation, spasticity, mental retardation, hyperuricemia and gout. LNS is associated with many types of mutations in the gene for the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) [1, 2]. This enzyme normally catalyzes the recycling of purines from degraded DNA and RNA. There is an almost complete deficiency of this enzyme in patients with the full syndrome. However, individuals with a partial deficiency in HGPRT generally have hyperuricemia and gout but not the full neurologic manifestations [3]. Treatment with allopurinol, which inhibits xanthine oxidase, reduces the levels of uric acid and the attendant symptoms of hyperuricemia but does not ameliorate the neurologic phenomena. Transgenic mouse models made to be genetically deficient in HGPRT or doubly deficient in HGPRT and in another purine salvage enzyme, adenine phosphoribosyltransferase (APRT), and thus devoid of any purine salvage pathways, do not show behavioral abnormalities reflective of the LNS phenotype [4]. However, administration of an inhibitor of APRT to HGPRT-deficient mice did produce self-injurious behavior [5]. Mice, in contrast to humans, exhibit uricase activity that might alter the consequences of high uric acid [6]. Other diseases associated with hyperuricemia, as in gout or phosphoribosylpyrophosphate synthetase deficiency, are not associated with the neurologic phenotype of LNS. Administration of dopamine agonists to rats with 6-*OH*-dopamine-induced lesions of catecholamine pathways or to primates with surgical lesions of the nigrostriatal pathway demonstrate stereotypical and self-injurious behaviors. These patterns appear related to supersensitivity of dopamine D1 receptors (see Ch. 12). Positron emission tomography with [^{18}F]-fluorodopa (see Ch. 58) has demonstrated abnormally few dopaminergic nerve terminals and cell bodies in the basal ganglia and frontal cortex of LNS patients, suggesting pervasive developmental abnormalities in dopaminergic systems [7]. Developmental dopaminergic neuronal system abnormalities may be related to the genetic alteration that occurs in the context of a multigene regulation pattern specific for the species [8, 9].

Mutations in the gene for adenylosuccinate lyase (ASL), inherited as an autosomal recessive disorder in purine metabolism, are associated with severe mental retardation and autistic behavior, but apparently not self-mutilation [10, 11]. This enzyme catalyzes two distinct reactions in the *de novo* biosynthesis of purines: the cleavages of adenylosuccinate (S-Ado) and succinylaminoimidazole carboxamide ribotide (SAICAR), both of which accumulate in plasma, urine and cerebrospinal fluid of affected individuals [12]. Measurements of these metabolites in urine is useful in screening neonates for this metabolic error [13]. **Deficiency of the muscle-specific myoadenylate deaminase (MADA)** is a frequent cause of exercise-related myopathy and is thought to be the most common cause of metabolic myopathy. MADA catalyzes the deamination of AMP to IMP in skeletal muscle and is critical in the purine nucleotide cycle. It is estimated that about 1–2% of all muscle biopsies submitted to medical centers for pathologic examination are deficient in AMP deaminase enzyme activity. MADA is 10 times higher in skeletal muscle than in any other tissue. Increase in plasma ammonia (relative to lactate) after ischemic exercise of the forearm may be low in this disorder, which is a useful clinical diagnostic test in patients with exercise-induced myalgia [14]. Genetta *et al.* [15] stated that AMPD deficiency is the most prevalent genetic disease in humans, the number of people heterozygous approaching 10% of Caucasians and individuals of African descent [16]. A small percentage of homozygous-deficient individuals, approximately 1.8% of the population, display symptoms of chronic fatigue and lost productivity as well as a predisposition to stress-related ailments, including heart disease and stroke [15, 16].

References

1. Nyhan, W. L. The recognition of Lesch–Nyhan syndrome as an inborn error of purine metabolism. *J. Inherit. Metab. Dis.* 20: 171–178, 1997.
2. Jinnah, H. A., Harris, J. C., Nyhan, W. L. and O'Neill, J. P. The spectrum of mutations causing HPRT deficiency: an update. *Nucleosides Nucleotides Nucleic Acids* 23: 1153–1160, 2004.
3. Puig, J. G., Torres, R. J., Mateos, F. A. *et al.* The spectrum of hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency. Clinical experience based on 22 patients from 18 Spanish families. *Medicine (Baltimore)* 80: 102–112, 2001.
4. Engle, S. J., Womer, D. E., Davies, P. M. *et al.* HPRT-APRT-deficient mice are not a model for Lesch–Nyhan syndrome. *Hum. Mol. Genet.* 5: 1607–1610, 1996.
5. Wu, C. L. and Melton, D. W. Production of a model for Lesch–Nyhan syndrome in hypoxanthine phosphoribosyltransferase-deficient mice. *Nat. Genet.* 3: 235–240, 1993.
6. Wu, X., Wakamiya, M., Vaishnav, S. *et al.* Hyperuricemia and urate nephropathy in urate oxidase-deficient mice *Proc. Natl Acad. Sci. U.S.A.* 91: 742–746, 1994.
7. Visser, J. E., Bar, P. R. and Jinnah, H. A. Lesch–Nyhan disease and the basal ganglia. *Brain Res. Brain Res. Rev.* 32: 449–475, 2000.
8. Smith, D. W. and Friedmann, T. Characterization of the dopamine defect in primary cultures of dopaminergic neurons from hypoxanthine phosphoribosyltransferase knockout mice. *Mol. Ther.* 1: 486–491, 2000.
9. Jinnah, H. A., Jones, M. D., Wojcik, B. E. *et al.* Influence of age and strain on striatal dopamine loss in a genetic mouse model of Lesch–Nyhan disease. *J. Neurochem.* 72: 225–229, 1999.
10. Stone, R. L., Aimi, J. and Barshop, B. A. *et al.* A mutation in adenylosuccinate lyase associated with mental retardation and autistic features. *Nat. Genet.* 1: 59–63, 1992.

Continued

BOX 17-1—cont'd
Inherited diseases of purine metabolism

11. Sivendran, S., Patterson, D., Spiegel, E., McGown, I., Cowley, D. and Colman, R. F. Two novel mutant human adenylosuccinate lyases (ASLs) associated with autism and characterization of the equivalent mutant Bacillus subtilis ASL. *J. Biol. Chem.* 279: 53789–53797, 2004.
12. Marinaki, A. M., Champion, M., Kurian, M. A. *et al.* Adenylosuccinate lyase deficiency – first British case. *Nucleosides Nucleotides Nucleic Acids* 23: 1231–1233, 2004.
13. Castro, M., Perez-Cerda, C., Merinero, B. *et al.* Screening for adenylosuccinate lyase deficiency: clinical, biochemical and molecular findings in four patients. *Neuropediatrics* 33: 186–189, 2002.
14. Fishbein, W. N., Armbrustmacher, V. W., Griffin, J. L., Davis, J. I. and Foster, W. D. Levels of adenylate deaminase, adenylate kinase, and creatine kinase in frozen human muscle biopsy specimens relative to type1/type2 fiber distribution: evidence for a carrier state of myoadenylate deaminase deficiency. *Ann. Neurol.* 15: 271–277, 1984.
15. Genetta, T., Morisaki, H., Morisaki, T. and Holmes, E. W. A novel bipartite intronic splicing enhancer promotes the inclusion of a mini-exon in the AMP deaminase 1 gene. *J. Biol. Chem.* 276: 25589–25597, 2001.
16. Sabina, R. L. Myoadenylate deaminase deficiency. A common inherited defect with heterogeneous clinical presentation. *Neurol. Clin.* 18: 185–194, 2000.

ischemia-reperfusion injury, perhaps by suppressing inflammation, preserves glial viability and promotes axon regeneration after injury.

The development of synthetic compounds that activate P_1 or P_2 receptors has been important for elucidating how these receptors function, because some of these compounds are more potent and selective than the parent purines, and most are more stable than the short-lived endogenous compounds adenosine and ATP.

The P-site of adenylyl cyclase inhibits cyclic AMP accumulation. Since P_1 and P_2 receptors are located on the cell surface, they bind purines or pyrimidines in the extracellular space. There also is an adenosine binding site located intracellularly on the enzyme adenylyl cyclase (see Ch. 21). This is referred to as the 'P-site' of adenylyl cyclase. Binding of adenosine and other purines, notably 3′AMP, 2′deoxy-3′-ATP and 2′,5′-dideoxyadenosine to this site, inhibits adenylyl cyclase activity [8]. The P-site of adenylyl cyclase and other intracellular purine binding sites are not classified as purinergic receptors.

There are four adenosine receptor subtypes. There are four subtypes of adenosine (P_1) receptor that have been cloned. These are referred to as A_1, A_{2A}, A_{2B} and A_3 (Fig. 17-4). All four of these belong to the superfamily of receptors that signal via guanosine triphosphate (GTP)-binding proteins (G proteins) and contain seven hydrophobic membrane-spanning segments, as illustrated in the case of the A_1 adenosine receptor in Figure 17-5 (see also Ch. 19). The receptors consist of a single subunit of molecular masses 35–46 kDa and have one or two consensus sequences for N-linked glycosylation (NXS/T) in their second extracellular loops. The human A_3 adenosine receptors also have two sites of N-linked glycosylation near the amino terminus. It is thought that these glycosylation sites may be involved in targeting receptors for expression on the cell surface. Adenosine receptors also contain multiple serine and threonine residues on the third intracellular loop or near the carboxyl terminus. In some cases, these may become phosphorylated and lead to receptor desensitization during prolonged exposure to adenosine. The greatest similarities in the structure of the adenosine receptor subtypes are found in the transmembrane segments, particularly in segments 2, 3 and 7. This suggests that these segments may be aligned next to each other in the plane of the membrane bilayer to form a ligand binding pocket. Three of the adenosine

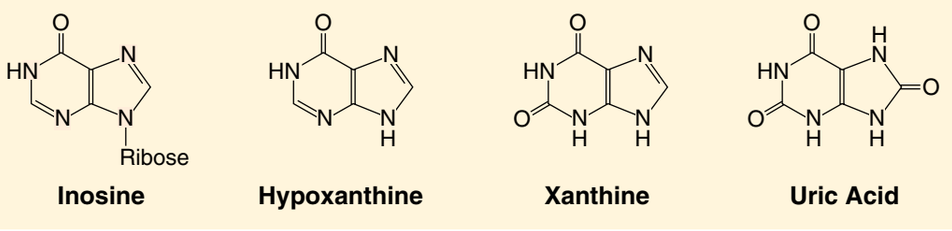

FIGURE 17-3 Adenosine metabolites. Adenosine is converted to inosine by adenosine deaminase. Removal of the ribose by nucleoside phosphorylase produces hypoxanthine, which is sequentially oxidized to xanthine and uric acid by xanthine oxidase.

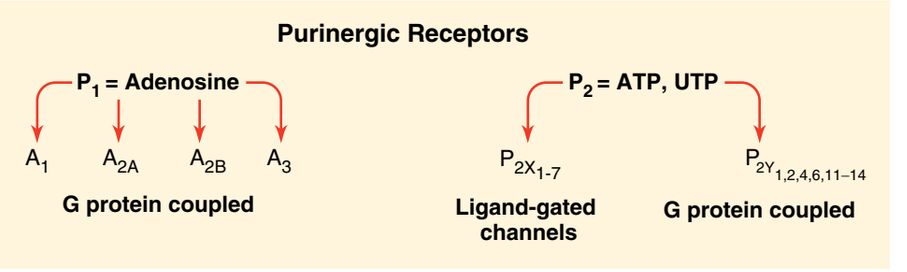

FIGURE 17-4 The purinergic receptor family. The purinergic receptors are divided into two major families, the P_1 or adenosine receptors and P2 receptors, which principally bind ATP, ADP, UTP or UDP. The P_{2Y14} receptor binds UTP-sugars. P_1 and P_{2Y} receptors are coupled to GTP binding proteins. P_{2X} subunits form trimeric ligand-gated ion channels.

receptors, A_1, A_{2B} and A_3, have a cysteine residue, thought to be palmitoylated, within 25 amino acids from the carboxyl terminus. The addition of this fatty acid may anchor the carboxyl terminal region of the receptor to the plasma membrane. As illustrated in Figure 17-6, the structure of the G-protein-coupled purinergic receptors varies little between species, and there is somewhat greater variability between subtypes, even within a single species. The adenosine receptor subtypes, their effectors and the identity of some selective agonists and antagonists are listed in Table 17-2.

Xanthines block P_1 but not P_2 receptors. One of the criteria initially used to distinguish between adenosine and ATP receptors was selective blockade of the former by xanthines (Fig. 17-7) such as caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine). These xanthines occur naturally in coffee, tea and chocolate, and their well known stimulant action has been attributed to blockade of adenosine receptors in the CNS. Recently, 8-phenylxanthines, 8-cycloalkylxanthines and nonxanthine synthetic antagonists have been synthesized and characterized as adenosine receptor antagonists that are thousands of times more potent and, in some cases, more selective for individual adenosine receptor subtypes than are caffeine and theophylline.

P_2 receptors are subdivided into ionotropic P_{2X} receptors and metabotropic P_{2Y} receptors. P_{2X} receptors are ionotropic ligand-gated ion channels ($P_{2X1–7}$). There are

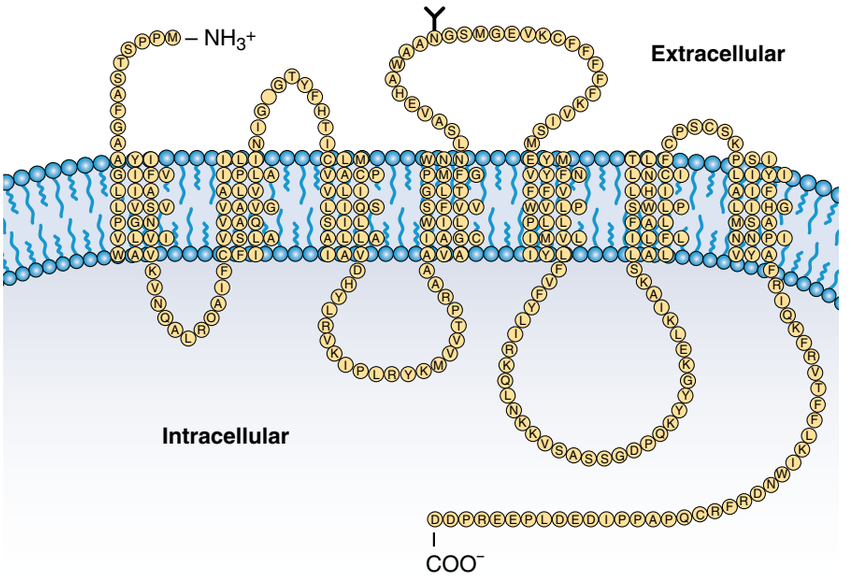

FIGURE 17-5 Deduced amino acid sequence and structure of the human A_1 adenosine receptor. This typical adenosine receptor is a member of the large family of guanine nucleotide-binding protein-coupled receptors that span the plasma membrane seven times. An unusual feature of adenosine receptors is the short amino terminus lacking N-linked glycosylation sites. These are found instead on extracellular loop 2 (as pictured). The ligand-binding pocket is thought to be formed by amino acids in transmembrane segments 2, 3, and 7. GTP-binding proteins are thought to interact with juxtamembranous regions of intracellular loops 2 and 3 and the carboxyl terminus.

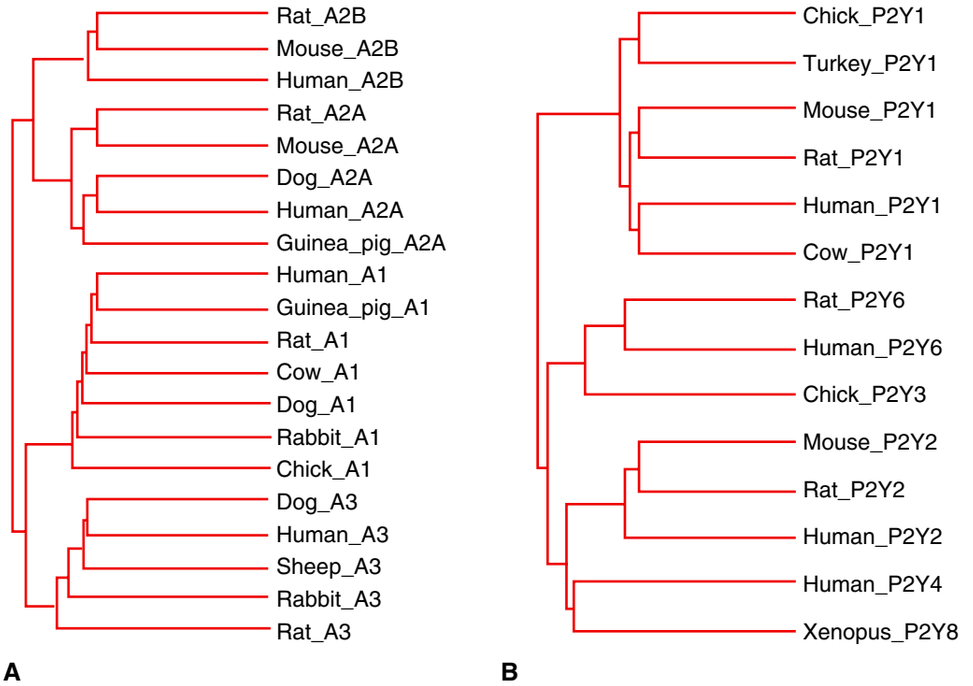

FIGURE 17-6 Dendrograms illustrating structural similarities among G-protein-coupled purinergic receptor subtypes. Distance along the horizontal axes is proportional to differences between species. (**A**) Adenosine receptor subtypes; the rat and rabbit A_3 receptors are 73.2% similar. (**B**) P_{2Y} receptor subtypes; the *Xenopus* P_{2Y8} and human P_{2Y4} receptors are 71.1% similar. P_{2Y3} is an avian receptor ortholog of human P_{2Y6}.

TABLE 17-2 Subtypes of adenosine receptor, their effectors and selective agonists and antagonists

Receptor (accession #)*	Effector†	Agonist	Antagonist	CNS location
A_1 (NM_000674)	Adenylyl cyclase (−) K^+ channels (+) Ca^{2+} channels (−) PI-PLC (+)	CPA	CPX	Widespread striatal cholinergic interneurons
A_{2A} (NM_000675)	Adenylyl cyclase (+)	ATL146e, CGS21680	ZM241385 SCH58261	Basal ganglia
A_{2B} (NM_000676)	Adenylyl cyclase (+) PI-PLC (+)	None	MRS-1754	Low levels
A_3 (NM_000677)	Adenylyl cyclase (−) PI-PLC (+)	IB-MECA	MRE3008F20	Low levels
Nonselective ligands		NECA	SPT	

* Genebank accession numbers are for human clones. †PI-PLC = phosphoinositide-specific phospholipase C.

CGS21680, 2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; CPA, N^6-cyclopenthyladenosine; CPX, 1,3-dipropyl-8-cyclopentylxanthine; MRE3008F20, 5N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (human only); NECA, 5′-*N*-ethylcarboxamidoadenosine; SPT, *p*-sulfophenyltheophylline; SCH58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-ε]-1,2,4-triazolo[1,5-c)pyrimidine; IB-MECA; N^6-(2-iodo)benzyl-5′-N-methylcarboxamidodiadenosine; ZM241385, 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-α][1,3,5]triazin-5-yl-amino]ethyl)phenol.

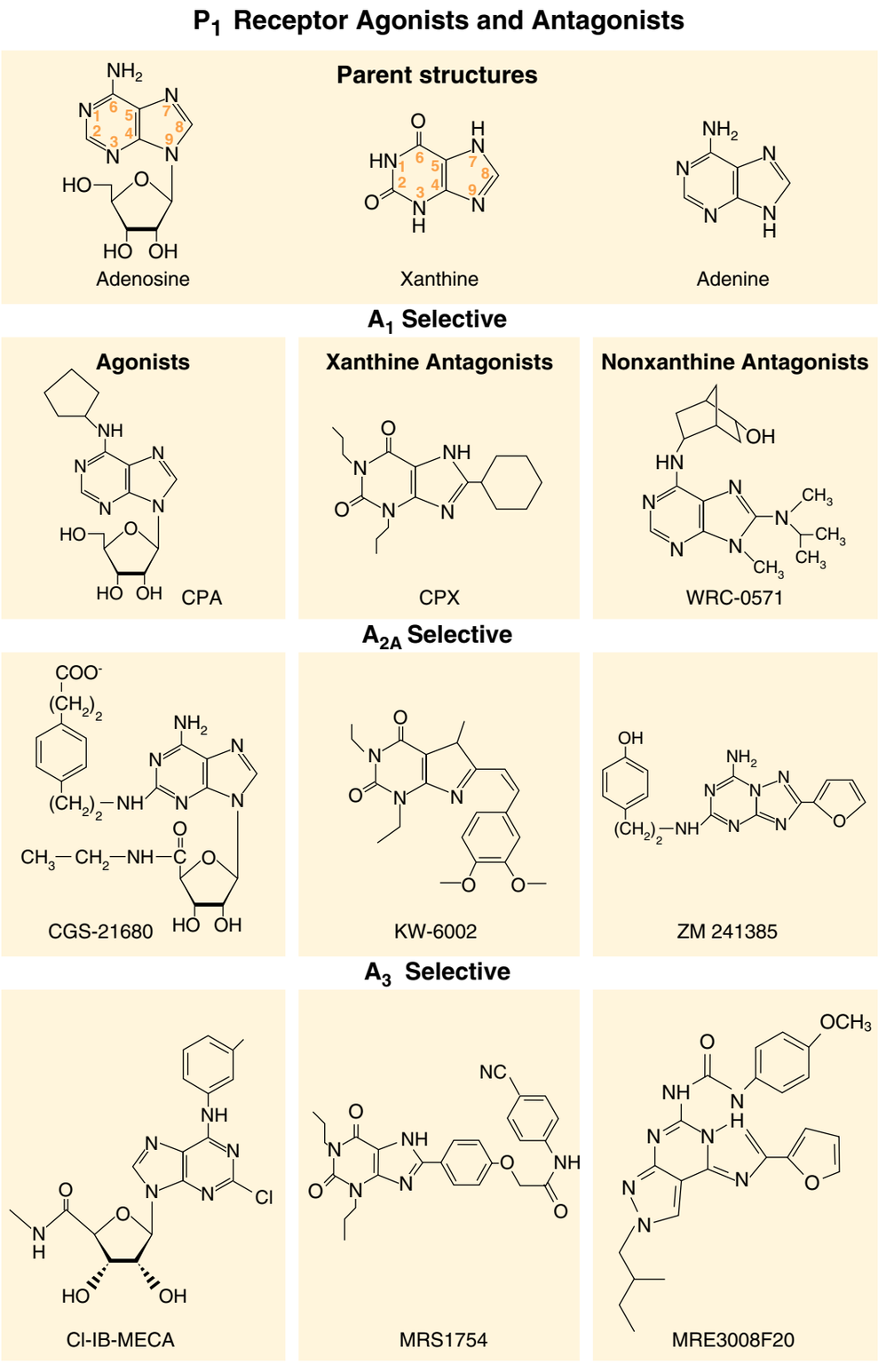

FIGURE 17-7 The structures of selective agonists and antagonists of adenosine receptors.

eight subtypes of metabotropic G-protein-coupled P_{2Y} receptors. The subtypes that have been cloned and identified to date as P_{2Y} receptors are P_{2Y1}, P_{2Y2}, P_{2Y4}, P_{2Y6} and $P_{2Y11–14}$. A putative receptor designated P_{Y15} was subsequently shown not be a purinergic receptor. The nomenclature is not sequential because of receptors that were either cloned from nonmammalian vertebrates or misidentified as purinergic receptors. It is likely that the number of P_{2Y} receptors will increase as the natural ligands for a group of cloned orphan receptors with highly similar sequence homology are identified. The P_{2Y} receptors can be subdivided into four categories based on their pharmacology: (a) P_{2Y1} and $P_{2Y11–13}$ receptors bind adenine-nucleotides, preferentially ATP and ADP; (b) P_{2Y6} receptors bind uracil-nucleotides, preferentially UTP and UDP; (c) P_{2Y4} and P_{2Y6} receptors bind ligands from both categories; and (d) P_{2Y14} receptors bind UDP-glucose, UDP-galactose or UDP-*N*-acetylglucosamine. They can also be grouped according to their G protein coupling, either to G_q (P_{2Y1}, P_{2Y2}, P_{2Y4}, P_{2Y6}, P_{2Y11}) with activation of phospholipase C and an inositol trisphosphate [IP_3]-dependent mobilization of intracellular Ca^{2+} (see Ch. 20) or to G_i ($P_{2Y12–14}$) with inhibition of adenylyl cyclase (see Ch. 21) [9]. The subset of receptors that respond to UTP was previously referred to as P2U receptors. The P_{2Y1} receptor is selectively activated by ADP and has the unusual feature of being blocked by ATP. This ADP receptor is found in platelets and was previously called $P2_T$ and P_{2YADP}. The P_{2Y2} receptor is activated by UTP and ATP with similar potency and is not activated by nucleoside diphosphates. Diadenosine tetraphosphate is a potent agonist at this receptor. The P_{2Y4} receptor is highly selective for UTP over ATP and is not activated by nucleoside diphosphates. The P_{2Y6} receptor is activated most potently by UDP and weakly or not at all by UTP, ADP and ATP.

The P_{2X} receptors are structurally unrelated to P_{2Y} receptors. They have two transmembrane domains with intracellular N and C termini separated by a large extracellular loop. P_{2X} receptors are heteromultimeric proteins that combine several subunits to form homomeric or heteromeric functional ion channels [10]. In general the P_{2X} receptors are activated by ATP and 2-methio-ATP. [^{35}S]ATPγS has been used as a radioligand for the direct labeling of cloned P_{2X} receptors. α,β-methylene-ATP is an agonist of P_{2X1} and P_{2X3} receptors but does not activate the other P_{2X} subtypes. ADP is a selective agonist of P_{2X5} and P_{2X6} receptors, and benzoyl-ATP is an agonist of the P_{2X7} receptor, an unusual receptor, previously called P2Z, that can cause cell lysis by promoting the formation of large pores. A problem that continues to hamper the study of P_2 receptors is the absence of receptor-subtype-selective antagonists (Table 17-3). The compounds that are used as P_2 receptor antagonists have low affinity for the receptors. These include reactive blue 2 (P_{2Y} receptors), suramin ($P_{2Y1} > P_{2Y2} > P_{2Y4}$), NF023 ($P_{2X}$ receptors) and pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; $P_{2X} > P_{2Y}$). ATP and ARL-66,096 are antagonists of P_{2Y1} receptors.

TABLE 17-3 Antagonists for P_{2X} receptors

Receptor	Antagonist (rat pIC_{50}, M)
P_{2X1}	NF 449 (9.5)
P_{2X2}	NF 279 (6.4)
P_{2X3}	TNP-ATP (9.5)
P_{2X4}	Protons (6.8)
P_{2X5}	PPADS (6.7)
P_{2X6}	None
P_{2X7}	Brilliant Blue G (8.0)

NF 279, 88′-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)) bis(1,3,5-naphthalenetrisulphonic acid); NF 449, 4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonyl imino)))tetra kis-benzene-1,3-disulfonic acid; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt; TNP-ATP, 2′- (or 3′)-*O*-(trinitrophenyl)adenosine 5′-triphosphate

The distribution of mRNAs for P_2 receptors in the nervous system of the adult rat is summarized in Table 17-4.

EFFECTS OF PURINES IN THE NERVOUS SYSTEM: ADENOSINE RECEPTORS

The adenosine that activates receptors in the nervous system is derived in part from adenine nucleotides packaged in the synaptic granules of nerves and in part from ATP and adenosine derived from sources other than synaptic granules. Adenosine release evoked by electrical stimulation is completely dependent on propagated electrical activity, is blocked by tetrodotoxin (TTX) and is largely Ca^{2+}-dependent. Adenosine release by anoxia is Ca^{2+}-independent and largely insensitive to TTX. Many of the central actions of adenosine can be attributed to inhibition of Ca^{2+}-dependent excitatory neurotransmitter release. Most of these effects appear to be presynaptic and are mediated by G proteins coupled to inhibition of N-type Ca^{2+} channels or to stimulation of K^+ channels. However, in some instances, adenosine produces excitatory effects in the CNS. For example, in the nucleus tractus solitarius (NTS), adenosine increases glutamate release and elicits excitatory cardiovascular effects [11]. Both excitatory and inhibitory effects of adenosine have been noted in hippocampal slices. In spinal cord, adenosine can be derived from capsaicin-sensitive, small-diameter primary afferent neurons and potentiates the antinociceptive action of norepinephrine [12]. Opiates have been shown to induce release of adenosine from brain slices, synaptosomes and the spinal cord, and adenosine receptor antagonists block some of the effects of opiates. It has also been suggested that some of the behavioral effects of alcoholic beverages may be mediated by adenosine because intoxicating concentrations of ethanol can block adenosine transport.

TABLE 17-4 Distribution of P_2 receptor mRNAs in the central nervous system

Receptor	Accession no.	Transcript location
P_{2X1}	NM_002558	Large motor neurons of the spinal cord; important for neurotransmission to smooth muscle
P_{2X2}	NM_174873	Sensory and autonomic ganglia, dorsal and ventral horn of the spinal cord, thalamus, hypothalamus, preoptic area, red nucleus, oculomotor nucleus, locus coeruleus and dorsal motor nucleus of the vagus
P_{2X3}	NM_002559	Dorsal root ganglion, superficial dorsal horn of spinal cord, a subset of small-diameter sensory neurons, nucleus of the solitary tract, spinal trigeminal nucleus (important for peripheral pain)
P_{2X4}	NM_175568	Widespread in brain and spinal cord
P_{2X5}	NM_175080	Proprioceptive neurons of mesencephalic trigeminal nucleus, sensory ganglia
P_{2X6}	NM_005446	Widespread in brain, spinal cord and sensory ganglia, predominantly found in skeletal muscle
P_{2X7}	NM_177427	Found in cells of the immune system but not in CNS
P_{2Y1}	NM_002563	Telencephalon, diencephalon, mesencephalon, cerebellum
P_{2Y2}	NM_176072	Found in pituitary
P_{2Y4}	X91852	Not detected in human brain
P_{2Y6}	NM_176796	Not found in neuronal tissue
P_{2Y12}	AF313449	Brain (glia), spinal cord
P_{2Y13}	AF406692	
P_{2Y14}	NM 133577	Brain (glia), peripheral immune cells

A_1 adenosine receptors are inhibitory in the central nervous system. A_1 receptors were originally characterized on the basis of their ability to inhibit adenylyl cyclase in adipose tissue. A number of other G-protein-mediated effectors of A_1 receptors have subsequently been discovered; these include activation of K^+ channels, extensively characterized in striatal neurons [13], and inhibition of Ca^{2+} channels, extensively characterized in dorsal root ganglion cells [14]. Activation of A_1 receptors has been shown to produce a species-dependent stimulation or inhibition of the phosphatidylinositol pathway in cerebral cortex. In other tissues, activation of A_1 receptors results in synergistic activation of the phosphatidylinositol pathway in concert with Ca^{2+}-mobilizing hormones or neurotransmitters [15]. The effectors of A_1 adenosine receptors and other purinergic receptor subtypes are summarized in Table 17-2.

A_1 adenosine receptors are widely distributed in the central nervous system (Fig. 17-8). These receptors have been extensively characterized in brain because they are expressed in high density (0.5–1.0 pmol/mg membrane protein) and because of the development of several high-affinity radioligands, including [^{3}H]CPX. In the periphery, adenosine A_1 receptors are found in the heart, where they produce negative inotropic, chronotropic and dromotropic responses; in adipose tissue where they inhibit lipolysis and enhance insulin-stimulated glucose transport; and in kidney where they constrict preglomerular arterioles and produce antidiuresis. At the neuromuscular junction, adenosine inhibits acetylcholine release by a prejunctional effect on A_1 adenosine receptors.

Effects of adenosine that have been attributed to activation of central A_1 receptors include sedation, anticonvulsant activity, analgesia and neuroprotection. Adenosine

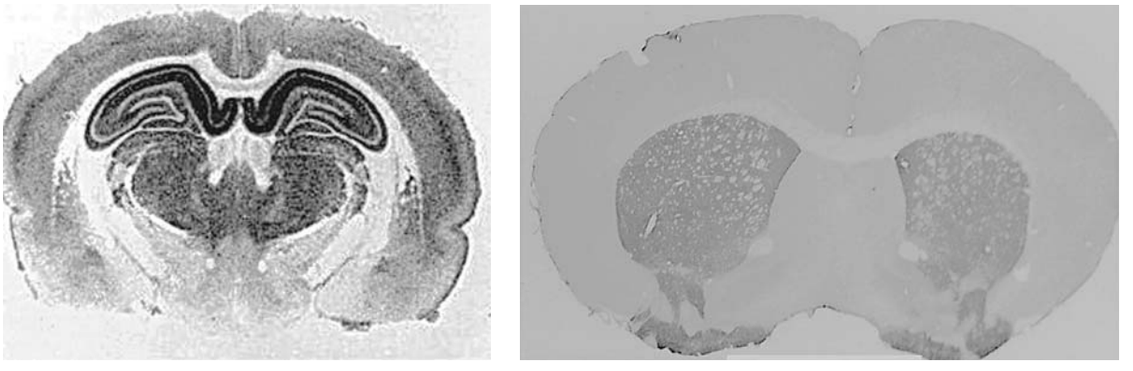

FIGURE 17-8 Distribution of A_1 and A_{2A} receptors in rat brain. (**A**) Autoradiograph of the binding of the A_1-selective radioligand [^{3}H]cyclohexyladenosine to a slice of rat brain. (Courtesy of Dr Kevin Lee, University of Virginia.) Note the widespread distribution of A_1 receptors and the particularly high density of receptors in the hippocampus. (**B**) Immunohistochemistry of A_{2A} adenosine in the mouse brain. Note the restricted striatal distribution.

modulates synaptic plasticity associated with distinct stimulation frequency patterns. Presynaptic A_1 adenosine receptors inhibit neurotransmitter release, especially at high neuronal stimulation frequency. Activation of A_1 receptors reduces long-term changes in synaptic efficiency (long-term potentiation) in various brain regions; e.g., activation of A_1 receptors reduces long-term potentiation of the population spikes evoked in the hippocampal CA1 area by stimulation of the Schaffer fibers. Release of glutamate and aspartate from nerve terminals in the CA1 region of the hippocampus is highly sensitive to inhibition by adenosine. Adenosine-induced antinociception is mediated in part by central A_1 receptors and by presynaptic A_1 receptor-induced inhibition of substance P- and CGRP-mediated release from terminals of sensory afferents, but mostly by activation of dorsal horn postsynaptic A_1 receptors located in lamina II of the spinal cord.

A_{2A} adenosine receptors are highly expressed in the basal ganglia. A_2 receptors were originally classified on the basis of their ability to stimulate cyclic AMP accumulation in neuronal tissues. Based on substantial differences in binding affinity for adenosine, these were divided into A_{2A} and A_{2B} subtypes, a subdivision that has subsequently been confirmed by molecular cloning. Adenosine analogs substituted on the 2-position, such as ATL146e and CGS21680, are agonists that bind with higher affinity to A_{2A} than to A_{2B} receptors. Caffeine, the most widely used psychoactive drug, has some selectivity as an antagonist of A_{2A} receptors. In transgenic mice that lack the A_{2A} receptors, caffeine reduces exploratory activity, an effect opposite to its usual one of stimulating exploratory activity. Potent and selective antagonists of A_{2A} receptors have been developed, including caffeine-like xanthines, such as CSC and KW-6002 and nonxanthine antagonists ZM241385 and SCH58261.

In the CNS, high-density expression of A_{2A} receptors is restricted to the striatum, nucleus accumbens and olfactory tubercle (Fig. 17-8B). In striatum, A_{2A} receptors are coexpressed in striatopallidal neurons with enkephalin and dopamine D_2 receptors. Blockade of A_{2A} receptors mimics the action of dopamine D_2 receptor agonists. Activation of A_{2A} adenosine receptors enhances cyclic AMP formation, whereas activation of D_2 dopamine receptors inhibits cyclic AMP formation. The administration of drugs that stimulate dopamine receptors, such as apomorphine and L-DOPA, to rodents with a unilateral lesion of the nigrostriatal pathway induces a turning behavior contralateral to the lesioned side, an effect due to the development of supersensitivity of dopamine receptors in the denervated striatum. Compounds that block adenosine receptors also produce such turning behavior and potentiate the effects of dopamine agonists. On the other hand, adenosine A_{2A} agonists inhibit D_2-mediated behaviors. Modulation by adenosine of striatal dopaminergic systems may contribute to the psychomotor depressant effects of adenosine agonists and to the psychomotor stimulatory effects of methylxanthines. The progressive degeneration of nigrostriatal neurons that occurs in Parkinson's disease results in a loss of dopaminergic input to striatal output neurons, enhanced striatopallidal GABAergic signaling and, hence, the motor disturbances that are characteristic of the disease (see Ch. 46). Replacement of dopaminergic input by administration of exogenous L-DOPA has been the primary therapeutic strategy for the treatment of Parkinson's disease for decades, but the complications associated with long-term L-DOPA treatment, particularly the 'wearing off' effects and the development of severe motor disturbances known as dyskinesias, have driven the search for alternative treatments. Adenosine A_{2A} receptors have become a target of therapeutic interest for a number of diseases, including Parkinson's disease [16, 17], mostly because of their discrete anatomical localization and their antagonistic interaction with dopamine D_2 receptors [18].

A_{2A}-selective adenosine receptor antagonists reverse the motor deficits in animal models of Parkinson's disease and potentiate the therapeutic effects of L-DOPA without producing dyskinesias. As such, they may have potential as adjunctive antiparkinsonian drugs. In addition, A_{2A} -receptor antagonists may also be useful in slowing the progression of neurodegeneration due to their neuroprotective effects in Parkinson's disease and other models of neurodegenerative disorders.

A_{2A} receptors that are present on sensory nerves in the carotid body, aortic body and elsewhere in the periphery produce excitatory sensory input. These receptors have been implicated in the production of pain associated with angina pectoris, ulcer and the human blister base preparation.

A_{2B} adenosine receptors regulate vascular permeability. A_{2B} receptors are found on endothelial cells, where they regulate vascular permeability, and on epithelial cells, where they regulate water secretion. These receptors are upregulated in the hippocampus following cerebral ischemic preconditioning and are thought to play a protective role. Both A_{2A} and A_{2B} receptors contribute to dilation of cerebral microvessels in response to adenosine.

A_3 adenosine receptors are few in the central nervous system. In the rat, abundant A_3 transcript is found exclusively in testes. However, the transcript for similar sheep and human receptors is modestly expressed throughout the brain and heavily expressed in pineal gland, lung and spleen.

EFFECTS OF PURINES IN THE NERVOUS SYSTEM: ATP RECEPTORS

P_{2X} subunits form trimeric ion-gated channels. As noted in Figure 17-4, there are seven genes encoding P_{2X}

channel subunits. Functional channels form when these receptors form homo- or heterotrimers [19]. The pharmacology of these channels is complex and may depend on the subunit composition of channel complexes.

P_{2X}-receptor-mediated responses in central and peripheral neurons indicate that ATP is a fast neurotransmitter that acts by opening ligand-gated ion channels [20]. P_2 receptors have a very wide tissue distribution. Excitatory effects of ATP on neurons in the CNS have been noted in the cuneate nucleus, cerebellar Purkinje cells, the cerebral cortex and cells in the sensory vestibular and trigeminal nuclei.. Effects of P_{2X} antagonists on excitatory postsynaptic currents in CA1 and CA3 pyramidal neurons suggest that P_2 receptors facilitate glutamate release. P_{2X} receptors may be important in central regulation of cardiorespiratory function; they have a critical role in peripheral chemoreceptor function and in modulating respiratory responses to hypoxia and hypercapnia. These receptors are also found in the gastrointestinal tract, veins and arteries, urinary bladder, vas deferens, nictitating membrane, cardiac muscle and pheochromocytoma (PC12) cells. In many instances, ATP has been noted to have biphasic or inhibitory effects, attributed in part to its breakdown to adenosine.

The release of ATP from a variety of cell types and its activation of P_{2X} receptors on sensory afferents is important in the initiation of pain that results from tissue trauma, inflammation or nerve injury. Adenosine, which may result from the rapid ectonucleotidase-mediated degradation of extracellular ATP, also modulates the pain pathways both centrally and peripherally. The P_{2X3} and heteromeric $P_{2X2/3}$ receptors in particular are present on a variety of nociceptive primary afferent neurons and may be involved in pain transmission. In addition, ATP can be released from spinal interneurons or by terminals of primary afferents in the spinal cord, where it modulates spinal nociceptive transmission by acting either on presynaptic P_{2X} receptors (1–3) to facilitate the release of glutamate or on postsynaptic P_{2X} receptors (homomeric P_{2X2}, P_{2X4}, and P_{2X6} and heteromeric $P_{2X4/6}$) on second-order neurons to stimulate fast excitatory transmission. If developed, potent and selective P_{2X} receptor antagonists, especially for P_{2X3} and $P_{2X2/3}$ receptors, could provide useful antinociceptive agents for the treatment of inflammatory, neuropathic and visceral pain.

P_{2Y} receptors are activated by adenine and uridine nucleotides. Most of the known P_{2Y} receptors have been detected in the nervous system [21]. The majority of P_{2Y} receptors inhibit neuronal N-type Ca^{2+} channels and M-type K^+ channels. P_{2Y1} receptors are found exclusively on platelets, on their precursor megakaryocyte cells and on certain other cultured hematopoietic cells, such as K562 leukemia cells. They can be distinguished from other P_2 receptors in that ADP is the most potent natural agonist and ATP is a competitive antagonist. ADP acts via a G protein to inhibit cyclic AMP accumulation, mobilize intracellular Ca^{2+} and stimulate granule secretion. ADP activates $P_{2Y}1$ and $P_{2Y}12$ receptors found on platelets and promotes platelet activity. Clopidogrel is metabolized in the liver to form a $P_{2Y}12$ antagonist that is used clinically to inhibit clotting.

Stable and potent P_{2Y2} receptor agonists are being administered by inhalation as a potential treatment for cystic fibrosis. P_{2Y14} receptors are unusual in that they are activated by UDP-glucose, UDP galactose and UDP-*N*-acetylglucosamine [22].

P_{2Y} receptors that are found on endothelial cells elicit a Ca^{2+}-dependent release of endothelium-dependent relaxing factor (EDRF) and vasodilation. A secondary activation of a Ca^{2+}-sensitive phospholipase A2 increases the synthesis of endothelial prostacyclin, which limits the extent of intravascular platelet aggregation following vascular damage and platelet stimulation. The P_{2Y}-mediated vasodilation opposes a vasoconstriction evoked by P_{2X} receptors located on vascular smooth muscle cells. The latter elicit an endothelial-independent excitation (i.e. constriction). P_{2Y} receptors are also found on adrenal chromaffin cells and platelets, where they modulate catecholamine release and aggregation respectively.

In the brain, P_{2Y} receptors may mediate the ability of ATP to enhance the postsynaptic actions of glutamate. Cotransmitter synergistic actions of ATP on the functional effects of glutamate may play an important role in learning and memory [23] (see also Ch. 53). Upregulation of P_{2Y14} receptors occurs in specific brain regions, including increased expression in astrocytes after immunological challenge, and suggests a mechanism for nervous system response to inflammation.

REFERENCES

1. Lazarowski, E. R., Boucher, R. C. and Harden, T. K. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol. Pharmacol.* 64: 785–795, 2003.
2. Zimmermann, H. Extracellular purine metabolism. *Drug Dev. Res.* 39: 337–352, 1996.
3. Resta, R. and Thompson, L. F. T cell signalling through cd73. *Cell Signal.* 9: 131–139, 1997.
4. Drury, A. N. and Szent-Gyorgyi, A. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J. Physiol. (Lond.)* 68: 213–237, 1929.
5. Berne, R. M. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *Am. J. Physiol.* 204: 317–322, 1963.
6. Burnstock, G. A basis for distinguishing two types of purinergic receptor. In L. Bolis and R. W. Straub (eds), *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* New York: Raven Press, pp. 107–118, 1978.
7. Maenhaut, C., Van Sande, J., Liebert, F. *et al.* RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* 173: 1169–1178, 1990.

8. Tesmer, J. J., Dessauer, C. W., Sunahara, R. K. *et al.* Molecular basis for P-site inhibition of adenylyl cyclase. *Biochemistry* 39: 14464–14471, 2000.
9. Illes, P. and Alexandre, R. J. Molecular physiology of P2 receptors in the central nervous system. *Eur. J. Pharmacol.* 483: 5–17, 2004.
10. Khakh, B. S., Burnstock, G., Kennedy, C. *et al.* International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol. Rev.* 53: 107–118, 2001.
11. Mosqueda-Garcia, R., Tseng, C.-J., Appalsamy, M., Beck, C. and Robertson, D. Cardiovascular excitatory effects of adenosine in the nucleus of the solitary tract. *Hypertension* 18: 494–502, 1991.
12. Sawynok, J., Reid, A. and Isbrucker, R. Adenosine mediates calcium-induced antinociception and potentiation of noradrenergic antinociception in the spinal cord. *Brain Res.* 524: 187–195, 1990.
13. Trussel, L. O. and Jackson, M. B. Adenosine-activated potassium conductance in cultured striatal neurons. *Proc. Natl Acad. Sci. U.S.A.* 82: 4857–4661, 1985.
14. Dolphin, A. C., Forda, S. R. and Scott, R. H. Calcium-dependent currents in cultured rat dorsal root ganglion neurons are inhibited by an adenosine analogue. *J. Physiol. (Lond.)* 373: 47–61, 1986.
15. Linden, J. Structure and function of the A_1 adenosine receptor. *FASEB J.* 5: 2668–2676, 1991.
16. Ferre, S., O'Connor, W. T., Snaprud, P., Ungerstedt, U. and Fuxe, K. Antagonistic interaction between adenosine A2A receptors and dopamine D2 receptors in the ventral striopallidal system. Implications for the treatment of schizophrenia. *Neuroscience* 63: 765–773, 1994.
17. Richardson, P. J., Kase, H. and Jenner, P.G. Adenosine A_{2A} receptor antagonists as new agents for the treatment of Parkinson's disease. *Trends Pharmacol. Sci.* 18: 338–344, 1997.
18. Ferré, S., Fredholm, B. B., Morelli, M., Popoli, P. and Fuxe, K. Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci.* 20: 482–487, 1997.
19. Chaumont, S., Jiang, L. H., Penna, A., North, R. A. and Rassendren, F. Identification of a trafficking motif involved in the stabilization and polarization of P2X receptors. *J. Biol. Chem.* 279: 29628–29638, 2004.
20. Edwards, F. A. and Gibb, A. J. ATP – a fast neurotransmitter. *FEBS Lett.* 325: 86–89, 1993.
21. Moore, D., Chambers, J., Waldvogel, H., Faull, R. and Emson, P. Regional and cellular distribution of the $P2Y_1$ purinergic receptor in the human brain: striking neuronal localisation. *J. Comp. Neurol.* 421: 374–384, 2000.
22. Abbracchio, M. P., Boeynaems, J. M., Barnard, E. A. *et al.* Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol. Sci.* 24: 52–55, 2003.
23. Wieraszko, A. and Ehrlich, Y. H. On the role of extracellular ATP in the induction of long-term potentiation in the hippocampus. *J. Neurochem.* 63: 1731–1738, 1994.

CHAPTER

18

Peptides

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NEUROPEPTIDES

Many neuropeptides were originally identified as pituitary or gastrointestinal hormones. Probably the first neuropeptide to be identified was vasopressin, a nine-amino-acid peptide secreted by the nerve endings in the neural lobe of the pituitary. The source of the vasopressin is the magnocellular neurons of the hypothalamus, which send axons to the neurohypophysis, which is the site of release into the blood, in classic neurosecretory fashion. Like vasopressin, a number of gastrointestinal peptides, such as cholecystokinin (CCK), are also found at high concentrations in the nervous system. In the gastrointestinal system, CCK is secreted into the blood by the duodenum and governs the delivery of digestive enzymes and bile acids into the intestine. In contrast to vasopressin and CCK, the hypothalamic releasing factors are peptides released into a special portal blood system that bathes the anterior pituitary, controlling the secretion of pituitary hormones (see Ch. 52). In this system, 'portal' means two successive capillary beds, one in the hypothalamus and one in the anterior pituitary. Substance P was first purified as a 'sialogogic peptide', causing salivation in a bioassay. Now substance P is recognized as a major bioactive peptide in many neuronal pathways, including pain signaling. Since there are so many peptides, this chapter focuses on the principles of how neuropeptides are synthesized, stored and released and how they act on the cells they regulate. Comparisons among peptides and smaller, 'fast-acting', 'classical' or 'conventional' neurotransmitters will be emphasized. It is significant to note that the number of known neuropeptides far exceeds the number of fast-acting conventional neurotransmitters.

Peptides can be grouped by structural and functional similarity. Although the list of neuropeptides is already quite long, as seen in the partial listing in Figure 18-1,

Selected Bioactive Peptides

Hypothalamic releasing factors

CRH: corticotropin releasing hormone
GHRH: growth hormone releasing hormone
GnRH: gonadotropin releasing hormone
Somatostatin
TRH: thyrotropin releasing hormone

Pituitary hormones

ACTH: adrenocorticotropic hormone
αMSH: α-melanocyte stimulating hormone
β-endorphin
GH: growth hormone
PRL: prolactin
FSH: follicle stimulating hormone
LH: luteinizing hormone
TSH: thyrotropin [thyroid stimulating hormone]

GI and brain peptides

CCK: cholecystokinin
Gastrin
GRP: gastrin releasing peptide
Motilin
Neurotensin
Substance K; substance P (tachykinins)

Circulating

Angiotensin
Bradykinin

Frog skin

Bombesin
Caerulein
Ranatensin

Opiate peptides

β-endorphin
Dynorphin
Leu-enkephalin
Met-enkephalin

Neurohypophyseal peptides

Oxytocin
Vasopressin

Neuronal and endocrine

ANF: atrial natriuretic peptide
CGRP: calcitonin gene-related peptide
VIP: vasoactive intestinal peptide

GI and pancreas

Glucagon
PP: pancreatic polypeptide
Ghrelin

Neurons only?

Galanin
Neuromedin K
NPY: neuropeptide Y
PYY: peptide YY

Endocrine only?

Calcitonin
Insulin
Secretin
Parathyroid hormone

FIGURE 18-1 Selected bioactive peptides are grouped by structural similarity or by tissue source.

additional neuropeptides are still being identified. Like GABA and glutamate or dopamine and norepinephrine, which differ by only a single carboxyl or hydroxyl group, yet have very different functions, many neuropeptides with similar structures have very different functions. Vasopressin and oxytocin are the two major neurohypophyseal peptides, and each consists of nine amino acids. These two peptides are identical at seven of those residues and are thought to be the result of gene duplication early in evolution. The actions of the two peptides are distinct: oxytocin causes milk letdown and uterine contraction, while vasopressin causes water retention in the kidney and blood vessel contraction. Likewise, the opiate peptides share a common Tyr–Gly–Gly–Phe–Met/Leu sequence at their NH_2-terminus and are potent endogenous opiates with distinct patterns of selectivity at the various classes of opiate receptor (see Ch. 56). The three glycoprotein hormones from the anterior pituitary, thyroid stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), share a common α subunit but have distinct β subunits, and only the αβ dimer is biologically active. The tachykinin group includes substance P and various frog skin peptides, all with similar core sequences and –Phe–X–Gly–Leu–Met–NH_2 at the COOH-terminus. The gastrointestinal peptides CCK and gastrin share a common COOH-terminal sequence (Trp–Met–Asp–Phe–NH_2) and are among the few peptides that undergo tyrosine sulfation. The sites of action of CCK and gastrin are distinct: gastrin stimulates gastric acid secretion, while CCK stimulates enzyme and bile acid delivery to the small intestine. Interestingly, the common COOH-terminal tetrapeptide, while inactive in the gastrointestinal tract, is abundant in the cerebral cortex and has important behavioral actions.

The function of peptides as first messengers is evolutionarily very old. In phylogenetic terms, neuropeptides were established very early as molecules effecting intercellular communication. In coelenterates, such as *Hydra*, there are many peptides used in neurotransmission, but many of the 'conventional' neurotransmitter systems, such as acetylcholine (ACh), catecholamines and serotonin,

covered in previous chapters, are not found [1]. The nerve net is strongly peptidergic in the lowest animal group with a nervous system, the cnidarians, which includes sea anemones, corals, jellyfishes and *Hydra*. Yeasts use bioactive peptides such as a- and α-mating factors to communicate.

Various techniques are used to identify additional neuropeptides. Bioassays are the oldest and surest way to identify biologically active peptides; more recently, with the advent of receptor-based assays, peptides have been identified by their ability to bind to a known receptor and thus to displace a ligand or to produce a biological response. The use of a relatively homogeneous tissue source, such as adrenal chromaffin granules, enabled identification of peptides derived from chromogranin A. Mass spectrometry, with its great sensitivity, is being used to characterize peptides from many sources. As noted above, peptides important in the nervous system were often identified first in some peripheral source, such as CCK in the gut, or even in unusual places, such as frog skin. Since α-amidation of the COOH-terminus of peptides has proven to be a signature of bioactive peptides, assays specific for COOH-terminal α-amides were developed and used to discover neuropeptide Y and several other peptides. Molecular biological approaches have also been used to discover many new peptides. Subtractive hybridization and differential display have been used to identify both the cocaine- and amphetamine-regulated transcript (CART) and RESP18, a dopamine-regulated transcript. Finally, screens using orphan receptors, which have no known ligand, were used to find natural ligands, such as orphanin FQ/nociceptin and orexin/hypocretin. Systematic scans of sequence databases can identify additional family members and potential peptide precursors, by taking into account key features of precursor structures.

The neuropeptides exhibit a few key differences from the classical neurotransmitters. First, neuropeptides are present in tissues at much lower concentrations than classical neurotransmitters. Consistent with this, neuropeptides are also active at receptors at correspondingly lower concentrations. For example, the concentration of ACh and NE in synaptic vesicles is in the 100–500 mmol/l range, while the concentration of neuropeptide in a large dense core vesicle is 3–10 mmol/l at most. Correspondingly, the affinity of ACh for its receptors is in the micromolar to millimolar range, while peptides typically bind to their receptors with affinities in the nanomolar to micromolar range.

Probably the most striking difference between neuropeptides and conventional neurotransmitters is in their biosynthesis (Fig. 18-2). Neuropeptides are derived from larger, inactive precursors that are generally at least 90 amino acid residues in length [2–4]. The simplest example is prolactin, a pituitary product. The signal sequence for

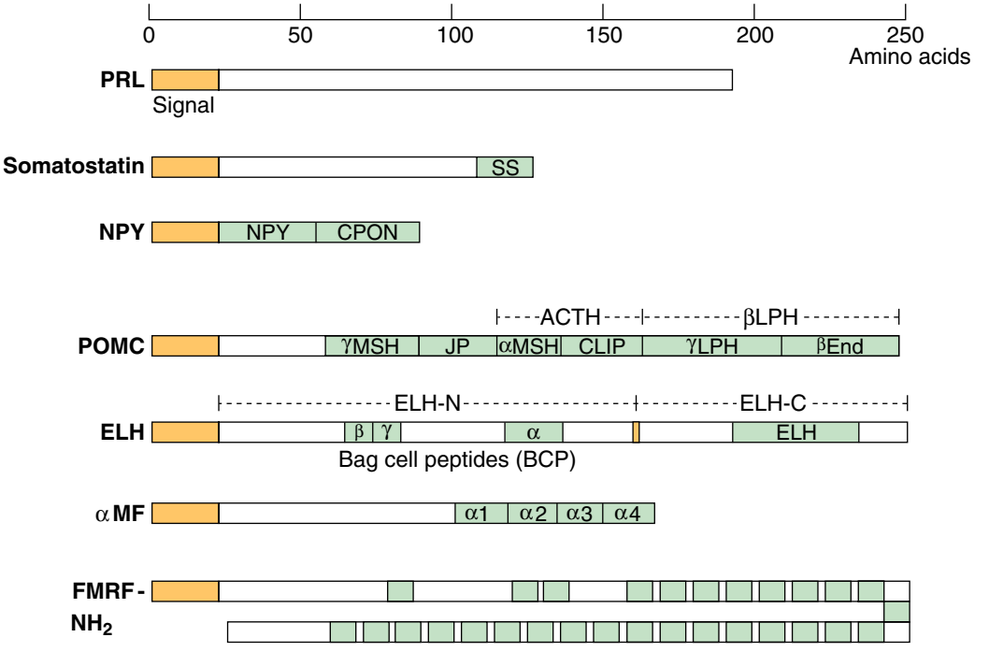

FIGURE 18-2 Structures of selected bioactive peptide precursors are diagrammed. The structures of prolactin (*PRL*), somatostatin, neuropeptide Y (*NPY*), pro-opiomelanocortin (*POMC*), egg-laying hormone (*ELH*), yeast α-mating factor (*αMF*) and FMRF-amide precursors (*FMRF-NH$_2$*) are indicated. Signal sequences are shaded and on the left of each precursor. *βEnd*, β-endorphin; *ACTH*, adrenocorticotropic hormone; *CLIP*, corticotropin-like intermediate lobe peptide; *CPON*, C-terminal flanking peptide of NPY; *JP*, joining peptide; *LPH*, lipotropin; *MSH*, melanocyte-stimulating hormone; *SS*, somatostatin.

prolactin must be removed and disulfide linkages must form, but no further enzymatic steps are necessary. The next simplest case is somatostatin, in which a single cleavage after signal peptide removal produces the bioactive peptide. Neuropeptide Y (NPY) is derived from proneuropeptide Y after signal peptide removal, cleavage between NPY and the C-terminal flanking peptide of NPY (CPON) and additional modifications, which are discussed below. The pro-opiomelanocortin (POMC) precursor includes several different bioactive peptides, as does the egg-laying hormone (ELH) precursor [2]. One interesting attribute common to peptide precursors in evolutionarily older species is the existence of multiple copies of the same bioactive peptide in one precursor; this is exemplified by the FMRF-NH_2 (Phe–Met–Arg–Phe–amide) precursor, with 29 copies of the active peptide [5]. Even in yeast, a similar process is used, so that four copies of α-mating factor are produced from the α-mating factor precursor. Precursors with multiple copies of bioactive peptide are much less common in evolutionarily more advanced species, although the rat TRH precursor contains five copies of the TRH tripeptide.

The supply of conventional neurotransmitters in small synaptic vesicles is replenished in nerve terminals by local synthesis, and many conventional neurotransmitters are recaptured after secretion. In striking contrast, neuropeptides are initially synthesized in the cell soma, sequestered within the lumen of the secretory pathway and transported down the axon while undergoing cleavages and other processing events, after which the peptide-containing, large dense-core vesicle (LDCV) is used once. After exocytosis, the membrane components of the LDCV must be reinternalized and either destroyed or transported back to the cell body for reuse. Thus, no re-use of either the neuropeptides or their immediate precursors occurs at the synapse.

Release is another area of difference: conventional neurotransmitters are secreted from small synaptic vesicles (SSVs) after cytosolic [Ca^{2+}] transiently reaches concentrations of 50–100 µmol/l, while peptides are released from LDCVs at lower concentrations of cytosolic [Ca^{2+}] (Fig. 18-3). Conventional neurotransmitter release is thought to occur very close to the site of Ca^{2+} entry (see Chs 9, 10), while neuropeptides are typically released at a distance from the site of Ca^{2+} entry (Fig. 18-4). Furthermore the Ca^{2+} that stimulates exocytosis from LDCVs may come from either internal stores or the extracellular fluid. Thus, the location of LDCVs relative to the site of Ca^{2+} influx has a very crucial impact on the intensity of stimulation necessary for secretion to occur.

Neuropeptides are often found in neurons with conventional neurotransmitters. As illustrated in Figure 18-3, both conventional neurotransmitters and neuropeptides are found at a majority of the synapses in the nervous system. Neuropeptide expression is extremely plastic, even in the adult. For example, the hypothalamic neurons that express vasopressin and those that synthesize

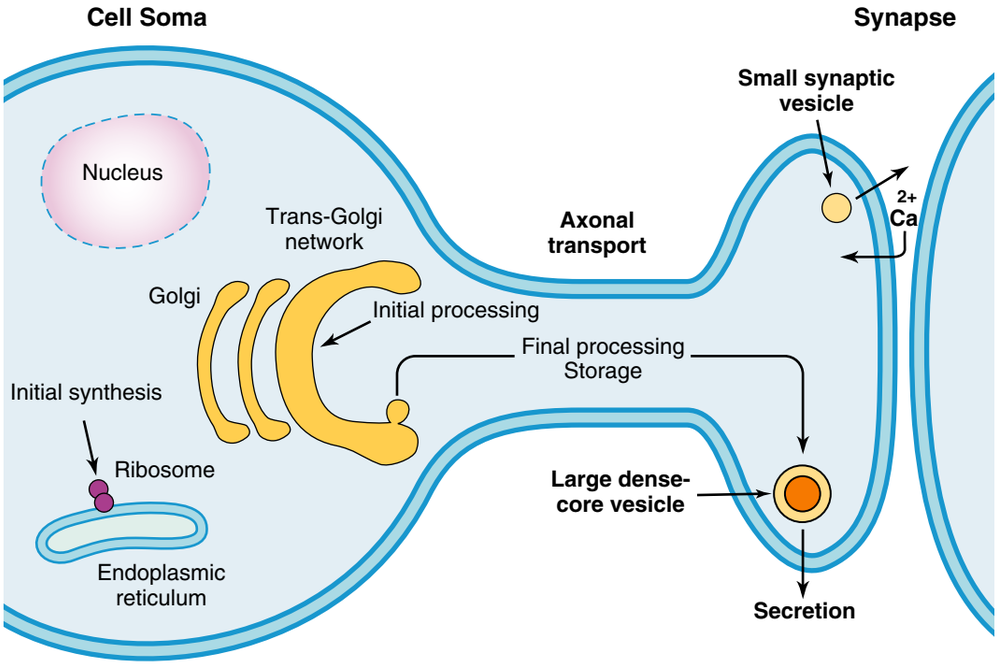

FIGURE 18-3 Intracellular pathway of bioactive peptide biosynthesis, processing and storage. Neuropeptide precursors are synthesized on ribosomes at the endoplasmic reticulum and processed through the Golgi. Axonal transport of the large dense-core vesicle to the synaptic site of release precedes the actual secretion.

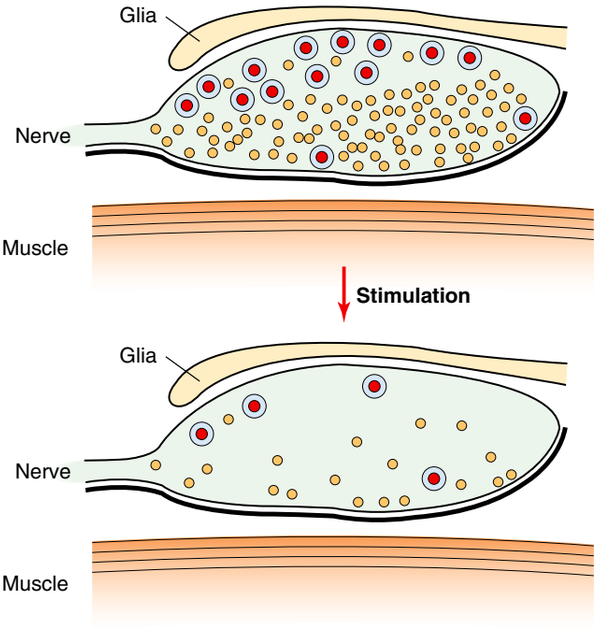

FIGURE 18-4 Neuropeptides and conventional neurotransmitters are released from different parts of the nerve terminal. A neuromuscular junction containing both large dense-core vesicles (containing the neuropeptide SCP) and also small synaptic vesicles (containing acetylcholine) was stimulated for 30 min at 12 Hz (3.5 s every 7 s). Depletion of the small clear vesicles at the muscle face and of the peptide granules at the nonmuscle face of the nerve terminal was observed. After stimulation, there was an increase in the number of large dense-core vesicles within one vesicle diameter of the membrane. (Adapted from reference [37].)

corticotropin-releasing hormone (CRH) are situated close to each other but constitute separate and virtually nonoverlapping populations of neurons in the normal animal. However, after glucocorticoid concentrations are lowered by blockade of adrenal cortical function or removal of the adrenal glands, vasopressin neurons begin to express CRH and CRH neurons begin to synthesize vasopressin. This adaptive response can be understood from a teleological point of view by knowing that CRH normally stimulates the adrenocorticotropic-hormone (ACTH)-producing cells of the anterior pituitary to secrete ACTH and that ACTH stimulates glucocorticoid production in the adrenal cortex. Vasopressin acts synergistically to increase ACTH secretion in times of need, such as following adrenalectomy. In addition to neuropeptides, many LDCVs contain ATP, just as many conventional neurotransmitter vesicles do, so that ATP is released along with neuropeptides. ATP and adenosine can have potent synaptic actions in their own right (see Ch. 17 for purinergic systems).

The biosynthesis of neuropeptides is fundamentally different from that of conventional neurotransmitters. To add to the complexity discussed above, the metabolism of neuropeptide precursors is tissue-specific, with a general rule that most precursors are expressed in more than one tissue and that the processing is not identical in different tissues (Fig. 18-5) [2–4]. For example, anterior pituitary corticotropes cleave POMC to ACTH(1–39), a molecule that stimulates adrenal glucocorticoid production. Neurons in the arcuate nucleus cleave ACTH and α-amidate, the smaller peptide to create ACTH(1–13)NH_2, which cannot stimulate the adrenal cortex but does have potent behavioral effects in the CNS. Intermediate pituitary melanotropes go one step further and α-*N*-acetylate the amidated peptide to produce α-melanocyte-stimulating hormone (αMSH). The skin-darkening activity of αMSH is especially important in lower vertebrates for which background color adaptation is protective. Similarly, corticotropes produce β-lipotropin (βLPH), which has no activity as an opiate peptide, while melanotropes and CNS neurons cleave βLPH to produce the potent opiate peptide β-endorphin(1–31). In some tissues, the β-endorphin may be shortened at the COOH-terminus, which decreases its opiate activity, or α-*N*-acetylated at its NH_2-terminus, which abolishes opiate activity. The cellular control of these different patterns of processing is beginning to be understood, with the identification of some of the enzymes that mediate these steps.

Other examples of tissue-specific processing include proenkephalin, proglucagon, procholecystokinin and prosomatostatin. Somatostatin neurons in the hypothalamus primarily produce a 14-residue form of the peptide, while somatostatin endocrine cells of the pancreas and intestine produce a 28-residue form derived from the same precursor. Proenkephalin is processed in the adrenal medulla to a set of opiate peptides of 15–35 residues, while proenkephalin in the brain is cleaved primarily to the pentapeptides met-enkephalin and leu-enkephalin. Procholecystokinin in the gut is processed to peptides of approximately 30 residues, which act on the pancreas and gallbladder, while smaller CCK-related peptides with behavioral effects are found in the brain. These smaller CCK-related peptides have no effects when applied to the pancreas or gallbladder. Understanding peptide biosynthesis and peptide structures is important, since many smaller peptide fragments are active, frequently exhibiting biological activities distinct from that of the larger parent peptide [6].

Many of the enzymes involved in peptide biogenesis have been identified. The most common steps in precursor processing and the enzymes involved are shown in Figure 18-6. The endoproteases involved are prohormone convertases 1 and 2 (PC1 and PC2), the exopeptidase is carboxypeptidase E (CPE) and the α-amidating enzyme is peptidylglycine α-amidating monooxygenase (PAM). The nomenclature for PCs is very confusing and is summarized in Table 18-1; this chapter uses the original names for the PCs. Many steps in their biosynthesis, such as signal peptide cleavage, disulfide bond formation, the addition and subsequent modification of N-linked and O-linked oligosaccharides, phosphorylation and sulfation,

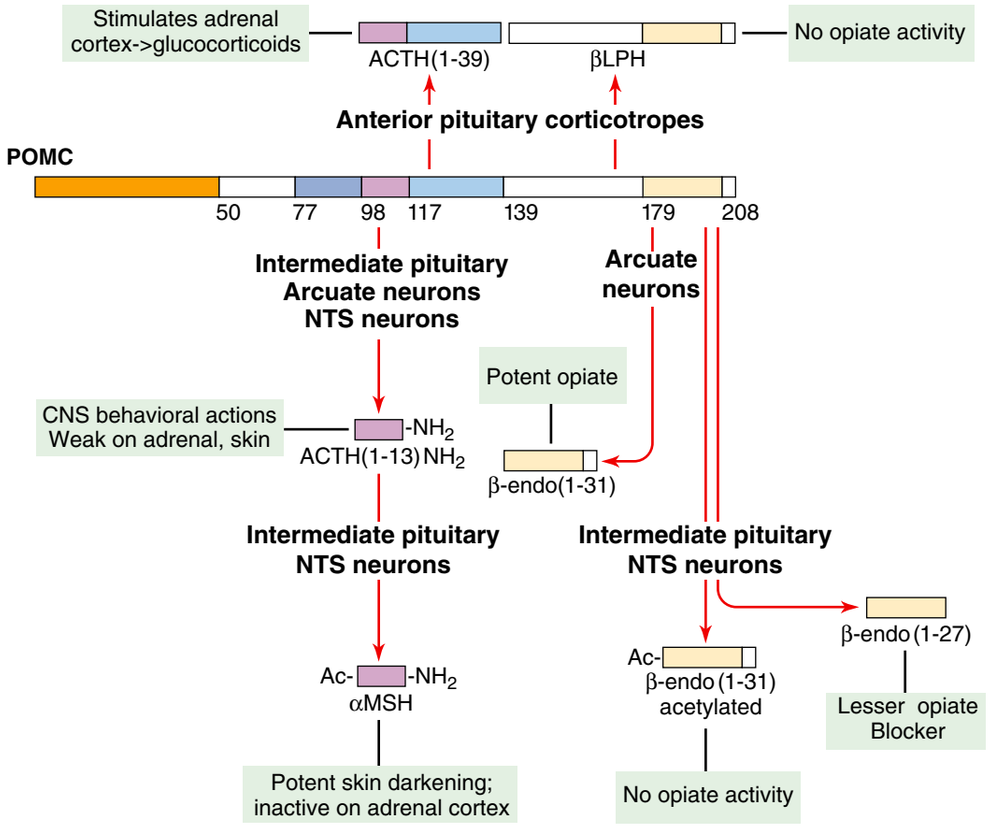

FIGURE 18-5 Tissue-specific processing of the pro-opiomelanocortin (*POMC*) precursor yields a wide array of bioactive peptide products. Processing of the POMC precursor varies in various tissues. In anterior pituitary, adrenocorticotropic hormone (*ACTH (1–39)*) and β-lipotropin (β-*LPH*) are the primary products of post-translational processing. Arcuate neurons produce the potent opiate β-endorphin (β-*endo (1–31)*) as well as ACTH(1–13)NH_2. Intermediate pituitary produces α-melanocyte-stimulating hormone (α*MSH*), acetylated β-endo(1–31) and β-endo(1–27). *NTS*, nucleus tractus solitarius.

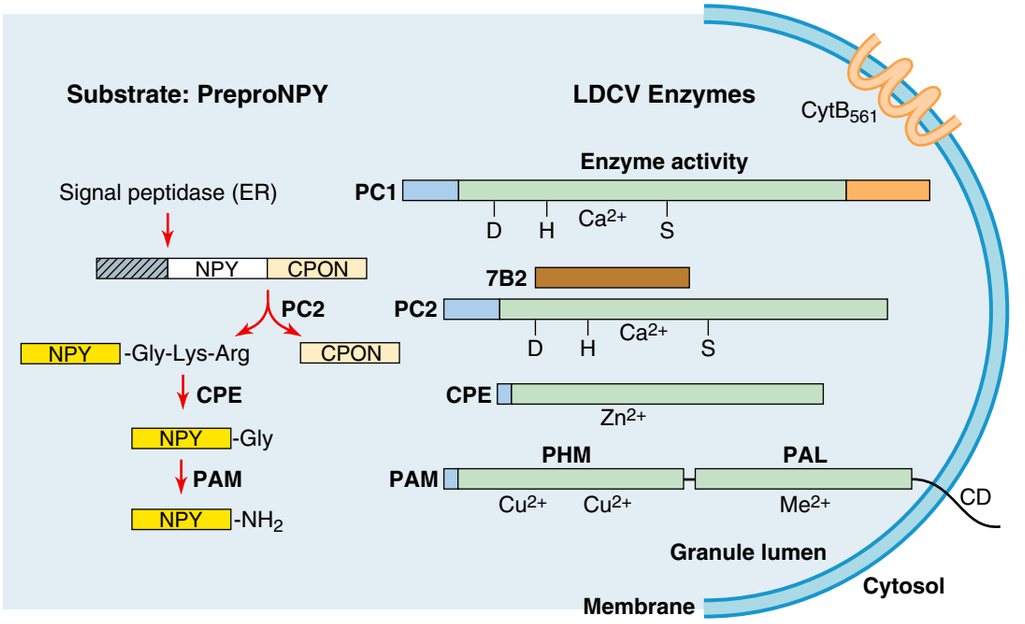

FIGURE 18-6 Sequential enzymatic steps lead from the peptide precursor to bioactive peptides. The neuropeptide Y (*NPY*) precursor shown at the left is processed sequentially by the enzymes of the large dense-core vesicles (*LDCV*) shown at the right. *CD*, cytoplasmic domain; *CPH*, carboxypeptidase E; *CPON*, C-terminal flanking peptide of NPY; *ER*, endoplasmic reticulum; Me^{2+}, divalent metal ion; *PAL*, peptidyl-α-hydroxyglycine α-amidating lyase; *PAM*, peptidylglycine α-amidating mono-oxygenase; *PC*, prohormone convertase; *PHM*, peptidylglycine α-hydroxylating mono-oxygenase.

TABLE 18-1 Prohormone convertases

Original name	SPC name	Other names
Furin	SPC1	PACE
PC2	SPC2	
PC1	SPC3	PC3
PACE4	SPC4	
PC4	SPC5	
PC6	SPC6	PC5
PC8	SPC7	PC7,LPC

are not unique to neuropeptides. As shown in Figure 18-3, many of the post-translational steps occur as the maturing neuropeptides travel down the axon toward the synapse in LDCVs. The later steps in neuropeptide biosynthesis (Fig. 18-6) are unique to neurons and endocrine cells.

Key enzymes in neuropeptide biosynthesis include endoproteases, exoproteases and enzymes modifying the ends of the peptides. The discovery and characterization of Kex2p, the endoprotease that cleaves yeast pro-α-mating factor to produce four copies of the pheromone α-mating factor (Fig. 18-2), were key to the discovery of the mammalian prohormone convertases, including furin, PC1, PC2, PC4, PC5, PC8 and PACE4 [3, 4, 7]. The prohormone convertases share homology with bacterial subtilisins at their NH_2-termini, and have an Asp–His–Ser catalytic triad, which consists of the three key amino acids involved in catalysis (denoted D, H and S in Fig. 18-6). Eukaryotic enzymes in this family all have an essential 'P' domain COOH-terminal to the subtilisin-related enzymatic domain, followed by a variable region [3]. The proregion of each enzyme (Fig. 18-6) must be present during biosynthesis for the protease to fold correctly but must be removed to yield an activated protease. For PC1 and furin, removal of the proregion occurs within a few minutes of biosynthesis while the enzyme is in the endoplasmic reticulum and is most probably an autocatalytic event. For the other prohormone convertases, removal of the proregion occurs much more slowly. Expression of active PC2 is thought to require coexpression of the peptide 7B2 (Fig. 18-6), which appears to perform a chaperone function and may also prevent expression of PC2 endoproteolytic activity until PC2 has been deposited into secretory granules [8]. Pro-SAAS may be a corresponding chaperone/inhibitor peptide for PC1.

The mammalian endoproteases most clearly involved in neuropeptide processing are PC1 and PC2, Ca^{2+}-dependent proteases found in secretory granules whose expression is limited to neurons and endocrine cells (Fig. 18-6). Several other members of this endoprotease family are more widely expressed, while still others are expressed in restricted locations distinct from neurons and endocrine cells. For example, furin is found in virtually all cells and is localized primarily to the *trans*-Golgi network; furin catalyzes cleavages important in peptide function, such as the initial cleavage of the precursors to ELH, nerve growth factor and parathyroid hormone, as well as cleavage within the insulin receptor precursor to produce the active αβ dimer form of that receptor. Furin may also be instrumental in the activation of some of the other processing enzymes, such as PC2 and CPE.

PC1 and PC2 cleave at selected pairs of basic amino acids in peptide precursors: Lys–Arg, Arg–Arg, Lys–Lys and Arg–Lys. PC1 may also catalyze cleavages at selected single Arg sites present in some precursors, such as prosomatostatin and procholecystokinin. Cleavages in LDCVs by PCs are tightly controlled, often occurring in a very orderly fashion (Fig. 18-7). The initial cleavages of POMC

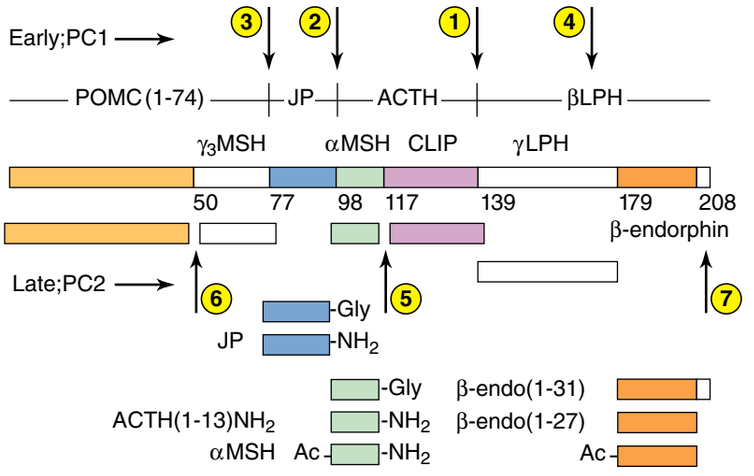

FIGURE 18-7 Processing of the pro-opiomelanocortin (*POMC*) precursor proceeds in an ordered, stepwise fashion. Cleavage of the POMC precursor occurs at seven sites, with some of the reactions being tissue-specific. The circled numbers indicate the temporal order of cleavage in tissues where these proteolytic events occur. *ACTH*, adrenocorticotropic hormone; *CLIP*, corticotropin-like intermediate lobe peptide; *JP*, joining peptide; *LPH*, lipotropin; *MSH*, melanocyte-stimulating hormone; *PC*, prohormone convertase.

occur in less than 1 hour (Fig. 18-7, steps 1 and 2, performed by PC1), while other cleavages occur only after several hours (Fig. 18-7, steps 6 and 7, performed by PC2). The endoproteolytic cleavage of propeptides is often the rate-limiting reaction in peptide biosynthetic processing.

The pattern of cleavages catalyzed by PC1, PC2 and furin when expressed in neurons and endocrine cells is much more selective than the pattern of cleavages seen in test tube assays with purified enzymes. For example, although prohormone convertases usually cleave at the COOH-terminus of a pair of basic residues in model peptide substrates, the cleavages in cells can be in the middle of the pairs of basic residues, as in the case of POMC cleavage (cleavage 6; Fig. 18-7), where the basic residues are separated and remain with the two resulting mature peptides [2]. It is likely that the Ca^{2+} concentration and internal pH of LDCVs are two variables used by neurons and endocrine cells to regulate endoproteolytic activity in LDCVs.

Additional endoproteases may be shown to play a role in neuropeptide biosynthesis. Leading candidates are the mammalian homolog of the yeast aspartyl protease-3 (YAP-3) and a thiol protease. The processing of proANF, which involves cleavage after a single Arg residue in proANF, cannot involve PC1 or PC2 since there are negligible amounts of these PCs in the heart.

CPE is a soluble protein found in virtually all LDCVs in neurons and endocrine cells (Fig. 18-6) [9]. It removes basic residues, Lys or Arg, from the COOH termini of peptide intermediates produced by the prohormone convertases, and has a pH optimum of 5.0–5.5, similar to the pH inside LDCVs. It was originally identified by its tissue distribution and substrate specificity, along with its specific inhibition by guanidinoethylmercaptosuccinic acid (GEMSA). CPE is a Co^{2+}- and Zn^{2+}-activated enzyme with a short proregion that is normally removed during maturation of the enzyme; unlike the prohormone convertases, CPE is active with the proregion attached. The carboxypeptidase function of peptide processing is not normally rate-limiting since peptide intermediates with COOH-terminal basic residues are detected only at extremely low concentrations in tissue or LDCV extracts. The structure of CPE has been modeled, based on the crystal structure of duck carboxypeptidase D domain II [10, 11]. The carboxypeptidase catalytic core has two domains, one of 300 residues with the one catalytic Zn^{2+} ion, and one with 80 residues. Additional carboxypeptidases were identified in mice lacking CPE, notably CPD, an integral membrane enzyme with two active and one inactive carboxypeptidase domains. The relative importance of CPE and these additional carboxypeptidases to neuropeptide processing *in vivo* is unclear. Given that cleavage at a pair of basic residues can be in the middle of the pair, there is good reason to think that an aminopeptidase will eventually be found in LDCVs.

PAM is a bifunctional enzyme found in nearly all LDCVs (Fig. 18-6) [12]. PAM acts on peptide substrates after endoproteolytic cleavage and exopeptidase action, when a COOH-terminal Gly residue is exposed, and converts the peptidyl-Gly into the corresponding peptide-NH_2. About half the known bioactive peptides are α-amidated, and α-amidation is generally crucial to biological potency. The peptidyl-Gly and peptide-COOH forms are usually inactive at physiological concentrations. The first step of the α-amidation reaction is performed by peptidylglycine α-hydroxylating monooxygenase (PHM), which is the NH_2-terminal portion of the bifunctional PAM protein; as with CPE, the pH optima for both enzymatic domains of PAM are around 5.5. PHM binds two Cu^{2+} atoms, which participate in catalysis by undergoing cycles of reduction and oxidation. PHM uses ascorbic acid as the reductant, with one atom of oxygen from O_2 incorporated into the peptide during the hydroxylation step. Thus, PHM is enzymatically very similar to dopamine β-hydroxylase (DBH), which converts dopamine to norepinephrine (see Ch. 12).

The crystal structure of the 315 residue catalytic core of PHM has been solved, revealing two domains of roughly equal size, each with one Cu^{2+} ion. The two copper ions are about 11 Å apart, with the peptide substrate relatively close to one of the two copper ions [12]. The second step of the α-amidation reaction is performed by a second enzymatic domain of PAM, peptidyl-α-hydroxyglycine α-amidating lyase (PAL). The PAL domain constitutes a novel, divalent metal ion-dependent enzyme. Neurons primarily express an integral membrane form of the bifunctional PAM protein (Fig. 18-6), while an additional mRNA-splicing event enables some endocrine cells to express soluble versions of the protein, lacking the transmembrane domain. In the integral membrane forms of PAM, the short COOH-terminal domain extends into the cytoplasm and participates in the routing of PAM into LDCVs and its recovery from the cell surface. The supply of reduced ascorbate in LDCVs is maintained by cytochrome b_{561}, a heme-containing protein that has six transmembrane domains and shuttles electrons from cytosolic ascorbate to ascorbate in the lumen of the LDCVs [13]. Cytochrome b_{561} is also found in catecholamine-containing vesicles, where it performs a similar function for DBH (see Ch. 12). Nervous and endocrine tissues have a high-affinity plasma membrane ascorbate uptake system to maintain concentrations of reduced ascorbate about 100-fold above the blood concentration of ascorbate, while many other tissues do not concentrate ascorbate significantly [14].

Several peptides have NH_2-terminal pyroglutamic acid residues, also termed cyclic glutamic acid (<Glu), which are essential to bioactivity. Prime examples are thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH). The enzyme responsible for this modification is glutaminyl cyclase, which converts the original NH_2-terminal Gln into Glu. Glutaminyl cyclase is a soluble, secretory pathway protein of 41 kDa, and requires divalent metal ion for full activity [15].

Another important but infrequent modification of peptides is α-*N*-acetylation (Figs 18-5, 18-7). During POMC processing, α-*N*-acetylation greatly increases the skin-darkening potency of ACTH(1–13)NH_2 while abolishing both the adrenal steroidogenic potency of ACTH and the opiate activity of β-endorphin [2]. The enzyme(s) responsible for this modification has not yet been purified or cloned.

As an example, Figure 18-7 shows the pattern of processing steps in the POMC system [2]. The initial endoproteolytic steps (Fig. 18-7, steps 1–4) are mediated by PC1 and occur in all POMC-producing neurons and endocrine cells, usually in the numerical order shown. It is clear that steps 1 and 2 are initiated in the *trans*-Golgi network and continue in LDCVs, while step 4 occurs only in LDCVs. Steps 5–7 occur only in LDCVs and seem to require PC2. In the adult anterior pituitary, corticotropes contain PC1 but not PC2 and perform only cleavages 1–4. However, during early postnatal development, corticotropes also express PC2 and cleavages 5–7 are transiently seen. In the rat, expression of PC2 and cleavage within ACTH (cleavage 5) decline simultaneously a few weeks after birth, at about the time that the adult pattern of ACTH control over adrenal steroidogenesis appears.

Melanotropes and CNS neurons making POMC express both PC1 and PC2 and, thus, the smaller peptide products are seen in these cells. PAM is expressed in all POMC-producing cells, so the α-amidation of joining peptide (JP), a small peptide with no clear biological function, occurs rapidly in all POMC cells (Fig. 18-7). In the melanotropes of the intermediate pituitary and the POMC neurons of the nucleus of the solitary tract, α-*N*-acetylation of ACTH(1–13)NH_2 and β-endorphin occurs. In melanotropes, α-*N*-acetylation of ACTH can occur before cleavage 5. As indicated in Figure 18-5, the particular cleavages made and the modifications made to the NH_2- and COOH-termini of the peptide products determine the mixture of bioactive peptides released.

Neuropeptides are packaged into large, dense-core vesicles. In many cases, the peptide products from the processing of a propeptide are packaged together in an equimolar fashion in LDCVs and the peptides and the soluble processing enzymes (PC1, PHM, CPE) are all released together in response to stimuli (Fig. 18-8) [2, 3]. By comparison, there are also examples where the products of propeptide processing are sorted into different LDCVs or are subject to degradation. In *Aplysia* bag cell neurons, ELH is formed from the COOH terminus of the ELH precursor (Fig. 18-2), while α, β and γ bag cell peptides (BCPs) are formed from the NH_2-terminal portion (Fig. 18-8). The initial cleavage of the pro-ELH precursor

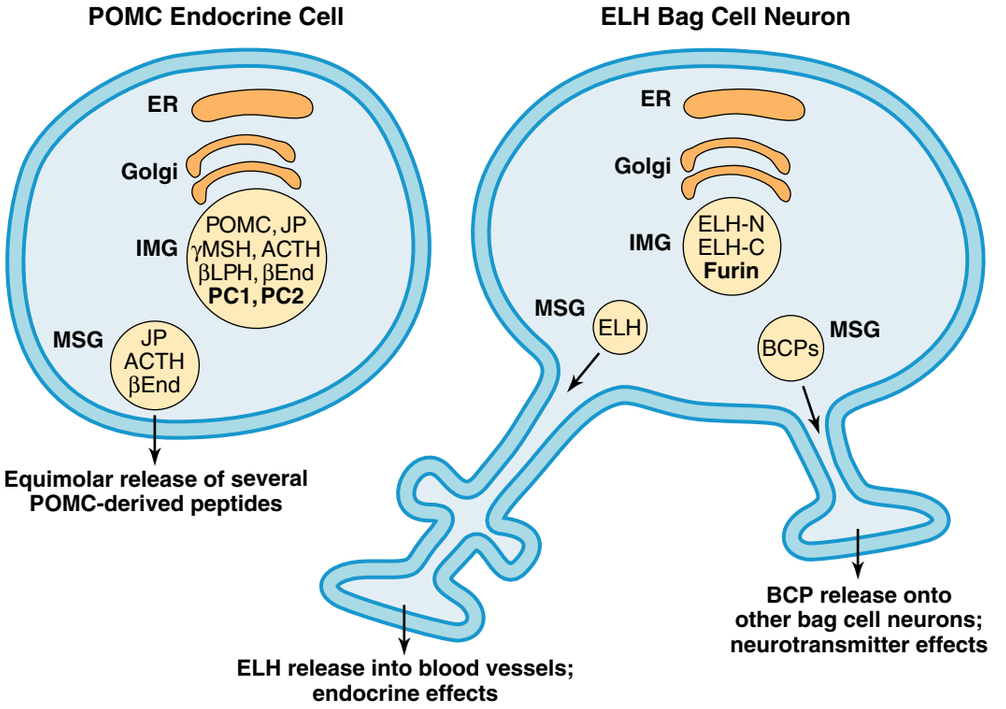

FIGURE 18-8 Cell-specific packaging of peptides into large dense-core vesicles can lead to very different patterns of peptide secretion. Sorting of neuropeptides into distinct mature secretory granules (*MSG*) is shown for bag cell neurons but does not occur for endocrine cells. *βEnd*, β-endorphin; *ACTH*, adrenocorticotropic hormone; *BCP*, bag cell peptide; *ELH*, egg-laying hormone, *POMC*, pro-opiomelanocortin; *ER*, endoplasmic reticulum; *IMG*, immature granules; *JP*, joining peptide; *LPH*, lipotropin; *MSH*, melanocyte-stimulating hormone; *PC*, prohormone convertase.

occurs in the *trans*-Golgi network and the peptides are then separated into two distinct types of LDCV, which are sent to different parts of the cell (Fig. 18-8). These two sets of peptides mediate a coordinated set of behaviors involved in egg laying. Similarly, in TRH neurons, the NH_2- and COOH-terminal domains of the pro-TRH precursor are separated from each other and stored in distinct LDCVs.

Diversity is generated by families of propeptides, alternative splicing, proteolytic processing and post-translational modifications. The huge number of biologically active peptides is the result of many factors. First, there are several families of genes which clearly evolved from a common ancestor (Fig. 18-1): examples include the three precursors to β-endorphin, dynorphin and the enkephalins; the precursors to gastrin and CCK; and the precursors to oxytocin and vasopressin. Second, there are several peptide precursors that yield multiple copies of bioactive peptide: examples include the ELH precursor, the α-mating factor precursor, the FMRF-NH_2 precursor and the enkephalin precursor. Likewise, several distinct biological activities are found within the POMC and ELH precursors. Third, there is alternative splicing of mRNAs encoding preprohormones, first discovered in the calcitonin and calcitonin gene-related peptide precursors but also seen in the case of the preprotachykinin precursor, which yields substance P, substance K and several other peptides, depending on the splicing pattern (Fig. 18-9) [16]. Finally, RNA editing can be involved, as in the case of the amphibian bombesin-like peptides, where nucleotides in the mRNA are changed and the final protein is not a direct reflection of the sequence encoded in the gene. RNA editing is also seen in the glutamate (Ch. 15) and serotonin receptors (Ch. 13 and Ch. 15) and probably will be found elsewhere as detection methods become more sophisticated.

NEUROPEPTIDE RECEPTORS

Most neuropeptide receptors are seven-transmembrane-domain, G-protein-coupled receptors. The first neuropeptide receptors characterized were those for substance P and neurotensin, and most have the general architecture shown in Figure 18-10. The basic rules for the function of these seven-transmembrane-domain receptors are the same as the rules established for similar serpentine receptors for conventional small molecule neurotransmitters, such as muscarinic, adrenergic and metabotropic glutamatergic receptors (Chs 10–16). In general, there are more receptors than peptides, and in mammals there are over 100 neuropeptides known; in *Drosophila*, current analyses recognize 22 neuropeptide prohormone precursors and 44 peptide receptor genes [17]. For many peptides, the extracellular domain plays a major role in ligand binding (Fig. 18-10) [18]. In addition, the key components of the

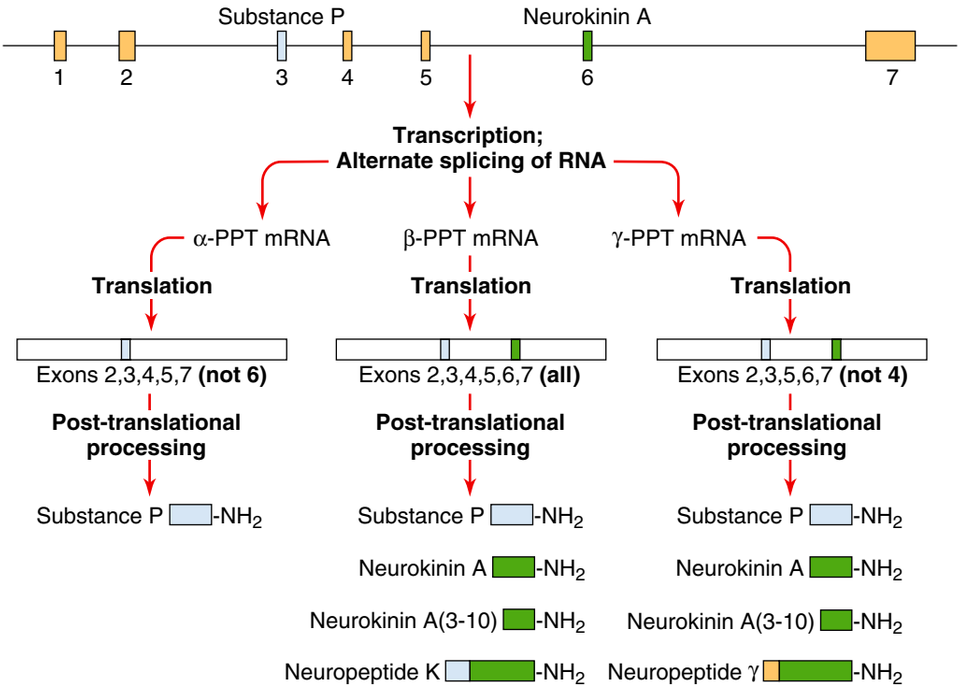

FIGURE 18-9 Several mechanisms through which the substance P gene gives rise to different bioactive peptides in different neurons. Alternative splicing of mRNA leads to translation of distinct precursors, and subsequent processing leads to unique mature peptides. *PPT*, pre-protachykinin. (Adapted from reference [16].)

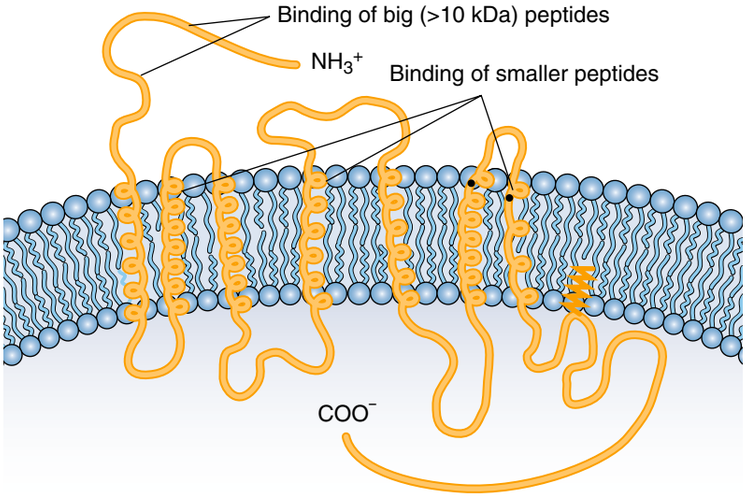

FIGURE 18-10 Serpentine (seven-transmembrane-domain) receptors for peptides have binding for their peptide ligands within the membrane and in the NH_2-terminal loop.

binding sites for many peptides also include the analogous residues important for binding small nonpeptide ligands to their receptors, within the transmembrane domains and in the extracellular loops [18].

The binding specificity of neuropeptide receptors for a given neuropeptide may vary considerably. Five somatostatin receptors have been identified; they bind somatostatin similarly and all inhibit adenylyl cyclase. However, these receptors differ substantially with regard to their interaction with various somatostatin analogs used for therapeutic purposes. For example, the receptor called SSTR2 binds a small peptide analog, octreotide, much more tightly than the other somatostatin receptors. This differential sensitivity is being used for therapeutic drug targeting [19]. Changing a few amino acids in transmembrane domains VI and VII of SSTR1 to the residues found in SSTR2 (Fig. 18-10) allows the mutant SSTR1 to bind the peptide analog as well as SSTR2. Similar changes in specificity are seen with several other peptide receptor families [16, 18, 20].

Neuropeptide receptors are not confined to synaptic regions. Peptidergic neurotransmission often operates on a slower time scale than conventional neurotransmitters, so it is not surprising that peptide receptors are often localized at a distance from the synapse. For example, although some substance P terminals contact membranes loaded with substance P receptor, only a small fraction of the substance P receptor-laden membrane is apposed to synaptic terminals. Substance P may diffuse a considerable distance from its release site and still find a receptor with which it can interact [21].

Expressions of peptide receptors and the corresponding peptides are not well matched. Neuropeptides can act at many sites. They may act directly on a postsynaptic target at the synapse; presynaptically on the terminal that released the peptide (autocrine effects); on an immediately adjacent cell (juxtacrine effects); or on a cell a few cell diameters away from the site of release (paracrine effects). In addition, peptides can exert their actions after traveling through the circulation to reach their target (endocrine effects), as in the case of hypothalamic releasing factors and neurohypophyseal hormones.

There is some correspondence of the peptide families and the families of peptide receptors, but it is certainly not simple. For example, there are three related opiate peptide precursors, POMC, proenkephalin and prodynorphin, and three major types of opiate receptor, μ, κ and δ. The best endogenous ligands for the μ receptor are β-endorphin and endomorphins-1 and -2 (Tyr–Pro–Phe/Trp–Phe–NH_2); enkephalins are the best ligands for δ receptors and dynorphins are best for κ receptors. However, the sites at which opiate peptides and opiate receptors are expressed in the brain do not show a simple 1:1 correspondence. Recently, an orphan receptor resembling the opiate receptors was expressed and used to identify its endogenous ligand, which was called orphanin FQ or nociceptin. This preprohormone is most similar to the dynorphin precursor and defines a fourth type of opiate-like peptide containing the sequence Phe–Gly–Gly–Phe instead of the Tyr–Gly–Gly–Phe sequence that originally defined the opiate peptide family. The orphanin FQ peptide is hyperalgesic in that it increases pain sensitivity, which is the opposite action to the opiate peptides. Reduction of the level of the orphanin FQ receptor in brain using antisense oligonucleotides leads to analgesia. In addition, endomorphins-1 and -2 show tighter binding and much greater selectivity for μ opiate receptors than other natural peptides [22, 23].

Similarly, five closely related melanocortin receptors that respond to various peptides derived from the POMC precursor have been identified (Fig. 18-7) [24]. As expected, the receptor on adrenal cortical cells responds best to ACTH, which normally stimulates adrenal steroidogenesis, and the receptor on melano cytes responds best to αMSH, which causes skin darkening. However, the pattern of melanocortin receptor expression in the brain is not simply explained by the known patterns of peptide expression in the brain or by the known effects of POMC-derived peptides when applied to various brain regions. With this number of peptide receptors, it is obvious that production of final peptide products must be precisely controlled and that different biosynthetic processing pathways can dramatically affect the biological activity observed (Figs 18-5, 18-7).

The amiloride-sensitive FMRF-amide-gated sodium ion channel is among the few peptide-gated ion channels identified. FMRF-amide induces a fast excitatory depolarizing response in selected neurons due to direct activation of an amiloride-sensitive sodium channel. Using cDNA from the snail *Helix aspersa* and a *Xenopus* oocyte expression system, an amiloride-sensitive, FMRF-amide-activated sodium channel was identified. The acid-sensing ion channels found in sensory neurons are also modulated by FMRF-NH_2 and by neuropeptide FF [25]. These channels have only two transmembrane domains and as such are similar in structure to the directly gated ion channels activated by some purinergic ligands (Ch. 17) and by hydrogen ions.

Neuropeptide receptors are becoming molecular targets for therapeutic drugs. The large number of neuropeptides and even larger number of neuropeptide receptors have raised the hope that neuropeptide receptors could become useful drug targets [26]. Radiolabeled peptides are being used to localize tumors and metastases [20]. Nonpeptidic antagonists acting at specific substance P receptors are being tested as antidepressants [27, 28]. Another major target is CRH, for a number of stress-related psychiatric disorders [28].

Many creatures use peptides to control their environments. Cone snails produce over a thousand different peptide toxins, which are exquisitely targeted to ion channels [29]. Similarly, insects produce peptide toxins to kill each other, with spiders being the champions at this endeavor. The ion channels affected by these toxins are becoming medically and agriculturally useful – for example, there are distinct calcium channels expressed in different tissues at various developmental times. The natural toxins, which are highly selective among closely related subtypes of ion channel, are being exploited for therapeutic purposes. Similarly, spider toxins are being adapted as insecticides.

NEUROPEPTIDE FUNCTIONS AND REGULATION

The study of peptidergic neurons requires a number of special tools. These tools include methods to detect the neuropeptides both in cells and after release, the enzymes specific to their biosynthesis and their cognate receptors. Since the actions of peptides require secretion, measurements of cell content (e.g. immunostaining) can be deceptive, with a decrease in content reflecting increased release.

Antibody-based detection methods include immunocytochemistry, which gives qualitative data but has very good spatial resolution. Radioimmunoassays provide a quantitative measure of release or content. One of the major limitations of all antibody-based methods is the potential for cross-reactivity among the many peptides. For example, some of the most sensitive 'gastrin' antisera also detect CCK, since the peptides share a common COOH-terminal tetrapeptide sequence. Methods for detection of the mRNAs encoding neuropeptides include Northern blots, which provide quantitative data and information on splice variants, but lack fine anatomical resolution. The more commonly used polymerase chain reaction, which can be quantitative but often is used in a more qualitative manner, provides great sensitivity. Alternatively, *in situ* hybridization preserves anatomical relationships and can be used to obtain both qualitative and quantitative data.

Direct methods for detection of neuropeptides include metabolic labeling with an appropriate radioactive amino acid and chemical isolation of peptides. Direct methods are difficult to apply to peptides or their receptors in the CNS, where the cells expressing the peptide are part of a large population of other neurons or endocrine cells. Reversed-phase high-pressure liquid chromatography or two-dimensional polyacrylamide gel electrophoresis, coupled with mass spectrometric identification, has revolutionized the identification of tiny amounts of peptides and receptors in tissue extracts. Similarly, the ability to mark living neurons producing a specific neuropeptide precursor with a fluorescent tag (e.g. green fluorescent protein expression driven by the promoter for the peptide precursor) has revolutionized the ability to study the functions of specific peptidergic neurons [30].

Peptide agonists and antagonists are becoming available for many peptides, and these are essential to a successful dissection of the function of peptidergic synapses, as they have been for conventional neurotransmitters (discussed in Chs 11–16). These approaches have the advantage that a physiologically important trait, binding of the ligand to the receptor, is being observed, and they can reveal binding to several related receptors, which are then distinguished by antisera, *in situ* hybridization, or drugs specific for receptor subtypes.

Peptides play a role in the plurichemical coding of neuronal signals. Many sets of neurons are chemically coded, giving rise to the concept of plurichemical transmission. For example, from the human lumbar paravertebral ganglion, adrenergic neurons efferent to blood vessels in skin and muscle usually contain NPY along with norepinephrine, while adrenergic neurons innervating hair follicles express primarily norepinephrine. The dual transmission on to blood vessels is important since the catecholamine effects undergo rapid tachyphylaxis, while sustained contraction of the blood vessels is mediated by NPY. It is likely that a similar but more complex mixture of phenotypes occurs in the CNS; for example, some CCK-containing neurons also secrete dopamine while others release glutamate [31]. Peptides are generally released by the same neurons that release fast-acting classical neurotransmitters, and each transmitter has its own unique effects on target tissues.

Neuropeptides make a unique contribution to signaling. The release of neuropeptides generally requires a more intense stimulus than that required for release of fast-acting classical neurotransmitters. The more intense stimulus presumably results in more entry of Ca^{2+} into the presynaptic terminal, allowing Ca^{2+} to diffuse from its entry site to the LDCVs (Figs 18-3, 18-4). As a result, the contribution of peptides vs. fast-acting conventional neurotransmitters to signaling can vary with the pattern of stimulation. For example, in *Aplysia*, several identified motor neurons devote about 10% of their total protein synthesis to their respective peptide neurotransmitters. Peptide release in each case has been shown to require extracellular Ca^{2+}, and release increases with the frequency of action potentials, in the range of frequencies seen in behaving animals. In addition to the peptides, several of the motor neurons secrete ACh. In these motor neurons, peptide release increases more strongly with action potential frequency than does release of ACh, so the ratio of peptide to ACh is dependent on frequency. In addition, there are unique postsynaptic responses, such as elevation of cAMP concentrations in muscles, which can be mimicked only by application of the peptides and not by the conventional neurotransmitters.

In other examples, the amount of peptide available for release can be depleted by repeated firing of a terminal since new peptide must arrive by axonal transport, while new conventional neurotransmitters are synthesized or recaptured locally and transported into small synaptic vesicles.

Regulation of neuropeptide expression is exerted at several levels. Control of neuropeptide function is mediated by factors controlling rates of prepropeptide gene transcription, translation, peptide degradation and secretion (Fig. 18-11). On the scale of seconds to minutes, peptide secretion is not always coupled lock-step with classical transmitter release (example above). Peptides are inactivated by diffusion and by proteolysis, so it would be expected that inhibition of specific extracellular proteases

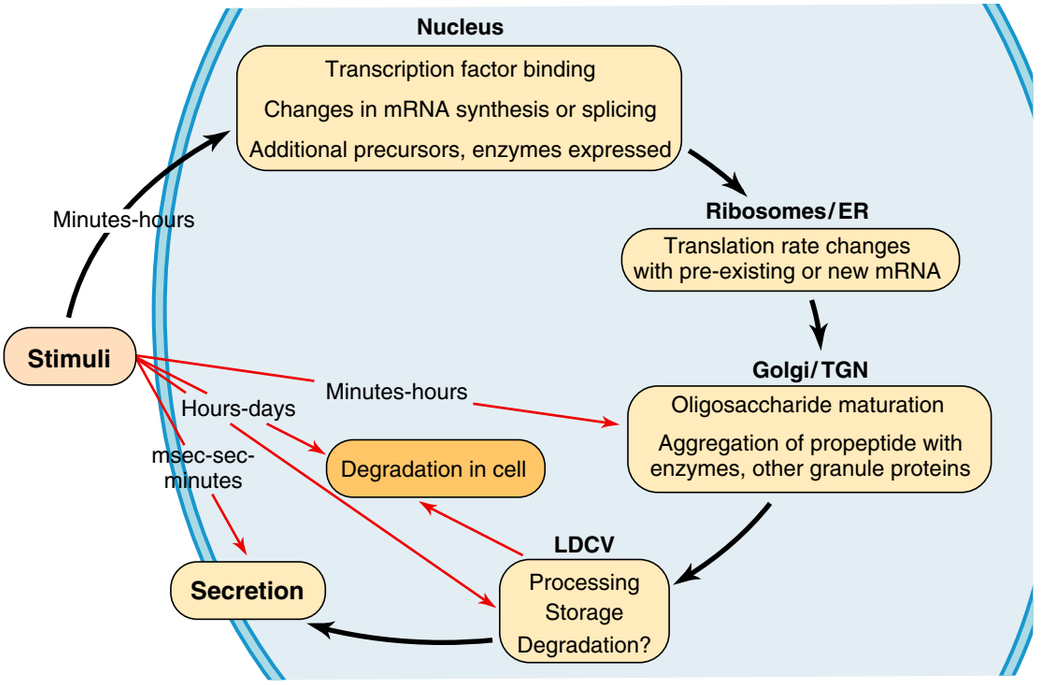

FIGURE 18-11 Regulation of neuropeptide expression is exerted at several levels. *ER*, endoplasmic reticulum; *LDCV*, large dense-core vesicle; *TGN*, *trans*-Golgi network.

could lead to increased effectiveness of their neuropeptide substrates, as has been shown in the cases of CCK and the enkephalins.

On a time scale of minutes to hours, the transcription of prepropeptide mRNA is regulated. Peptide mRNAs are often immediate early genes, showing extremely rapid responses to stimuli that are not blocked by inhibitors of protein translation. The rate of translation of existing prepropeptide mRNAs can also change dramatically within a few minutes of stimulation. Since processing enzymes must also be synthesized and inserted into the same LDCVs as the peptide precursors, the rate of production of these enzymes might be expected to show changes under physiological conditions. They do show such changes but usually more slowly than the peptide precursors. As seen above (Figs 18-5–18-8), the peptide products produced are crucially dependent on the mixture of processing enzymes in the LDCVs.

On a scale of hours to days, dramatic changes can be seen in cells producing peptides that are subjected to chronic stimulation or inhibition. POMC production by intermediate pituitary melanotropes is inhibited by treatment of rats with dopaminergic agonists or exposure of frogs to a light background; POMC production is stimulated by treatment of rats with dopaminergic antagonists or exposure of frogs to a dark background. The expression of processing enzymes, the number of LDCVs and the rate of cell division are also responsive to these treatments. Similarly, the processing of different regions of the pro-TRH precursor responds to thyroid hormone status, and it is possible that the routing of ELH and the BCPs is regulated (Fig. 18-8). In response to the changes in steroid hormone concentrations that occur during the estrous cycle in female rats, the concentration of CCK mRNA varies more than twofold in a subset of CCK-producing CNS neurons.

PEPTIDERGIC SYSTEMS IN DISEASE

Diabetes insipidus occurs with a loss of vasopressin production in the Brattleboro rat model. In the Brattleboro rat, a single-base deletion in the vasopressin gene alters the COOH terminus of preprovasopressin so that it cannot leave the endoplasmic reticulum; as a result, no neurons make vasopressin. Intriguingly, a frameshift mutation occurs in postmitotic neurons, most often an additional deletion of two nucleotides, so that the neurons somewhat regain the ability to produce vasopressin. In the *hpg* mouse, a spontaneous mutation in the gonadotropin-hormone-releasing hormone gene results in loss of gonadal function in homozygous animals. For both vasopressin and growth hormone, dominant negative mutations occur which block successful expression of the normal gene – these dominant negative phenotypes are presumably due to defects in folding in the endoplasmic reticulum or packaging into large dense-core granules.

Mutations and knockouts of peptide-processing enzyme genes cause a myriad of physiological problems. A single-base change in the CPE gene changes Ser^{202} to Pro, yielding a CPE molecule that is malfolded, inactive and incapable of exiting from the endoplasmic reticulum [9]. The phenotype observed in these *fat*/*fat* mice, which includes obesity and late-onset diabetes mellitus, is caused in part by high concentrations of inactive proinsulin in the blood, with very little mature, functional insulin (Fig. 18-12). In the brains of these *fat*/*fat* mice, there is an excess of immature, biologically inactive neuropeptide fragments, such as neurotensin–Lys–Arg and thyrotropin-releasing hormone intermediates. It is still unclear how the loss of CPE function causes all the biochemical effects observed, with selective loss of processing of several peptides, while other peptide systems remain relatively quite normal.

The fact that the naturally occurring CPE knockout (*fa*/*fa*) is viable might be taken as a surprise, but this has led to the search for additional CPE-like enzymes, which were indeed found [9]. Mouse knockouts for furin die at about embryonic day 11, while knockouts for PACE4 and PAM die at or before embryonic day 15 [32]. The surprising result is that knockouts for PC1, PC2 and the PC2 chaperone-inhibitor 7B2 are viable, although not normal. The PC1 knockout mouse has symptoms due to hyperproinsulinemia, the excessive secretion of uncleaved and inactive proinsulin, while human PC1 nulls exhibit early-onset severe obesity, perhaps due to the loss of MSH in the hypothalamus [32]. The PC2 knockout has deficits with glucagon and MSH biosynthesis, but is fairly normal. Surprisingly, the 7B2 knockout has far more severe problems than the PC2 knockout, exhibiting very high ACTH levels and a lethal form of Cushing's disease [33]. As expected, the 7B2 knockout mice have little or no detectable PC2 activity in any tissues.

Neuropeptides play key roles in appetite regulation and obesity. Many genes for neuropeptides and neuropeptide receptors have been implicated in obesity and cachexia, anorexia and bulimia [34]. For example, NPY administration into the CNS causes overeating and obesity. A second peptide involved in obesity is leptin, a product of adipocytes and the stomach. The leptin gene is defective in the *ob*/*ob* mouse but in normal mice leptin binds to its receptor in the hypothalamus, causing a decrease in the synthesis and release of hypothalamic NPY.

Control of feeding behavior involves peripheral peptides (insulin, ghrelin, leptin) plus several peptides in the CNS (orexins/hypocretins, CCK, galanin, MSH, neuropeptide Y, CRH, cocaine-and-amphetamine-regulated transcript (CART)) [35, 36]. Some of the same peptides are involved in reward systems crucial to drug addiction. Specific receptor blockers are being tested for many of these peptide-receptor systems, with the hope of very selective actins with minimal side effects [35]. For example, there are two CCK receptor subtypes, CCK-A and

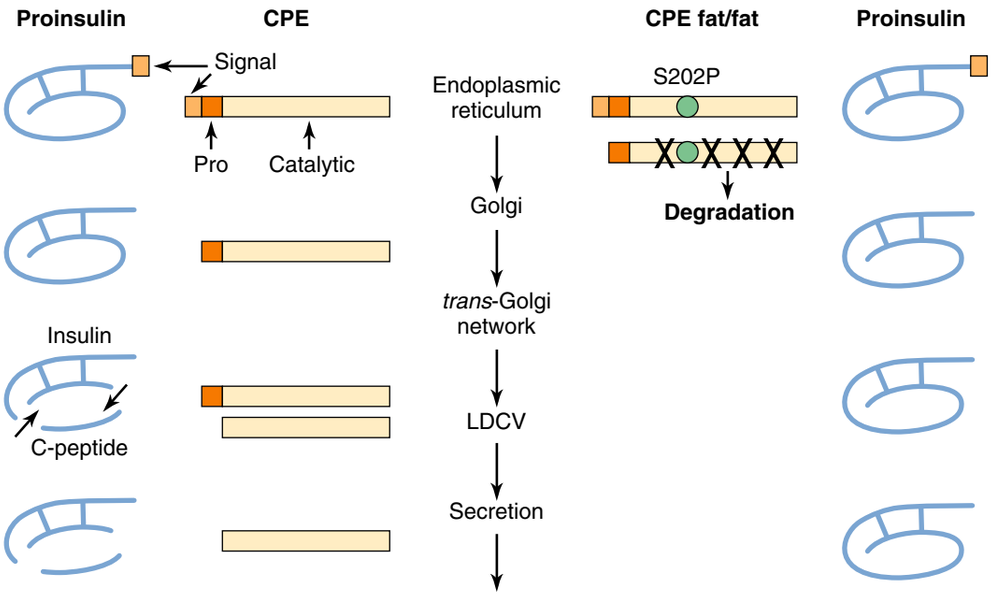

FIGURE 18-12 The *fat/fat* mutation in carboxypeptidase E (*CPE*) leads to secretion of proinsulin, not mature insulin, and results in diabetes. The S202P mutation within CPE results in degradation of the enzyme and defective insulin processing in the *fat/fat* heterozygous mouse. *LDCV*, large dense-core vesicle.

CCK-B. Protease inhibitors that slow the degradation and inactivation of endogenous CCK promote satiety via CCK-A receptor. By contrast, the CCK-B receptor is important in mediating anxiety and panic attacks, and CCK antagonists are in clinical use to treat these symptoms.

Enkephalin knockout mice reach adulthood and are healthy. When the proenkephalin gene, one of the three endogenous opiate peptide precursor genes, was eliminated from the mouse genome by genetic engineering, the knockout mice exhibited a normal hotplate/tail-flick responses; in normal mice, the tail-flick response (moving the tail away from a hot light) is diminished by prior exposure to another noxious stimulus, an unavoidable electrical foot-shock. This indicates that enkephalin plays no role in the normal tail-flick response but does play a role in centrally mediated analgesia. Similarly, β-endorphin knockout mice also show a loss in centrally mediated analgesia. Behavioral abnormalities in the enkephalin knockout mice include reduced exploratory activity in an unfamiliar environment and increased aggressiveness in the resident–intruder test. In both enkephalin and β-endorphin knockout mice, there are no compensatory increases in the concentrations of the remaining endogenous opiate peptides.

Neuropeptides are crucial to pain perception. Substance P is released in the spinal cord by a subset of dorsal root ganglion neurons; Substance P is crucial to the sensation of noxious stimuli. Substance P binds to the neurokinin-1 receptor, which gets rapidly internalized and recycled to the plasma membrane of the neurons [21]. When substance P receptors are partially ablated, the sensitivity to noxious stimuli decreases. In conditions of chronic pain, the pattern of peptide expression can also change. For example, there is a dramatic increase in the expression of the opiate peptide dynorphin in the spinal cord, while dynorphin is undetectable in the normal animal.

REFERENCES

1. Grimmelikhuijzen, C. J. P., Hauser, F., Eriksen, F. F. and Williamson, M. Invertebrate neurohormones and their receptors. *Results Probl.Cell Differ.* 26: 339–362, 1999.
2. Mains, R. E. and Eipper, B. A. The tissue-specific processing of pro-ACTH/endorphin. *Trends Endocrinol. Metab.* 1: 388–394, 1990.
3. Zhou, A., Webb, G., Zhu, X. and Steiner, D.F. Proteolytic processing in the secretory pathway. *J. Biol. Chem.* 274: 20745–20748, 1999.
4. Seidah, N. G. and Chretien, M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Res.* 848: 45–62, 1999.
5. Li, C., Kim, K. and Nelson, L. S. FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans. Brain Res.* 848: 26–34, 1999.
6. Hallberg, M. and Nyberg, F. Neuropeptide conversion to bioactive fragments–an important pathway in neuromodulation. *Curr. Protein Pept. Sci.* 4: 31–44, 2003.
7. Muller, L. and Lindberg, I. The cell biology of the prohormone convertases PC1 and PC2. *Prog. Nucleic Acid Res. Mol. Biol.* 63: 69–108, 1999.

8. Muller, L., Zhu, P., Juliano, M. A., Juliano, L. and Lindberg, I. A 36-residue peptide contains all of the information required for 7B2-mediated activation of prohormone convertase 2. *J. Biol. Chem.* 274: 21471–21477, 1999.
9. Fricker, L. D. and Leiter, E. H. Peptides, enzymes and obesity: new insights from a 'dead' enzyme. *Trends Biochem. Sci.* 24: 390–393, 1999.
10. Aloy, P., Companys, V., Vendrell, J. *et al.* The crystal structure of the inhibitor-complexed carboxypeptidase D domain II and the modeling of regulatory carboxypeptidases. *J. Biol. Chem.* 276: 16177–16184, 2001.
11. Gomis-Ruth, F. X., Companys, V., Qian, Y. *et al.* Crystal structure of avian carboxypeptidase D domain II: a prototype for the regulatory metallocarboxypeptidase subfamily. *EMBO J.* 18: 5817–5826, 1999.
12. Prigge, S. T., Mains, R. E., Eipper, B. A. and Amzel, L. M. New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *Cell Mol. Life Sci.* 57: 1236–1259, 2000.
13. Takigami, T., Takeuchi, F., Nakagawa, M., Hase, T. and Tsubaki, M. Stopped-flow analyses on the reaction of ascorbate with cytochrome b561 purified from bovine chromaffin vesicle membranes. *Biochemistry* 42: 8110–8118, 2003.
14. Takanaga, H., Mackenzie, B. and Hediger, M. A. Sodium-dependent ascorbic acid transporter family SLC23. *Pflugers Arch.* 10: 1007/s00424–003–1104–1, 2003.
15. Schilling, S., Hoffmann, T., Rosche, F., Manhart, S., Wasternack, C. and Demuth, H. U. Heterologous expression and characterization of human glutaminyl cyclase: evidence for a disulfide bond with importance for catalytic activity. *Biochemistry* 41: 10849–10857, 2002.
16. Helke, C. J., Krause, J. E., Mantyh, P. W., Couture, R. and Bannon, M. J. Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors, and regulatory mechanisms. *FASEB J* 4: 1606–1615, 1990.
17. Taghert, P. H. and Veenstra, J. A. *Drosophila* neuropeptide signaling. *Adv. Genet.* 49: 1–65, 2003.
18. Brothers, S. P., Janovick, J. A. and Conn, P. M. Unexpected effects of epitope and chimeric tags on gonadotropin-releasing hormone receptors: implications for understanding the molecular etiology of hypogonadotropic hypogonadism. *J. Clin. Endocrinol. Metab.* 88: 6107–6112, 2003.
19. Van Der Hoek, J., De Herder, W. W., Feelders, R. A., Van Der Lely, A. J. and Lamberts, S. W. A single-dose comparison of the acute effects between the new somatostatin analog SOM230 and octreotide in acromegalic patients. *J. Clin. Endocrinol. Metab.* 89: 638–645, 2004.
20. Reubi, J. C. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocrinol. Rev.* 24: 389–427, 2003.
21. Mantyh, P. W. Neurobiology of substance P and the NK1 receptor. *J. Clin. Psychiatry* 63(Suppl. 11): 6–10, 2002.
22. Zadina, J. E. Recent advances in the search for the μ-opioidergic system: isolation and distribution of endomorphins in the central nervous system. *Jpn. J. Pharmacol.* 89: 203–208, 2002.
23. Mizoguchi, H., Tseng, L. F., Suzuki, T., Sora, I. and Narita, M. Recent advances in the search for the μ-opioidergic system: differential mechanism of g-protein activation induced by endogenous μ-opioid peptides, endomorphin and β-endorphin. *Jpn. J. Pharmacol.* 89: 239–244, 2002.
24. Marks, D. L. and Cone, R. D. The role of the melanocortin-3 receptor in cachexia. *Ann. N.Y. Acad. Sci.* 994: 258–266, 2003.
25. Xie, J., Price, M. P., Berger, A. L. and Welsh, M. J. DRASIC contributes to pH-gated currents in large dorsal root ganglion sensory neurons by forming heteromultimeric channels. *J. Neurophysiol.* 87: 2835–2843, 2002.
26. Strand, F. L. Neuropeptides: general characteristics and neuropharmaceutical potential in treating CNS disorders. *Prog. Drug Res.* 61: 1–37, 2003.
27. Herpfer, I. and Lieb, K. Substance P and Substance P receptor antagonists in the pathogenesis and treatment of affective disorders. *World J. Biol. Psychiatry* 4: 56–63, 2004.
28. Gorman, J. M. New molecular targets for antianxiety interventions. *J. Clin. Psychiatry* 64(Suppl. 3): 28–35, 2003.
29. Terlau, H. and Olivera, B. M. Conus venoms: a rich source of novel ion channel-targeted peptides. *Physiol. Rev.* 84: 41–68, 2004.
30. Gong, S., Zheng, C., Doughty, M. L., Losos, K., Hatten, M. E. and Heintz, N. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* 425: 917–925, 2003.
31. Hokfelt, T., Blacker, D., Broberger, C., Herrera-Marschitz, M. and Snyder, G. Some aspects on the anatomy and function of central cholecystokinin systems. *Pharmacol. Toxicol.* 91: 382–386, 2002.
32. Czyzyk, T. A., Morgan, D. J., Peng, B. *et al.* Targeted mutagenesis of processing enzymes and regulators: implications for development and physiology. *J. Neurosci. Res.* 74: 446–455, 2003.
33. Laurent, V., Jaubert-Miazza, L., Desjardins, R., Day, R. and Lindberg, I. Biosynthesis of proopiomelanocortin-derived peptides in prohormone convertase 2 and 7B2 null mice. *Endocrinology* 145: 519–528, 2004.
34. Damcott, C. M., Sack, P. and Shuldiner, A. R. The genetics of obesity. *Endocrinol Metab. Clin. North Am.* 32: 761–786, 2004.
35. Zorrilla, E. P., Tache, Y. and Koob, G. F. Nibbling at CRF receptor control of feeding and gastrocolonic motility. *Trends Pharmacol. Sci.* 24: 421–427, 2003.
36. Sakurai, T. Orexin: a link between energy homeostasis and adaptive behavior. *Curr. Opin. Clin. Nutr. Metab. Care* 6: 353–360, 2003.
37. Karhunen, T., Vilim, F. S., Alexeeva, V., Weiss, K. R. and Church, P. J. Targeting of peptidergic vesicles in cotransmitting terminals. *J. Neurosci.* 21: RC127, 2001.

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—III—

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CHAPTER

19

G Proteins

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G proteins comprise several families of diverse cellular proteins that subserve an equally diverse array of cellular functions. These proteins derive their name from the fact that they bind the guanine nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP) and possess intrinsic GTPase activity. G proteins play a central role in signal transduction as well as in a myriad of cellular processes, including membrane vesicle transport, cytoskeletal assembly, cell growth and protein synthesis (see Chs 5, 8 and 9).

Mammalian G proteins can be divided into two major categories: heterotrimeric G proteins and small G proteins. This chapter reviews the types of G protein that exist in the nervous system and the ways in which they regulate signal transduction and other processes essential for brain function.

HETEROTRIMERIC G PROTEINS

The family of heterotrimeric G proteins is involved in transmembrane signaling in the nervous system, with certain exceptions. The exceptions are instances of synaptic transmission mediated via receptors that contain intrinsic enzymatic activity, such as tyrosine kinase or guanylyl cyclase, or via receptors that form ion channels (see Ch. 10). Heterotrimeric G proteins were first identified, named and characterized by Alfred Gilman, Martin Rodbell and others close to 20 years ago. They consist of three distinct subunits, α, β and γ. These proteins couple the activation of diverse types of plasmalemma receptor to a variety of intracellular processes. In fact, most types of neurotransmitter and peptide hormone receptor, as well as many cytokine and chemokine receptors, fall into a superfamily of structurally related molecules, termed G-protein-coupled receptors. These receptors are named for the role of G proteins in mediating the varied biological effects of the receptors (see Ch. 10). Consequently, numerous effector proteins are influenced by these heterotrimeric G proteins: ion channels; adenylyl cyclase; phosphodiesterase (PDE); phosphoinositide-specific phospholipase C (PI-PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2); and phospholipase A_2 (PLA_2), which catalyzes the hydrolysis of membrane phospholipids to yield arachidonic acid. In addition, these G proteins have been implicated in

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several other intracellular processes, such as vesicular transport (Ch. 9) and cytoskeletal assembly (Chs 8 and 28).

Multiple forms of heterotrimeric G proteins exist in the nervous system. Three types of heterotrimeric G protein were identified in early studies. G_t, termed transducin, was identified as the G protein that couples rhodopsin to regulation of photoreceptor cell function (see Ch. 49), and G_s and G_i were identified as the G proteins that couple plasma membrane receptors to the stimulation and inhibition, respectively, of adenylyl cyclase, the enzyme that catalyzes the synthesis of cAMP (see Ch. 21).

Since that time, over 35 heterotrimeric G protein subunits have been identified by a combination of biochemical and molecular cloning techniques [1–4]. In addition to G_t, G_s and G_i, the other types of G protein in brain are designated G_o, G_{olf}, G_{gust}, G_z, G_q and $G_{11–16}$. Moreover, for most of these G proteins, multiple subtypes show unique distributions in the brain and peripheral tissues. G proteins are now divided into four main categories (Table 19-1): the G_s family stimulates adenylyl cyclase; the G_i family (which includes G_o, G_{gust}, G_z) can inhibit adenylyl cyclase, activate a certain type of K^+ channel, inhibit voltage-gated Ca^{2+} channels, activate the MAP-kinase pathway or activate phosphodiesterase; the G_q family activates PI-PLC; and the G_{12} family activates a group of proteins termed Rho-GEFs (guanine nucleotide exchange factors, which are described in greater detail below).

Each G protein is a heterotrimer composed of single α, β and γ subunits. The different types of G protein contain distinct α subunits, which confer part of the specificity of functional activity. G protein α subunits, listed in Table 19-1, are categorized on the basis of their structural and functional homologies. Current nomenclature identifies several subfamilies of G protein α subunit: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12}$. The M_r of these proteins varies between 38,000 and 52,000. As a first approximation, these distinct types of α subunits share common β and γ subunits. However, multiple subtypes of β and γ subunit are known: five β subunits of M_r 35,000–36,000 and 11 γ subunits of M_r 6,000–9,000. These proteins show distinct cellular distributions, and differences in their functional properties are becoming increasingly apparent [1–4].

TABLE 19-1 Heterotrimeric G protein α-subunits in brain

Family	M_r*	Toxin-mediated ADP-ribosylation	Effector protein(s)†
G_s			
$G_{\alpha s1}$	52,000	Cholera	Adenylyl cyclase (activation)
$G_{\alpha s2}$	52,000		
$G_{\alpha s3}$	45,000		
$G_{\alpha s4}$	45,000		
$G_{\alpha olf}$	45,000		
G_i			
$G_{\alpha i1}$	41,000	Pertussis	Adenylyl cyclase (inhibition)
$G_{\alpha i2}$	40,000		K^+ channel (activation)
$G_{\alpha i3}$	41,000		Ca^{2+} channel (inhibition)
			?PI-Phospholipase C (activation)
			?Phospholipase A_2
$G_{\alpha o1}$	39,000	Pertussis	K^+ channel (activation)
$G_{\alpha o2}$	39,000		Ca^{2+} channel (inhibition)
$G_{\alpha t1}$	39,000	Cholera and pertussis	Phosphodiesterase (activation) in rods and cones
$G_{\alpha t2}$	40,000		
$G_{\alpha gust}$	41,000	Unknown	Phosphodiesterase (activation) in taste epithelium
$G_{\alpha z}$	41,000	None	?Adenylyl cyclase (inhibition)
G_q	41,000–43,000		
$G_{\alpha q}$		None	PI-Phospholipase C (activation)
$G_{\alpha 11}$		Unknown	
$G_{\alpha 14}$			
$G_{\alpha 15}$			
$G_{\alpha 16}$			
G_{12}	44,000	None	Rho-GEFs (activation)
$G_{\alpha 12}$			
$G_{\alpha 13}$			

Question marks indicate that the association between the particular G proteins and effector proteins shown in the table remains tentative.

*Values shown reflect apparent M_r by gel electrophoresis in most cases. Values shown for $G_{\alpha gust}$, $G_{\alpha z}$ and $G_{\alpha 11–16}$ reflect calculated M_r based on amino acid sequence.

†For several of the effector proteins listed, the functional regulation of the effector is mediated, in part or in full, by the βγ subunits associated with the indicated α subunit.

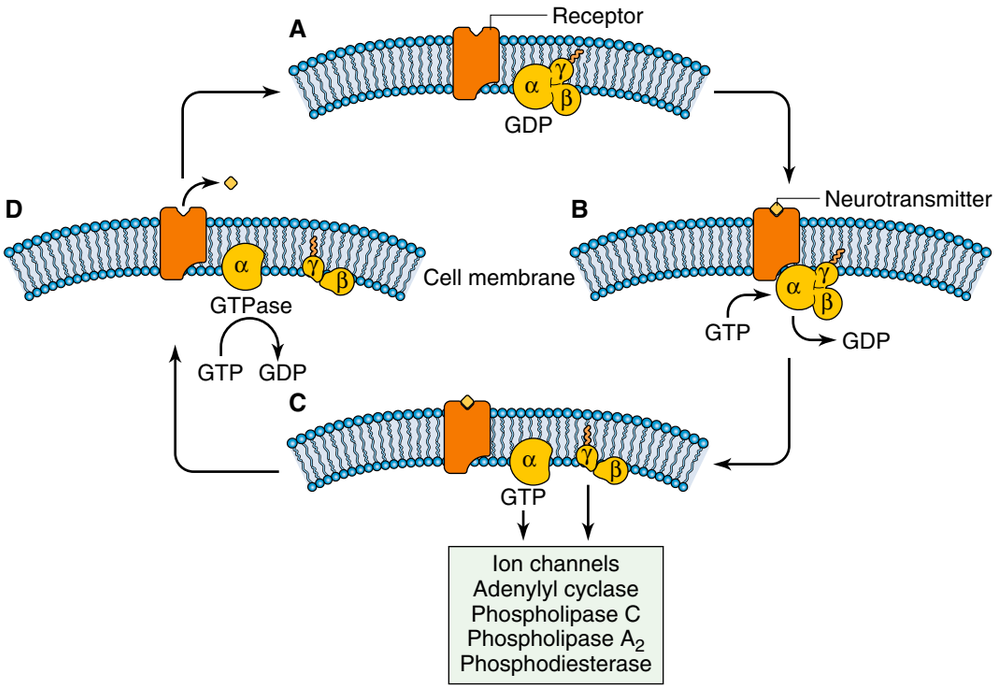

FIGURE 19-1 Functional cycle of heterotrimeric G proteins. (**A**) Under basal conditions, G proteins exist in cell membranes as heterotrimers composed of single α, β and γ subunits and are associated only loosely with neurotransmitter receptors. In this situation, GDP is bound to the α subunit. (**B**) Upon activation of the receptor by its ligand, such as a neurotransmitter, the receptor physically associates with the α subunit, which leads to the dissociation of GDP from the subunit and the binding of GTP instead. (**C**) GTP binding induces the generation of free α subunit by causing the dissociation of the α subunit from its β and γ subunits and the receptor. Free α subunits (bound to GTP) and free βγ subunit dimers are functionally active and directly regulate a number of effector proteins, which, depending on the type of subunit and cell involved, can include ion channels, adenylyl cyclase, phospholipase C, phospholipase A_2 and phosphodiesterase. (**D**) GTPase activity intrinsic to the α subunit degrades GTP to GDP. This leads to the reassociation of the α and βγ subunits, which, along with the dissociation of ligand from receptor, leads to restoration of the basal state. (With permission from Hyman and Nestler [36].)

The functional activity of G proteins involves their dissociation and reassociation in response to extracellular signals. This G protein cycle is shown schematically in Figure 19-1. In the resting state, G proteins exist as heterotrimers that bind GDP and are associated with extracellular receptors (Fig. 19-1A). When a ligand binds to and activates the receptor, it produces a conformational change in the receptor, which in turn triggers a dramatic conformational change in the α subunit of the G protein (Fig. 19-1B). This conformational change leads to (a) a decrease in the affinity of the α subunit for GDP, which results in the dissociation of GDP from the α subunit and the subsequent binding of GTP because the cellular concentration of GTP is much higher than that of GDP; (b) dissociation of a βγ subunit dimer from the α subunit; and (c) release of the receptor from the G protein (Fig. 19-1B,C). This process generates a free α subunit bound to GTP as well as a free βγ subunit dimer, both of which are biologically active and regulate the functional activity of effector proteins within the cell. The GTP-bound α subunit is also capable of interacting with the receptor and reducing its affinity for ligand. The system returns to its resting state when the ligand is released from the receptor and the GTPase activity that resides in the α subunit hydrolyzes GTP to GDP (Fig. 19-1D). The latter action leads to reassociation of the free α subunit with the βγ subunit complex to restore the original heterotrimers.

The structural basis of the interactions among the α, β, and γ subunits of G proteins and between the subunits and the associated receptor has become increasingly understood as the three-dimensional structure of these proteins has been determined [5, 6]. Each α subunit has two identifiable domains. One contains the GTPase activity and the GTP-binding site. This domain also appears to be most important in binding βγ subunits as well as various effector proteins. The function of the other domain remains unknown, but it may be involved in the dramatic conformational shift that occurs in the protein upon exchanging GTP for GDP. The ability of the heterotrimeric G protein to bind to a receptor is thought to depend on sites located within all three G protein subunits. Thus, the different α subunits, as well as subtypes of β and γ subunits, seem to be responsible for targeting a particular type of G protein to a particular type of receptor.

G proteins couple some neurotransmitter receptors directly to ion channels. It is now clearly established that G protein subunits, when released from their G protein-receptor interactions, can directly gate (i.e. open or close) specific ion channels [2, 7–9]. One of the best examples of this mechanism in brain is the coupling of many types of receptor to the activation of an inward-rectifying K^+ channel (GIRK) via pertussis-toxin-sensitive G proteins, i.e. subtypes of G_i, in many types of neurons. The coupled receptors include opioid, α_2-adrenergic, D_2-dopaminergic, muscarinic cholinergic, 5-HT_{1A}-serotonergic and $GABA_B$. In initial studies, it was controversial as to whether the free α subunit or the free $\beta\gamma$ dimer was responsible for this action. Based on the results of elegant studies in which cloned channel and G protein subunits have been expressed in a variety of cell types, the general consensus is now that the $\beta\gamma$ complex is the more important mechanism [7, 8]. The region of the GIRK that binds to the $\beta\gamma$ complex has been identified. Moreover, it seems that particular combinations of β and γ subtypes are more effective at opening this channel than others. In addition, some subtypes of α_i can open the channel, although not to the same extent as the $\beta\gamma$ subunits.

These same neurotransmitter receptors also are coupled via pertussis-toxin-sensitive G proteins to voltage-gated Ca^{2+} channels. In this case the channels are inhibited by the interaction. Available evidence supports a primary role for $\beta\gamma$ in mediating this effect, although there is some evidence that α_i (including α_o) subunits also can be involved [9]. Binding of the G protein subunits to the Ca^{2+} channels reduces their probability of opening in response to membrane depolarization. This mechanism is best established for L-type Ca^{2+} channels, which are also inhibited by dihydropyridine antihypertensive drugs, such as verapamil, but may also operate for other types of voltage-gated Ca^{2+} channel (see also Chs 6 and 22).

G proteins regulate intracellular concentrations of second messengers. G proteins control intracellular cAMP concentrations by mediating the ability of neurotransmitters to activate or inhibit adenylyl cyclase. The mechanism by which neurotransmitters stimulate adenylyl cyclase is well known. Activation of those neurotransmitter receptors that couple to G_s results in the generation of free $G_{\alpha s}$ subunits, which bind to and thus directly activate adenylyl cyclase. In addition, free $\beta\gamma$-subunit complexes activate certain subtypes of adenylyl cyclase (see Ch. 21). A similar mechanism appears to be the case for $G_{\alpha olf}$, a type of G protein structurally related to $G_{\alpha s}$ that is enriched in olfactory epithelium and striatum (Ch. 50).

The mechanism by which neurotransmitters inhibit adenylyl cyclase and decrease neuronal levels of cAMP has been more difficult to delineate. By analogy with the action of G_s, it was proposed originally that activation of neurotransmitter receptors that couple to G_i results in the generation of free $G_{\alpha i}$ subunits, which bind to and, thereby, directly inhibit adenylyl cyclase. Initially, such direct inhibition of adenylyl cyclase by $G_{\alpha i}$ was difficult to demonstrate in some cell-free reconstitution experiments. Nevertheless, there is now strong evidence, including insight coming from the crystalline structures of these proteins, that certain subtypes of adenylyl cyclase are directly inhibited by $G_{\alpha i}$. For other adenylyl cyclase isoforms, free $\beta\gamma$-subunit complexes, generated by the release of $G_{\alpha i}$, appear to mediate inhibition of the enzyme (Ch. 21). In addition to G_i, there is evidence that $G_{\alpha z}$, which is considered a subtype of the G_i family on the basis of sequence homologies, also can mediate neurotransmitter inhibition of adenylyl cyclase.

The transducin family of G proteins mediate signal transduction in the visual system (Ch. 49) by regulating specific forms of phosphodiesterase that catalyze the metabolism of cyclic nucleotides (Ch. 21). $G_{\alpha t}$ activates PDE via direct binding to the enzyme. Gustducin ($G_{\alpha gust}$) shares a high degree of homology with $G_{\alpha t}$. It is enriched in taste epithelium and is believed to mediate signal transduction in this tissue via the activation of a distinct form of phosphodiesterase.

The ability of many neurotransmitters and their G-protein-coupled receptors to stimulate the phosphoinositide second-messenger pathway is mediated by the activation of PI-PLC, which, as mentioned above, catalyzes the hydrolysis of PIP_2 to form the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (Ch. 20). Such receptor-induced activation of PI-PLC is mediated via G proteins and involves the regulation specifically of the β isoform of PI-PLC [10, 11]. (By contrast, it is the γ isoform of PI-PLC that is regulated by growth factors (Ch. 27) and their tyrosine kinase receptors (Ch. 24).) In most cases, G_q mediates neurotransmitter regulation of PI-PLC_β, and it is thought that $G_{\alpha q}$ and related α subunits bind to and directly activate the enzyme. In some cell types, $\beta\gamma$ subunits released from G_i proteins directly bind and activate PI-PLC_β. The mechanism by which G proteins mediate neurotransmitter regulation of arachidonic acid metabolism (see Ch. 33), via the activation or inhibition of PLA_2, is less well established but also may involve $\beta\gamma$ dimers released from subtypes of G_i [1].

G proteins have been implicated in membrane trafficking. In addition to mediating signal transduction at the plasma membrane, certain heterotrimeric G proteins have been implicated in several processes that involve the trafficking of cell membranes, although the precise mechanisms involved remain incompletely understood. For example, the $G_{\alpha i}$ subunit has been detected at relatively high concentrations in intracellular membranes, including the Golgi complex, *trans*-Golgi network and endoplasmic reticulum [12, 13]. Experiments that involve activation or inhibition of this subunit with various guanine nucleotides suggest that $G_{\alpha i}$ may regulate the budding of membrane vesicles through these organelles. It also has been suggested that $G_{\alpha i}$ could be involved in the process by

which portions of the plasma membrane are vesicularized into the cytoplasm via endocytosis. Synaptic vesicle trafficking is discussed in detail in Chapter 9.

G protein βγ subunits subserve numerous functions in the cell. In early studies, G protein βγ subunits were thought to be inactive proteins that merely sequestered active α subunits or anchored them to the plasma membrane. However, it has become clear that βγ subunits, acting as dimers, are highly active biological molecules that play important roles in several cellular functions.

As mentioned above, βγ subunits directly bind to and activate a class of K^+ channels called GIRKs and bind to and modulate the activity of PI-PLC_β and certain classes of adenylyl cyclase. The βγ subunits also bind to several other proteins, including certain protein kinases as well as phosducin and Ras-GEFs (see below). The ability of such diverse cellular proteins to bind βγ subunits has led to a search for a common structural motif in these various target proteins that is responsible for binding to the βγ subunits. One possibility is that the target proteins contain, within their primary structures, a specific amino acid sequence termed the pleckstrin homology (PH) domain, which binds βγ with high affinity.

One class of protein kinase that binds βγ subunits is called G -protein-receptor kinases (GRKs). These kinases phosphorylate G-protein-coupled receptors that are occupied by ligand and thereby mediate one form of receptor desensitization (Ch. 23). It now appears that βγ subunits play a key role in this process [14]. As shown in Figure 19-2, the GRK is normally a cytoplasmic protein that does not come into appreciable contact with the plasma membrane receptor under basal conditions. Ligand binding to the receptor activates the associated G protein, which results in the generation of free α and βγ subunits. The βγ subunits, which remain membrane-bound (as will be described below), are now free to bind to the C-terminal domain of the GRK. This draws the GRK into close physical proximity with the receptor and enables receptor phosphorylation. In this way, the βγ subunits direct GRKs, which have constitutive catalytic activity, to those receptors that are ligand-bound.

Another important role for βγ subunits is regulation of the mitogen-activated protein kinase (MAP-kinase) pathway [15]. MAP-kinases are the major effector pathway for growth factor receptors (see Chs 10, 23 and 27). However, signals that act through G-protein-coupled receptors, particularly those coupled to G_i, can modulate growth factor activation of the MAP-kinase pathway. This is mediated via βγ subunits. Activation of the receptors leads to the generation of free βγ subunits, which then activate the MAP-kinase pathway at some early step in the cascade. Some possibilities include direct action of the βγ subunits on Ras (see below) or on one of several 'linker' proteins between the growth factor receptor itself and activation of Ras.

Recent research has implicated $G_{\beta\gamma}$ subunits in mediating G-protein signaling in the absence of activation of the G protein's associated receptor. This mechanism involves a newly discovered modulatory protein, called GoLoco, which triggers the release of free βγ dimers from G-protein–receptor complexes without receptor activation and leads to βγ regulation of its several effector proteins. This GoLoco mechanism is described further in the next section of this chapter.

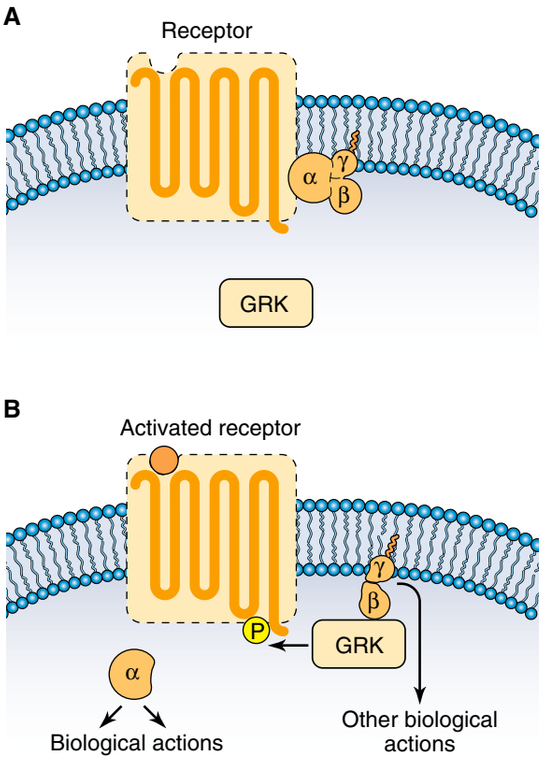

FIGURE 19-2 Schematic illustration of the role of G protein βγ subunits in intracellular targeting of proteins. (**A**) Under resting conditions, the receptor is associated loosely with a heterotrimeric G protein and G protein-receptor kinases (*GRK*) are cytoplasmic and therefore unable to phosphorylate the receptor. (**B**) Upon activation of the receptor and G protein, free α subunit is generated, which can lead to a variety of physiological effects. In addition, a free βγ subunit dimer is generated, which can bind to the GRK and draw it toward the membrane, where it can phosphorylate the ligand-occupied receptor. In this way, βγ subunits can direct GRKs specifically to the targets, which are those receptor molecules occupied by ligand. Free βγ also produces other physiological effects by interacting with other cellular proteins. The βγ complex is tethered to the membrane by an isoprenyl group on the γ subunit, as depicted.

The activity of G protein βγ subunits is modulated by another protein termed phosducin [16]. Phosducin is a cytosolic protein enriched in retina and pineal gland but also expressed in brain and other tissues. Phosducin binds to G protein βγ subunits with high affinity. The result is prevention of βγ subunit reassociation with the α subunit. In this way, phosducin may sequester βγ subunits, which initially may prolong the biological activity of the α subunit. However, eventually this sequestration may inhibit G protein activity by preventing the direct biological

effects of the βγ subunits as well as preventing regeneration of the functional G protein heterotrimer. How phosducin functions in intact cells remains incompletely understood, although the ability of phosducin to bind to βγ subunits is altered upon its phosphorylation by cAMP- or Ca^{2+}-dependent protein kinases (see Ch. 23). This raises the possibility that phosducin may be an important physiological modulator of G protein function.

The molecular specificity of subtypes of β and γ subunits is an area of intense research [4, 17]. The five known forms of G protein β subunit, whose structures are highly similar, are divided into two families comprising $G_{Aβ1–4}$ and $G_{β5}$. $G_{β5}$ appears unique in its selective association with a particular type of G protein modulatory protein known as RGS proteins (see 'Regulators of G protein signaling', below) [18]. The 11 forms of γ subunit are more divergent structurally. Some show striking regional distributions in the brain: for example, $G_{Aγ7}$ is highly enriched in striatum [19]. Different forms of β and γ subunit interact with each other with widely varying abilities in *in vitro* expression systems. Identifying which forms of βγ subunit complexes occur *in vivo* and the specificity of these complexes for various target proteins, such as adenylyl cyclases, K^+ channels, GRKs and others, remains an important area of investigation.

The functioning of heterotrimeric G proteins is modulated by other proteins. It has been known for years that the activity of small G proteins is modulated by proteins that bind to the G proteins and stimulate their intrinsic GTPase activity (see below). These are termed *GTPase-activating proteins* (GAPs; Fig. 19-3). Now, analogous proteins have been identified also for heterotrimeric G proteins [20]. These GAPs, first identified in yeast but subsequently found in mammalian tissues, are termed *RGS proteins*. RGS proteins bind to G protein α subunits and stimulate their GTPase activity. This action hastens the hydrolysis of GTP to GDP and more rapidly restores the inactive heterotrimer; thus, RGS proteins inhibit the biological activity of G proteins. In addition, RGS proteins impart important scaffolding properties that alter the function of multiprotein signaling complexes at the plasma membrane.

Some 20 forms of mammalian RGS protein are now known, and most are expressed in brain with highly specific regional patterns [21]. All RGS proteins act on the G_i and G_q family of G proteins, with varying degrees of specificity. Figure 19-4 shows the categorization of mammalian RGS proteins based on their structural properties [20]. All RGS proteins contain a core RGS domain, which is responsible for regulating G protein α subunit GTPase activity. However, several other domains contained within the RGS protein subtypes differ and confer other diverse functions on these proteins, in addition to the proteins' GAP activity. One example is provided by the R7 subfamily of RGS proteins. This subfamily, which uniquely associates with $G_{β5}$, contains a GGL ($G_γ$-like) domain

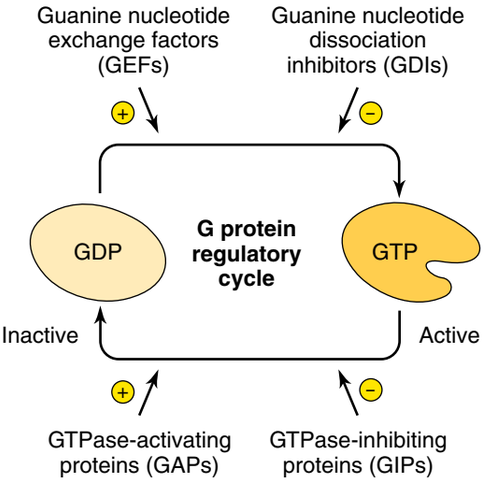

FIGURE 19-3 Schematic illustration of proteins that modulate the functioning of G proteins. The functional activity of G proteins is controlled by cycles of binding GDP versus GTP. This is associated with a major conformational change in the protein, as depicted. There are several proteins that regulate this cycle and thereby regulate the functional activity of G proteins. Analogous modulator proteins exist for heterotrimeric G protein α subunits and for small G proteins. There are proteins that facilitate the release of GDP from the G protein and thereby enhance G protein function. Examples of such guanine nucleotide exchange factors (*GEFs*) are receptors for heterotrimeric G proteins or a large number of GEFs specific for various small G proteins. There are proteins, GTPase-activating proteins (*GAPs*), that activate the GTPase activity intrinsic for the G proteins and thereby inhibit G protein function. Examples are the regulators of G protein-signaling (RGS) proteins for heterotrimeric G proteins and a series of GAPs specific for various small G proteins. There also may be GTPase-inhibitory proteins (GIPs) that exert the opposite effects. Heterotrimeric βγ subunits can be viewed as such; analogous proteins have been proposed for the small G proteins. Phosducin, by binding to βγ subunits, would represent yet another regulatory protein that modulates G protein function.

that may enable these RGS subtypes to substitute for $G_γ$ subunits in regulation of G-protein-coupled receptor function.

Another example is provided by the R12 subfamily of RGS proteins [22]. These subtypes contain the GoLoco motif described earlier. The name of the motif comes from the fact that it interacts with G proteins and the fact that the *Drosophila* homolog of RGS12 is called Loco. GoLoco binds directly to $G_{αi}$ and stabilizes it in its GDP-bound form. At the same time, it leads to the dissociation of $G_{βγ}$ subunit dimers, which then activate numerous effectors. This occurs independent of ligand activation of the associated G-protein-coupled receptor. In this way, RGS12 proteins can stimulate receptor-independent G protein signaling. Several other types of protein contain Go-Loco motifs and similarly regulate G protein signaling. Examples are the AGS (activators of G protein signaling) proteins [23].

These are just some examples of the diverse functions of RGS proteins in mammalian cells. Given the number and diversity of RGS proteins, and their region-specific expression in brain, there is considerable interest in

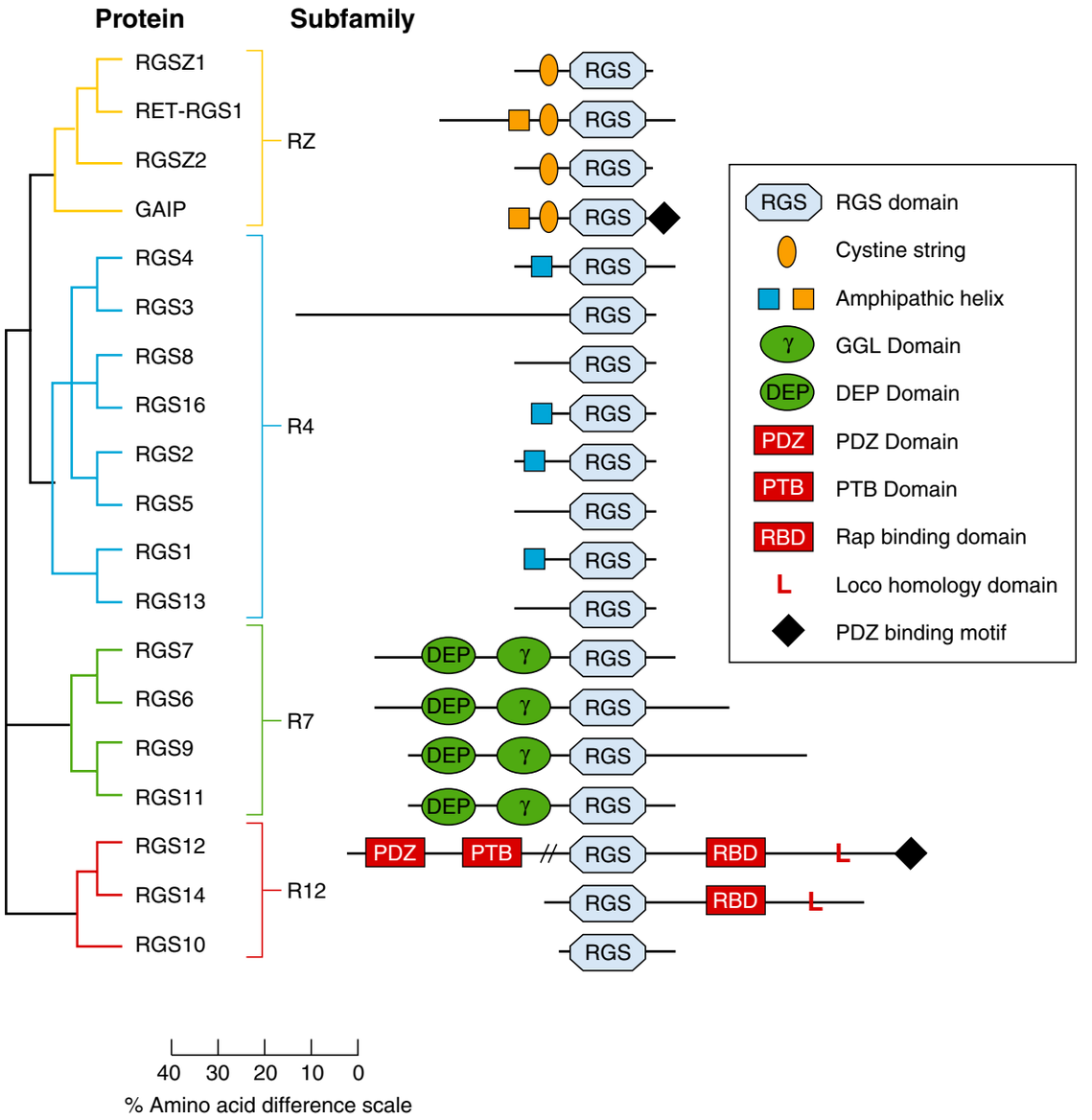

FIGURE 19-4 Schematic illustration of the structure and classification of mammalian RGS proteins. All the proteins contain a highly conserved RGS domain that has GAP activity. Most of the proteins contain additional domains that mediate other functions. The figure does not include several other types of homologous proteins, which lack the RGS domain but nevertheless are considered members of the RGS superfamily.

targeting them in the development of new pharmacotherapeutic agents for neuropsychiatric disorders. There is also growing evidence that alterations in the activity of specific RGS proteins, for example via changes in their expression or phosphorylation, modulate the activity of specific G proteins and, consequently, the sensitivity of specific G-protein-coupled receptors. Such mechanisms have been implicated, for example, in disorders as diverse as drug addiction (Ch. 56) and schizophrenia (see also Ch.54) [24, 25].

G proteins are modified covalently by the addition of long-chain fatty acids. Addition of these lipid groups appears to modify the ability of the G proteins to interact with other proteins or with the plasma membrane. Several fatty acid modifications have been demonstrated: myristoyl groups, which consist of 14 carbon chains (C_{14}); farnesyl groups (C_{15}); palmitoyl groups (C_{16}); and geranylgeranyl, or isoprenyl, groups (C_{20}) [26, 27]. Myristoyl groups are added via amide links to N-terminal glycine residues present in certain proteins. Farnesyl and isoprenyl groups are added to cysteine residues of specific C-terminal motifs. Farnesyl and isoprenyl groups are reduced and, therefore, the addition of these groups to G proteins is also reduced in the presence of inhibitors of cholesterol synthesis in *in vitro* models. Palmitoyl groups also are added to cysteine groups within specific consensus amino acid sequences.

All G protein α subunits are modified in their N-terminal domains by palmitoylation or myristoylation. These modifications may regulate the affinity of the α subunit for its βγ subunits and, thereby, the likelihood of dissociation or reassociation of the heterotrimer. The modifications also may help determine whether the α subunit, released upon ligand-receptor interaction, remains associated with the plasma membrane or diffuses into the cytoplasm. This could have important consequences on the types of effector protein regulated. It is also possible that palmitoylation, but not myristoylation, is regulated dynamically. There is evidence that palmitoylation can be regulated by ligand binding, which makes this a potentially important control point. However, very little is known about palmitoyl transferases and depalmitoylases, the enzymes responsible for palmitoylation. In contrast, myristoylation appears to be a one-time event in the life cycle of an α subunit.

G protein γ subunits are modified on their C-terminal cysteine residues by isoprenylation [26, 27]. There is now strong evidence that this modification plays a key role in anchoring the γ subunit and its associated β subunit to the plasmalemma. The importance of this anchoring is illustrated in Figure 19-2, which shows that the ability of βγ subunits to direct GRKs to ligand-bound receptors depends on this membrane localization.

The functioning of G proteins may be influenced by phosphorylation. G proteins, as well as their associated receptors and RGS proteins, have been reported to undergo phosphorylation by a host of protein serine/threonine kinases and protein tyrosine kinases. While the ramifications of receptor phosphorylation are becoming increasingly well understood (see Chs 23 and 24), the effect of phosphorylation of G proteins and RGS proteins, and its role in the regulation of physiological processes, have been more difficult to establish with certainty. This remains an important area of future investigation.

SMALL G PROTEINS

In addition to the heterotrimeric G proteins, other forms of G protein play important roles in cell function. These proteins belong to a large superfamily often referred to as 'small G proteins' because of their low M_r (20,000–35,000) [28]. The small G proteins, like the heterotrimeric G proteins, bind guanine nucleotides, possess intrinsic GTPase activity and cycle through GDP- and GTP-bound forms (as shown in Fig. 19-1). One unifying feature of the various classes of G protein is that the binding of GTP versus GDP dramatically alters the affinity of the protein for some target molecule, apparently by inducing a large conformational change. Small G proteins appear to function as molecular switches that control several cellular processes. Examples of small G proteins and their possible functional roles are given in Table 19-2.

TABLE 19-2 Examples of small G proteins

Class	Proposed cellular function
Ras	Signal transduction (control of growth factor and MAP-kinase pathways)
Rac, CDC42	Signal transduction (control of cellular stress responses and MAP-kinase pathways)
Rab	Localized to synaptic vesicles, where it regulates vesicle trafficking and exocytosis
Rho	Assembly of cytoskeletal structures (e.g. actin microfilaments)
ARF	ADP-ribosylation of $G_{\alpha s}$; assembly and function of Golgi complex
EFTU	Associated with ribosomes, where it regulates protein synthesis
Ran	Nuclear-cytoplasmic trafficking of RNA and protein

ARF, ADP-ribosylation factor; EFTU, eukaryotic elongation factor.

The best characterized small G protein is the Ras family, a series of related proteins of ≈21 kDa proteins. Ras proteins were identified originally as the oncogene products of Harvey and Kirsten rat sarcoma viruses. Subsequently, normal cellular homologs, also known as proto-oncogenes, of viral Ras were identified. Mammalian Ras proteins are encoded by three homologous genes: the photo-oncogene for Harvey Ras virus (H-*ras*), the proto-oncogene for Kirsten Ras virus (K-*ras*) and neural Ras (N-*ras*), although all three forms of Ras are found in diverse mammalian tissues, including brain. All three forms of Ras are membrane-associated proteins [28].

The activity of Ras is highly regulated by a variety of associated proteins, as shown in Figure 19-3 [28]. Guanine nucleotide exchange factors (GEFs) stimulate the release of GDP from inactive Ras, which facilitates the binding of GTP. Thus, GEFs increase the activity of Ras. Multiple forms of GEFs have been identified, some specific for Ras and others for different small G proteins. In contrast, GAPs bind to Ras and activate its intrinsic GTPase activity, thereby reducing the functional activity of Ras. There also may be GTPase-inhibitory proteins (GIPs) that bind Ras and inhibit this GTPase activity, although these remain poorly described. In addition, small G proteins are modified by guanine nucleotide dissociation inhibitors (GDIs), which reduce the rate of exchange of GDP for GTP.

The analogy between Ras and its related proteins, on the one hand, and heterotrimeric G proteins and their related proteins, on the other, is striking (Fig. 19-3). G-protein-coupled receptors, in their ligand-bound form, essentially function as GEFs for the heterotrimeric G proteins, whereas RGS proteins function as GTPase-activating proteins and βγ subunits as GDIs. One major difference between the systems is that the intrinsic GTPase activity of Ras is far lower than that of heterotrimeric G protein α subunits. As a result, GAPs exert a much more profound effect on the functioning of Ras, essentially turning it on and off.

Upon binding GTP, a major conformational change occurs in Ras, which is thought to be responsible for its functional activation. Ras has long been known to be a major control point for cell growth and some of the effector molecules through which Ras produces its myriad effects have been identified. Numerous types of cell signal, including many and perhaps most growth factors, converge on Ras to regulate MAP-kinase pathways and, thereby, to produce their diverse effects on cell function (see Chs 27 and 24) [28, 29]. Briefly, it appears that activation of growth factor receptors results in the activation of a GEF, termed Sos, which in turn activates Ras. Activated Ras then binds to the N-terminal domain of a protein kinase called Raf, the first protein kinase in the MAP-kinase pathway. Ras appears to activate Raf via an indirect mechanism. Ras, which is membrane-bound, draws Raf to the plasmalemma, where it (Raf) is activated through other means that are not yet completely understood. Anchoring of Ras in the plasmalemma may be mediated by isoprenylation, another point of analogy between Ras and heterotrimeric G proteins (Fig. 19-2).

Although the major mechanism governing Ras activation is through growth factors, as outlined above, Ras function can also be modulated by heterotrimeric G protein and second-messenger pathways [28, 29]. The ability of free βγ subunits, particularly those released from G_i, to activate Ras, was mentioned earlier. In addition, the cAMP cascade inhibits Ras activity in several systems, although it is unknown whether this occurs via direct phosphorylation by cAMP-dependent protein kinase of Ras or a closely associated protein. 'Cross-talk' such as this among these various intracellular cascades emphasizes that the multitude of intracellular messengers are highly integrated to coordinate the response of a cell to a myriad of extracellular signals.

Rab is a family of small G proteins involved in membrane vesicle trafficking. Mammalian tissues contain around 30 forms of Rab, which specifically associate with the various types of membrane vesicles and organelles that exist in cells [30, 31]. Rab proteins, named originally as *ras*-related proteins in brain, are isoprenylated and associate with membranes, as do isoprenylated Ras and G protein γ subunits. However, unlike these other G proteins, the GTP and GDP binding to Rab appears to regulate its association with membrane compartments.

Subtypes of Rab, particularly Rab3, have been implicated in the regulation of exocytosis and neurotransmitter release at nerve terminals (see also Chs 9 and 10) [30, 31]. One possible scheme by which this might occur is shown in Figure 19-5. In its GTP-bound form, Rab associates with synaptic vesicles and interacts with other membrane proteins to create a complex unfavorable for vesicle docking and perhaps fusion. Upon depolarization of the nerve terminal, a Rab GAP is activated, which results in dissociation of the GDP form of Rab from the vesicle membrane. This enables the synaptic vesicle to proceed with docking and fusion. The mechanism by which depolarization leads to activation of a Rab GAP and subsequently to release of Rab from the vesicle remains unknown, but Ca^{2+} is believed to be involved. To complicate matters further, several other proteins are involved in this Rab-synaptic vesicle cycle, including a Rab GDP dissociation inhibitor, which regulates the ability of Rab to bind to synaptic vesicles, and rabphilin, one of several synaptic vesicle proteins that can bind Rab (see Ch. 9).

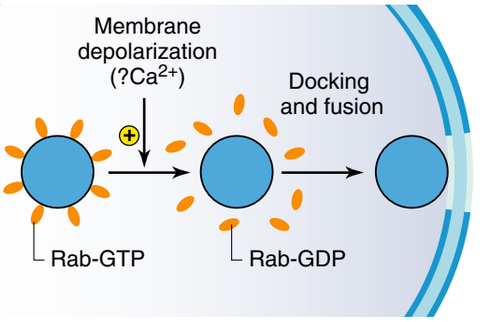

FIGURE 19-5 Schematic illustration of the proposed role of Rab in neurotransmitter release. Under basal conditions, GTP is bound to Rab3, which allows Rab3 to associate with other proteins on synaptic vesicles. This creates conditions unfavorable for the docking and fusion of vesicles at the nerve terminal plasma membrane. Membrane depolarization results in hydrolysis of the GTP bound to Rab, possibly via a Ca^{2+}-induced activation of Rab-GTPase activity. Rab then dissociates from the vesicle, creating a condition more favorable for docking and fusion. A large number of other synaptic vesicle proteins contribute to this process.

OTHER FEATURES OF G PROTEINS

G proteins can be modified by ADP-ribosylation catalyzed by certain bacterial toxins. Among the tools that facilitated the discovery and characterization of G proteins were the bacterial toxins cholera and pertussis, which were known to influence adenylyl cyclase activity. Subsequently, it was shown that the actions of these toxins are achieved by their ability to catalyze the addition of an ADP-ribose group donated from nicotinamide adenine dinucleotide (NAD) to specific amino acid residues in certain heterotrimeric G protein α subunits [1].

Cholera toxin catalyzes the ADP-ribosylation of a specific arginine residue in $G_{\alpha s}$ and $G_{\alpha t}$. This covalent modification inhibits the intrinsic GTPase activity of these α subunits and thereby 'freezes' them in their activated, or free, state (Fig. 19-1C). By this mechanism, cholera toxin stimulates adenylyl cyclase activity and photoreceptor transduction mechanisms. The ability of cholera toxin to ADP-ribosylate $G_{\alpha s}$ may require the presence of a distinct protein, ADP-ribosylation factor (ARF). ARF, which is itself a small G protein (Table 19-2), also is ADP-ribosylated by cholera toxin. ARF is implicated in controlling membrane vesicle trafficking (see Ch. 9).

In contrast, pertussis toxin catalyzes the ADP-ribosylation of a specific cysteine residue in $G_{\alpha i}$, $G_{\alpha o}$ and $G_{\alpha t}$ [1]. Only α subunits bound to their βγ subunits can undergo this modification. Pertussis-toxin-mediated ADP-ribosylation inactivates these α subunits such that they cannot exchange GTP for GDP in response to receptor activation (Fig. 19-1B). By this mechanism, pertussis toxin blocks the ability of neurotransmitters to inhibit adenylyl cyclase or to influence the gating of K^+ and Ca^{2+} channels in target neurons. However, since $G_{\alpha z}$ is not a substrate for pertussis toxin, the toxin may not be able to block neurotransmitter-mediated inhibition of adenylyl cyclase in all cases. The G_q and G_{11-16} types of G protein α subunit are not known to undergo ADP-ribosylation.

A third type of bacterial toxin, diphtheria toxin, catalyzes the ADP-ribosylation of eukaryotic elongation factor (EFTU), a type of small G protein involved in protein synthesis (Table 19-2). The functional activity of the elongation factor is inhibited by this reaction. Finally, a botulinum toxin ADP-ribosylates and disrupts the function of the small G protein Rho, which appears to be involved in assembly and rearrangement of the actin cytoskeleton (Table 19-2). These toxins may be involved in neuropathy (see Ch. 36) and membrane trafficking (see Ch. 9).

G proteins are implicated in the pathophysiology and treatment of disease. The best characterized examples of G protein abnormalities involved in a disease state are endocrinopathies caused by mutations in $G_{\alpha s}$ [32, 33]. There are both 'gain of function' and 'loss of function' mutations. Examples of gain of function mutations, which result in increased or constitutive activity of $G_{\alpha s}$, include forms of acromegaly, which involves hypersecretion of growth hormone, and McCune–Albright syndrome, which involves hyperossification of bone, *café-au-lait* skin hyperpigmentation and mixed endocrine hyperfunction. Examples of loss of function mutations include Albright's hereditary osteodystrophy, characterized by abnormal bone formation, as well as forms of pseudohypoparathyroidism, a rare hereditary disease in which target tissues are resistant to the physiological actions of parathyroid hormone despite the existence of a normal number of functionally active hormone receptors. In addition, mutations in $G_{\alpha i}$ have been implicated in certain forms of ovarian and adrenal cortical tumor.

Neurofibromatosis type 1, a familial disorder characterized by multiple benign tumors of certain glial cells, is due to a mutation in the gene that codes for one form of GAP that regulates Ras [34]. The mutation in GAP that leads to neurofibromatosis renders GAP unable to activate GTPase activity of Ras. This means that the GTP-bound form of Ras remains active for abnormally long periods of time, which leads to abnormal cellular growth.

Of course, the critical importance of Ras and most other small G proteins in cell growth and differentiation is highlighted by the consideration, stated above, that several forms of these proteins are proto-oncogenes (see also Ch. 25). This means that mutations in these proteins that result in alterations in their regulatory properties can lead to oncogenesis. Ras in particular has been implicated in several human cancers [32]. It has been estimated that as many as 30% of all human cancers contain mutations in one of the three Ras genes. While the frequency of Ras mutations in some types of human cancer is very low, its frequency in certain cancers, such as squamous cell carcinoma, lymphatic cancers and colorectal adenocarcinoma, is very high.

In addition to their involvement in specific disease states, concentrations of heterotrimeric G protein subunits, as well as of particular RGS protein subtypes, are altered in specific regions of the central nervous system in response to chronic exposure to many types of psychoactive drugs. This has been shown for the antidepressant drugs lithium, which is used in the treatment of mania and depression (Ch. 55), and for several drugs of abuse [24, 25, 35] (see also Ch. 56). Evidence has been presented to suggest that drug-induced alterations in G protein and RGS protein levels influence signal transduction pathways in the brain and, thereby, contribute to the therapeutic or addictive actions of these drugs.

An increasing number of G proteins and RGS proteins have been deleted or overexpressed in genetic mutant mice. Not surprisingly, some of these mice exhibit complex behavioral abnormalities and in some cases abnormal responses to psychotropic drugs. These data further highlight the importance of G protein signaling in the brain in health and disease and open new paths for investigation in the years ahead.

REFERENCES

1. Neer, E. J. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 80: 249–257, 1995.
2. Wickman, K. and Clapham, D. E. Ion channel regulation by G proteins. *Physiol. Rev.* 75: 865–885, 1995.
3. Gilman, A. G. G proteins and regulation of adenylyl cyclase. *Biosci. Rep.* 15: 65–97, 1995.
4. Preininger, A. M. and Hamm, H. E. G protein signaling: insights from new structures. *Sci. STKE 2003* 218: re3, 2004.
5. Onrust, R., Herzmark, P. and Chi, P. Receptor and βγ binding sites in the α subunit of the retinal G protein transducin. *Science* 275: 381–384, 1997.
6. Hatley, M. E., Lockless, S. W., Gibson, S. K., Gilman, A. G. and Ranganathan, R. Allosteric determinants in guanine nucleotide-binding proteins. *Proc. Natl Acad. Sci. U.S.A.* 100: 14445–14450, 2003.
7. Yi, B. A., Minor, D. L. Jr, Lin, Y. F., Jan, Y. N. and Jan, L. Y. Controlling potassium channel activities: interplay between the membrane and intracellular factors. *Proc. Natl Acad. Sci. U.S.A.* 98: 11016–11023, 2001.
8. Sadja, R., Alagem, N. and Reuveny, E. Gating of GIRK channels: details of an intricate, membrane-delimited signaling complex. *Neuron* 39: 9–12, 2003.
9. Dolphin, A. C. G protein modulation of voltage-gated calcium channels. *Pharmacol. Rev.* 55: 607–627, 2003.

10. Rhee, S. G. Regulation of phosphoinositide-specific phospholipase C. *Rev. Biochem.* 70: 281–312, 2001.
11. Ghosh, M. and Smrcka, A. V. Assay for G protein-dependent activation of phospholipase C beta using purified protein components. *Methods Mol. Biol.* 237: 67–75, 2004.
12. Helms, J. B. Role of heterotrimeric GTP binding proteins in vesicular protein transport: indications for both classical and alternative G protein cycles. *FEBS Lett.* 369: 84–88, 1995.
13. Yamaguchi, T., Nagahama, M., Itoh, H., Hatsuzawa, K., Tani, K. and Tagaya, M. Regulation of the Golgi structure by the alpha subunits of heterotrimeric G proteins. *FEBS Lett.* 470: 25–28, 2000.
14. Lodowski, D. T., Pitcher, J. A., Capel, W. D., Lefkowitz, R. J. and Tesmer, J. J. Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gβγ. *Science.* 300: 1256–1262, 2003.
15. Schmitt, J. M. and Stork, P. J. Gα and Gβ γ require distinct Src-dependent pathways to activate Rap1 and Ras. *J. Biol. Chem.* 277: 43024–43032, 2002.
16. Schulz, R. The pharmacology of phosducin. *Pharmacol. Res.* 43: 1–10, 2001.
17. Albert, P. R. and Robillard, L. G protein specificity: traffic direction required. *Cell. Signal.* 14: 407–418, 2002.
18. Witherow, D. S. and Slepak, V. Z. A novel kind of G protein heterodimer: the G β5-RGS complex. *Receptors Channels* 9: 205–212, 2003.
19. Schwindinger, W. F., Betz, K. S., Giger, K. E., Sabol, A., Bronson, S. K. and Robishaw, J. D. Loss of G protein gamma 7 alters behavior and reduces striatal alpha (olf) level and cAMP production. *J. Biol. Chem.* 278: 6575–6579, 2003.
20. Ross, E. M. and Wilkie, T. M. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem.* 69: 795–827, 2000.
21. Gold, S. J., Ni, Y. G., Dohlman, H. and Nestler, E. J. Regulators of G protein signaling: Region-specific expression of nine subtypes in rat brain. *J. Neurosci.* 7: 8024–8037, 1997.
22. Kimple, R. J., De Vries, L., Tronchere, H. *et al.* RGS12 and RGS14 GoLoco motifs are Gαi interaction sites with guanine nucleotide dissociation inhibitor activity. *J. Biol. Chem.* 276: 29275–29281, 2001.
23. Blumer, J. B. and Lanier, S. M. Accessory proteins for G protein-signaling systems: activators of G protein signaling and other nonreceptor proteins influencing the activation state of G proteins. *Receptors Channels* 9: 195–204, 2003.
24. Rahman, Z., Schwarz, J., Zachariou, V. *et al.* RGS9 modulates dopamine signaling in striatum. *Neuron* 8: 941–952, 2003.
25. Mimics, K., Middleton, F. A., Stanwood, G. D., Lewis, D. A. and Levitt, P. Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia. *Mol. Psychiatry* 6: 293–301, 2001.
26. Wedegaertner, P., Wilson, P. and Bourne, H. R. Lipid modifications of trimeric G proteins. *J. Biol. Chem.* 270: 503–505, 1995.
27. Ross, E. M. Palmitoylation in G protein signaling pathways. *Curr. Biol.* 5: 107–109, 1995.
28. Marshall, C. J. Ras effectors. *Curr. Opin. Cell Biol.* 8: 197–204, 1996.
29. Raabe, T. and Rapp, U. R. Ras signaling: PP2A puts Ksr and Raf in the right place. *Curr. Biol.* 13: R635–R637, 2003.
30. Sudhof, T. C. The synaptic vesicle cycle revisited. *Neuron* 28: 317–320, 2000.
31. Spang, A. Vesicle transport: a close collaboration of Rabs and effectors. *Curr. Biol.* 14: R33–R34, 2004.
32. Schnabel, P. and Bohm, M. Mutations of signal-transducing G proteins in human disease. *J. Mol. Med.* 73: 221–228, 1995.
33. Spiegel, A. M. Genetic basis of endocrine disease. Mutations in G proteins and G protein-coupled receptors in endocrine disease. *J. Clin. Endocrinol. Metab.* 81: 2434–2442, 1996.
34. Dasgupta, B. and Gutmann, D. H. Neurofibromatosis 1: closing the GAP between mice and men. *Curr. Opin. Genet. Dev.* 13: 20–27, 2003.
35. Donati, R. J. and Rasenick, M. M. G protein signaling and the molecular basis of antidepressant action. *Life Sci.* 73: 1–17, 2003.
36. Hyman, S. E. and Nestler, E. J. Initiation and adaptation: a paradigm to understand psychotropic drug action. *Am. J. Psychiatry* 153: 151–162.

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CHAPTER

20

Phosphoinositides

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A large variety of ligands, including neurotransmitters, neuromodulators and hormones, exert their physiological action via an intracellular second-messenger system in which the activated receptor–ligand complex stimulates the turnover of inositol-containing phospholipids, resulting in the formation of the second messengers inositol trisphosphate and diacylglycerol. The original demonstration that phosphoinositide turnover was under neurohumoral control dates back to the pioneering studies of the Hokins in the early 1950s [1]. Although it was recognized for many years that alterations in Ca^{2+} homeostasis and protein phosphorylation accompanied these changes in inositol lipid turnover, it was not until some 30 years later that inositol 1,4,5-trisphosphate and diacylglycerol were identified as second messenger molecules capable of mobilizing intracellular Ca^{2+} and activating protein kinase C, respectively. For a historical perspective, the reader is referred to a review by Irvine [2]. In this chapter, the biochemical and cellular bases of this ubiquitous pathway, as well as its pharmacological significance, are examined in the context of the nervous system. To better understand the events leading to the enhanced inositol lipid turnover, it is necessary first to review the underlying structural chemistry.

THE INOSITOL LIPIDS

The three quantitatively major phosphoinositides are structurally and metabolically related. These consist of phosphatidylinositol (PI) and the two polyphosphoinositides, namely phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)* (Fig. 20-1). PI consists of a diacylglycerol (DAG) moiety, which is phosphodiesterified to myo-inositol, a 6-carbon polycyclic alcohol (Fig. 20-1). myo-inositol, one of nine possible isomers of hexahydroxycyclohexane, has one axial and five equatorial hydroxyls and is by far the most prevalent isomer in nature. myo-inositol is particularly enriched in neural tissues (5–20 mmol/l), where it serves not only as a precursor molecule for inositol lipid synthesis but also as a physiologically important osmolyte [3]. The distinctive configuration of myo-inositol can be more readily understood by making use of Agranoff's turtle, in

*The 'Chilton Conference' nomenclature for inositol lipids is used throughout this chapter, e.g. PI, PI4P, $PI(4,5)P_2$ for phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate respectively. Note that the IUB-recommended nomenclature for these lipids is PtdIns, PtdIns4P and $PtdIns(4,5)P_2$ (see Ch. 3).

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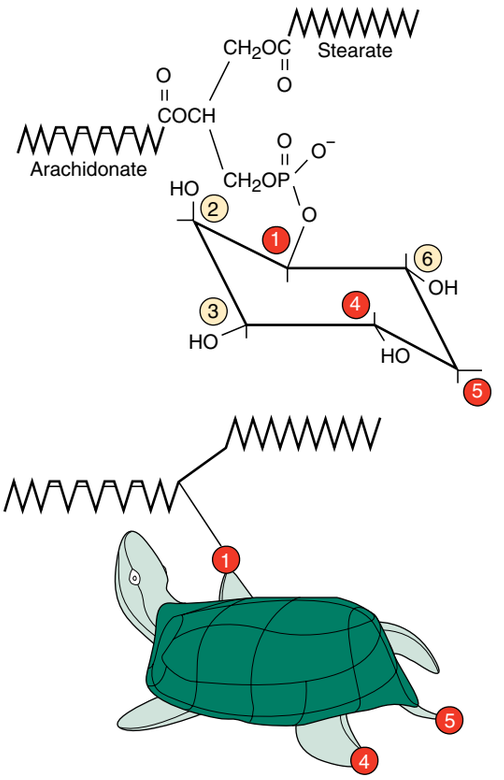

FIGURE 20-1 Stereochemistry of the quantitatively major inositol lipids. Inositol lipids characteristically contain stearic acid (18:0) and arachidonic acid (20:4 ω6) esterified to the 1 and 2 position of sn-glycerolphosphate, respectively. The phosphate (colored circle) is diesterified to the 1 position of D-myo-inositol. myo-inositol in its favored chair conformation has five equatorial hydroxyls and one axial hydroxyl. Looking at the chair from above and counting counterclockwise, the axial hydroxyl is then in position 2. As indicated by the drawing, the inositol molecule can be conveniently viewed as a turtle [4], in which the diacylglycerol phosphate moiety is attached to the right front leg (position 1), next to the raised head (the axial hydroxyl in position 2). The other equatorial hydroxyls are represented by the remaining limbs and the tail. Phosphatidylinositol (PI) can be phosphorylated at position 4 (the rear left leg), or the rear left leg as well as the tail (positions 4 and 5 as shown), to yield phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) respectively.

which the cyclohexane chair configuration is viewed as a turtle with the axial hydroxyl as the head, and hydroxyls in the five equatorial positions serving as the four limbs and tail (Fig. 20-1; [4]). At present, there are no known brain phosphoinositides containing cyclitols other than myo-inositol, nor are there as yet examples of inositol lipids in which the inositol is diesterified to DAG at a position other than D-1.

There exists an unusual uniformity in the fatty acid composition of the inositol lipids. All three of the major phosphoinositides are enriched in the 1-stearoyl, 2-arachidonoyl ('ST/AR') sn-glycerol species (≈80% in brain). The polyphosphoinositides (PI4P and $PI(4,5)P_2$) are present in much lower amounts than PI. $PI(4,5)P_2$ has been shown to be predominantly, although not exclusively, localized to the plasmalemma [5]. Because of its high complement of plasmalemmas, the brain is one of the most enriched sources of the polyphosphoinositides.

The phosphoinositides are synthesized from the glycerolipid precursor phosphatidic acid (PA) via the formation of the liponucleotide intermediate cytidine diphosphate diacylglycerol (CDP-DAG; Fig. 20-2B; see also Ch. 3). This reaction is catalyzed by CDP-DAG synthase, a 51 kDa protein localized to both mitochondrial and endoplasmic reticulum membranes. Subsequently, PI synthase, an extremely hydrophobic 24 kDa protein primarily localized to the endoplasmic reticulum and Golgi, catalyzes the formation of PI. The latter is then phosphorylated to PI4P in a reaction requiring ATP and catalyzed by PI 4-kinase (PI4K; Fig. 20-2A).

Cloning studies have revealed the presence of three distinct isoforms of PI4K of 97, 110 and 230 kDa. The 97 kDa enzyme is inhibited by adenosine and by the monoclonal antibody 4C5G. In contrast, neither the 110 kDa nor the 230 kDa isoform is markedly inhibited by these agents. Also in contrast to the 97 kDa isozyme, both are sensitive to the fungal metabolite wortmannin, albeit at relatively high concentrations. The ability of wortmannin to block the sustained agonist-induced turnover of inositol lipids has led to the suggestion that wortmannin-inhibitable isoforms of PI4K are involved in the regulation of hormone-sensitive pools of phosphoinositides [6].

PI4P is further phosphorylated via PIP kinase to $PI(4,5)P_2$ (Fig. 20-2A). This enzyme is found both in brain membranes and cytosol and does not phosphorylate PI. Two distinct classes of PIP kinase can be identified, types I and II, each with three distinct subfamilies (α, β and γ) [7]. Although the type I enzyme preferentially phosphorylates PI(4)P to $PI(4,5)P_2$, it can also phosphorylate PI3P (a 3′-phosphoinositide, see below) at either the 4′ or 5′ positions of the inositol ring to yield $PI(3,4)P_2$ and $PI(3,5)P_2$. There is also a PI 5-kinase, further adding to the complexity of phosphoinositide metabolism. The type II PIP kinase exhibits a preference for PI(5)P over PI(4)P and will phosphorylate at the 4-position of the inositol ring to yield $PI(4,5)P_2$. As with the type I enzyme, type II PIP-kinases will also phosphorylate 3′-phosphoinositides. Both $PI(4,5)P_2$ and PI4P can be dephosphorylated by phosphatases, with most information available regarding the 5-phosphatases. Of these, the major members are synaptojanins and the product of the oculocerebrorenal syndrome gene. These are discussed in more detail in the following section.

The quantitatively minor 3′-phosphoinositides are synthesized by phosphatidylinositol 3-kinase. These lipids (principally, PI3P, $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$), documented by Cantley and colleagues in the mid-1980s, are present in both neural and non-neural tissues at concentrations well below those found for either PI4P or $PI(4,5)P_2$ (for review, see [8]). In resting cells, the concentration of PI3P exceeds, by an order of magnitude, that of

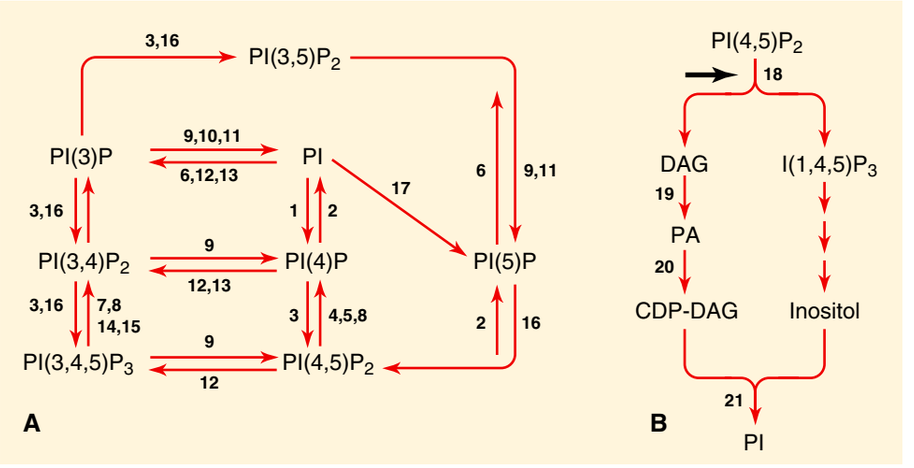

FIGURE 20-2 Pathways of inositol lipid metabolism. (**A**) Interrelationship between routes of synthesis and degradation of the 'canonical' inositol lipids (PI, PI(4)P and PI(4,5)P_2) and the 3-phosphoinositides. Numbers refer to the following enzymes (for explanation of abbreviations, see text): 1, PI 4-kinase; 2, PI 4-phosphatase; 3, PIP kinase I; 4, OCRL gene product; 5, synaptojanin; 6, PI 3-kinase (III); 7, pharbin. 8, PIPP; 9, PTEN; 10, myotubularin; 11, sac1p phosphatase; 12, PI 3-kinase (I); 13, PI 3-kinase (II); 14, GIP; 15, SHIP; 16, PIP kinase II; 17, PI 5-kinase. (**B**) Regenerative cycle of phosphoinositide resynthesis following receptor–ligand-mediated breakdown of PI(4,5)P_2 (bold arrow) and generation of the second messengers DAG and I(1,4,5)P_3. Numbers refer to the following enzymes: 18, phosphoinositide-specific phospholipase C; 19, DAG kinase; 20, CDP-DAG synthase; 21, PI synthase.

the other 3-phosphoinositides. However, whereas the concentration of PI3P is relatively stable in cells, the mass of PI(3,4)P_2 and PI(3,4,5)P_3 can increase dramatically upon receptor activation [8]. The metabolic interrelationships of the 3-phosphoinositides are shown in Figure 20-2A.

The key enzyme involved in the synthesis of the 3-phosphoinositides is PI 3-kinase (PI3K). Three major classes of PI3K have been described, based upon lipid substrate specificity and regulation. Class I PI3Ks are able to phosphorylate PI, PI4P and PI(4,5)P_2 in vitro to form PI3P, PI(3,4)P_2 and PI(3,4,5)P_3 respectively, although their preferred substrate in vivo is PI(4,5)P_2. The catalytic subunits of this class of PI3K form heterodimeric complexes with adaptor or regulatory proteins that link the enzyme to upstream signaling events.

Class I PI3K catalytic subunits can be further subdivided. Class I A catalytic subunits (110–130 kDa) interact with PI3K regulatory proteins (p85) that contain two SH2 domains. The presence of the latter enables the enzyme to more readily associate with phosphorylated tyrosine residues present on upstream proteins, such as receptors with intrinsic tyrosine kinase activity or, alternatively, nonreceptor tyrosine kinases, such as src- or JAK kinases, thereby linking PI3K to tyrosine kinase signaling pathways (see Ch. 24). Phosphotyrosine binding to the regulatory domain of PI3K is thought to permit the translocation of the enzyme from cytosol to plasmalemma.

Class I B catalytic subunits (110 kDa) are stimulated by βγ subunits derived from G proteins (see Ch. 19). A regulatory protein (p101) that associates tightly with the catalytic subunit has been isolated. Both type A and type B catalytic subunits of class I PI3K interact with a small GTP-binding protein, Ras, although the significance of this interaction remains uncertain. Class I PI3Ks can be potently inhibited by either wortmannin, a fungal metabolite, or by LY294002, a quercetin analog. Both inhibitors bind to the catalytic subunit of PI3K and inhibit kinase activity. Class II and class III PI3Ks have a more restricted substrate specificity; both exhibit a strong preference for PI as a substrate but class II enzymes can also phosphorylate PI(4)P, at least in vitro. Unlike class I PI3Ks, neither class II nor class III PI3Ks appear to be regulated by extracellular stimuli.

The 3-phosphoinositides can be degraded via the action of three structurally distinct families of enzymes: 3′-or 4′-phosphatases from the CX5R families, which share a limited consensus sequence, CXXXXXR, in their catalytic core domain; and the 5′-phosphatases. Of the 3′-phosphatases, myotubularin, Sac1p phosphatase and PTEN (phosphatase and TENsin homolog) are major examples. Myotubularin was originally thought to be a tyrosine phosphatase responsible for myotubular myopathy, but was subsequently demonstrated to be a lipid phosphatase, whose substrate specificity is restricted to PI3P. Sac1p phosphatase, originally identified in yeast as a suppressor of actin mutations, also demonstrates a restricted substrate specificity in that it will not hydrolyze 3-phosphoinositides that possess vicinal phosphates, e.g. PI(3,4)P_2 or PI(3,4,5)P_3. However, it will hydrolyze PI3P or PI(3,5)P_2, with PI3P being preferred. Like myotubularin, PTEN was originally described as a tyrosine phosphatase that exhibited tumor suppressive properties, but it was subsequently recognized that the enzyme was primarily a lipid phosphatase, the latter activity being

essential for its ability to regulate cell proliferation. PTEN will dephosphorylate all 3-phosphoinositides and $I(1,3,4,5)P_4$ in vitro, and $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in vivo.

Although relatively little is known about the 4-phosphatases, two such enzymes have been cloned and sequenced. These enzymes can also dephosphorylate inositol phosphates. Degradation of $PI(3,5)P_2$ and $PI(3,4,5)P_3$ can also be accomplished via the action of type II 5-phosphatases. The type II enzyme hydrolyzes both lipids and inositol phosphates, while type I 5′-phosphatases hydrolyze only the water-soluble inositol phosphates. At least four members of the type II 5-phosphatase family can be identified: the GAP-containing inositol 5-phosphatases (GIPs), Sac1p-containing inositol 5-phosphatases (SCIPs), SH2-domain-containing inositol 5-phosphatases (SHIPs) and proline-rich-domain-containing inositol 5-phosphatases (PIPs). The GIPs will dephosphorylate both $PI(4,5)P_2$ and $PI(3,4,5)P_3$, as well as the corresponding inositol phosphates. A type II 5-phosphatase gene, which is disrupted in the human X-chromosome-linked developmental disorder Lowe's oculocerebrorenal (OCRL) syndrome, has been identified as a GIP homolog. The wild type gene product is reportedly enriched in the Golgi or lysosomal fractions and preferentially hydrolyzes $PI(4,5)P_2$, but also has activity toward $PI(3,4,5)P_3$, $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$. Lowe's syndrome, which results in mental retardation, congenital cataracts and renal tubular acidosis, is the only known inborn error of phosphoinositide metabolism.

Two synaptojanins are members of the SCIP family: synaptojanin 1 is restricted in distribution to neurons and plays a role in synaptic vesicle recycling, whereas synaptojanin 2 is ubiquitously expressed (see Ch. 9). Both synaptojanins are phosphoinositide 5-phosphatases capable of hydrolyzing $PI(4,5)P_2$ and $PI(3,4,5)P_3$. Thus, they could conceivably metabolize one bioactive lipid, namely $PI(3,4,5)P_3$, to yield an alternate, i.e. $PI(3,4)P_2$, a phosphoinositide implicated in the regulation of Akt (see 'Phosphoinositides and cell regulation', below). Two members of the SHIP family have been identified (SHIP1 and SHIP2), as well as two splice variants of SHIP1. These enzymes are characterized by an N-terminal SH2 domain and a C-terminal proline-rich domain. The preferred substrate of both SHIP1 and SHIP2 is $PI(3,4,5)P_3$, but both will also hydrolyze $I(1,3,4,5)P_4$. The PIPs are characterized by an N-terminal proline-rich domain, and examples include pharbin and PIPP (proline-rich inositol polyphosphate 5-phosphatase). Both enzymes will hydrolyze $PI(3,4,5)P_3$, as well as $PI(4,5)P_2$, $PI(3,5)P_2$ and corresponding soluble inositol phosphates.

In contrast to their more highly expressed counterparts, the 3-phosphoinositides do not serve as substrates for phospholipase C (PLC), the enzyme known to be activated in stimulated phosphoinositide turnover. This observation indicates that the 3-phosphoinositides themselves, rather than their breakdown products, are likely to be the intracellular mediators of biological activity. PI3P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ can bind to specific domains within proteins, thereby facilitating protein–protein interactions. As will be detailed below ('Phosphoinositides and cell regulation'), roles for 3-phosphoinositides in the regulation of membrane trafficking, cell survival and maintenance of the cytoskeleton have been proposed.

Phosphoinositides are cleaved by a family of phosphoinositide-specific phospholipase C isozymes. PLC catalyzes the phosphodiesteratic breakdown of $PI(4,5)P_2$, a reaction activated by a large number of pharmacologically distinct CNS receptors [9] (Table 20-1). The breakdown of $PI(4,5)P_2$ generates two second messenger molecules: (a) diacylglycerol, which is released into the plane of the plasmalemma and can then activate protein kinase C, and (b) inositol 1,4,5-trisphosphate ($I(1,4,5)P_3$), which is released into the cytosol and mobilizes the release of intracellular Ca^{2+} following the activation of $I(1,4,5)P_3$ receptors (see below and Ch. 22). The metabolism of these second messengers occurs in either membrane (DAG) or cytosolic ($I(1,4,5)P_3$) compartments of the cell (Fig. 20-2B). Thus DAG is phosphorylated by DAG kinase to form PA, the precursor molecule for PI and polyphosphoinositide synthesis, these events occurring in plasmalemma and other intracellular sites. In contrast, $I(1,4,5)P_3$ undergoes a series of sequential dephosphorylations, catalyzed by cytosolic or membrane-bound phosphatases, which ultimately yield free myo-inositol. The latter is then available for the resynthesis of PI, in the presence of CDP-DAG and PI synthase.

Four isoforms of PLC (β, γ, δ and ε; Fig. 20-3) have been identified in the brain, with the nomenclature based upon the chronology of their discovery. The α isoform was subsequently identified as a proteolytic fragment of PLCδ (for reviews, see [10, 11]). Because the lower eukaryotes such as yeast and slime molds possess only the δ isoform, it has been suggested that the other isoforms evolved

TABLE 20-1 Examples of ligand-activated phosphoinositide hydrolysis in neural tissues

Muscarinic	Neuropeptide Y
Adrenergic	Neurotensin
Histaminergic	Gastrin-releasing peptide
Serotonergic	Bombesin
Glutamatergic	Substance P
Endothelin	Oxytocin
Purinergic	Eledoisin
Thromboxane	Neurokinin
Prostaglandin	Vasointestinal peptide
Bradykinin	Angiotensin
Vasopressin	Gonadotropin-releasing hormone
Nerve growth factor	Platelet-activating factor
Cholecystokinin	Thyrotropin-releasing hormone

For the identities of the individual receptor subtypes that couple to phosphoinositide hydrolysis, the reader is referred to the individual chapters on neurotransmitters in this volume.

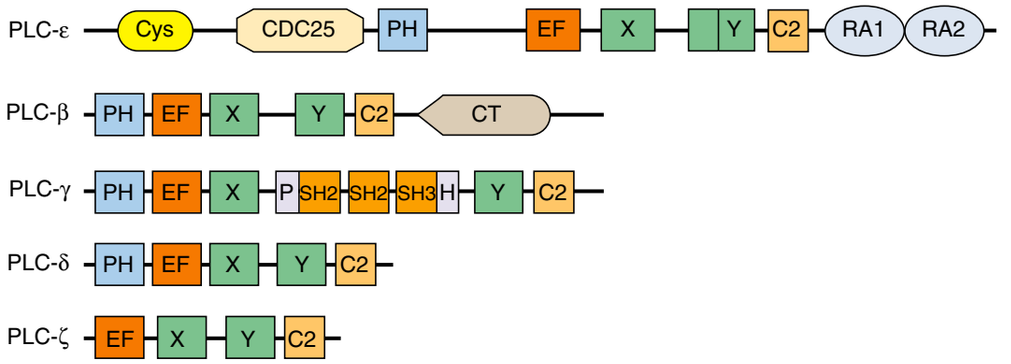

FIGURE 20-3 Linear representation of the five classes of PLC isoforms that can be distinguished by the presence of different domains. (Modified with permission from reference [11].)

from an archetypal PLCδ. The β, γ and δ isoforms of PLC have molecular weights of approximately 130–155, 145 and 85 kDa respectively. Each has been extensively purified, their complementary DNA (cDNA) sequences elucidated and antibodies have been raised to the recombinant proteins. PLCε has been identified in the brain and is of larger size (210 kDa). One additional isoform of PLC found only in sperm has been described – PLC_ζ. A number of isozymes of each subtype of PLC have been identified such that at least 12 distinct isozymes are thought to exist. Isozymes of PLC require the presence of Ca^{2+} for full activation and will hydrolyze the inositol lipids with a selectively for $PI(4,5)P_2$ over PI.

Despite the similar functions of each isozyme, only two regions of amino acid homology exist (X and Y), one of 150 and a second of 120 amino acid residues, which are 54% and 42% identical among the isozymes but are differentially localized within each enzyme (Fig. 20-3). The X and Y domains form the catalytic core of the enzyme. A characteristic of the β and δ isoforms is that relatively few amino acids (40–110) separate the X and Y entities, whereas a much larger separation is observed for the PLCγ isoform (approx. 400). In addition, in PLCγ, the region between X and Y contains amino acid sequences that are found in nonreceptor tyrosine kinases (SH_2 and SH_3 domains). All four isoforms possess pleckstrin homology (PH) domains. The latter are considered to enable the enzyme to become tethered to the plasmalemma via an interaction with $PI(4,5)P_2$. In addition, all PLC isoforms possess an E-F hand domain, which is located between PH and X domains, and a C2 domain, which is located close to the Y domain.

The γ and δ forms of PLC can be distinguished from PLCβ by the virtual absence of a 400–500 amino acid consensus sequence present in the C-terminal region of PLCβ. Consistent with the absence of putative transmembrane spanning domains, most PLC activity is localized to the cytoplasm, although a significant amount of activity is associated with membrane fractions.

Four distinct mechanisms of activation of PLC have been identified. As shown in Figure 20-4, agonist occupancy of receptors that possess intrinsic tyrosine kinase activation (e.g. platelet derived growth factor) can result in receptor dimerization and autophosphorylation of the cytoplasmic tails on tyrosine residues (mechanism I) (see Ch. 24). The presence of these phosphorylated tyrosine residues serves to 'recruit' proteins (such as PLCγ) that possess SH2 domains. In doing so, the cytosolic effector enzyme PLCγ is brought into close proximity to the plasmalemma, the site of its substrate, $PI(4,5)P_2$, and of its activation by phosphorylation. Other receptors activate PLC via intervening guanine-nucleotide-binding (G) proteins. The interaction of a ligand with a receptor (e.g. muscarinic cholinergic) results in the activation and dissociation of G_q, a pertussis- toxin-insensitive G protein, to liberate α_q (see Ch. 19) (mechanism II). The latter then may activate $PLC\beta_1$ and/or $PLC\beta_3$. In a third mechanism, the ligand–receptor interaction results in the activation and dissociation of a pertussis-toxin-sensitive G protein (G_i or G_o), which in turn liberates α_i or α_o and βγ subunits (Ch. 19). In this case, it is the βγ subunits, but not the α subunit, that can then activate $PLC\beta_3$ (or, in the case of hematopoietic cells, $PLC\beta_2$). Different regions of the PLC molecule appear to be required for α_q or βγ activation. Thus, whereas cleavage of the carboxyl terminal portion of $PLC\beta_1$ renders it refractory to α_q regulation, the amino terminal region of the molecule is required for βγ activation.

The discovery of PLCε reveals a fourth mechanism whereby the enzyme can be activated (Fig. 20-5). PLCε possesses two Ras-binding (RA) domains in its carboxyl terminal region, and occupancy of these by Ras-GTP results in activation of the enzyme. In addition, the enzyme possesses a CDC-25 domain at its N-terminus, which serves as a guanine nucleotide exchange factor (GEF) for small GTP-binding proteins such as Ras or Rap1A. Thus PLCε can not only activate the GDP-bound forms of these small GTP-binding proteins but can also be

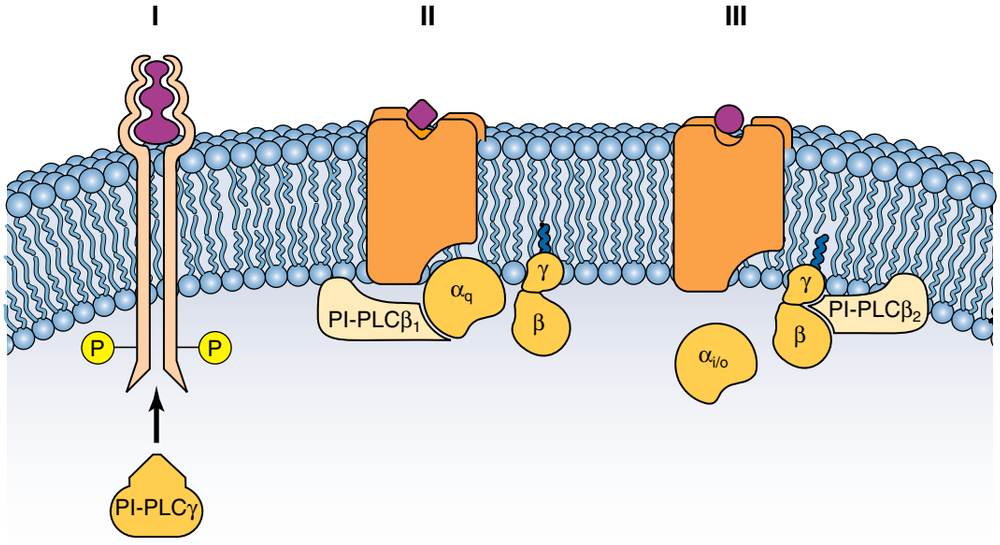

FIGURE 20-4 Three of four distinct mechanisms for the activation of phosphoinositide-specific phospholipase C (PI-PLC). In mechanism I, the ligand activates a receptor that possesses an intrinsic tyrosine kinase. Following dimerization of the receptor, it becomes autophosphorylated on tyrosine residues present in the cytoplasmic domain of the receptor. PI-PLCγ is then recruited to the plasmalemma, via interaction of its SH2 domains with the phosphorylated tyrosine residues on the receptor, and is activated by phosphorylation. In mechanism II, the ligand-receptor interaction results in the dissociation of a pertussis-toxin-insensitive G-protein (G_q), to liberate α_q. The latter then activates either PI-PLCβ_1 or PI-PLCβ_3. In mechanism III, the ligand–receptor interaction results in the dissociation of a pertussis-toxin-sensitive G-protein (e.g. G_i or G_o), which in turn liberates α_i/α_o and $\beta\gamma$ subunits. The latter can then activate either PI-PLCβ_2 or PI-PLCβ_3. Note that, although $\beta\gamma$ subunits derived from G_q could also theoretically activate this pathway, this possibility is less likely, given the abundance of G_i/G_o relative to G_q.

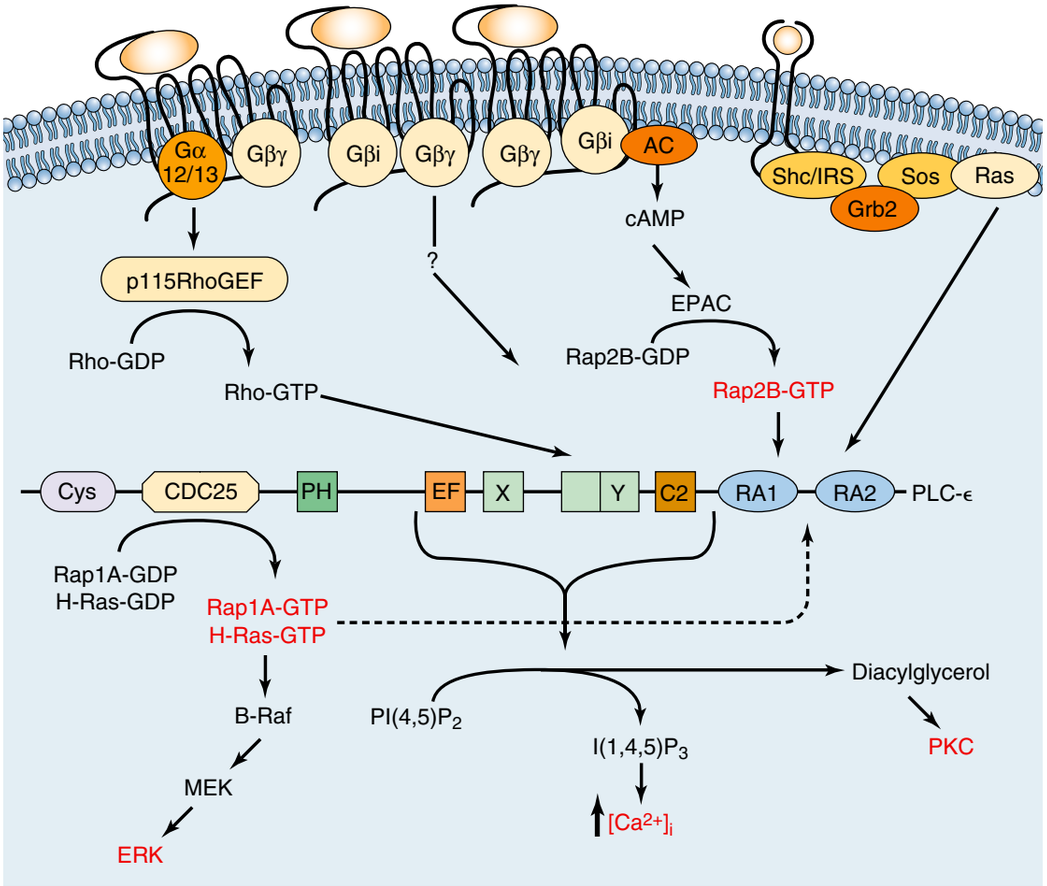

FIGURE 20-5 Activation of PLC-ε by heterotrimeric ($G_{\alpha 12/13}$, $G_{\beta\gamma}$, $G_{\alpha s}$) and small G protein (Ras, Rho and Rap) signaling pathways (see text for details). (Modified with permission from reference [11].) Note that Ras, activated via the mitogen-activated protein kinase (MAPK) pathway, may also activate PLCε. For details of the MAPK pathways and abbreviations, see Chapter 24.

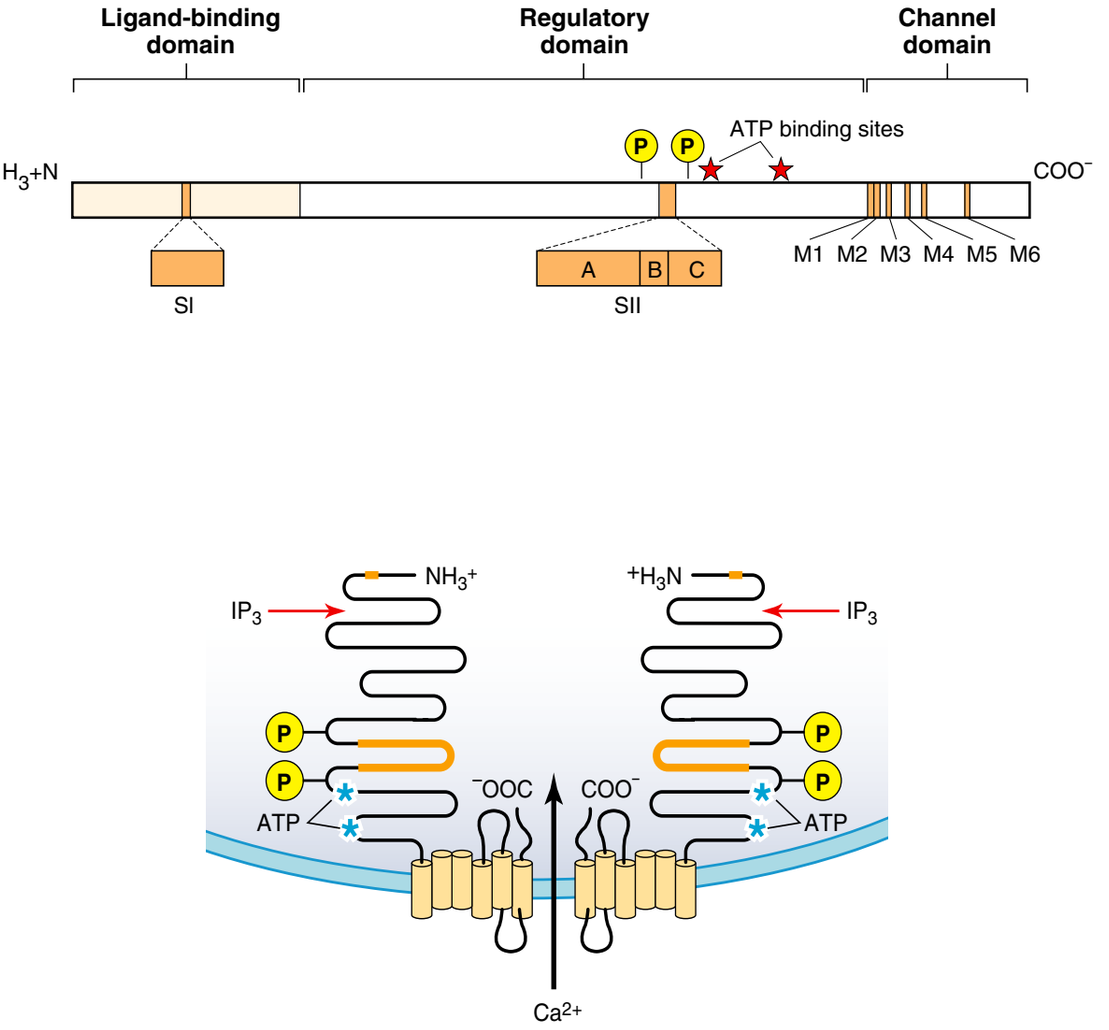

FIGURE 20-6 (**A**) Linear representation of the IP_3 receptor showing the three functional domains. P, phosphorylation sites; SI and SII isoforms produced by alternative RNA splicing; MI–M6, membrane-spanning regions. (**B**) Model of IP_3 receptor showing transmembrane topology. One half of the homotetrameric structure is shown. *, ATP-binding sites. (With permission from reference [16].)

activated by the GTP-bound forms of either Ras or Rap1A. An additional small GTP-binding protein, Rho, can also directly activate PLCε, although via interaction with a site distinct from the Ras binding domains. Rho may become active following ligand occupancy of receptors coupled to $G_{\alpha 12/13}$ and the subsequent activation of a Rho GEF. βγ subunits derived from G_i are also able to activate PLCε, and thus this highly versatile enzyme may serve as a 'nexus' for both heterotrimeric and small G protein signaling pathways [11]. In addition, it appears that cyclic AMP can indirectly activate PLCε, via the cyclic nucleotide's ability to activate EPAC (a GEF), which in turn will activate Rap2B. The latter (like Ras and Rap1A) can then bind to the Ras domain and activate PLCε.

It should be noted that the process whereby $PI(4,5)P_2$ is cleaved by PLC and subsequently resynthesized is metabolically expensive. Thus, for each mole of $PI(4,5)P_2$ hydrolyzed and resynthesized, 3 moles of ATP and 1 mole of CTP are consumed. ATP is consumed at the DAG kinase, PI4-kinase and PIP-kinase steps, whereas CTP is utilized by CDP-DAG synthase. Consequently, it is likely that, under physiological conditions, $PI(4,5)P_2$ breakdown is kept to a minimum. Since $PI(4,5)P_2$ represents only a minor fraction of the total inositol lipid pool, its resynthesis would be a prerequisite for both acute and sustained signaling. Despite the obvious physiological importance, mechanisms that regulate the resynthesis of $PI(4,5)P_2$ have not been well-defined. However, some evidence has

been presented in favor of the regulation of PI4-kinase and PI(4)P5-kinase by a diverse array of agents, including PA, GTP and protein kinase C [12].

THE INOSITOL PHOSPHATES

D-*myo*-inositol 1,4,5-trisphosphate ($I(1,4,5)P_3$) is a second messenger that liberates Ca^{2+} from the endoplasmic reticulum via intracellular receptors. The unique ability of $I(1,4,5)P_3$ to mobilize intracellular Ca^{2+} was first demonstrated by Berridge and colleagues in the early 1980s [13]. When directly injected into cells, or added to permeabilized cells or membrane fractions, $I(1,4,5)P_3$ elicits increased release of Ca^{2+} from a store that has been associated with the endoplasmic reticulum (Ch. 22). That specific receptor sites mediate the action of $I(1,4,5)P_3$ was first inferred from the presence of binding sites in membrane fractions obtained from selected brain regions, notably the cerebellum, the brain region most enriched in these receptors. The purified receptor is a glycoprotein with a molecular weight of 260–313 kDa and it is highly selective for $I(1,4,5)P_3$ (for reviews, see [14, 15]). The IP_3-receptor (IP_3-R) consists of three distinct regions (Fig. 20-6A). A ligand-binding domain is located at the amino terminal region of the molecule. A regulatory (coupling) domain contains potential phosphorylation sites for protein kinase A and protein kinase C, an immunophilin binding site, a Ca^{2+}-sensor binding site and two ATP-binding sites, which regulate the affinity with which IP_3 binds to the receptor. The receptor channel domain through which Ca^{2+} is released consists of six transmembrane spanning segments.

The native IP_3-R exists as a homotetramer with both amino and carboxyl termini facing the cytoplasm. Upon binding of an IP_3 molecule to each of the four subunits, a conformational change in the receptor is presumed to occur that results in the opening of the intrinsic Ca^{2+} channel (Fig. 20-6B). A critical feature of IP_3-Rs is that their opening is regulated by cytosolic Ca^{2+}, the latter acting as a 'coagonist' at the IP_3-R. Thus, paradoxically, the ability of $I(1,4,5)P_3$ to mobilize intracellular Ca^{2+} is itself regulated by Ca^{2+}, with nanomolar concentrations of Ca^{2+} being a prerequisite for Ca^{2+} channel opening. This sensitivity to Ca^{2+} allows IP_3-Rs to act as Ca^{2+}-induced Ca^{2+} release (CICR) channels that promote the amplification of smaller triggering events (see further details in Ch. 22). Activation of PLC-linked receptors frequently results in oscillations of the intracellular Ca^{2+} signal. The relationship between IP_3 production and signal oscillations is discussed in Chapter 22.

It has been observed that the IP_3-R can be activated in the absence of its ligand, $I(1,4,5)P_3$, by a group of proteins termed Ca^{2+}-binding proteins (CaBPs), a subfamily of E-F hand-containing proteins known as neuronal Ca^{2+} sensors [17]. These proteins are exclusively expressed in neurons and are particularly enriched in synaptic/dendritic fields. The addition of CaBPs can directly activate the IP_3-R in a Ca^{2+}-dependent manner, via interaction with the N-terminus region of the receptor. This raises the possibility that CaBPs can act on IP_3-Rs to potentiate the Ca^{2+} signals that arise from other sources, e.g. ligand-gated ion channels.

At least three distinct forms of the IP_3-R have been identified, and these share an overall amino acid homology of 60–80%. Type I predominates in the cerebellum and has been most extensively studied. It is the largest of the 3 forms of the receptor and, unlike type II and III receptors, the gene possesses a 120 nucleotide insert. Type II IP_3-Rs are found mainly in non-neural tissues, whereas type III receptors occur in both neural and non-neural tissues. In response to chronic activation, IP_3-Rs are degraded via the ubiquitin–proteasome pathway [14] (see also Ch. 2).

The metabolism of inositol phosphates leads to regeneration of free inositol. $I(1,4,5)P_3$ can be metabolized either by a membrane-bound enzyme, 5-phosphatase, to yield inositol 1,4-bisphosphate ($I(1,4)P_2$) or by a cytosolic 3-kinase to form inositol 1,3,4,5-tetrakisphosphate ($I(1,3,4,5)P_4$) (Fig. 20-7; see [18] for review). Both of these activities may be regarded as 'off signals', terminating the action of $I(1,4,5)P_3$. The $I(1,4)P_2$ that results from 5-phosphatase action is dephosphorylated further by an inositol bisphosphatase to inositol 4-monophosphate (I(4)P)] and then by the action of inositol monophosphatase to free inositol. $I(1,3,4,5)P_4$, which itself has been suggested to possess a second-messenger function in facilitating the entry of Ca^{2+} into cells, also serves as a substrate for the same 5-phosphatase that acts on $I(1,4,5)P_3$, with the resultant formation, in this instance, of inositol 1,3,4-trisphosphate ($I(1,3,4)P_3$). Unlike the 1,4,5-trisphosphate isomer, $I(1,3,4)P_3$ is ineffective at mobilizing intracellular Ca^{2+}. $I(1,3,4)P_3$ can be further metabolized to inositol 1,3-bisphosphate ($I(1,3)P_2$) or inositol 3,4-bisphosphate ($I(3,4)P_2$) (Fig. 20-7). These compounds are then dephosphorylated by 4- or 3-phosphatases to yield inositol 3- or 1-monophosphates respectively.

The inositol polyphosphate 5-phosphatases belong to a family of enzymes that terminate the signals generated by inositol lipid kinases and PLC. To date, two major types of 5-phosphatase have been identified, both of which share a common '5-phosphatase' domain of approximately 300 amino acids, with several highly conserved motifs. Type-I enzymes are 43–65 kDa and preferentially hydrolyze $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$, with the attendant formation of $I(1,4)P_2$ and $I(1,3,4)P_3$, but have little or no activity towards membrane-bound phosphoinositides. The prototypic form of a type-I 5-phosphatase is a 43 kDa protein that is post-translationally modified by farnesylation of the carboxyl terminus CAAX motif; this modification juxtaposes the enzyme with the membrane. Type-II enzymes are larger (75–160 kDa) and will hydrolyze both water-soluble inositol phosphates and lipids that

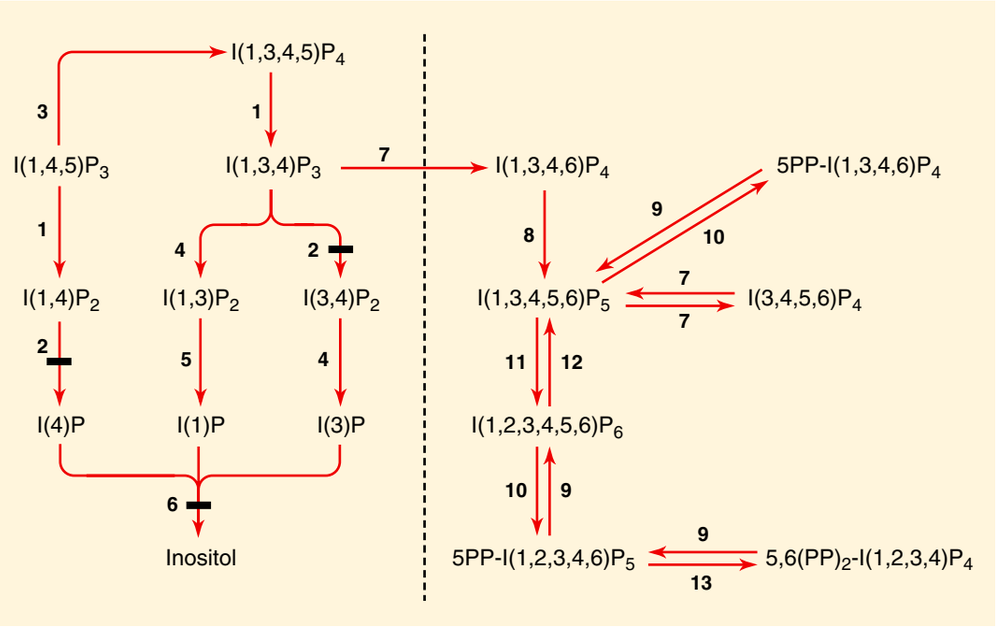

FIGURE 20-7 Pathways of inositol phosphate metabolism. Note that the metabolism of the second messenger $I(1,4,5)P_3$ is shown to the left of the dashed line, while the interconversions of the higher inositol phosphates are shown to the right of the dashed line. Only the quantitatively major established pathways are depicted. Li^+ is known to block the dephosphorylation reactions indicated by the (*black*) bars. Numbers refer to the following enzymes: 1, inositol polyphosphate 5-phosphatase (I); *2*, inositol polyphosphate 1-phosphatase; *3*, $I(1,4,5)P_3$ 3-kinase; *4*, inositol polyphosphate 4-phosphatase; *5*, inositol polyphosphate 3-phosphatase; *6*, inositol monophosphate phosphatase; *7*, $I(1,3,4)P_3$ 6-kinase/$I(3,4,5,6)P_4$ 1-kinase; *8*, Ipmk; *9*, DIPP; *10*, IP_6 kinase; *11*, Ipk 1; *12*, MIPP; *13*, PP-IP_5 kinase.

possess a phosphate group at the 5-position of the inositol ring. For further details regarding these type II phosphatases, see the section on the metabolism of the 3-phosphoinositides.

A single enzyme, inositol monophosphatase, leads to loss of the remaining phosphate and the regeneration of free inositol. This enzyme exhibits similar affinities for all five of the equatorial inositol monophosphate hydroxyls. Inositol 2-phosphate, which is not produced in this degradative pathway, is a poor substrate, a property that is probably attributable to its axial configuration. The enzyme is inhibited by Li^+ in an uncompetitive manner; i.e. the degree of inhibition is a function of substrate concentration. The putative link between the ability of Li^+ to interfere with phosphoinositide turnover and its therapeutic efficacy in the treatment of bipolar disorders is discussed in Box 20-1 and Chapter 55. It should be noted that unlike most other tissues, brain can synthesize inositol de novo by the action of inositol monophosphate synthase, which cyclizes glucose 6-phosphate to form I(3)P. The enzyme has been localized immunohistochemically to the brain vasculature.

The action of Li^+ on inositol monophosphatase has greatly facilitated the use of [^{3}H]inositol in the study of stimulated phosphoinositide turnover [19]. Berridge and colleagues demonstrated that in the presence of both Li^+ and a phosphoinositide-linked ligand, the amount of labeled intracellular inositol phosphates that accumulates following a preincubation with [^{3}H]inositol can be stimulated as much as 50-fold. In contrast, the presence of either Li^+ or ligand alone has very little effect on [^{3}H]inositol monophosphate accumulation. Although ligand-activated turnover is initiated by $PI(4,5)_2$ breakdown and the generation of $I(1,4,5)P_3$, this product is quickly degraded. As an index of receptor activation, it is thus more convenient to use an indirect measurement, the accumulation of labeled inositol monophosphates in the presence of Li^+, than to attempt to measure the transient appearance of labeled $I(1,4,5)P_3$ within a few seconds of ligand addition.

Highly phosphorylated forms of *myo*-inositol are present in cells. $I(1,2,3,4,5,6)P_6$ (phytic acid) is the most abundant species [3]. IP_6 had long been known as the principal phosphate storage molecule in plants; it has also been found ubiquitously expressed in animal cells at concentrations of 10–60 µmol/l. Biosynthesis of IP_6 occurs by a complex series of reactions (Fig. 20-7; for review, see [20]), starting with the phosphorylation of $I(1,4,5)P_3$ by IP_3 3-kinase and subsequent dephosphorylation of IP_4 to $I(1,3,4)P_3$. The latter serves as a substrate for $I(1,3,4)P_3$ 6-kinase, a 46 kDa enzyme that also phosphorylates $I(3,4,5,6)P_4$ at the 1-position to form IP_5. This versatile enzyme can also act on $I(1,3,4,5,6)P_5$ as a 1-phosphatase, an activity that may be of physiological significance, since there is evidence that the product, $I(3,4,5,6)P_4$, is an intracellular signaling molecule, modulating the activity of chloride channels in

BOX 20-1

Does the Action of Li^+ on the Phosphoinositide Labeling Cycle Explain the Therapeutic Action of Li^+ in Manic Depressive Psychosis?

Given the striking effect of Li^+ on the metabolism of the inositol phosphates (see above) and the clinical effectiveness of Li^+ in bipolar mental illness (see Ch. 55), it is tempting to speculate that the therapeutic effects of Li^+ are mediated via a regulation of inositol lipid turnover. It has been suggested that the uncompetitive inhibition of inositol monophosphatase by Li^+ mediates its therapeutic action in affective disorders [1, 2]. The inositol depletion hypothesis proposes that monophosphatase inhibition in vivo lowers inositol levels in cells that are most actively producing inositol monophosphate, i.e. have maximally activated their phosphoinositide-linked receptors. This selectivity is the result of the uncompetitive nature of the Li^+ inhibition, since the degree of inhibition is proportional to the amount of substrate, i.e. inositol monophosphate. PI synthase is then proposed to be slowed by lack of inositol substrate. Thus, overactive cells are thought to be selectively inhibited. Although clinically therapeutic doses of Li^+ do achieve sufficiently high levels of Li^+ in the brain (about 1 mmol/l) to inhibit the monophosphatase, experiments in rats with even higher doses show only a 25% lowering of brain inositol. This degree of lowering of brain inositol would not appear to be sufficiently near the K_m of PI synthase to slow PI synthesis. It has also been observed that Li^+ must be administered for many days before a therapeutic effect is seen. This suggests that delayed effects of Li^+, such as regulation of enzymes, might better correlate with the clinical effect. While the inositol depletion hypothesis appears attractive in many respects, its validity remains to be demonstrated. A number of other biochemical sites of Li^+ action have been reported [3,4], and it may be that multiple actions of Li^+ are necessary for its clinical effectiveness.

References

1. Berridge, M. J., Downes, C. P. and Hanley, M. R. Neural and developmental actions of lithium: a unifying hypothesis. Cell 59: 411–419, 1989.
2. Agranoff, B. W. and Fisher, S. K. Inositol, lithium, and the brain. Psychopharmacol. Bull. 35: 5–18, 2001.
3. Jope, R. S. Anti-bipolar therapy: mechanism of action of lithium. Mol. Psychiatry 4: 117–128, 1999.
4. Phiel, C. J. and Klein, P. S. Molecular targets of lithium action. Annu. Rev. Pharmacol. Toxicol. 41: 789–813, 2001.

epithelial cells. $I(1,3,4,6)P_4$ is converted to IP_5 following phosphorylation at the 5-position by inositol phosphate multikinase (Ipmk), an enzyme capable of phosphorylating inositol at multiple positions in vitro, although not all of these activities may be relevant in vivo. IP_5 may play a role in cell proliferation. The phosphorylation of IP_5 at the 2-position to yield IP_6 is carried out by a homolog of the yeast 2-kinase (Ipk1), which is enriched in brain.

An additional layer of complexity was introduced upon the discovery of the existence of diphosphorylated inositol phosphates, which contain energy-rich pyrophosphoryl (PP) residues (see [3] for review). These include $PP\text{-}IP_4$ ($5PP\text{-}I(1,3,4,6)P_4$), IP_7 ($5PP\text{-}I(1,2,3,4,6)P_5$) and IP_8 ($5,6(PP)_2\text{-}I(1,2,3,4)P_4$). The formation of the latter is catalyzed by IP_7 kinase, which has not yet been cloned. Formation of the other two pyrophosphoryl derivatives is catalyzed by IP_6 kinase, for which three isoforms (46–49 kDa) have been cloned from animals. The activity of both IP_6 and IP_7 kinases is reversible in vitro, thereby acting as ATP synthases. This property led to the suggestion that IP_7 and IP_8 might function as high energy phosphate donors, although the physiological significance of this reverse reaction has yet to be demonstrated. In addition to this reverse kinase route for dephosphorylation of the diphosphoinositol polyphosphates, two other activities have been described: a multiple inositol polyphosphate phosphatase (MIPP), which is enriched in brain, and a group of enzymes known as diphosphoinositol polyphosphate phosphatase (DIPP).

Multiple physiological roles for IP_6 and the diphosphoinositol polyphosphates have been proposed, including effects on endocytosis and mRNA transport [3]; however, definitive evidence for many of these functions is lacking. Such studies are complicated by possible nonspecific effects of this highly negatively charged molecule. Of note is the report of an IP_6/IP_7-dependent protein kinase activity that phosphorylates pacsin/syndapin I, a protein involved in synaptic vesicle recycling [21].

DIACYLGLYCEROL

Protein kinase C is activated by the second messenger diacylglycerol. This unique family of protein kinases was originally identified in the late 1970s by Nishizuka and colleagues [22]. At least 14 isoforms of protein kinase C (PKC) (70–80 kDa) may exist in mammalian tissues. Four conserved (C) and five variable (V) regions can be discerned (Fig. 20-8). PKC isoforms can be distinguished by the presence or absence of motifs that dictate the cofactor requirements for maximal catalytic activity. The C1 region contains two cysteine-rich repeats and represents the site at which DAG regulates the enzyme. A 'pseudosubstrate' sequence is also present in this region of the molecule and blocks kinase activity until the enzyme becomes activated by DAG. The C2 region confers the Ca^{2+} sensitivity of PKC, whereas the C3 and C4 domains are, respectively, the ATP- and substrate binding sites. The 'conventional'

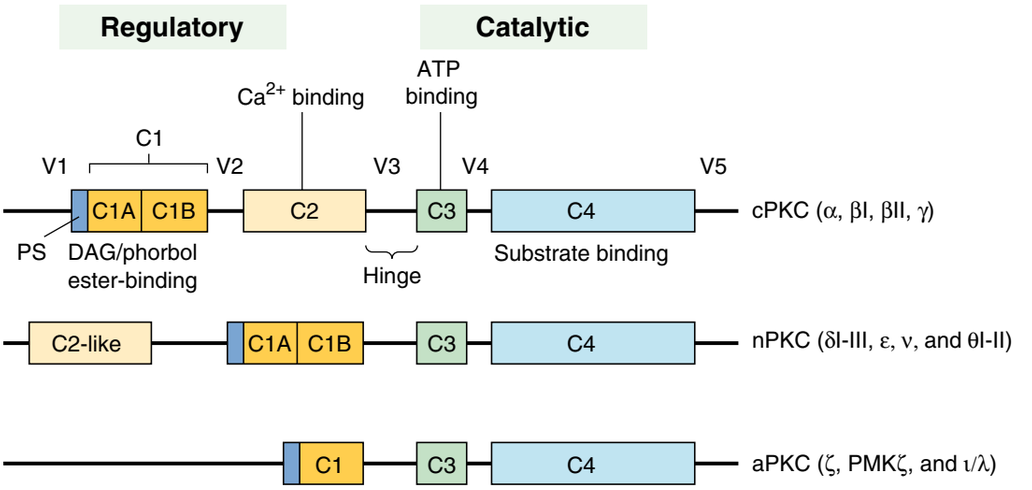

FIGURE 20-8 Linear representation of PKC isozymes. See text for details. Reproduced with permission from Tan, S. L. and Parker, P. J. Biochemical Journal 376: 545–552, 2003 [23] © The Biochemical Society.

isoforms of PKC (cPKC: α, β and γ) are Ca^{2+} sensitive, whereas the 'novel' isoforms (nPKC: δ, ε, η and σ) lack the conventional C2 region and are accordingly Ca^{2+}-insensitive. An additional variation in PKC structures is observed for the ζ and ι/λ isoforms, which possess only one cysteine-rich repeat. These isoforms are not regulated by DAG, are constitutively active and are termed 'atypical' (aPKC). The V3 'hinge' region of the molecule is susceptible to proteolysis following persistent activation of PKC.

All isoforms of PKC are predominantly localized to the cytosol and, upon activation, undergo translocation to either plasma or nuclear membranes. However, newly synthesized PKCs are localized to the plasmalemma and are in an 'open' conformation in which the autoinhibitory pseudosubstrate sequence is removed from the substrate binding domain. The 'maturation' of PKC isoforms is effected by phosphoinositide-dependent kinase-I (PDK-I), which phosphorylates a conserved threonine residue in the activation loop of the catalytic (C4) domain [24]. This in turn permits the autophosphorylation of C-terminus threonine and serine residues in PKC, a step which is a prerequisite for catalytic activity (see also Chs 22 and 23). The phosphorylated enzyme is then released into the cytosol, where it is maintained in an inactive conformation by the bound pseudosubstrate. It was originally thought that 3-phosphoinositides such as $PI(3,4)P_2$ and $PI(3,4,5)P_3$ could directly activate PKCs. However, it now seems more likely that these lipids serve to activate PDK-1 (a frequent contaminant of PKC preparations).

Although many cellular proteins are potential targets for phosphorylation by PKC, the myristoylated alanine-rich protein kinase C substrate (MARCKS) appears to be a major in vivo substrate. MARCKS is an acidic filamentous actin cross-linking protein that is found in high concentrations at presynaptic junctions and that is directed to the plasmalemma via a myristoylated amino-terminal membrane binding domain. This interaction keeps MARCKS in close proximity to PKC, thereby facilitating efficient phosphorylation. Upon phosphorylation MARCKS undergoes a translocation from the membrane to the cytoplasm, and its ability to cross-link actin filaments is lost. Other PKC substrates include vinculin, talin, adducins and annexins. Since each of these proteins is involved in linking plasma or intracellular membranes to cytoskeletal structures, an important aspect of PKC signaling appears to be the regulation of membrane cytoskeleton interactions.

From overexpression studies, it can be inferred that individual isoforms of PKC are precisely directed to distinct subcellular locations (e.g. PKCα to the endoplasmic reticulum and PKCδ to the Golgi). Directing PKC isozymes to specific subcellular loci appears to occur via interaction of the enzyme with localized intracellular binding proteins. Such proteins may or may not be substrates for PKC. An example of the latter category would be RACK (receptors for activated C kinase) 1. RACKs are thought to interact only with activated PKCs and to direct translocated PKCs to specific loci.

The phorbol esters are useful for studying the function of PKC since they mimic the stimulatory effects of DAG on the enzyme. These tumor-promoting plant products and their synthetic derivatives are able to penetrate intact cells. Many inferences regarding the intracellular actions of PKC are based on results of studies on whole-cell preparations with the phorbol esters. These substances, like DAG, may produce feedback inhibition of signal transduction at a number of metabolic levels. Results of experiments using phorbol esters in whole cells are thus often complex and must be interpreted cautiously. Notwithstanding this consideration, based upon

experiments with phorbol esters there is ample evidence to implicate PKC in a number of complex processes, including long-term potentiation (see Ch. 53).

Since I(1,4,5)P_3 production leads to increased intracellular Ca^{2+}, which in turn can stimulate PKC action, the two messengers DAG and I(1,4,5)P_3 often act in concert. DAG can, however, arise in cells from sources other than the phosphoinositides. For example, DAG can be generated from the action of a phosphatidylcholine (PC)-specific phospholipase C or from the transfer of phosphorylcholine from PC to ceramide in the synthesis of sphingomyelin (see Ch. 3). If the DAG released from stimulated phosphoinositide turnover specifically activates cellular PKC, one might expect ST/AR DAG to be a particularly effective activator of the enzyme. It turns out to be only marginally better than other long-chain fatty acyl DAGs; however, chemical analysis indicates that brain DAG is enriched in the ST/AR species. It would therefore seem that ST/AR DAG is de facto the principal activator of PKC in vivo, even though the enzyme shows little specificity for it.

As a second messenger, DAG is rapidly metabolized under normal conditions, and the predominant route is via phosphorylation to PA, a reaction catalyzed by DAG kinase, for which multiple forms of 64–140 kDa have been identified and characterized. All possess a C-terminal catalytic domain and two or three cysteine-rich repeat sequences. The α, β and γ forms possess E-F hands and are thus likely to be regulated by changes in the concentration of cytosolic Ca^{2+}. Expression of the mRNA for the α, β, γ, ζ and θ forms of DAG kinase appears to be highest in the brain. Once DAG is phosphorylated to PA, this in turn can be converted into PI via CDP-DAG (Fig. 20-2B).

PHOSPHOINOSITIDES AND CELL REGULATION

Inositol lipids can serve as mediators of other cell functions, independent of their role as precursors of second messengers. Although the major focus of studies of the phosphoinositides has centered on the role played by these lipids in transmembrane signaling events, in particular the generation of second messengers linked to Ca^{2+} homeostasis and protein phosphorylation, it is now abundantly clear that inositol lipids are important mediators of a diverse array of cell functions. This, in large part at least, can be attributed to the fact that the D-3 phosphoinositides and polyphosphoinositides (in particular PI(4,5)P_2) can interact with protein modules that direct them to specific targets. Many proteins have evolved lipid-binding domains that exhibit some degree of specificity for different classes of phosphoinositides [25] (Table 20-2). Through interaction of these particular high-affinity lipid domains (and others that have yet to be fully characterized), phosphoinositides can recruit specific proteins and regulate multiple cell functions both in the CNS and in non-neural cells. Putative functions for phosphoinositides in membrane trafficking, maintenance of the cytoskeleton, cell death/survival and regulation of ion channels are discussed below. The role of phosphoinositides in anchoring certain cell surface proteins via a PI-glycan linkage is discussed in Chapter 3.

TABLE 20-2 Examples of domains within proteins that specifically interact with inositol lipids

Domain	Lipid
PH	PI(4,5)P_2 or PI(3,4)P_2 or PI(3,4,5)P_3
FYVE	PI(3)P
C2	Anionic phospholipids
ENTH	PI(4,5)P_2 or PI(3,4,5)P_3
FERM	PI(4,5)P_2
MARCKS	PI(4,5)P_2 or PI(3,4)P_2

C2, protein kinase C domain; ENTH, epsin N-terminal homology; FERM, band 4.1, exrin, radixin, moesin; FYVE, Fab1, YOTB, Vac1, EEA1; MARCKS, myristoylated alanine-rich protein kinase C substrate; PH, pleckstrin homology.

Membrane trafficking. A direct role for the quantitatively major phosphoinositides, i.e. PI4P and PI(4,5)P_2, in the exocytosis of neurosecretory granules was first proposed by Eberhard et al., who observed that the ATP requirement for Ca^{2+}-regulated exocytosis in chromaffin cells could be attributed to the generation of PI(4,5)P_2 (see details of exocytosis in Ch. 9). Subsequently, studies in PC12 cells revealed that three cytosolic proteins (termed 'priming of exocytosis proteins' (PEPs)) were demonstrated to be prerequisites for exocytosis. PEP1 and PEP3 were identified as PI4P 5-kinase and the PI-transfer protein respectively, while PEP2 represents PI 4-kinase. It was subsequently observed that the introduction of antibodies to PI(4,5)P_2 into permeabilized PC12 cells blocked the secretion of catecholamines. Thus, for catecholamine exocytosis to occur, a series of reactions culminating in the synthesis of PI4P and PI(4,5)P_2 is required.

The requirement for PIP_2 in these membrane trafficking events reflects the ability of the lipid, which is localized in the plasmalemma, to recruit synaptotagmin, a putative Ca^{2+} sensor for exocytosis. The latter protein is located in the membrane of the secretory organelle and interacts with PI(4,5)P_2 via its C2 domains. It is thought that the synaptotagmin-PI(4,5)P_2 interactions facilitate the close apposition of the vesicle and target membranes [27]. PI(4,5)P_2 also regulates the clathrin-dependent endocytosis of synaptic vesicles by binding to key endocytic proteins, including the clathrin adaptor AP-180, dynamin and epsin (via PH or ENTH domains; Table 20-2).

The interaction of PI(4,5)P_2 (or PI(3,4,5)P_3)) with these cytosolic proteins facilitates their recruitment to the plasmalemma and, in the case of dynamin, also stimulates the GTPase activity [28]. Synaptic PI(4,5)P_2 (and PI(3,4,5)P_3))

is dephosphorylated by synaptojanin. A loss of synaptojanin function results in an increase in $PI(4,5)P_2$ content and an accumulation of clathrin-coated vesicles – which suggests that $PI(4,5)P_2$ may also be required for efficient uncoating. The 3-phosphoinositides also appear to play a role in late endocytic events, since inhibition of PI 3-kinase (PI3K) with wortmannin prevents the postendosomal sorting of platelet-derived growth factor receptor (PDGF-R). Moreover, mutations of the PDGF-R that prevent its association with PI3K result in receptors that fail to undergo trafficking from the Golgi to lysosomes. Through its interaction with the FYVE domain (Table 20-2) of the clathrin adapter protein-2 (AP-2), PI3P may also play a role in the assembly of clathrin-coated pits at the plasmalemma. A much lower affinity binding occurs between AP-2 and either $PI(3,4)P_2$ or $PI(3,4,5)P_3$.

Maintenance of the cytoskeleton. $PI(3,4)P_2$, $PI(3,4,5)P_3$, PI4P and $PI(4,5)P_2$ all avidly bind to a group of 'capping' proteins that regulate actin assembly, e.g. gelsolin and profilin(see also Ch. 9). Upon the binding of inositol lipids to these proteins, the barbed ends of the actin filaments become uncapped and the addition of actin monomers is permitted, resulting in the production of filamentous (F-) actin. Because the 3-phosphoinositides are present at relatively low concentrations, it is probable that PI4P and $PI(4,5)P_2$ play the major role in regulation of actin assembly. Direct evidence for phosphoinositide regulation of the actin cytoskeleton has been obtained from experiments with permeabilized platelets [29]. The addition of $PI(4,5)P_2$ (or PI4P, $PI(3,4)P_2$ or $PI(3,4,5)P_3$)) to these preparations results in an increase in the number of barbed ends and in the proportion of F-actin. Moreover, the time-course of increases in $PI(4,5)P_2$ concentration in stimulated platelets parallels the appearance of F-actin. Remodeling of the actin cytoskeleton also occurs in parallel with membrane rearrangements during endocytosis. $PI(4,5)P_2$ appears to play a role since disruption of synaptojanin function leads not only to deficits in endocytosis but also to abnormalities in the presynaptic cytoskeleton.

$PI(4,5)P_2$ also plays a role in the formation of membrane adhesion complexes – sites where the actin cytoskeleton is linked to the extracellular matrix (focal adhesions) or to cell-cell contacts through interactions with integrins or cell adhesion molecules (CAMs) (see Chs 2 and 7). Many proteins in these adhesion complexes bind $PI(4,5)P_2$ and, by doing so, have increased binding to each other. For example, when vinculin binds to $PI(4,5)P_2$, there is a conformational change in the molecule, which then allows the binding of talin, actin and VASP, thereby allowing newly synthesized actin filaments to be linked to integrins. Similarly, members of the ERM family of proteins (ezrin, radixin and moesin) display enhanced binding to CAMs in the presence of $PI(4,5)P_2$, following the interaction of the lipid with the FERM domain (Table 20-2).

Cell death and cell survival. The 3-phosphoinositides play a key role in the control of cell death (apoptosis) or survival, via their ability to regulate the activity of a kinase that is pivotal in these processes – Akt (PKB), a serine-threonine kinase (Chs 23 and 35). Following PI3K activation, $PI(3,4,5)P_3$ (or $PI(3,4)P_2$)) recruits Akt to the plasmalemma via its PH domain [30]. Phosphoinositide-dependent kinase-1 (PDK-1), which also possesses a PH domain with high affinity for $PI(3,4,5)P_3$, is directed to the same region of the membrane and phosphorylates ^{308}Threonine on Akt. Additional phosphorylation of Akt on ^{473}Serine by a PDK-2 kinase allows for full activation of Akt. When the concentrations of 3-phosphoinositides decline, the activated Akt is released from the membrane and is able to phosphorylate multiple target proteins.

The ability of 3-phosphoinositides to stimulate cell proliferation/survival via activation of Akt is countered by the 3-phosphatase PTEN, which hydrolyzes $PI(3,4)P_2$ and $PI(3,4,5)P_3$. A link between PTEN activity and 3-phosphoinositide content in cells is evident from the observations that: (a) overexpression of PTEN results in a dramatic reduction in the cellular content of these lipids, and (b) 3-phosphoinositide concentrations are greatly elevated in mammalian PTEN-null cell lines [28]. Cells in which PTEN activity is reduced have increased tumorigenic properties, since Akt inhibits apoptosis and promotes cell survival. Conversely, PTEN activity programs the fate of the cell toward apoptosis. Mutations of PTEN have been shown to occur in a wide range of tumor types, but with a particularly high frequency in glioblastomas.

Regulation of ion channel activity. Evidence that $PI(4,5)P_2$ can regulate ion channel activity has been obtained from experiments in which the lipid was reconstituted into artificial membranes or, alternatively, by the addition of $PI(4,5)P_2$ to excised membrane patches [31] (see also Ch. 6). The addition of $PI(4,5)P_2$ can either facilitate the opening of some channels, e.g. plasmalemmal Ca^{2+} pump, P/Q- and N-type Ca^{2+} channels, inwardly rectifying K^+ channels, ryanodine-sensitive Ca^{2+} release channels and Na^+/H^+ exchanger or, conversely, inhibit the activity of other channels, e.g. cyclic-nucleotide ion gated channels in rods and $I(1,4,5)P_3$-gated calcium release channel. Other systems, probably the majority, appear to be completely insensitive to $PI(4,5)P_2$, e.g. voltage-gated Na^+ and K^+ channels and Na^+/K^+ pump. Little is known of the mechanism whereby $PI(4,5)P_2$ regulates ion channel activity, except that the lipid is presumed to bind directly to cationic sites on the channels, and that these are distinct from the phosphoinositide binding domains listed in Table 20-2.

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REFERENCES

1. Hokin, L. E. and Hokin, M. R. Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices. Biochim. Biophys. Acta 18: 102–110, 1955.
2. Irvine, R. F. 20 years of Ins(1,4,5)P_3, and 40 years before. Nat. Rev. Mol. Cell Biol. 4: 586–590, 2003.
3. Fisher, S. K., Novak, J. E. and Agranoff, B. W. Inositol and higher inositol phosphates in neural tissues: homeostasis, metabolism and functional significance. J. Neurochem. 82: 736–754, 2002.
4. Agranoff, B. W. Cyclitol confusion. Trends Biochem. Sci. 3: N283–N285, 1978.
5. Micheva, K. D., Holz, R. W. and Smith S. J. Regulation of presynaptic phosphatidylinositol 4,5-biphosphate by neuronal activity. J. Biol. Chem. 154: 355–368, 2001.
6. Balla, T. Phosphatidylinositol 4-kinases. Biochim. Biophys. Acta 1436: 69–85, 1998.
7. Toker, A. Phosphoinositides and signal transduction. Cell. Mol. Life Sci. 59: 761–779, 2002.
8. Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K. et al. Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. 70: 535–602, 2001.
9. Fisher, S. K., Heacock, A. M. and Agranoff, B. W. Inositol lipids and signal transduction in the nervous system: an update. J. Neurochem. 58: 18–38, 1992.
10. Katan, M. Families of phosphoinositide-specific phospholipase C: structure and function. Biochim. Biophys. Acta 1436: 5–17, 1998.
11. Wing, M. R., Bourdon, D. M. and Harden, T. K. PLC-ε: a shared effector protein in ras-, rho-, and Gαβγ-mediated signaling. Mol. Interv. 3: 273–280, 2003.
12. Fisher, S. K. Homologous and heterologous regulation of receptor-stimulated phosphoinositide hydrolysis. Eur. J. Pharmacol. 288: 231–250, 1995.
13. Streb, H., Irvine, R. F., Berridge, M. J. et al. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306: 67–69, 1983.
14. Taylor, C. W., Genazzani, A. A. and Morris, S. A. Expression of inositol trisphosphate receptors. Cell Calcium 26: 237–251, 1999.
15. Bultynck, G., Sienaert, I., Parys, J. B. et al. Pharmacology of inositol trisphosphate receptors. Pflügers Arch. 445: 629–642, 2003.
16. Mikoshiba, K. Inositol 1,4,5-trisphosphate receptors. Trends Pharmacol. Sci. 14: 86–89, 1993.
17. Yang, J., McBride, S., Mak, D. O. et al. Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor $Ca^{(2+)}$ release channels. Proc. Natl Acad. Sci. U.S.A. 99: 7711–7716, 2002.
18. Irvine, R. F. and Schell, M. J. Back in the water: the return of the inositol phosphates. Nat. Rev. Mol. Cell Biol. 2: 327–338, 2001.
19. Berridge, M. J., Downes, C. P. and Hanley, M. R. Lithium amplifies agonist dependent phosphatidylinositol responses in brain and salivary glands. Biochem. J. 206: 587–595, 1982.
20. Shears, S. B. How versatile are inositol phosphate kinases? Biochem J. 377: 265–280, 2004.
21. Hilton, J. M., Plomann, M., Ritter, B. et al. Phosphorylation of a synaptic vesicle-associated protein by an inositol hexakisphosphate-regulated protein kinase. J. Biol. Chem. 276: 16341–16347, 2001.
22. Nishizuka, Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9: 484–496, 1995.
23. Tan, S. L. and Parker, P. J. Emerging and diverse roles of protein kinase C in immune cell signaling. Biochem. J. 376: 545–552, 2003.
24. Newton, A. C. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem. J. 370: 361–371, 2003.
25. DiNitto, J. P., Cronin, T. C. and Lambright, D. G. Membrane recognition and targeting by lipid-binding domains. Sci. STKE 2003 re16, 2003.
26. Eberhard, D. A., Cooper, C. L., Low, M. G. et al. Evidence that inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. Biochem. J. 268: 15–25, 1990.
27. Bai, J., Tucker, W. C. and Chapman, E. R. PIP_2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nat. Struct. Mol. Biol. 11: 36–44, 2004.
28. Osborne, S. L., Meunier, F. A. and Schiavo, G. Phosphoinositides as key regulators of synaptic function. Neuron 32: 9–12, 2001.
29. Hartwig, J. H., Bokoch, G. M. et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 82: 643–653, 1995.
30. Leslie, N. R., Biondi, R. M. and Alessi, D. R. Phosphoinositide-regulated kinases and phosphoinositide phosphatases. Chem. Rev. 101: 2365–2380, 2001.
31. Hilgemann, D. W., Feng, S. and Nasuhoglu, C. The complex and intriguing lives of PIP_2 with ion channels and transporters. Sci. STKE 2001 re19, 2001.

CHAPTER

21

Cyclic Nucleotides in the Nervous System

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INTRODUCTION: THE SECOND MESSENGER HYPOTHESIS

The mechanisms by which extracellular molecules, such as neurotransmitters and circulating hormones that act on plasmalemma receptors, alter intracellular processes have been intensively investigated for several decades. One of the seminal advances in this field came in the late 1950s when Earl Sutherland and his colleagues demonstrated that epinephrine induces glycogenolysis in the liver by stimulating the synthesis of an intracellular second messenger, adenosine 3′-5′-cyclic monophosphate (cyclic AMP or cAMP). Since that time, cAMP and other small molecules (e.g. cGMP, Ca^{2+}, nitric oxide and the metabolites of phosphatidylinositol (Ch. 20) and of arachidonic acid (Ch. 33)) have been shown to serve a myriad of second messenger roles in the nervous system. These messengers mediate many of the actions of most types of extracellular signal on diverse aspects of neuronal function (Fig. 21-1). This chapter reviews the proteins involved in the synthesis and metabolism of the cyclic

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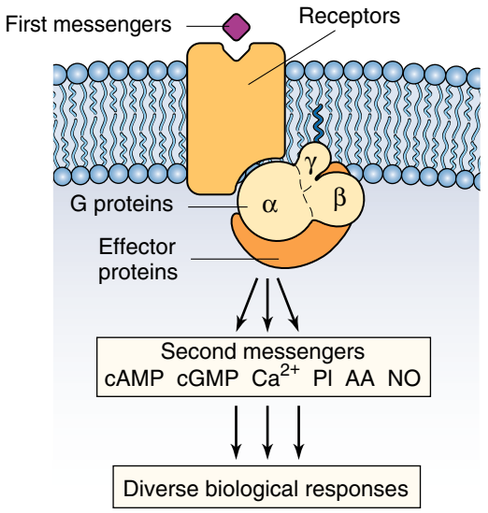

FIGURE 21-1 Schematic illustration of the second messenger hypothesis. This hypothesis, supported by decades of research, states that many types of first messengers in the brain, through the activation of specific plasma membrane receptors and G proteins, stimulate the formation of intracellular second messengers, which mediate many of the biological responses of the first messengers in target neurons. Prominent second messengers in the brain include cAMP, cGMP, Ca^{2+}, the metabolites of phosphatidylinositol (*PI*) (e.g. inositol triphosphate and diacylglycerol) and of arachidonic acid (*AA*) (e.g. prostaglandins, prostacyclins, thromboxanes, leukotrienes), and nitric oxide (*NO*).

nucleotide second messengers, cAMP and cGMP, in the nervous system.

ADENYLYL CYCLASES

Regulation of cAMP formation is determined by the activity of adenylyl cyclase. This enzyme activity is itself regulated by the action of neurotransmitter receptors mediated via intracellular messengers. Adenylyl cyclase can also be activated directly by forskolin, a plant diterpene that has been useful in studies of enzyme regulation and purification. The substrate for adenylyl cyclase is a complex of Mg^{2+} and ATP. In addition, free divalent cation (e.g. free Mg^{2+}), in excess of ATP, is a requisite cofactor for enzyme activity. As shown in Figure 21-2, adenylyl cyclase forms cAMP by creating a cyclic phosphodiester bond with the α-phosphate group of ATP, and the concomitant release of pyrophosphate provides energy for the reaction.

Biochemical and molecular cloning studies indicate the existence of nine separate and unique forms of adenylyl cyclase (AC), which comprise a distinct enzyme family, referred to as AC1–AC9 [1, 2]. These members of the adenylyl cyclase superfamily are all membrane-bound. There is also an additional soluble form, sAC, that has catalytic activity similar to the others but is genetically the most divergent member of the family. All the membrane-bound forms of adenylyl cyclase are activated by the stimulatory G protein $G_{\alpha s}$ (see Ch. 19), and all with the exception of AC9 are stimulated by forskolin. The soluble form sAC is not stimulated by either $G_{\alpha s}$ or forskolin but is sensitive to bicarbonate levels. All known forms of adenylyl cyclase are inhibited by P-site inhibitors, which are adenosine analogs that act at the catalytic site of the enzyme. The different forms of adenylyl cyclase exhibit distinct patterns of expression in brain and peripheral tissues and are differentially regulated by Ca^{2+}/calmodulin (see Ch. 23), by the G protein subunits $G_{\alpha i}$ and $G_{\beta\gamma}$ (see Ch. 19) and by phosphorylation.

Multiple forms of adenylyl cyclase exist in the nervous system. AC1 is expressed exclusively in brain, adrenal and retina, and is the only neural-specific adenylyl cyclase [3–5]. In brain, AC1 is expressed at high levels in the hippocampus, cerebral cortex, cerebellum, olfactory system and pineal gland. AC8 is expressed in brain, as well as in lung and parotid gland. In brain, AC8 is enriched in the olfactory bulb, thalamus, habenula, cerebral cortex and hypothalamic nuclei. The catalytic activity of AC1 and AC8 is stimulated by Ca^{2+}/calmodulin and thereby could be regulated through activation of NMDA glutamate receptors that permit Ca^{2+} entry into neurons and through voltage-sensitive Ca^{2+} channels (see Chs 6, 15). This type of regulation has been implicated in several forms of neural plasticity. Further evidence for a role of AC1 and AC8 in synaptic plasticity is the finding that mice lacking these forms of adenylyl cyclase have deficits in spatial memory as well as in long-term potentiation, which is a cellular electrophysiologic model of learning and memory that can be measured in the hippocampus and other brain regions [6, 7] (see Ch. 53).

AC2 is expressed at high levels in many brain regions but it is also found at lower levels in skeletal muscle, lung and heart [4, 5]. The highest levels are found in

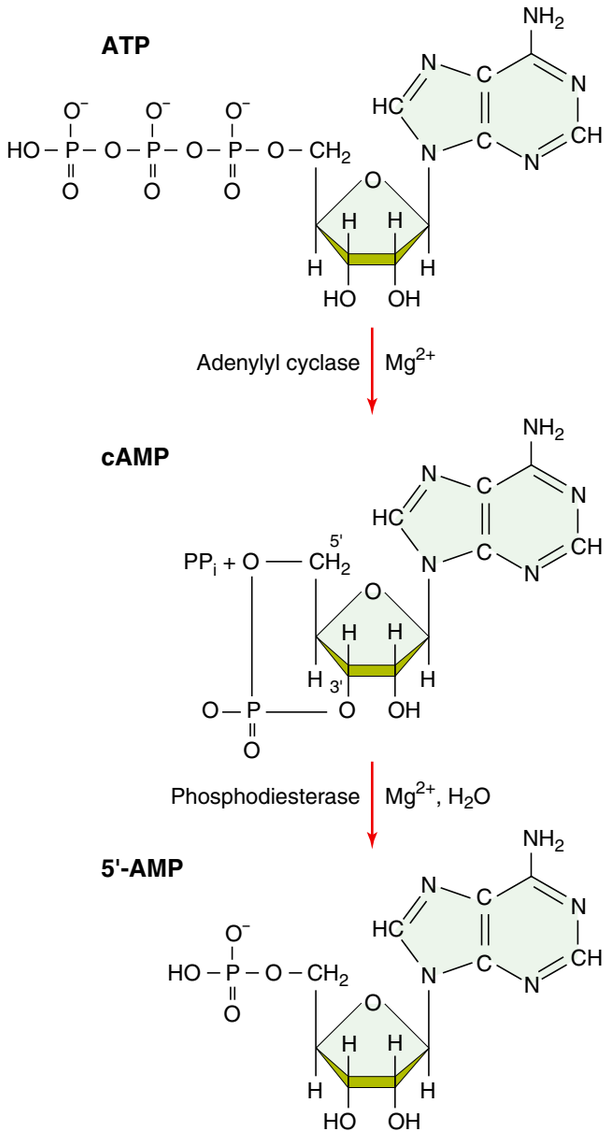

FIGURE 21-2 Chemical pathways for the synthesis and degradation of cAMP. cAMP is synthesized from ATP by the enzyme adenylyl cyclase with the release of pyrophosphate, and is hydrolyzed into 5′-AMP by the enzyme phosphodiesterase. Both reactions require Mg^{2+}. Analogous reactions underlie the synthesis and degradation of cGMP (not shown). PP_i, inorganic pyrophosphate.

hippocampus, hypothalamus and cerebellum, while moderate levels are found in neocortex, piriform cortex and amygdala. AC3 is highly enriched in olfactory epithelium and is expressed at lower levels in other brain regions, as well as in male germ cells, pancreas, brown adipose tissue, uterus, lung and heart [8]. AC4 is widely distributed in brain and has been identified in most peripheral tissues tested to date [9].

AC5 is enriched in the brain and is especially localized to the striatum and related structures (e.g. nucleus accumbens, olfactory tubercle) that are innervated by dopaminergic fibers (see Ch. 46). As a result, it is often referred to as 'striatal adenylyl cyclase' [10, 11]. AC5 is also expressed in heart and kidney (where it is associated with blood vessels), the anterior lobe of the pituitary and the retina. These tissues share the common feature of having dopamine innervation and it has been suggested that AC5 is associated uniquely with dopamine actions [10]. AC6 is structurally similar to the AC5 and both are inhibited by free Ca^{2+} at physiological concentrations (0.1–1.0 mmol/l) [12]. AC6 is enriched in brain and heart but is ubiquitous with low levels of expression seen in other tissues (e.g. testes, muscle, kidney and lung).

AC7 is found in several tissues, with highest levels in lung and spleen, moderate levels in heart, and low levels in brain, kidney and skeletal muscle [13, 14]. In brain, the highest levels are found in cerebellar granule cells, while lower levels are seen in hippocampus, neocortex and striatum. AC9 is expressed in brain and skeletal muscle but also displays widespread distribution in other tissues. The soluble isoform sAC is enriched in testes but is also found in other tissues.

The membrane forms of adenylyl cyclase are similar in their amino acid sequence and putative topographical structure. Their structure is illustrated in Figure 21-3 [1, 2]. Hydropathicity profiles of their amino acid sequences indicate that the adenylyl cyclases contain two regions (M1 and M2), each of which consists of six putative membrane-spanning hydrophobic domains. At the N-terminus, there is a short variable amino terminus preceding the membrane-spanning domain. In addition, there are two large cytoplasmic domains (C1 and C2), one between the two hydrophobic regions and the other at the carboxy terminus of the protein. These cytoplasmic domains also contain the ATP binding/catalytic domains. The predicted molecular weight of AC1 is 124 kDa [15], although other types of analysis indicate a native molecular mass of more than 200 kDa. This suggests that adenylyl cyclases may exist *in situ* as a dimer or as a complex with other regulatory proteins.

The two cytoplasmic domains are the most highly conserved portions of the known forms of adenylyl cyclase and share approximately 40% sequence homology across all membrane-bound forms of adenylyl cyclase. In addition, C1 and C2 within a given enzyme share an even greater degree of homology. C1 is larger (360–390 amino acids) than C2 (255–330 amino acids). These cytoplasmic regions have been subdivided into a and b domains (Fig. 21-3). C1a and C2a are similar to each other and to certain sequences of both membrane-bound and soluble guanylyl cyclases (the enzyme that catalyzes the synthesis of cGMP – see below). These contain the nucleotide-binding sites and are thought to be the catalytic domains of the enzyme. Both C1 and C2 appear to be necessary for catalytic activity: there is no enzymatic activity when only one of the domains is expressed, but activity is reinstated when the two halves are co-expressed [9].

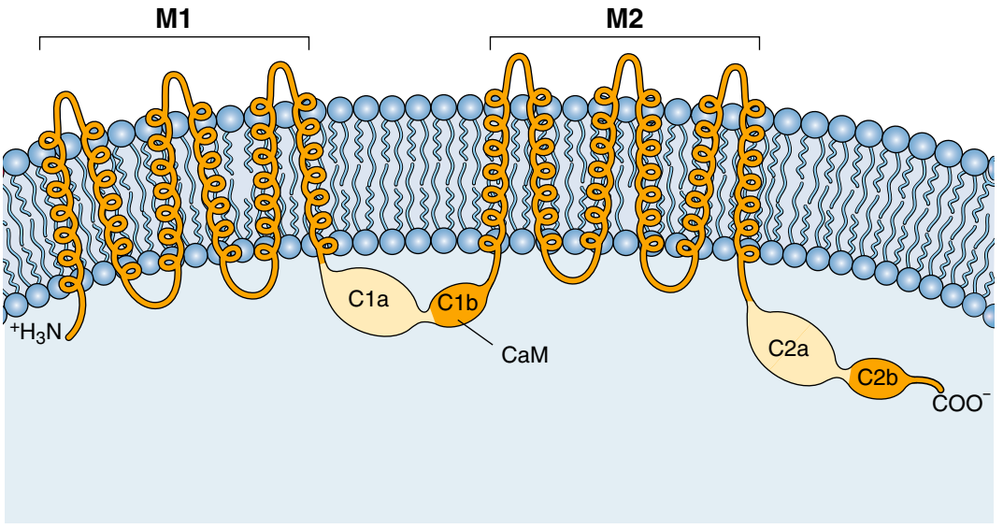

FIGURE 21-3 Schematic illustration of the proposed topographical structure of adenylyl cyclase (AC). Hydropathicity profiles predict that different forms of AC contain two hydrophobic regions (M1 and M2), each of which contain six membrane spanning regions, and two relatively less hydrophobic regions (C1 and C2), which are thought to be located in the cytoplasm. The catalytic domains may be located within C1 and C2, and both are necessary for functional activity of the enzyme. The carboxy (COO^-) portion of the C1 and C2 domains determines whether βγ subunit complexes inhibit AC1 or stimulate AC2 and AC4. The C1b domain contains a calmodulin-binding site and is thought to mediate Ca^{2+}/calmodulin activation of certain forms of the enzyme.

Studies of the crystalline structure of adenylyl cyclase, together with functional mutation studies, indicate that the C1 and C2 domains form the catalytic core of adenylyl cyclases [16, 17]. The forskolin-binding site is located in a hydrophobic pocket created by the interface of C1 and C2. Likewise, $G_{\alpha s}$ interacts with both domains, the majority of the contact being made with C2, and the binding results in rotation of the C1 domain around the C2 region, presumably to position the active site for catalysis [16, 17]. This site is probably also the site for binding of the other G protein subunits, such as the inhibitory $G_{\alpha i}$, which could act to stabilize the C1 domain of certain isoforms (e.g. AC5 and AC6) and thereby block rotation of C1 around C2 and block formation of the active catalytic site. Substitution or mutation studies demonstrate that the amino acid residues contacting the substrate are conserved in all types of adenylyl cyclase. The substrate specificity can be switched from ATP to GTP, the substrate for guanylyl cyclases, by substituting specific amino acids in this region of the catalytic domains.

The 12-transmembrane-spanning domain topology of the adenylyl cyclase enzymes is similar to that found in the ABC family of transporters (see Ch. 5), which includes the cystic fibrosis transmembrane rectifier and the P-glycoprotein. However, there is currently no convincing evidence of a transporter or channel function for mammalian adenylyl cyclases. The structural similarity may indicate that these functionally divergent protein families are derived in an evolutionary sense from related proteins.

Adenylyl cyclases have multiple modes of regulation. Adenylyl cyclases and the cAMP second messenger system have been implicated in diverse cellular functions in brain, including learning, memory, cognition and mood to name just a few (see Chs 53 and 55). The involvement of adenylyl cyclases in these essential and complex functions dictates that the control of cAMP formation should be finely controlled. Experimental evidence supports this conclusion, demonstrating that the adenylyl cyclase isoforms are differentially regulated by multiple forms of $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\beta\gamma}$, free Ca^{2+} and Ca^{2+}/calmodulin, as well as by covalent modifications (e.g. protein phosphorylation and glycosylation).

In general, the 10 different forms of adenylyl cyclase can be divided into three major and two minor groups based on their functional attributes as well as on their sequence homology. The three major families are: (1) the Ca^{2+}/calmodulin-stimulated isoforms, which include AC1, AC3, and AC8; certain of these enzymes are inhibited by $G_{\beta\gamma}$; (2) the $G_{\beta\gamma}$-stimulated isoforms, which include AC2, AC4, and AC7; and (3) the Ca^{2+} and $G_{\alpha i}$ inhibited isoforms, which are AC5 and AC6. The other two categories each have a single member: (4) AC9, which is the most divergent of the membrane-bound isoforms, is stimulated by $G_{\alpha s}$, inhibited by calcineurin (a protein phosphatase), and is insensitive to forskolin; (5) sAC, the only soluble isoform, is the most divergent and is most similar to the adenylyl cyclase found in Cyanobacteria.

Adenylyl cyclases are regulated by $G_{\alpha s}$ and $G_{\alpha l}$. Each of the different forms of adenylyl cyclase can be stimulated by activated $G_{\alpha s}$ (i.e. $G_{\alpha s}$ bound to GTP). In this way $G_{\alpha s}$ couples endocrine and neurotransmitter receptors that stimulate cAMP formation to adenylyl cyclases

in many cellular systems. As described in Chapter 19, activation by G-protein-coupled receptors occurs when a neurotransmitter or hormone binds to the receptor and catalyzes the exchange of GDP for GTP at the G protein α subunit. This promotes the dissociation of the α and βγ subunits, which allows for activated $G_{\alpha s}$ to interact with and stimulate adenylyl cyclase. The GTP-bound form has 10-fold higher affinity than the GDP-bound form of $G_{\alpha s}$ for adenylyl cyclase. GTPase activity contained within the α subunit hydrolyzes GTP to GDP and thereby leads to the reassociation with βγ. This intrinsic GTPase activity provides a time dependent switch for turning off the active form of $G_{\alpha s}$ and adenylyl cyclase activity, as well as $G_{\beta\gamma}$ that is deactivated by its reassociation with $G_{\alpha s}$. The GTPase activity can be accelerated by a specific RGS/GAP (regulator of G protein signaling/GTPase activator protein), PX1-RGS, which speeds up the termination of $G_{\alpha s}$-stimulated adenylyl cyclase activity.

It is also notable that AC1 and AC8 are activated synergistically by $G_{\alpha s}$ and Ca^{2+}/calmodulin. This finding suggests that these forms of the enzyme are capable of responding to receptors coupled to $G_{\alpha s}$ as well as to those that increase intracellular Ca^{2+} levels. This may be of particular importance to the function of these proteins in integrating the consequences of multiple extracellular stimuli as well as in synaptic plasticity, as mentioned earlier.

There are also many neurotransmitter and hormone receptors that contribute to the fine control of cAMP formation by inhibition of adenylyl cyclase. The action of inhibitory receptors is mediated by several different forms of the $G_{\alpha i}$ family, specifically the $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$ and $G_{\alpha z}$ subtypes. The G_{α} subunits of these isoforms can inhibit the catalytic activity of adenylyl cyclase when the enzyme is activated by either $G_{\alpha s}$ or forskolin. The inhibition of catalytic activity does not occur via competition with $G_{\alpha s}$ but appears to occur by an interaction at a symmetric site on the AC molecule. $G_{\alpha i}$-mediated inhibition of adenylyl cyclase is most dramatic for AC5 and AC6. A few other forms of adenylyl cyclase, most notably AC1, can be inhibited by $G_{\alpha o}$ but this effect is not as potent as the inhibition of AC5 and AC6 by $G_{\alpha i}$ isoforms. The GTPase activity of $G_{\alpha i}$ family members can be accelerated by a large family of RGS proteins (see Chapter 19).

Adenylyl cyclase subtypes are also regulated by βγ subunits. The regulation by $G_{\beta\gamma}$ varies dramatically with the different forms of adenylyl cyclase [1, 2, 18]. In the presence of $G_{\alpha s}$, the addition of $G_{\beta\gamma}$ complexes inhibits AC1 and AC8 but stimulates AC2, AC4, and AC7. The concentration of $G_{\beta\gamma}$ required for its inhibitory effect on AC1 is higher than the concentration of free $G_{\alpha s}$ required to activate this enzyme. This indicates that dissociation of $G_{\beta}\gamma$ from other, more abundant G proteins (e.g. $G_{\alpha i}$ and $G_{\alpha o}$) would be the primary source of $G_{\beta\gamma}$ for this type of inhibition to occur in brain that expresses relatively high levels of these G protein subtypes. The effect of $G_{\beta\gamma}$ on AC1 and AC8 is notable in that the inhibition is more potent than either $G_{\alpha i}$ or $G_{\alpha o}$. There is evidence that AC5 and AC6 are also inhibited by $G_{\beta\gamma}$, but this may be via indirect mechanisms and not direct interactions with enzyme molecules.

Stimulation of AC2, AC4, and AC7 by $G_{\beta\gamma}$ complexes is not only dependent on the presence of $G_{\alpha s}$ but leads to synergistic activation of these AC enzymes. As is the case for inhibition of AC1, the concentration of $G_{\beta\gamma}$ required for activation of AC2, AC4, and AC7 is higher than for $G_{\alpha s}$. This implies that activation of neurotransmitter receptors coupled to $G_{\alpha s}$ or $G_{\alpha i}/G_{\alpha o}$ may be able to activate adenylyl cyclase subtypes, and these responses could be synergistic under certain conditions. AC2, AC4 and AC7 are not directly influenced by $G_{\alpha i}$ or $G_{\alpha o}$.

Studies with chimeras of the different enzymes indicate that the stimulatory effect of $G_{\beta\gamma}$ depends on the carboxy half of the adenylyl cyclase molecule [9]. These studies have utilized 'noncovalent' chimeras, where the amino half of AC1 (containing the first membrane-spanning and cytoplasmic domains) is co-expressed in cultured cells with the carboxy half of either AC1 or AC2 (containing the second membrane-spanning and cytoplasmic domains). Depending on whether the carboxy portion is from AC1 or AC2, the coexpressed chimera is either inhibited or stimulated, respectively, by $G_{\beta\gamma}$ complexes. The binding site in the AC enzymes that are stimulated by $G_{\beta\gamma}$ corresponds to amino acid residues 956–982 of AC2, which sequence is located near the middle of the second catalytic domain. This sequence also contains a putative consensus $G_{\beta\gamma}$-binding site that is found in other enzymes known to be regulated by $G_{\beta\gamma}$, including G-protein-coupled receptor kinase, inward-rectifying K^+ channels and phospholipase C_{β}. These data highlight the key role of $G_{\beta\gamma}$ in the regulation of multiple signaling molecules important for neuronal function.

In addition to the selective responses of adenylyl cyclases to $G_{\beta\gamma}$ subunits, it is likely that different forms of G_{β} and G_{γ} subunits influence the various forms of adenylyl cyclase in different ways [19]. There are five known forms of G_{β} subunits and 11 known forms of G_{γ} subunits (see Ch. 19). Differential expression and regulation of these subunits could provide still additional mechanisms for selectively controlling adenylyl cyclase catalytic activity in specific neuronal cell types.

Adenylyl cyclases show differential regulation by Ca^{2+}. The regulation of the various forms of adenylyl cyclase by Ca^{2+} provides an important mechanism by which receptors that influence ligand- or voltage-stimulated Ca^{2+} channels, or receptors that influence intracellular Ca^{2+} levels via coupling to $G_{\alpha q}$, exert control over the cAMP signaling cascade (see Ch. 22) [1, 2]. AC1 and AC8, and possibly AC3, are activated by Ca^{2+}/calmodulin. This effect is synergistic with $G_{\alpha s}$, while AC2, AC4, AC5, and AC6 are insensitive to Ca^{2+}/calmodulin. AC1 and AC8 are activated by voltage-stimulated Ca^{2+} channels but not by receptors that couple to $G_{\alpha q}$ and release of intracellular Ca^{2+}.

The concentration of Ca^{2+} required for half-maximal activation of AC1 is approximately 150 mmol/l, while AC8 is five times less sensitive. The C1b domain of adenylyl cyclase appears to mediate the activation of the enzyme by Ca^{2+}/calmodulin (see Fig. 21-3). Activation of AC1 by Ca^{2+}/calmodulin can be blocked by a peptide fragment of the C1b portion of this enzyme. This peptide is also capable of binding Ca^{2+}/calmodulin itself.

Although AC5 and AC6 are not influenced by Ca^{2+}/calmodulin, these two forms of the enzymes are inhibited by free Ca^{2+}. The concentrations of Ca^{2+} required for this inhibition are in the physiological range (0.1–1.0 mmol/l) that would be observed upon activation of voltage-sensitive Ca^{2+} channels in neurons [1]. The localization of AC5 and AC6 in the brain indicates that this form of Ca^{2+} influx is the primary means of Ca^{2+} regulation of these enzymes.

Adenylyl cyclase are regulated upon phosphorylation. In addition to direct regulation by binding G protein subunits and Ca^{2+}, certain forms of adenylyl cyclase are regulated upon their phosphorylation by second-messenger-dependent protein kinases (see Ch. 23). The protein kinases that have been most extensively studied are cAMP-dependent protein kinase (protein kinase A), protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinase. Activation of protein kinase A inhibits AC5 and AC6 [20],while activation of protein kinase C inhibits AC4 and AC6. In contrast, AC1, AC2, AC3 and AC5 are phosphorylated and activated by the activation of protein kinase C. Although AC1, AC3 and AC8 are directly activated by Ca^{2+}/calmodulin, AC1 and AC3 are inhibited by activation of Ca^{2+}/calmodulin-dependent protein kinase. This counter regulation most likely represents a negative feedback inhibition of these AC isoforms by Ca^{2+} signaling (see also Ch. 22). Although these initial observations are exciting, much further work is needed to define the physiological role of adenylyl cyclase phosphorylation by second-messenger-dependent protein kinases and perhaps by other protein kinases.

Diversity of adenylyl cyclases leads to dramatic cell-to-cell differences in regulation of the cAMP pathway. The differential sensitivity of AC1–AC8 to G protein α and $\beta\gamma$ subunits and to Ca2+ provides potentially complex mechanisms for the regulation of cAMP formation. The three major categories of adenylyl cyclases, based on their regulatory properties discussed above are illustrated in Figure 21-4.

While the schemes for regulation are hypothetical, certain features do suggest different patterns of regulation of cAMP formation in cells that are consistent with the different forms of adenylyl cyclase expressed in the cell, or their phenotype. For example, in cells that contain AC1, AC3 or AC8 (Fig. 21-4A), cAMP formation would be stimulated by extracellular signals that activate receptors coupled to $G_{\alpha s}$ as well as by those signals that increase Ca^{2+} entry into the cells. Neurons expressing AC1 may have inherently high levels of cAMP formation due to basal stimulation by Ca^{2+}/calmodulin. In fact, the high levels of adenylyl cyclase enzymatic activity known to be present in brain, relative to other tissues, may be partly explained in this way. This high level of enzyme activity could also underlie the requirement for additional mechanisms by which this type of adenylyl cyclase is inhibited: cAMP formation would be inhibited in these cells not only by signals that activate receptors coupled to $G_{\alpha i}$ but also by additional signals that activate receptors coupled to other G proteins (e.g. $G_{\alpha o}$ and $G_{\alpha q}$) via the release of $G_{\beta\gamma}$ subunits. This could provide a mechanism for keeping 'in check' the high cAMP synthetic capacity present in brain, as well as providing multiple mechanisms for the regulation of cAMP formation through a variety of extracellular signals.

A very different situation would exist in cells that primarily express AC2, AC4 and AC7 (Fig. 21-4B). In these cells, enzyme activity would not be stimulated by Ca^{2+}/calmodulin but would be increased by signals that activate receptors coupled to $G_{\alpha s}$, as well as by additional signals that activate receptors coupled to other G proteins through the generation of $G_{\beta\gamma}$ subunit complexes. This would provide a mechanism by which cAMP formation is regulated in an integrated manner by multiple extracellular stimuli. In fact, there are several examples in brain of interactions between receptors coupled to $G_{\alpha s}$ and those coupled to other G proteins. For example, stimulation of adenylyl cyclase activity in cerebral cortex by activation of β-adrenergic receptors (which are coupled to $G_{\alpha s}$) can be potentiated by activation of α_1-adrenergic or $GABA_B$ receptors (which coupled to other G proteins), which alone have little or no effect on cAMP formation [21]. This potentiation could result from the release of free $\beta\gamma$ complexes from the G proteins coupled to the α_1-adrenergic or $GABA_B$ receptors (e.g. $G_{\alpha o}$ and/or $G_{\alpha q}$). However, these potentiating effects are dependent on extracellular Ca^{2+} and could also, therefore, be mediated by activation of Ca^{2+}-dependent intracellular pathways (see Ch. 22).

The third situation would be cells that express AC5 and AC6. In these cells, adenylyl cyclase would be activated potently by $G_{\alpha s}$ (Fig. 21-4C). However, this activation would be tightly controlled, since the same signals would lead to activation of protein kinase A, which would phosphorylate and inhibit these adenylyl cyclases and thereby inhibit further cAMP formation. Activation of AC5 and AC6 would be further controlled by receptors coupled to $G_{\alpha i}$ and those that increase levels of free Ca^{2+}, both of which exert an inhibitory effect on these enzymes. In addition, receptors coupled to $G_{\alpha q}$, which would lead to activation of protein kinase C, would also be expected to inhibit AC5 and AC6. These types of mechanism may be of particular relevance to psychotropic drugs that act on dopamine-rich brain regions (i.e. neostriatum and nucleus accumbens), including drugs of abuse and antipsychotic drugs, since AC5 is highly enriched in these brain areas as mentioned above (see Chs 12, 46 and 56).

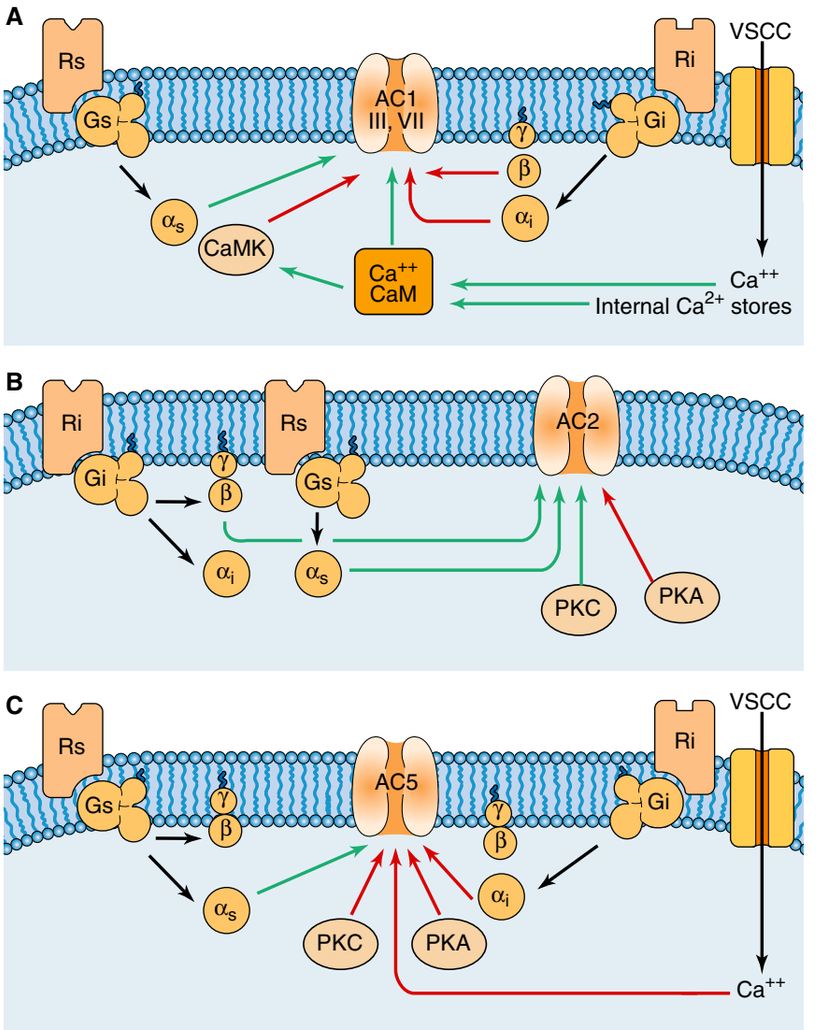

FIGURE 21-4 Schematic illustration of the mechanisms by which the activity of different types of adenylyl cyclase (*AC*) may be regulated. Whereas all types of AC are activated by $G_{\alpha s}$ (αs) and forskolin, different types of the enzyme can be distinguished by their regulation by Ca^{2+} and G protein subunits. Activation and inhibition are indicated by green and red arrows, respectively. (**A**) Since AC1, AC3, and AC8 are stimulated by Ca^{2+}/calmodulin (Ca^{2+}/CaM), an increase in cellular Ca^{2+} levels, which can result from increased entry of Ca^{2+} into the cell either via voltage sensitive Ca^{2+} channels (VSCC) or via NMDA receptor channels, would be expected to activate these enzymes. The actions of Ca^{2+}/CaM are synergistic with $G_{\alpha s}$. In addition, in the presence of activated $G_{\alpha s}$, AC1 is inhibited by $\beta\gamma$ subunits. The potency of $G_{\alpha s}$ to activate the enzyme is some 10- to 20-fold higher than that of $\beta\gamma$ complexes to inhibit it, so that activation of enzyme activity is the predominant effect when only stimulatory receptors and Gs are activated. $G_{\alpha i}$ mediates neurotransmitter inhibition of these AC, and enzyme activity is decreased by Ca^{2+}/CaM-stimulated kinase (CaMK). (**B**) AC2, AC4 and AC7AC4 are stimulated by $\beta\gamma$ complexes, but only in the presence of activated $G_{\alpha s}$. The receptors (*Rx*) and G protein α subunits that provide the $\beta\gamma$ subunits for this type of regulation could conceivably involve receptors coupled to several types of G protein (e.g. G_i, G_o, G_q, etc.). The activation by $\beta\gamma$ is synergistic with $G_{\alpha s}$. Note: while the same $\beta\gamma$ complexes are shown for all the G proteins listed, there are several known subtypes of β and γ subunits, which may well influence the various types of AC in different ways. These enzymes can also be regulated by protein kinase A (PKA) or protein kinase C (PKC) as indicated. (**C**) AC5 and AC6 are inhibited by free Ca^{2+}. In addition, these enzymes are inhibited upon phosphorylation by protein kinase A (PKA) or protein kinase C (PKC). AC5 and AC6 are also inhibited by $G_{\alpha i}$, but are not influenced by $\beta\gamma$ subunits.

Adenylyl cyclase in learning and memory. The cAMP-signal transduction cascade has been demonstrated to play a role critical to the formation of long-term memory in both cellular models and in animals. The specific adenylyl cyclases involved are being identified by current research. In AC1 mutant mice there is a partial disruption of long-term potentiation (LTP), a cellular model of learning and memory [22]. This effect is specific to the mossy fiber pathway of the hippocampus, indicating that LTP in other pathways such as the Schaffer collaterals may involve other types of adenylyl cyclase. Complete elimination of LTP in the mossy fiber pathway was observed in AC1 and AC8 double knockout mice, demonstrating a role for both Ca^{2+}/calmodulin-sensitive forms of adenylyl

cyclase [23]. The AC1 and AC8 double mutant mice also display loss of contextual and passive avoidance long-term memory in models of fear-associated learning as well as spatial memory [23]. In these models the double knockout mice are able to learn a task but the memory only lasts 5–10 minutes, unlike the long-term memory displayed by wild type mice.

Adenylyl cyclase is subject to long-term regulation in the nervous system. It has been known for many years that prolonged exposure of cells to a receptor agonist typically leads to desensitization of receptor function, whereas prolonged exposure to an antagonist can lead to sensitization of receptor function. Increasing evidence indicates that such desensitization and sensitization can be achieved, depending on the receptor and cell type involved, through alterations in the receptors themselves as well as through alterations in the series of proteins that are distal to the receptor and that mediate receptor function, including adenylyl cyclase [24]. Possible mechanisms underlying adaptations in adenylyl cyclase involved in desensitization and sensitization of receptor function could conceivably include phosphorylation of the enzyme as well as alterations in its expression at transcriptional, translational, and post-translational levels. Investigations into these mechanisms have just begun.

Processes of receptor desensitization and sensitization are best established for G-protein-coupled receptors coupled (positively or negatively) to the cAMP system. Agonist- and antagonist-dependent changes in these receptors themselves are discussed elsewhere in this text (Chs 12 and 13). However, adaptations in postreceptor signaling proteins also play an important role in controlling receptor sensitivity under a variety of physiological and pathological conditions. One example in which adaptations in adenylyl cyclase have been related to long-term adaptations in receptor function is opiate tolerance and dependence (see also Ch. 56). In this case, chronic exposure to opiates leads to coordinate upregulation in the activity of the cAMP cascade (including higher levels of adenylyl cyclase and protein kinase A and in some cases lower levels of inhibitory G proteins) in specific opiate-responsive brain regions [24, 25]. Upregulation of the cAMP cascade contributes to opiate tolerance and dependence by opposing the acute actions of opiates (which stimulate receptors coupled to $G_{\alpha i}$). Increased expression of adenylyl cyclase, which in some brain regions is seen selectively for the AC1 and AC8, may be achieved at the level of gene expression, since higher levels of enzyme mRNA are observed as well [26, 27]. More specifically, there is increasing evidence that opiate induction of AC8 in these regions is mediated by the transcription factor CREB (cAMP response element binding protein; see Ch. 26).

In addition to drug regulation of adenylyl cyclase mediated through agonist or antagonist effects on receptors, some drugs produce direct effects on the adenylyl cyclase protein. One example is forskolin, which, as stated earlier, directly stimulates the catalytic activity of all known forms of the enzyme. Another example is lithium, the drug of choice in the treatment of bipolar disorder. The lithium ion inhibits adenylyl cyclase acutely, by interfering with the Mg^{2+} binding sites on the enzyme. This action accounts for some of lithium's side-effects, such as inhibition of thyroid hormone production, which is dependent on the cAMP cascade. However, chronic lithium administration, which is required for its beneficial clinical effects, leads to increases in adenylyl cyclase expression in brain [28], which may represent a compensatory response to acute inhibition of the enzyme. Whether this upregulation of adenylyl cyclase contributes to lithium's clinical effects remains unknown (see Ch. 55).

GUANYLYL CYCLASE

Guanylyl cyclase (GC) catalyzes the synthesis of cGMP from GTP in a reaction analogous to that shown in Figure 21-2 for adenylyl cyclase. Like cAMP, cGMP is known to regulate many cellular proteins, such as protein kinases, ion channels and phosphodiesterases. Two major classes of GC are known to exist, identified originally on the basis of their subcellular distribution: membrane-bound and soluble [29–32]. The general types of mechanism by which these GC types are thought to be regulated by extracellular signals are shown in Figure 21-5.

Membrane-bound forms of guanylyl cyclase act as plasma membrane receptors. The transmembrane forms of GC proteins possess extracellular surface domains that function as neuropeptide receptors, a single transmembrane domain, an intracellular kinase homology domain and a catalytic domain. The activation of membrane-bound GC requires a complex series of steps involving oligomerization of the receptor, ligand binding and modulation of intracellular domains. Activation of enzyme activity requires phosphorylation of the kinase homology domain, which also contains an ATP-binding site that is required for GC activity. Indeed, the binding of ATP to the site may play a role in transducing the binding of ligand at the receptor domain to the activation of enzyme activity in the catalytic domain. This would be functionally homologous to the role of GTP-binding to G proteins in transducing the binding of ligand to G-protein-coupled receptors in the regulation of adenylyl cyclase.

There are seven membrane forms of GC, designated GC-A to GC-G [33]. Two forms, GC-A and GC-B (M_r ≈120 kDa), serve as receptors for atrial natriuretic peptide (ANP) and related peptides. ANP is a 28-amino-acid peptide isolated originally from cardiac atria as an important factor in the regulation of sodium excretion and blood pressure. GC-A binds ANP, as well as brain natriuretic peptide (BNP), and is located in vascular tissue and kidney.

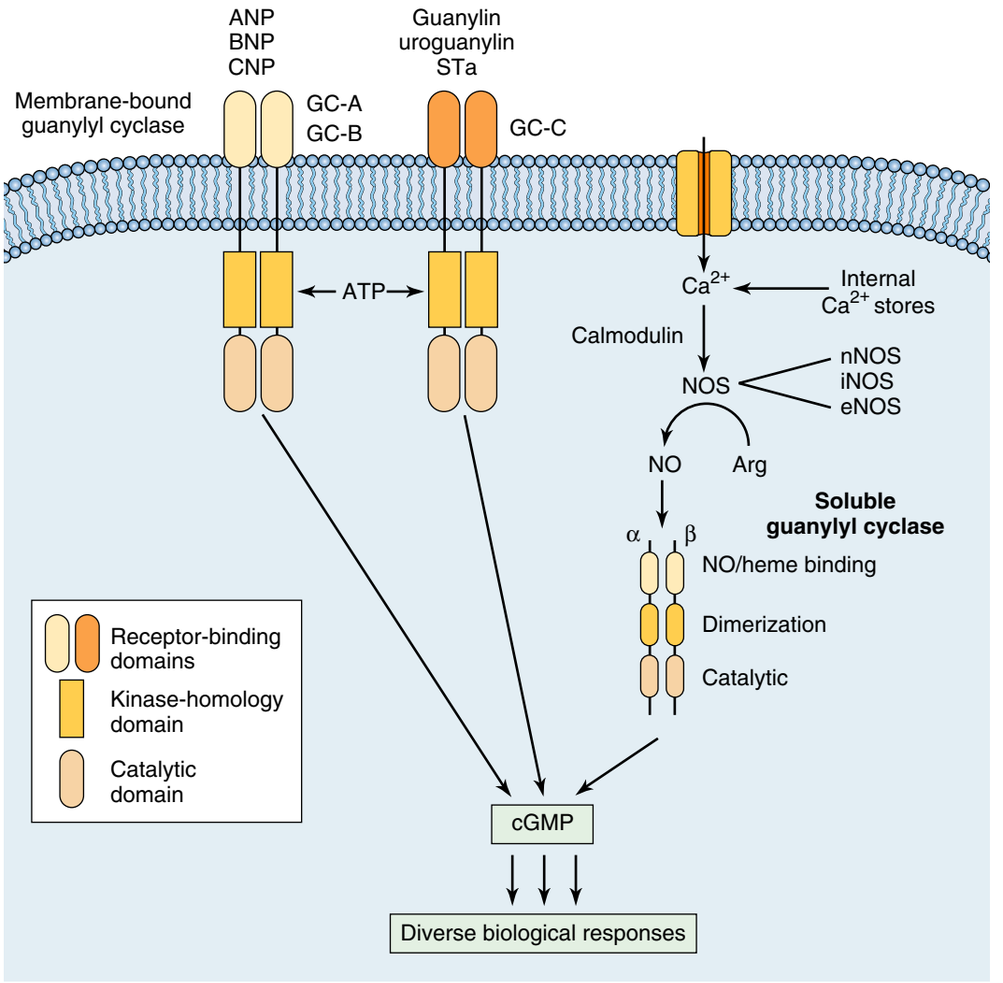

FIGURE 21-5 Schematic illustration of the mechanisms by which first messengers stimulate guanylyl cyclase. Two major classes of guanylyl cyclase are known: membrane-bound and soluble. The membrane-bound forms (*GC-A*, *GC-B* and *GC-C*) contain extracellular receptor domains that recognize specific peptide ligands (atrial natriuretic peptide and related peptides): GC-A binds atrial natriuretic peptide (*ANP*) and brain natriuretic peptide (*BNP*) and GC-B binds C-type natriuretic peptide (*CNP*). GC-C binds the endogenous peptides guanylin and uroguanylin, as well as a heat stable bacterial enterotoxin (*STa*). The membrane-bound receptors contain an intracellular kinase-like domain that binds ATP and a catalytic domain that synthesizes cGMP from GTP. The soluble forms contain the catalytic domains only (α and β subunits), and are activated by nitric oxide (*NO*). Catalytic activity of soluble guanylyl cyclase is dependent on the presence of both α and β subunits. First messengers lead to activation of NO synthesis by increasing cellular levels of Ca^{2+}, which in conjunction with calmodulin activates NO synthase (*NOS*). First messengers increase cellular Ca^{2+} levels in most cases by depolarizing neuronal membranes and thereby activating voltage-dependent Ca^{2+} channels and increasing the flux of Ca^{2+} into the cell (e.g. nerve impulses, glutamate, acetylcholine, substance P). In some cases, Ca^{2+} can enter the cell directly via ligand-gated ion channels (e.g. as with NMDA glutamate receptors). First messengers can also regulate cellular Ca^{2+} levels by stimulating Ca^{2+} release from intracellular stores. There are three forms of NOS: neuronal (*nNOS*), inducible (*iNOS*) and endothelial (*eNOS*).

GC-B is expressed in neuronal tissue and binds the C-type natriuretic peptide (CNP). There is also a truncated form of GC-B that lacks the intracellular kinase and catalytic domains. The truncated form may act to bind and clear the peptide ligands. Increasing evidence suggests that these peptides play important roles as extracellular signals in diverse tissues including brain. Activation of GC and the consequent increased levels in cellular cGMP mediate some of the cellular actions of these peptides on target tissues. A third and related GC type, GC-C, is localized to the intestine and binds the endogenous peptides guanylin and uroguanylin. GC-C can also be activated by a bacterial enterotoxin.

It is worth mentioning that membrane-bound forms of GC, which can be considered 'signal transducing enzymes', are structurally homologous to other signal transducing enzymes, such as certain protein tyrosine kinases and phosphatases, which also possess receptor moieties in their extracellular (amino terminus) domain and enzyme catalytic activity in their intracellular domain (see Ch. 24). Activation of many of these receptors occurs upon ligand-induced dimerization of the receptors, and a similar

mechanism may occur for the membrane-bound forms of guanylyl cyclase.

The ligands for the other forms of membrane-bound GC are less well characterized. In some cases the functions of these GC forms have not been identified. GC-D is expressed in olfactory sensory neurons but its function has not been determined. GC-E and GC-F are expressed in photoreceptor cells in the retina and play a role in phototransduction. GC-G is widely expressed in peripheral tissues and brain but its ligand binding domain is similar to that for GC-A/GC-B, suggesting that it may be regulated by an ANP/BNP-like ligand [34].

Soluble forms of guanylyl cyclase are activated by nitric oxide. These enzymes are homologous to the catalytic domains of the membrane-bound forms of GC. They are considered heterodimers because they appear to exist, under physiological conditions, as complexes of α and β subunits, each with M_r of 70–80 kDa. Both types of soluble GC contain three primary domains: an amino-terminus heme domain responsible for binding nitric oxide (NO), a dimerization domain and a carboxy terminus catalytic domain. The αβ heterodimer is required for enzyme activity [35]. This can be seen as similar to the situation for AC, which contains two catalytic entities within a single polypeptide chain (Fig. 21-5).

There are multiple isoforms of the α and β subunits of soluble GC that exhibit distinct tissue and cellular distributions. Most of the isoforms are expressed in brain, including α_{1-3} and β_1 and β_3. The β_2 subunit is found primarily in lung. This isoform also contains a consensus sequence at its carboxy terminus for isoprenylation or carboxymethylation, which could serve to anchor the protein to the plasma membrane. It has been proposed that specific isoforms of the α and β subunits of GC may form heterodimers with distinct functional and regulatory properties, although this remains to be established with certainty.

A major advance in our understanding of the regulation of soluble guanylyl cyclase was the demonstration that the activity of these enzymes is potently activated by NO [36, 37]. NO activates these enzymes via interactions with the heme prosthetic groups located in the amino-terminus. This conclusion is supported by the finding that removal of the heme group results in an enzyme that cannot be activated by NO. However, there are additional sites that are known to be regulated by NO (i.e. vicinal thiols) that could also contribute to the actions of NO. The synthesis of NO is controlled by NO synthase (NOS), which in turn is regulated by Ca^{2+}/calmodulin as described below. Thus, the generation of NO and activation of sGC is stimulated by numerous types of neurotransmitters that lead to increased intracellular Ca^{2+} levels, including glutamate, acetylcholine, substance P, histamine and bradykinin. Similarly, all organic nitrate drugs (e.g. nitroglycerin, nitroprusside), which are used in the treatment of ischemic heart disease, induce vasodilatation via the activation of soluble GC and increased levels of cGMP.

Nitric oxide functions as an intracellular second messenger. NOS converts L-arginine into free NO and citrulline in a reaction that requires a tetrahydrobiopterin cofactor and NADPH [36, 37]. Three types of NOS have been identified by molecular cloning [30, 38]. Type I NOS, also referred to as neuronal NOS (nNOS), is found at high concentrations in nervous tissue, as well as other tissues. Ca^{2+}/calmodulin binds to nNOS and increases its catalytic activity (Fig. 21-5). Neurotransmitters that increase cellular Ca^{2+} levels would thereby be expected to stimulate sGC activity in those neurons that contain this form of NO synthase. Type II NOS is referred to as macrophage NOS or inducible NOS (iNOS). It is expressed at low levels under basal conditions but is induced in response to immunologic or other challenges. Type III NOS is referred to as endothelial NOS (eNOS), and it is also activated by Ca^{2+}/calmodulin. In addition, this form of NOS is associated with the plasmalemma and is associated with the production of endothelium-derived relaxing factor (EDRF). Despite this nomenclature, Type II and type III NOS, in addition to type I, contribute to signal transduction in the brain.

Free NO also acts as an intercellular messenger. According to this scheme, NO, generated in response to an increase in intracellular Ca^{2+} levels in one neuronal cell type, can diffuse out of the cell and into a second neuron, where it would stimulate guanylyl cyclase activity and produce specific physiological effects. Such a role for NO has been proposed as contributing to the mechanisms underlying long-term potentiation [39].

It is important to note that, in addition to guanylyl cyclase, NO may directly regulate other types of cellular protein. Further work is needed to identify these NO substrates.

PHOSPHODIESTERASES

Given the significant role of cyclic nucleotides in signal transduction pathways, it is not surprising that the metabolism, like the synthesis, of these second messengers is highly regulated. Such metabolism is achieved by a large family of enzymes, the phosphodiesterases (PDEs), which catalyze the conversion of cAMP and cGMP into 5′-AMP and 5′-GMP, respectively, via hydrolysis of the 3′-phosphoester bonds. PDEs usually act as homodimers.

There are multiple forms of phosphodiesterases in brain. Eleven major subfamilies/types of PDE, listed in Table 21-1, have been delineated, based primarily on three criteria: (1) the amino acid sequence homology; (2) the kinetic properties of each enzyme for hydrolyzing cAMP and cGMP; and (3) the mechanisms for regulation of PDE activity [40, 41]. The kinetic properties are characterized according to the affinity of each enzyme for cAMP or cGMP. The affinity is derived from the K_m values: a low K_m signifies that the enzyme has a high affinity for

TABLE 21-1 Classification and selected properties of cyclic nucleotide phosphodiesterases

	Family regulatory/kinetic characteristics	Genes described	Inhibitors*
1	Ca^{2+}/calmodulin-stimulated PDEs PDE1A/1B, low affinity, cAMP/cGMP PDE1C, high affinity for cAMP	PDE1A PDE1B PDE1C	Trifluoperazine Vinpocetine SCH51866† (0.1 µmol/l)
2	cGMP-stimulated PDEs Regulated by cGMP Low affinity for cAMP and cGMP	PDE2 (soluble, particulate)	EHNA, erytho-9-(2-hydroxy-3-nonyl) adenine
3	cGMP-inhibited PDEs Regulated by phosphorylation and cGMP High affinity, cGMP > cAMP	PDE3A PDE3B	Milrinone Enoximone Amrinome‡
4	cAMP-specific PDEs Regulated by phosphorylation and cAMP High affinity, cAMP >>> cGMP	PDE4A PDE4B PDE4C PDE4D	Rolipram Ro20–1724
5	cGMP-binding, cGMP-specific PDEs Regulated by phosphorylation, cGMP High affinity, cGMP >>> cAMP	PDE5A	Sildenafil Zaprinast Dipyridamole SCH51866† (0.1 µmol/l)
6	Retina cGMP specific PDEs Regulated by transducin High affinity, cGMP >>> cAMP	PDE6A PDE6B PDE6C	Zaprinast Zaprinast Zaprinast
7	cAMP-specific PDE Rolipram-insensitive	PDE7A PDE7B	–
8	cAMP-specific PDEs IBMX- and rolipram-insensitive	PDE8A PDE8B	–
9	cGMP-specific PDE IBMX-insensitive	PDE9	SCH51866† (1.5 µmol/l)
10	Dual substrate, cAMP-inhibited PDE High affinity, cAMP >>> cGMP	PDE10	SCH51866† (1.0 µmol/l) Dipyridamole
11	Dual substrate PDE cGMP>cAMP	PDE11A	Dipyridamole

*In addition to the relatively specific inhibitors listed in this table, there are a number of compounds, particularly the methylxanthines (e.g. theophylline, isobutylmethylxanthine (IBMX), papaverine, caffeine), that inhibit most major forms of PDE except the newer forms).
†SCH51866 is not selective and inhibits PDE1, PDE5, PDE9, and PDE10 with IC50 values shown.
‡The compounds listed here are among a large number that have been developed as specific inhibitors of PDE3.

substrate. For example, a low K_m PDE typically has a K_m of <5 µmol/l.

Ca^{2+}/calmodulin-stimulated phosphodiesterases (PDE1). The PDE1 family is stimulated by Ca^{2+}/calmodulin and consists of several soluble subtypes in brain and peripheral tissues. Two isozymes of PDE1,of 61 kDa and 63 kDa, account for more than 90% of total brain PDE activity. Separate genes for each of these isozymes have been identified and are referred to as PDE1A (61 kDa) and PDE1B (63 kDa). Both enzymes exhibit relatively low affinity for cGMP and for cAMP and are referred to as dual substrate PDEs. PDE1A is expressed at highest levels in cerebral cortex and hippocampus and at moderate levels in amygdala [42]. Expression of PDE1B is high in brain regions innervated by dopaminergic fibers (e.g. striatum, nucleus accumbens, olfactory tubercle), with moderate levels of expression found in hippocampus and cerebral cortex [42]. An additional isozyme, PDE1C (73 kDa), also encoded by a distinct gene, has been identified in brain and testes. Interestingly, PDE1C has a high affinity for cAMP, in contrast to the low affinity that PDE1A and PDE1B exhibit for cyclic nucleotides.

The activity of PDE1 isozymes can be regulated under physiological conditions by those extracellular signals that influence intracellular Ca^{2+} levels. This may be particularly relevant, in a physiological sense, for PDE1C, given its high affinity for cAMP. There is also evidence that these enzymes are regulated by protein phosphorylation [16, 43]. The 61 and 63 kDa isozymes are good substrates for protein kinase A and Ca^{2+}/calmodulin-dependent protein kinase, respectively. Such phosphorylation decreases the functional capacity of the enzymes by decreasing their affinity for Ca^{2+}/calmodulin complexes.

cGMP-regulated phosphodiesterases (PDE2 and PDE3). There are two PDE families that are regulated by cGMP, one that is stimulated (PDE2) and one that is inhibited (PDE3). Both these PDEs are found in soluble and membrane-associated forms. cGMP-stimulated PDE2 has low affinity for cAMP and cGMP and, like PDE1, is a dual substrate PDE. There are at least two cyclic nucleotide binding sites on PDE2: one is the catalytic site, the other is a high affinity site that allosterically regulates the catalytic activity of the enzyme. This second site has homology with an evolutionarily conserved sequence known as a

GAF domain, named after different classes of proteins that contain this site, including cGMP-specific and cGMP-regulated PDEs, adenylyl cyclase (cyanobacterial) and *Escherichia coli* transcription factor FhlA [44]. Similar sites are found in PDE5, PDE6, PDE9, PDE10 and PDE11. PDE2 can be activated 10–50-fold by concentrations of cGMP that are found in cells. However, stimulation is transient since cGMP is also a substrate for the enzyme and is therefore metabolized rapidly. Localization studies demonstrate that PDE2 expression is largely restricted to brain and adrenal gland [45]. Within brain, the highest levels of the enzyme are seen in cerebral cortex, striatum, and hippocampus. PDE2 may be a primary effector for the physiological actions of cGMP in cells that do not express appreciable levels of cGMP-dependent protein kinase. In contrast to PDE2, there is currently no evidence for the presence of cGMP-inhibited PDE3 in brain. In contrast to PDE2, PDE3 is inhibited by cGMP and has a low K_m for cAMP. PDE3 is enriched in heart and vascular tissues, where it regulates cardiac and smooth muscle contraction.

cAMP-specific phosphodiesterases (PDE4, PDE7 and PDE8). There are three different PDEs that show a high affinity specifically for cAMP: PDE4, PDE7 and PDE8. Members of the PDE4 family are found in many tissues, in both soluble and membrane-associated forms, and are abundant in the central nervous system. There are four members of the PDE4 family, PDE4A, PDE4B, PDE4C and PDE4D, each encoded by a separate gene and each with multiple splice variants. PDE4A and PDE4B are expressed at relatively high levels in hippocampus, cerebral cortex and striatum and represent the majority of the membrane-bound form of PDE4 in these brain regions [46]. PDE4D represents the majority of the soluble PDE4 in brain. Expression of PDE4C is relatively low in nervous tissue. The four PDE4 genes are highly conserved across several mammalian species [41]. The PDE4 isozymes are regulated by phosphorylation and by binding cAMP. In addition, the expression of certain PDE4 genes is significantly regulated by activation of the cAMP intracellular pathway. Indeed, this is considered a primary mechanism for enhancing the function of these isozymes in some cell types.

PDE7 and PDE8 are two relatively new families of high affinity cAMP-specific PDEs. There are known two members of each family, PDE7A and PDE7B, and PDE8A and PDE8B [47, 48]. Unlike PDE4, PDE7 and PDE8 are not inhibited by rolipram, a well-characterized and specific inhibitor of PDE4. PDE7A is expressed at high levels in the immune system but is also expressed throughout the brain, with highest levels seen in hippocampus, olfactory system, thalamus and certain brainstem nuclei [49]. PDE7B is expressed in several peripheral tissues, including pancreas, heart, thyroid, skeletal muscle and liver, but is also expressed at relatively high levels in certain brain regions, including hippocampus.

A unique feature of PDE8 and of PDE9 (see below) is that they are insensitive to isobutylmethylxanthine (IBMX), which inhibits most other forms of PDE. The amino terminus of PDE8 also contains a PAS domain, named for the proteins first discovered to contain this moiety (Per, Arnt and Sim proteins). PAS domains have been shown to mediate homomeric or heteromeric protein-protein interactions and can influence the subcellular distribution of proteins [50]. The two forms of PDE8 display unique expression patterns in peripheral tissues and in brain. PDE8A is expressed at highest levels in liver and testes but at very low levels in brain, where it is undetectable by in situ hybridization analysis. PDE8B is expressed primarily in the brain with highest levels in the hippocampus and cerebral cortex. Expression of PDE8B has also been observed in thyroid gland of human, but not rat.

cGMP-specific phosphodiesterases (PDE5, PDE6 and PDE9). PDE5, PDE6 and PDE9 are soluble PDE isozymes with high affinity for cGMP that hydrolyze this substrate to the monoester. The PDE5, PDE6 and PDE9 isozymes are also referred to as the cGMP-specific PDEs since they display a 50-fold selectivity for cGMP relative to cAMP. Although PDE5 and PDE6 both have high affinity for cGMP, they are structurally distinct. PDE5 and PDE6 also contain GAF domains in the N-terminus that influence phosphorylation and interaction of the catalytic subunits respectively. PDE5 is enriched in smooth muscle, including lung and erectile tissue, but is also expressed in brain at lower levels and has been implicated in learning and memory [51]. PDE5 inhibitors, such as sildenafil, have proved to be effective for the treatment of erectile dysfunction.

There are several members of the PDE6 family, all of which are light-activated photoreceptor isozymes. Activation of photoreceptor PDE6 in rod and cone outer segments is mediated by transducin, a G protein specific to retina (see Ch. 49). PDE6 is a multimeric enzyme complex, composed of α, β, γ and δ subunits. The functional complex is a heterodimer comprised of α and β subunits and the inhibitory γ subunit (two copies). The δ subunit aids in solubilizing PDE6. PDE6 is inactive in the dark; light results in its activation via a complex biochemical cascade analogous to mechanisms for activation of G-protein-coupled receptors (see Ch. 49 for complete description).

PDE9 has the highest affinity/lowest K_m of the cGMP-specific PDEs. Because of its high affinity for cGMP, it is thought that PDE9 may be a key regulator for levels of this cyclic nucleotide. It is widespread in brain with highest levels of expression in the cerebellar Purkinje cells, striatum, nucleus accumbens, cerebral cortex and hippocampus [51]. The pattern of PDE9 is similar to that of soluble GC, suggesting a functional coupling of PDE9 and sGC for regulation of cGMP levels. PDE9 has five splice variants (PDE9A1–5), and some of these are expressed at high levels in the immune system, as well as intestine and prostate.

PDE10 and PDE11. PDE10 and PDE11 are relatively new members of the PDE family. Both are dual substrate PDEs although they differ in their selectivity for cAMP and cGMP. PDE10 has a higher affinity for cAMP than cGMP (K_m values are 0.05 and 3.0 µmol/l respectively) [48] while PDE11A has higher affinity for cGMP than cAMP (K_m values are 0.5 and 1.0 µmol/l respectively) [52]. Although PDE10A has higher affinity for cAMP, it has a fivefold greater V_{max} for cGMP, suggesting that cAMP, by occupying the catalytic site, may act as an inhibitor of cGMP hydrolysis. Both PDE10A and PDE11A have sequence homology in the N-terminus with PDE2, PDE5 and PDE6 that encode GAF domains. The GAF domains could serve as noncatalytic cGMP binding sites for allosteric modulation of enzyme activity. PDE10A is expressed in testis and brain, where it is enriched in striatum. PDE11A is expressed in skeletal muscle, prostate, liver, kidney, pituitary and salivary gland, with relatively low levels in brain.

Phosphodiesterase enzymes show a distinctive molecular structure. Members of each of the seven PDE families have been characterized by molecular cloning techniques [40, 41]. All the enzymes thus far identified in mammalian tissues contain a highly conserved region of approximately 300 amino acids at the carboxy terminus. Deletion studies have confirmed that this region represents the catalytic domain. Within this region there are a number of conserved histidine residues that appear to play a role in folding of the proteins and are required for their catalytic activity. It has also been suggested that the histidine residues may bind zinc, given the structural similarities of the PDEs with zinc hydrolases.

The regions outside the catalytic domain, particularly the amino terminus, are much more variable across the different PDE families. These regions contain many of the regulatory sites that control PDE activity (Fig. 21-6). For example, this region contains the Ca^{2+}/calmodulin

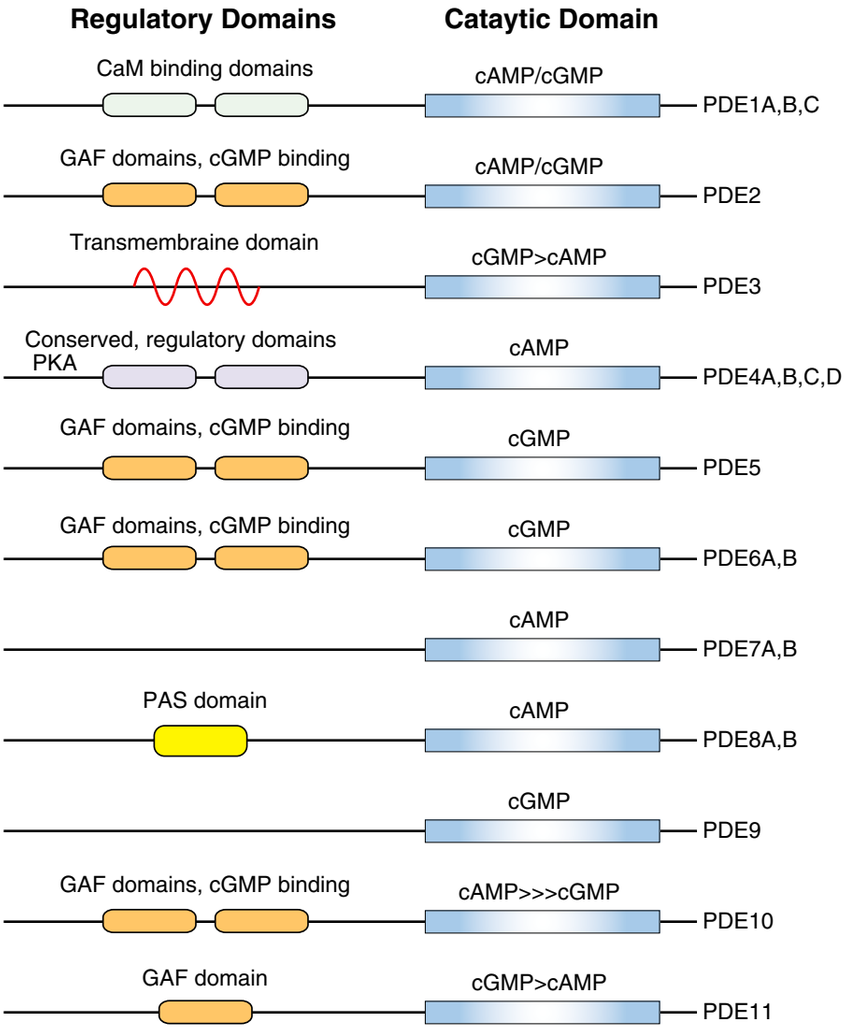

FIGURE 21-6 Schematic illustration of the overall structure and regulatory sites of eleven different phosphodiesterase subtypes. The catalytic domain of the phosphodiesterases are relatively conserved, and the preferred substrate(s) for each type is shown. The regulatory domains are more variable and contain the sites for binding of Ca^{2+}/calmodulin (*CaM*) and cGMP, as well as GAF and PAS domains. The regulatory domains also contain sites of phosphorylation by cAMP-dependent protein kinase (*PKA*).

binding site on PDE1. Similarly, those PDEs that possess high affinity cGMP-binding sites that apparently serve an allosteric function (i.e. PDE2, PDE5, PDE6, PDE10 and PDE11) share a GAF domain. There are also several phosphorylation sites within this region in PDE1, PDE3, PDE4 and PDE6. There is evidence for an additional phosphorylation site in the carboxy terminus of certain PDE4 isozymes that is a substrate for the extracellularly regulated protein kinase (ERK).

The association of PDE isozymes with the cell membrane is mediated by a conserved, hydrophobic sequence in the amino terminus of the proteins. This has been most convincingly demonstrated for PDE4A: when the amino terminus is removed PDE4A is no longer localized to the membrane fraction. A similar amino terminus sequence is found in the membrane-bound forms of PDE2, and may also mediate the membrane-association of certain PDE3 isozymes.

In addition to the numbers of distinct genes that encode the 11 described families of PDEs, there is evidence that multiple protein products can be derived from individual genes. This has been established for the PDE1, PDE2, PDE4, PDE8 and PDE9 genes. These multiple forms provide yet another mechanism to increase the diversity of PDEs expressed in different tissues or under various biological conditions. For example, the four PDE4 genes give rise to at least 17 variants as a result of alternative splicing or the presence of multiple promoter start sites within the genes [41]. Many of these variants are conserved across species, which suggests that they may have functional importance.

While the physiological significance of most of these PDE4 variants has not been fully appreciated, certain variants have been shown to differ dramatically with respect to their regulatory properties. Short and long forms of PDE4D are generated by alternative splicing. Expression of the short forms (D1 and D2, 67–72 kDa) results from activation of an intronic promoter, whereas expression of the long form (D3, 93 kDa) results from activation of another promoter located further upstream. The long form, but not the short forms, contains a site for phosphorylation and is regulated by protein kinase A [53]. Although the short forms are not regulated by phosphorylation, their expression is increased at the transcriptional level by cAMP [54]. The PDE4A-C genes also have splice variants that give rise to short and long forms that may be regulated in a similar manner. The activity of the long, but not the short, form is enhanced by phosphatidic acid and phosphatidylserine.

One of the primary mechanisms for regulation of phosphodiesterase enzyme activity is phosphorylation. PDE1 is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II), which results in decreased affinity of this enzyme for Ca^{2+}/calmodulin and an increase in the concentration of Ca^{2+} needed for its activation. PDE1 is also phosphorylated by protein kinase A, which likewise decreases its binding to Ca^{2+}/calmodulin. The inhibition of PDE1 by protein kinase A could serve to sustain intracellular cAMP levels under certain physiological conditions.

Protein kinase A also phosphorylates PDE2, PDE3 and PDE4, although the function of these enzymes is thereby influenced in different ways. Only the particulate form of PDE2 is phosphorylated by the protein kinase, but this is not known to influence enzyme activity. Phosphorylation of PDE3 by protein kinase A in rat adipocytes stimulates the catalytic activity of the enzyme. PDE3 in these cells is also phosphorylated and activated by an insulin-activated kinase, which has not yet been identified with certainty.

PDE4 is similarly activated upon phosphorylation by protein kinase A. As discussed above, only the long form of PDE4 is phosphorylated; the short forms lack the amino terminus that contains the phosphorylation site. This provides a mechanism for rapid and readily reversible activation of PDE4 (by phosphorylation) as well as more long-term and sustained regulation of PDE4 (by gene expression). Certain PDE4 isozymes are reported to be phosphorylated by protein kinase C and MAP-kinases, and in response to insulin, although these phosphorylation reactions are incompletely characterized and the functional consequences are not yet well-established.

Phosphodiesterase inhibitors show promise as pharmacotherapeutic agents. This may not be surprising given the widespread role of cyclic nucleotides in the regulation of cell function [40, 41]. The best examples of drugs that influence PDEs are the methylxanthines; these drugs are used therapeutically in the treatment of obstructive pulmonary disease and are the mild stimulants present in coffee, tea and related substances. Inhibition of PDE contributes to some of the clinical effects of these drugs. The widespread use of PDE5 inhibitors in the treatment of erectile dysfunction was mentioned earlier.

Other examples of PDE inhibitors with possible clinical usefulness are inhibitors of PDE3 or PDE4 (Table 21-1). Based on the localization of PDE3 to heart and vascular tissue and the role of cAMP in regulating heart muscle contraction and smooth muscle relaxation, a large number of PDE3 inhibitors have been developed for possible clinical applications in cardiovascular medicine.

Inhibitors of PDE4 have been developed as cognitive-memory enhancing agents and as possible antidepressants. As discussed for adenylyl cyclases, experimental evidence *in vitro* and *in vivo* demonstrates a role for the cAMP cascade in learning and memory, and inhibition of PDE4 has proved to be an effective approach to enhancing learning and memory in these models [55] (see Ch. 53). Further evidence for the importance of PDE4 isozymes in neuronal function comes from the *dunce* mutation in *Drosophila*. This mutation, which results in learning and memory deficits, involves loss of function of a cAMP-specific PDE that is functionally homologous to PDE4.

The rationale for the use of PDE4 inhibitors for the treatment of depression comes from the observations that

many types of antidepressant treatments appear to increase cAMP function in brain (see also Ch. 55). It is speculated that persistent increases in cAMP function may lead to some of the long-term adaptive changes in the brain thought to underlie the antidepressant effects of these agents [56]. Although early clinical trials demonstrated that PDE4-specific inhibitors have antidepressant efficacy, these drugs also produce unwanted side-effects that have limited their use. The presence of multiple PDE4 subtypes in brain raises the possibility that there may be developed more selective inhibitors that retain antidepressant actions and avoid unwanted side-effects.

FUNCTIONAL ROLES FOR CYCLIC cAMP AND CYCLIC cGMP

Over the last four decades, cAMP has been shown to serve as an intracellular second messenger for numerous extracellular signals in the nervous system. In fact, the number of functional processes regulated by cAMP is too large to enumerate here in detail. It is important, however, to review the general types of effects that cAMP exerts in neurons.

Cyclic AMP can be viewed as subserving two major functions in the nervous system. First, cAMP mediates some short-term aspects of synaptic transmission: some rapid actions of certain neurotransmitters on ion channels that do not involve ligand-gated channels are mediated through cAMP.

Second, cAMP, along with other intracellular messengers, plays a central role in mediating other aspects of synaptic transmission: virtually all other effects of neurotransmitters on target neuron functioning, both short-term and long-term, are achieved through intracellular messengers. This includes regulation of the general metabolic state of the target neurons, as well as modulatory effects on their neurotransmitter synthesis, storage and release, neurotransmitter receptor sensitivity, cytoskeletal organization and structure, and neuronal growth and differentiation. This also includes those long-term actions of neurotransmitters that are mediated through alterations in neuronal gene expression.

It is important to emphasize that such a role for cAMP and other intracellular messengers is not limited to actions of neurotransmitters mediated via G-protein-coupled receptors. Thus, although activation of ligand-gated ion channels leads to initial changes in membrane potential independent of intracellular messengers, activation of ligand-gated ion channels also leads to numerous additional (albeit slower) effects that are mediated via intracellular messengers. For example, activation of certain glutamate receptors (which are ligand-gated ion channels) leads rapidly to membrane depolarization and more slowly to increases in cellular levels of cAMP (e.g. by activation of Ca^{2+}/calmodulin-sensitive forms of adenylyl cyclase). cAMP then mediates several other effects of glutamate on the neurons. By virtue of numerous interactions between cAMP and other intracellular messenger cascades, these pathways play the central role in coordinating a myriad of neuronal processes and adjusting neuronal function to environmental cues [57].

Most of the effects of cyclic AMP on cell function are mediated via protein phosphorylation. By far the most important mechanism by which cAMP exerts its myriad physiological effects is through the specific activation of protein kinase A. This was first demonstrated by Edwin Krebs and his co-workers for cAMP regulation of glycogenolysis and shortly thereafter shown to be a widespread mechanism in nervous system by Paul Greengard and his colleagues. Indeed, cAMP-dependent protein kinase is now known to phosphorylate virtually every major class of neural protein; this accounts for the ability of cAMP to influence so many diverse aspects of neuronal function. The ability of cAMP to activate protein kinases, and the role of protein phosphorylation in the regulation of neuronal function, is covered in greater detail in Chapter 23.

How do a wide variety of neurotransmitters and hormones produce tissue- and cell-specific biological responses, if many such responses are mediated by the same intracellular messengers, cAMP and cAMP-dependent protein kinase? Specificity is achieved at two levels: at the level of tissue-specific receptors for the neurotransmitter or hormone and at the level of tissue-specific substrate proteins for the protein kinase. Only tissues that possess specific receptors will respond to a certain neurotransmitter or hormone. Moreover, since all cells contain very similar catalytic subunits of protein kinase A (see Ch. 23), the nature of the proteins that are phosphorylated in a given tissue depends on the types and amounts of proteins expressed in that tissue and on their accessibility to the protein kinase.

There are important exceptions to the rule that the physiological effects of cAMP in mammals are achieved via the activation of protein kinase A. The most prominent is a class of ion channels, referred to as HCN (hyperpolarization cyclic nucleotide gated) channels, that directly bind, and are thereby gated by, cAMP. Several forms of HCN channel are widely expressed in brain and peripheral tissues and have been implicated in the regulation of many facets of cell function.

The mechanisms by which cyclic GMP produces its physiological effects are more varied. It has been more difficult to identify second messenger actions of cGMP compared to cAMP. This probably reflects the lower concentrations of cGMP in most tissues and the likelihood that cGMP plays a less widespread role in cell function.

Nevertheless, physiological actions of cGMP are being identified. The best studied action is in the retina (see above), where cGMP mediates the effects of light on cation channels in rod outer segments by directly binding

to and gating the channels. In addition, cGMP has been shown to activate and inhibit specific forms of PDE, as discussed earlier in this chapter, also through direct binding to the enzymes.

In addition to such direct actions of cGMP on effector proteins, many physiological effects of cGMP are probably mediated via the activation of cGMP-dependent protein kinase (protein kinase G) and the subsequent phosphorylation of specific substrate proteins (see Ch. 23). For example, the ability of neurotransmitters to influence certain ion channels in target neurons is mediated through increased cellular levels of cGMP, the activation of protein kinase G and the subsequent phosphorylation of the channels (or some associated protein) by the protein kinase. As another example, in certain neuronal cell types, neurotransmitters that increase cGMP levels, through the activation of protein kinase G and the phosphorylation and activation of dopamine-and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32, an inhibitor of protein phosphatase1 discussed in Ch. 23), would alter the phosphorylation state of the numerous proteins dephosphorylated by this protein phosphatase.

FUTURE PERSPECTIVES

Although tremendous progress has been made in characterizing the enzymes that control the synthesis and metabolism of cyclic nucleotides in the nervous system, our understanding of the regulation and interaction of these systems is far from complete. Work is now needed to definitively characterize, for each of these enzyme subtypes, its unique distribution pattern in the brain as well as its distinct functional and regulatory properties.

Future studies of cyclic nucleotide-mediated and other signal transduction pathways in the brain will utilize recent advances in molecular and cellular neurobiology to determine the role of these pathways in coordinating the functions of individual neurons and of synaptic connections among neurons. These approaches will be combined with gene mutation technology to more fully probe the function of specific signal transduction proteins in neuronal function and complex behavior. Characterization of these signal transduction pathways will ultimately elucidate how dysfunction of these systems contributes to psychiatric and neurological disorders.

REFERENCES

1. Cooper, D., Mons, N. and Karpen, J. W. Adenylyl cyclases and the interaction between calcium and cAMP signaling. *Nature* 374: 421–424, 1995.
2. Sunahara, R., Dessauaer, C. W. and Gilman, A. G. Complexity and diversity of mammalian adenylyl cyclases. *Ann. Rev. Pharmacol. Toxicol.* 36: 461–480, 1996.
3. Cali, J., Zwaagstra, J. C., Mons, N., Cooper, D. M. F. and Krupinski, J. Type VIII adenylyl cyclase-A Ca^{+2}/calmodium-stimulated enzyme expressed in discrete regions of rat brain. *J. Biol. Chem.* 269: 12190–12195, 1994.
4. Feinstein, P., Schrader, K. and Bakalyar, H. Molecular cloning and charactirization of a Ca^{+2}/calmodulin-insensitive adenylyl cyclase from rat brain. *Proc. Natl Acad. Sci. U.S.A.* 88: 10173–10177, 1991.
5. Mons, N., Yoshimura, M. and Cooper, D. Discrete expression of Ca^{+2}/calmodulin-sensitive and Ca2+-insensitive adenylyl cyclases in the rat brain. *Synapse* 14: 51–59, 1993.
6. Wang, H. and Storm, D. R. Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. *Mol. Pharmacol.* 63: 463–468, 2003.
7. Wu, Z., Thomas, S. A., Villacres, E., Xia, Z. and Simmons, M. Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. *Proc. Natl Acad. Sci. U.S.A.* 92: 220–224, 1995.
8. Bakalyar, H. and Reed, R. R. Identification of a specialized adenylyl cyclase that may mediate odorant detection. *Science* 250: 1403–1406, 1990.
9. Gao, B. and Gilman, A. G. Cloning and expression of a widely distributed (type IV) adenylyl cyclase. *Proc. Natl Acad. Sci. U.S.A.* 88: 10178–10182, 1991.
10. Glatt, C. and Synder, S. H. Cloning and expression of an adenylyl cyclase localized to the corpus striatum. *Nature* 361: 536–538, 1993.
11. Pieroni, J., Miller, D., Premont, R. T. and Lyengar, R. Type-5 adenylyl cyclase distribution. *Nature* 363: 679, 1993.
12. Krupinski, J., Lehman, T. C., Frankenfield, C. D., Zwaagstra, J. C. and Watson, P. A. Molecular diversity in the adenylyl cyclase family. *J. Biol. Chem.* 267: 24858–24862, 1992.
13. Hellevuo, K., Yoshimura, M., Mons, N., Hoffman, P. L., Cooper, D. M. F. and Tabakoff, B. The characterization of a novel human adenylyl cyclase which is present in brain and other tissues. *J. Biol. Chem.* 270: 11581–11589, 1995.
14. Watson, P., Krupinski, J., Kempinski, A. M. and Frankenfield, C. D. Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 lymphoma cells. *J. Biol. Chem.* 269: 28893–28898, 1994.
15. Krupinski, J., Coussen, F. and Bakalyar, H. Adenylyl cyclase amino acid sequence; possible channel- or transporter-like structure. *Science* 244: 1558–1564, 1989.
16. Sharma, R. and Wang, J. H. Differential regulation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozzymesdby cyclic AMP-dependent protein kinase and calmodulin-dependent protein phosphatase. *Proc. Natl Acad. Sci. U.S.A.* 82: 2603–2607, 1986.
17. Sunahara, R. and Taussig, R. Isoforms of mammalian adenylyl cyclase. *Mol. Interv.* 2: 168–184, 2002.
18. Tang, W. and Gilman, A. G. Type-specific regulation of adenylyl cyclase by protein βγ subunits. *Science* 254: 1500–1503, 1991.
19. Wickman, K. and Clapman, D. E. Ion channel regulation by G proteins. *Physiol. Rev.* 75: 865–685, 1995.
20. Premont, R., Jacobowitz, O. and Iyengar, R. Lowered responsiveness of the catalyst of adenylyl cyclase to stimulation by Gs in heterologous desensitization: a role for cAMP dependent phosphorylation. *Endocrinology* 131: 2774–2783, 1992.

21. Duman, R. and Enna, S. J. Modulation of receptor-mediated cyclic AMP production in brain. *Neuropharmacology* 26: 981–986, 1987.
22. Villacres, E., Wong, S. T., Chavkin, C. and Storm, D. R. Type I adenylyl cyclase mutant mice have impaired mossy fiber long-term potentiation. *J. Neurosci.* 18: 3186–3194, 1998.
23. Wang, H., Pineda, V. V., Chan, G. C. K., Wong, S. T., Muglia, L. J. and Storm, D. R. Type 8 adenylyl cyclase is targeted to excitatory synapses and required for mossy fiber long-term potentiation. *J. Neurosci.* 23: 9710–9718, 2003.
24. Watts, V. Molecular mechanisms for heterologous sensitization of adenylate cyclase. *J. Pharmacol. Exp. Ther.* 302: 1–7, 2002.
25. Nestler, E. Molecular basis of neural plasticity underlying addiction. *Nat. Rev. Neurosci.* 2: 119–128, 2001.
26. Chao, J. R., Ni, Y. G., Bolanos, C. A. *et al.* Characterization of the mouse adenylyl cyclase type VIII gene promoter: Regulation by cAMP and CREB. *Eur. J. Neurosci.* 16: 1284–1294, 2002.
27. Matsuok, I., Maldonado, R., Defer, N., Noel, F., Hanoune, J. and Roques, B. Chronic morphine administration causes region-specific increase of brain type VIII adenylyl cyclase mRNA. *Eur. J. Pharmacol.* 268: 215–216, 1994.
28. Coli, S., Chang, H.-C., Mollner, S. *et al.* Chronic lithium regulates the expression of adenylate cyclase and Gia in rat cerebral cortex. *Proc. Natl Acad. Sci. U.S.A.* 88: 10634–10637, 1991.
29. Koesling, D., Bohme, E. and Schultz, G. Guanylyl cyclases, a growing family of signal-transducing enzymes. *FASEB J.* 5: 2785–2792, 1991.
30. McDonal, L. and Murad, F. Nitric oxide and cyclic GMP signaling. *Soc. Exp. Biol. Med.* 1–6,1995.
31. Nakane, M. and Murad, F. Cloning of guanylyl cyclase isoforms. *Adv. Pharmacol.* 26: 7–18, 1994.
32. Yuen, P. and Garbers, D. L. Guanylyl cyclase-linked receptors. *Physiol. Rev.* 75: 865–885, 1995.
33. Tremblay, J., Desjardins, R., Hum, D., Gutkowska, J. and Hamet, P. Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. *Mol. Cell. Biochem.* 230: 31–47, 2002.
34. Kuhn, M. Structure, regulation and function of mammalian membrane guanylyl cyclase receptors, with a focus on guanylyl cyclase-A. *Circ. Res.* 93: 700–709, 2003.
35. Krumenacker, J., Hanafy, K. A. and Murad, F. Regulation of nitric oxide and soluble guanylyl cyclase. *Brain Res. Bull.* 62: 505–515, 2004.
36. Bredt, D. and Snyder, S. H. Nitric oxide, a novel neuronal messenger. *Neuron* 8: 3–11, 1992.
37. Hope, B., Michael, G. J., Knigge, K. M. and Vincent, S. R. Neuronal NADPH diaphorade is a nitric oxide synthase. *Proc. Natl Acad. Sci. U.S.A.* 88: 2811–2814, 1991.
38. Marlett, M. Nitric oxide synthase: aspects concerning structure and catalysts. *Cell* 78: 927–930, 1994.
39. Garthwaite, J. and Boulton, C. Nitric oxide signaling in the central nervous system. *Ann. Rev. Physiol.* 57: 683–706, 1995.
40. Beavo, J. Cyclic nucleotide phosphodiesterases: function implications of multiple isoforms. *Physiol. Rev.* 75: 725–748, 1996.
41. Conti, M., Nemoz, G., Sette, C. and Vicini, E. Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocr. Rev.* 16: 370–389, 1995.
42. Yan, C., Bentley, J. K., Sonnenberg, W. K. and Beavo, J. A. Differential expression of the 61 kDa and 63 kDa calmodulin-dependent phosphodiesterases in the mouse brain. *J. Neurosci.* 14: 973–984, 1994.
43. Hashimoto, Y., Sharma, R. K. and Soderling, T. R. Regulation of Ca^{+2}/calmodulin-dependent cyclic nucleotide phosphodiesterase by the autophosphorylated form Ca^{+2}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* 264: 10884–10887, 1989.
44. Martinez, S., Beavo, J. A. and Hol, W. G. J. GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides. *Mol. Interv.* 2: 317–323, 2002.
45. Repaske, D., Corbin, J. G., Conti, M. and Goy, M. F. A cyclic GMP-stimulated cyclic nucleotide phosphodiesterase gene is highly expressed in the limbic system of the rat brain. *Neuroscience* 56: 673–686, 1993.
46. McPhee, I., Pooley, L., Lobban, M., Bolger, G. and Houslay, M. D. Identification, charactirization and regional distribution in brain of RPDE-6 (RNPDE4A5), a novel splice variant of the PDE4A cyclic AMP phosphodiesterase family. *Biochem. J.* 310: 965–974, 1995.
47. Hetman, J., Soderling, S. H., Glavas, N. A. and Beavo, J. A. Cloning and characterization of PDE7B, a cAMP-specific phosphodiesterase. *Proc. Natl Acad. Sci. U.S.A.* 97: 472–476, 1999.
48. Soderling, S., Bayuga, S. and Beavo, J. A. Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc. Natl Acad. Sci. U.S.A.* 96: 7071–7076, 1999.
49. Miro, X., Perez-Torres, S., Palacios, J. M., Puigdomenech, P. and Mengod, G. Differential distribution of cAMP-specific phosphodiesterase 7A mRNA in rat brain and peripheral organs. *Synapse* 40: 201–214, 2001.
50. Soderling, S. and Beavo, J. A. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr. Opin. Cell Biol.* 12: 174–179, 2000.
51. Van Staveren, W., Steinbusch, H. W. M., Markerink-Van Ittersum, M. *et al.* mRNA expression patterns of the cGMP-hydrolysing phosphodiesterases types 2, 5, and 9 during development of the rat brain. *J. Comp. Neurol.* 467: 566–580, 2003.
52. Fawcett, L., Baxendale, R., Stacey, P. *et al.* Molecular cloning and characterrization of a distinct human phosphodiesterase gene familly: PDE11A. *Proc. Natl Acad. Sci. U.S.A.* 97: 3702–3707, 2000.
53. Sette, C., Vicini, E. and Conti, M. The rat PDE3/IVd phosphodiesterase gene codes for multiple proteins differentially activated by cAMP-dependent protein kinase. *J. Biol. Chem.* 269: 18171–18174, 1994.
54. Swinne, J., Tsikalas, K. E. and Contim, M. Properties and hormonal regulation of two structurally related cAMP phosphodiesterases from the rat Sertoli cell. *J. Biol. Chem.* 266: 18370–18377, 1991.
55. Bara, M., Bourtchouladze, R., Winder, D. G., Golan, H. and Kandel, E. Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. *Proc. Natl Acad. Sci. U.S.A.* 95: 15020–15025, 1998.
56. Duman, R., Heninger, G. R. and Nestler, E. J. A molecular and cellular theory of depression. *Arch. Gen. Psychiatry* 54: 597–606, 1997.
57. Hyman, S. and Nestler, E. J. *The molecular basis of psychiatry*. American Psychiatric Press, Washington, DC, 1993.

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CHAPTER

22

Calcium

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THE CONCEPT OF Ca^{2+} AS A CELLULAR SIGNAL

In most eukaryotic cells, a large electrochemical gradient for Ca^{2+} exists across the plasma membrane. The −70 to −90 mV potential across the membrane favors the accumulation of cations, yet the cytoplasmic concentration of free ionized calcium ($[Ca^{2+}]_i$) is less than one-10,000th of that in the extracellular milieu. There are also intracellular organelles, such as the endoplasmic reticulum (ER) and secretory granules, that contain 1,000–10,000-fold greater concentrations of Ca^{2+} than the cytoplasm. It is likely that these gradients for Ca^{2+} evolved partly because of the incompatibility of a phosphate-based energy economy with the high Ca^{2+} concentrations in seawater. The system that has evolved permits rapid changes in $[Ca^{2+}]_i$ as a cellular signaling mechanism. This chapter focuses on the mechanisms of Ca^{2+} storage and compartmentation in cells and the mechanisms by which cellular Ca^{2+} signaling occurs in neurons and other cell types.

MEASUREMENT OF CELLULAR Ca^{2+} CONCENTRATIONS AND MOVEMENTS

From the earliest measurements of tissue calcium, it was clear that total calcium is largely a measure of stored calcium. Through the years, scientists have used a variety of indirect measures of $[Ca^{2+}]_i$. These include shortening of or tension in muscles; secretion from secretory cells; the activity of Ca^{2+}-dependent enzymes, most notably glycogen phosphorylase; and flux of K^+, or K^+ currents, as a reflection of Ca^{2+}-activated K^+ channels. In addition, investigators often use the radioactive calcium ion [$^{45}Ca^{2+}$] as an indirect indicator of Ca^{2+} concentrations and Ca^{2+} movements.

The development of fluorescent Ca^{2+} indicators that can be introduced easily into almost any vertebrate cell revolutionized calcium signaling research in the 1980s (Fig. 22-1) [1]. The majority of these compounds are

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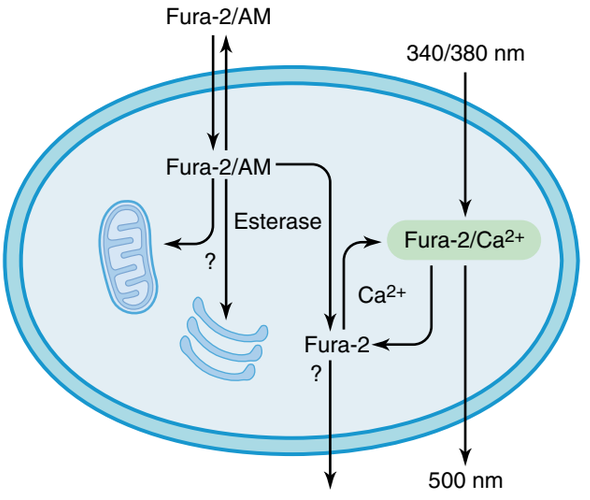

FIGURE 22-1 Introduction of esterified fluorescent Ca^{2+} indicators into cells. The fluorescent indicator, in this example fura-2, is presented to cells as the acetoxymethyl ester (*fura-2/AM*). This relatively lipid-soluble chemical permeates into the cytoplasm of cells, where esterases cleave the acetoxymethyl ester groups, liberating the Ca^{2+} binding moiety (*fura-2*). Fura-2 then reversibly combines with Ca^{2+} and, when excited alternatively with light of wavelengths 340 and 380 nm, gives rise to fluorescence at 500 nm, which can be used for quantitation of $[Ca^{2+}]_i$. Potential complications with the use of these fluorescent dyes, indicated in this figure by ?, are uptake into intracellular organelles and leak of dye from the cells. (Redrawn and modified from reference [4] with permission of Landes Bioscience.)

derivatives of the calcium ion chelator 'BAPTA', 1,2-bis (*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. A major advantage is that the wavelengths of their fluorescence maxima shift upon binding of calcium ions. This allows one to utilize these indicators in fluorescence microscope systems to obtain reasonably accurate quantitation of intracellular calcium ion concentrations.

Prior to the introduction of these synthetic Ca^{2+} indicators, a few laboratories in the 1970s employed the Ca^{2+}-activated photoproteins aequorin and obelin to study calcium dynamics. At the time, methodology restricted the broad use of this technique as a research tool. In particular it was challenging or impossible to introduce these photoproteins into the cytoplasm of most cells. However, these studies laid the groundwork for developing genetically engineered fluorescent proteins that overcome these obstacles. The introduction of green fluorescent protein (GFP) and its derivatives as genetically encoded proteins has again revolutionized the study of intracellular calcium [2]. Fluorescent proteins have been designed to incorporate many of the properties of the organic fluorescent indicators, including families with different calcium sensitivities. The same fluorescent microscope systems can be used to detect them. However, unlike the organic fluorophors, the fluorescent proteins can be introduced into cells with specific promoters and coupled to specific targeting signals, so that optical imaging using these calcium-sensitive proteins can be studied not just in single cells but also in intact tissues or organisms. The level of control in targeting these fluorescent proteins is exquisite, where expression can be restricted to a specific tissue within an organism and further restricted to a selected compartment within a cell. These genetically encoded proteins provide an important experimental approach to define intricate spatiotemporal calcium signaling processes that may often only be fully appreciated in an intact multicellular system.

Yet another method of assessing calcium ion movements in cells is by direct measurement of the current that such movement generates. Originally such currents were detected by impaling cells with microelectrodes, but most of the understanding of Ca^{2+} channel function has come from more recent work utilizing the powerful patch-clamp technique (see Ch. 6). In the whole-cell configuration, the investigator is afforded the opportunity of measuring calcium currents while manipulating both the intracellular and extracellular milieus. With excised patches, the behavior of single calcium channels can be investigated in similarly controlled environments. This approach, described in Chapter 6, has been very useful for describing voltage-activated Ca^{2+} channels [3].

Ca^{2+} REGULATION AT THE PLASMA MEMBRANE

Although there are a number of mechanisms in cells for buffering or sequestering Ca^{2+} to prevent untoward or inappropriate rises in $[Ca^{2+}]_i$, in the long term, it is the activity of plasma membrane transport processes that determines the steady-state $[Ca^{2+}]_i$. This is because the plasma membrane acts as a Ca^{2+} buffer of essentially infinite capacity. This results from *in vivo* clamping of the extracellular concentration of Ca^{2+} by dietary and endocrine mechanisms. In *in vitro* experiments, this results from incubation volumes very much larger than the cell volume.

Two distinct mechanisms for controlling $[Ca^{2+}]_i$ at the plasma membrane are provided by a Ca^{2+}-ATPase pump and a Na^+/Ca^{2+} exchanger. The basic properties of both the pump and the exchanger are discussed in Chapter 5. Of the two mechanisms, the plasma membrane Ca^{2+} pump is ubiquitous in eukaryotic cells. Na^+/Ca^{2+} exchanger expression is more restricted: it is found in excitable cells, such as nerve and muscle. As its name implies, the exchanger can, at least in theory, drive extrusion of Ca^{2+} from the cell by a process coupled to the inward movement of Na^+ down its concentration gradient. However, because in resting cells the outward electrochemical gradient is so very large, there is debate as to the extent to which the exchanger actually contributes to the lowering of $[Ca^{2+}]_i$, at least under resting conditions. This debate hinges on assumptions about the stoichiometry and

driving forces for the exchanger in intact cells. It is clear that turnover of Ca^{2+} through the exchanger can be substantial, and this may be important in the rapid control of $[Ca^{2+}]_i$ during activity. The exchanger is of pharmacological interest because of the possibility that digitalis glycosides, which disturb the normal transmembrane Na^+ gradient, augment cardiac contraction by facilitating Ca^{2+} entry or delaying Ca^{2+} exit via the exchanger (see Ch. 5). It also has been suggested that the exchanger provides a rapidly responding buffer that can allow an inward movement of Ca^{2+} under conditions requiring restoration of Ca^{2+} to intracellular storage organelles [4].

The plasma membrane Ca^{2+}-ATPase pump effects outward transport of Ca^{2+} against a large electrochemical gradient for Ca^{2+}. The mechanism of the pump involves its phosphorylation by ATP and the formation of a high-energy intermediate. This basic mechanism is similar for both the plasma membrane and ER pumps; however, the structures of these distinct gene products are substantially different. As discussed below, the ER pump, sometimes called a sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, is inhibited potently by certain natural and synthetic toxins that do not affect the plasma membrane pump. The plasma membrane pump, but not the SERCA pump, is controlled in part by Ca^{2+} calmodulin, allowing for rapid activation when cytoplasmic Ca^{2+} rises.

Ca^{2+} STORES AND Ca^{2+} POOLS

Within cells, the major calcium storing, buffering and signaling organelle appears to be the ER or, as discussed below, a specialized compartment of it. Other specialized structures that may be involved in calcium storage include the mitochondria and the nucleus. There was, for a time, a rather widely held belief that mitochondria represented the major source of signaling calcium that was released by hormone or neurotransmitter receptor activation. Perhaps the most convincing demonstration that mitochondria were not involved primarily in hormonal responses came with the discovery of inositol 1,4,5-trisphosphate (IP_3, or, to distinguish it from other positional isomers, $I(1,4,5)P_3$), as the mediator of intracellular release of Ca^{2+} (reviewed in [5, 6] and discussed below; see also Ch. 20). IP_3 was clearly shown to act only on nonmitochondrial stores. As endoplasmic reticulum is the principal nonmitochondrial calcium storage compartment, this was taken as evidence that ER is the critical storage site for signaling calcium. Whether this is a property of generic ER or of a specialized ER component is still a matter of some debate and is discussed in more detail in a subsequent section.

The only known mechanism for Ca^{2+} accumulation by the endoplasmic reticulum is by means of sarcoendoplasmic reticulum Ca^{2+}-ATPase SERCA pumps. Inside the lumen of the ER, the Ca^{2+} storage capacity is enhanced substantially by one or more low-affinity calcium binding proteins. In muscle, this protein is calsequestrin, while in most other cells the major protein is calreticulin. Each molecule of calreticulin has multiple Ca^{2+}-binding sites with an affinity in the high micromolar range and one site with an affinity in the nanomolar range. The significance of this high-affinity site is not known.

The functions of the calcium-storage capacity of the ER are at least threefold: the association of Ca^{2+} with Ca^{2+}-binding proteins in the ER is part of a chaperone function that is essential for normal protein synthesis; the rapid rate of Ca^{2+} uptake by endoplasmic pumps provides short-term cytoplasmic Ca^{2+} buffering that resists untoward and transient changes in $[Ca^{2+}]_i$; and, finally, many signaling pathways employ elevated $[Ca^{2+}]_i$ to activate physiological processes. Extensive Ca^{2+} release from ER is coupled to activation of Ca^{2+} entry across the plasma membrane, a process known as capacitative calcium entry, which is discussed below.

Mitochondria may accumulate Ca^{2+} by an energy-dependent process. Unlike the ER, mitochondria do not employ a specific Ca^{2+} transporter, but rather Ca^{2+} is accumulated via an electrogenic Ca^{2+} channel. The energy for driving accumulation of Ca^{2+} comes from the substantial mitochondrial membrane potential generated by the proton gradient established by the electron transport chain that also couples respiration to ATP synthesis. Experimentally, Ca^{2+} accumulation can be driven either by mitochondrial substrate oxidation or by ATP hydrolysis. In the latter case, H^+ accumulation and membrane potential generation occurs through reversal of the ATP synthetase. Drugs that interfere with electron transport, such as antimycin, or that collapse the proton gradient, such as protonophors, also block mitochondrial Ca^{2+} uptake. Experiments employing either subcellular fractions, permeable cells or *in situ* imaging of sequestered Ca^{2+} have all demonstrated that the kinetics of Ca^{2+} uptake do not permit significant accumulation of Ca^{2+} by mitochondria under resting conditions (Fig. 22-2). However, once the threshold for uptake is reached, the driving force for Ca^{2+} accumulation is substantial, and if $[Ca^{2+}]_i$ is elevated for a prolonged period of time, mitochondria can accumulate Ca^{2+} to remarkable concentrations. In recent years, Ca^{2+}-sensitive proteins, based on aequorin and GFP, have been specifically directed to mitochondria. These studies have revealed that mitochondria can accumulate Ca^{2+} rapidly and efficiently without any apparent prolonged period of $[Ca^{2+}]_i$ elevation. In reconciling these observations with the physiology of the mitochondrial Ca^{2+} uptake mechanism, it is suggested that there can be close apposition of mitochondria to ER Ca^{2+} release channels [7]. In these cases the local $[Ca^{2+}]_i$ is higher than that measured in the bulk of the cytoplasm and is above the threshold for uptake into the mitochondria. Such a spatial relationship may also exist between mitochondria and the plasma membrane. These latter observations suggest

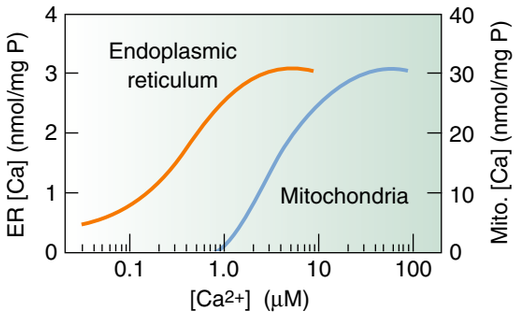

FIGURE 22-2 ATP-dependent uptake of calcium into endoplasmic reticulum and mitochondria as a function of extraorganellar Ca^{2+} concentration. As $[Ca^{2+}]_i$ was raised, ATP-dependent Ca^{2+} uptake into the endoplasmic reticulum (ER) and mitochondrial pools increased. Data for ER uptake were determined as the amount of Ca^{2+} [$^{45}Ca^{2+}$] taken up by saponin-permeabilized hepatocytes after addition of ATP and in the presence of mitochondrial inhibitors. The curve for mitochondrial uptake was obtained by subtracting the ATP-dependent uptake in the presence of these inhibitors from that in the absence of inhibitors. For additional details consult Burgess *et al.* [31]. Stylized data taken from results originally reported in Burgess *et al.* [31]. (Redrawn and modified from reference [4] with permission of Landes Bioscience.)

potential physiological roles of mitochondrial Ca^{2+} uptake, such as protecting the cell from deleterious effects of prolonged $[Ca^{2+}]_i$ elevation, or shaping spatiotemporal Ca^{2+} responses during receptor activation. Although Ca^{2+} uptake by mitochondria is electrogenic, Ca^{2+} efflux is neutral, an exchange for either $2H^+$ or $2Na^+$, and proceeds more slowly than cytoplasmic and plasma membrane buffering. Thus, Ca^{2+} accumulated during periods of prolonged stress is released to the cytoplasm at a sufficiently slow rate that the excess can be removed from the cell without significantly elevating $[Ca^{2+}]_i$.

Calcium is stored at other significant sites in the cell. These include secretory granules, lysosomes and the nucleus. The Ca^{2+} in secretory granules generally has been considered to be relatively inert and to function largely in a structural capacity. However, evidence exists that this Ca^{2+} can be released during cell stimulation and can participate in the control of Ca^{2+} signaling (see NAADP discussion below) and in secretion. The nuclear pores generally prevent the development of substantial gradients of Ca^{2+} between the nucleoplasm and cytoplasm. Nonetheless, calcium does signal to the nucleus, having effects on gene transcription and cell growth, with elevations of nuclear Ca^{2+}. Although originally thought to occur by diffusion through nuclear pores from the bulk cytoplasm, it is now recognized that extensions of endoplasmic reticulum, complete with Ca^{2+} release sites, occur in the nuclear envelope. A reticular network of nuclear calcium stores has been identified in an epithelial cell line [8]. The locality of such Ca^{2+} stores in and around the nucleus suggests that cell stimulation can lead to discharge of Ca^{2+} directly into the nucleoplasm, allowing local control of calcium signals and nuclear function independent of changes in cytoplasmic $[Ca^{2+}]_i$.

Ca^{2+} SIGNALING

The processes of calcium signaling consist of the molecular and biophysical events that link external stimuli to the expression of appropriate intracellular responses by means of increases in cytoplasmic Ca^{2+}. The external signal is most commonly a neurotransmitter, hormone or growth factor but, in the case of excitable cells, the initial chemical stimuli may bring about membrane excitation, which in turn activates a calcium-signaling pathway.

Release of intracellular Ca^{2+} is mediated primarily via inositol 1,4,5-trisphosphate receptors and ryanodine receptors. The calcium ions that give rise to a $[Ca^{2+}]_i$ signal can come either from intracellular stores or from external Ca^{2+} entering across the plasma membrane. Typically, both sources are utilized. The most ubiquitous of the intracellular Ca^{2+} release mechanisms involves the phosphoinositide specific phospholipase C (PI-PLC)-derived second messenger IP_3, which acts by binding to a specific receptor on the ER or a specialized component of the ER. The IP_3 receptor/channel appears to be a homotetramer containing four binding sites for IP_3. Distinct subtypes of the receptor exist, representing products of at least three distinct genes, and additional forms arise as a result of alternative splicing. The origin of IP_3 and the characteristics of IP_3 are discussed in Chapter 20.

The interaction of IP_3 with its receptor involves complex and poorly understood regulatory interactions among the receptor, IP_3 and Ca^{2+} – the latter apparently exerting influence from both the cytoplasmic and luminal aspects of the receptor. Ca^{2+} in the lumen of the ER appears to sensitize the receptor to IP_3. On the cytoplasmic surface, low concentrations of Ca^{2+} sensitize while higher concentrations are inhibitory. These actions may contribute to the 'all-or-none' oscillatory behavior of $[Ca^{2+}]_i$ signals seen in some cell types. This phenomenon will be discussed in more detail below.

The other major type of intracellular Ca^{2+}-mobilizing receptor is the ryanodine receptor. The ryanodine receptor is named for a toxin that binds to the molecule with high affinity and was employed in its purification and characterization. Like the IP_3 receptor, it is a homotetramer, and these receptors share considerable structural homology [9]. In its most specialized setting, in skeletal muscle, the ryanodine receptor is gated by a direct conformational interaction with a dihydropyridine receptor in the *t*-tubule membrane (see Ch. 43). This coupling permits rapid release of stored Ca^{2+} when an action potential invades the *t*-tubule system. However, the physiological ligand for the ryanodine receptor is usually Ca^{2+} itself; i.e. it is considered to be a calcium-induced calcium release (CICR) Ca^{2+} channel (see also Ch. 20). Because Ca^{2+} can

sensitize the IP_3 receptor to IP_3, the IP_3 receptor also can exhibit CICR behavior. However, some IP_3 is always required for its action, whereas the ryanodine receptor can function as a 'pure' CICR receptor. Although the ryanodine receptor is thought to be regulated primarily by CICR, a small water-soluble molecule, cyclic adenosine diphosphate ribose (cADPr), functions as a regulatory ligand for at least some forms of the ryanodine receptor [10]. cADPr functions in a manner somewhat similar to IP_3: its principal site of action is the ER and it appears to increase the opening probability of the ryanodine receptor channel by increasing its sensitivity to Ca^{2+}. Another water-soluble molecule, synthesized by the same family of enzymes that generate cADPr, is nicotinic acid-adenine dinucleotide phosphate (NAADP). NAADP can also mobilize intracellular Ca^{2+} but, unlike cADPr, it does not appear to modulate CICR channels. The principal locations of the NAADP receptor appear to be secretory granules and/or lysozymes. While the NAADP receptor does not exhibit CICR activity itself, the Ca^{2+} released from this Ca^{2+} pool may promote a CICR response by interacting with calcium release channels on the ER. It is tempting to speculate that Ca^{2+} signaling in neurons may be regulated by changing concentrations of cADPr and NAADP, and there is limited evidence for this suggestion [11]. In at least one cell type, the IP_3 receptor, cADPr-sensitive ryanodine receptor and NAADP receptor seem to function in a cooperative manner to promote neurosecretion at the frog neuromuscular junction [12].

Ca^{2+} can enter cells via voltage- or ligand-dependent channels and by capacitative entry. These three fundamental mechanisms of regulated calcium ion entry across the plasma membrane involve, respectively, voltage-dependent Ca^{2+} channels, ligand-gated Ca^{2+} channels and capacitative Ca^{2+} entry associated with phospholipase C-coupled receptors.

Voltage-dependent Ca^{2+} channels are found in a variety of excitable cell types, including neurons, myocytes, endocrine cells and neuroendocrine, such as chromaffin, cells [3] but they are almost never found in classical 'nonexcitable' cells, such as epithelial cells, leukocytes and fibroblasts. By definition, voltage-dependent channels can be activated by membrane depolarization. In addition, they are subject to complex combinations of voltage-dependent activation and voltage-dependent or calcium-mediated inactivation mechanisms similar to the voltage-dependent regulation of Na^+ channels described by Hodgkin and Huxley (see Ch. 6). These are the Ca^{2+} channels that provide the activator Ca^{2+} for cardiac contractility, for contraction of some smooth muscle types and, generally, for discharge of neurotransmitters by Ca^{2+}-dependent exocytosis (see Ch. 9) and synaptic transmission (see Ch. 10). In most instances, their activation is initiated by membrane depolarization, whether by propagated action potential or by the opening of other ligand-gated channels. However, in some cases, the channels are activated by removing an inhibitory influence, such as a decrease in a hyperpolarizing K^+ conductance, or by sensitizing the channels to activation at resting membrane potential through their phosphorylation.

There are at least five different types of voltage-dependent Ca^{2+} channel molecules, differing in their gating kinetics, modes of Ca^{2+}-inactivation and Ca^{2+}-iregulation, and sensitivity to specific marine toxins [13] (see Ch. 6). The distinctions between the types of channel are of considerable interest because the different subtypes are believed to subserve different cellular functions. For example, the control of neurotransmitter release in peripheral sympathetic neurons appears to be under the predominant control of N-type calcium channels.

Ligand-gated ion channels are numerous, and some permit sufficient entry of Ca^{2+} for cellular activation. Here, ligand-gated channels denote channels that are gated directly by agonist binding rather than through the generation of second messengers. Generally the ligand-binding sites are located on the channel proteins, which are invariably nonspecific cation channels; as yet no receptor-gated channels specific for Ca^{2+} have been clearly identified. Ligand-gated ion channels are discussed in detail in Chapters 10, 11, 13, 15–17.

Capacitative Ca^{2+} entry is the predominant mode of regulated Ca^{2+} entry in nonexcitable cells but it also occurs in a number of excitable cell types. This pathway of Ca^{2+} entry is usually associated with the activation of phospholipase C, which mediates the formation of IP_3 (see Ch. 20). Intracellular application of IP_3 mimics the ability of hormones and neurotransmitters to activate calcium ion entry, and activation of calcium ion entry by hormones and neurotransmitters can be blocked by intracellular application of low-molecular-weight heparin, which potently antagonizes IP_3 binding to its receptor. There is considerable evidence for the presence of an IP_3 receptor in the plasma membrane of some cells types. $I(1,3,4,5)P_4$, a product of IP_3 phosphorylation, has been shown in some cells to augment this action of IP_3 in activating PM calcium ion entry, but in others IP_3 alone is clearly sufficient.

However, the current view of the regulation of calcium ion entry into the cytoplasm by PLC-linked stimuli holds that activation occurs not as a direct result of the action of IP_3 on the plasma membrane but indirectly, as a result of depletion of calcium ions from an intracellular store by IP_3 [14]. In the context of this 'capacitative model', the actions of intracellularly applied IP_3 and heparin reflect the effects of these maneuvers on intracellular release process from ER into cytosol, rather than via the plasma membrane. The reported actions of $I(1,3,4,5)P_4$, if in fact they do represent physiological control mechanisms, may reflect an ability of $I(1,3,4,5)P_4$ to augment the calcium-releasing ability of IP_3, rather than a distinct and

specific action at the plasma membrane. Originally the capacitative model for calcium ion entry was proposed on the basis of circumstantial evidence about the relative rates of emptying and refilling of intracellular stores of calcium. More direct tests of the model have arisen from the discovery of reagents, such as thapsigargin and cyclopiazonic acid, that selectively inhibit the ATP-dependent Ca^{2+} pumps that maintain the stores of intracellular calcium in the IP_3-sensitive pool (Fig. 22-3). These reagents can deplete this pool of Ca^{2+} without stimulating the formation of inositol phosphates. Numerous reports have demonstrated that Ca^{2+} entry across the plasma membrane is activated in cells treated with such agents[15]. Importantly, this Ca^{2+} entry is not affected by concomitant stimulation of inositol phosphate production.

Two general mechanisms have been considered by which a depleted intracellular Ca^{2+} pool might communicate with the plasma membrane [4]. There is evidence that the IP_3 receptor is associated with the cytoskeleton, and this association may tether the IP_3 receptor to the plasma membrane. Depletion of intracellular calcium stores might cause a conformational change in the IP_3 receptor, which could be conveyed to the plasma membrane via the cytoskeleton or by a more direct protein–protein interaction. Alternatively, signaling could occur through the action of a diffusible messenger released by the depleted intracellular calcium store and acting on calcium channels in the plasma membrane.

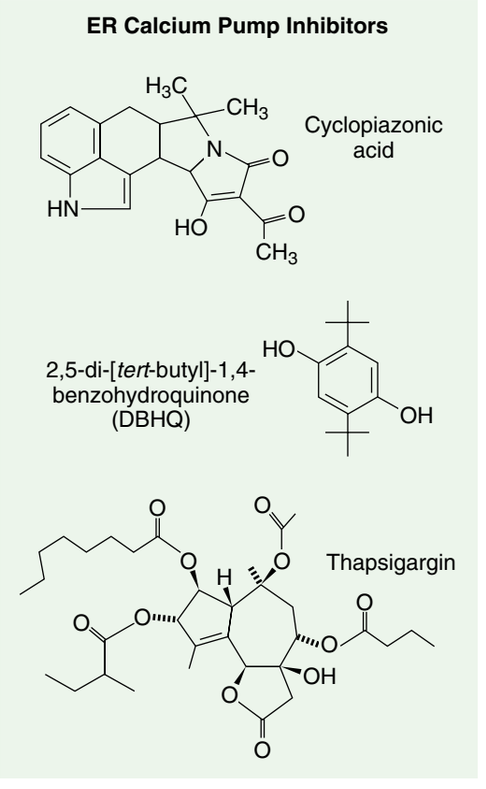

FIGURE 22-3 Structures of compounds that inhibit sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) calcium pumps.

Channels associated with capacitative calcium ion entry have been characterized electrophysiologically. In leukocytes, the current associated with the depletion of intracellular Ca^{2+} stores is highly Ca^{2+}-selective and, on the basis of noise analysis, is believed to involve minute single channels [16] (see Ch. 6). This is the calcium release-activated calcium current (I_{CRAC}). In other cell types, currents with significantly different properties have been identified, including in some instances store-operated nonselective cation channels. These marked electrophysiological distinctions may be indicative of distinct channel types mediating capacitative calcium ion entry in different cell types.

While the molecular identity of the capacitative Ca^{2+} entry channel is not known, a candidate is a homolog of the *Drosophila* mutant *trp*. This photoreceptor mutant is incapable of maintaining a sustained photoreceptor potential. This phenotype could be mimicked by the calcium entry blocker lanthanum, and it was suggested that the related defect is a failure of Ca^{2+} entry.

Now described as a *trp* superfamily, more than 20 vertebrate *trp* homologs have been identified and these can be divided phylogenetically into three subfamilies: *trpC* (termed 'canonical' as they are most closely related to *Drosophila trp*), *trpV* and *trpM*. Some of these proteins are potential contenders for the capacitative calcium ion entry channel. In particular, expression of members of the TRPC and TRPV families in mammalian cells has been shown to augment capacitative calcium ion entry, but their role is still a matter for debate [17].

A major function of capacitative calcium ion entry is to provide for sustained Ca^{2+} signaling. However, it also importantly provides a means for rapid replenishment of intracellular stores following their release by IP_3 and the cessation of agonist activation. How is this accomplished in those excitable cells that do not express capacitative calcium ion entry? The answer may be that excitable cells generally do, while nonexcitable cells generally do not, express a rapidly turning over sodium–calcium exchange transporter (see Ch. 5). This transporter could provide an energy-efficient route for rapidly adjusting cytoplasmic Ca^{2+} in response to rapid uptake demands of a depleted intracellular Ca^{2+} store. Because the resting turnover of this exchanger is relatively fast, it may be that such a pathway provides kinetic continuity of Ca^{2+} stores with extracellular Ca^{2+}, and thus serves to replenish intracellular stores in the absence of any store-dependent regulation. The relation of plasmalemma Na/Ca exchange transporter to local cytosolic Ca^{2+} concentrations and Ca^{2+} stores is discussed in Chapter 5.

Periodic temporal and spatial patterns of Ca^{2+} signaling give rise to calcium oscillations and waves. As discussed above, the IP_3 receptor is subject to complex and only

partially understood regulation by both IP_3 and calcium ion. The complicated kinetic behavior of the IP_3 receptor is believed to underlie calcium oscillations and waves. In excitable cells, oscillations in $[Ca^{2+}]_i$, reflecting periodic fluctuations in membrane electrical activity, have been known for some time. The clearest example results from the rhythmic cardiac action potentials that drive bursts of Ca^{2+} release, entry and extrusion and thereby maintain the pumping activity of the heart. However, $[Ca^{2+}]_i$ often will oscillate in cells through mechanisms that have nothing to do with surface membrane excitability. Calcium oscillations have been shown to be important regulators of cell function, including specific gene regulation. Thus, much effort has been devoted to understanding the control mechanisms giving rise to $[Ca^{2+}]_i$ waves and oscillations [18].

Intracellular calcium oscillations generally fall into one of two categories involving different mechanisms: baseline transients, or spikes, and sinusoidal oscillations. Figure 22-4 illustrates these two oscillatory patterns.

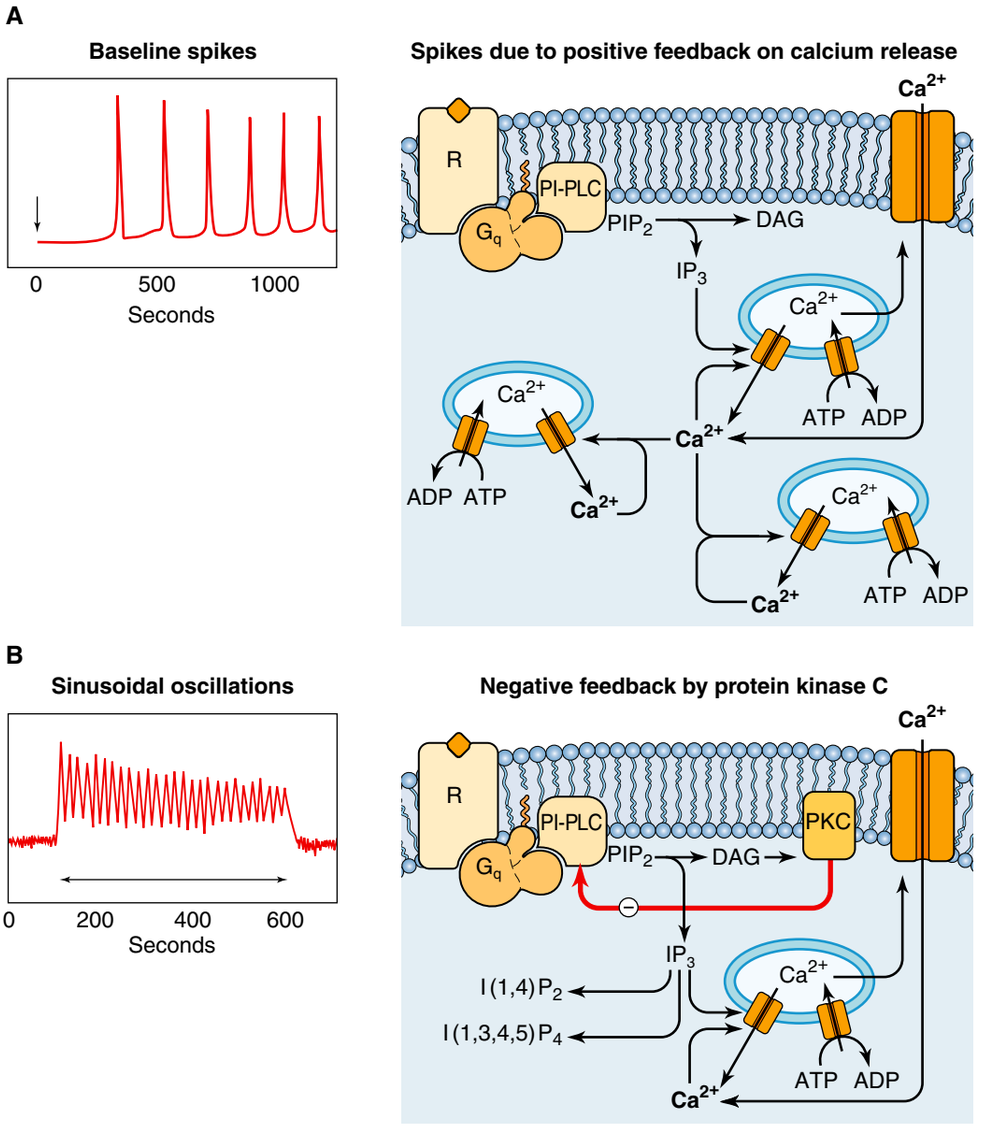

FIGURE 22-4 Patterns and mechanisms for major types of $[Ca^{2+}]_i$ oscillation. (**A**) **Left.** A single rat hepatocyte treated with vasopressin exhibits low-frequency baseline spikes of $[Ca^{2+}]_i$. **Right.** The mechanism for baseline $[Ca^{2+}]_i$ spiking is seen as being initiated by a small release of Ca^{2+} from a 'calciosome' by IP_3, and then, in the presence of a sensitizing concentration of IP_3, Ca^{2+} regeneratively activates release at neighboring release sites, leading to an all-or-none $[Ca^{2+}]_i$ wave propagating through the cell. (**B**) **Left.** A single mouse lacrimal acinar cell treated with methacholine exhibits higher frequency sinusoidal oscillations superimposed on an elevated baseline. **Right.** The mechanism underlying sinusoidal oscillations is suggested to occur as follows: phosphoinositide-specific phospholipase C (*PI-PLC*) activation leads to production of IP_3 and mobilization of Ca^{2+}, but also to protein kinase C (*PKC*) activation. With some delay, the latter inhibits PI-PLC, causing a fall in IP_3 and an attenuation of the $[Ca^{2+}]_i$ signal. The inhibition of PI-PLC also attenuates the PKC signal, leading to a cyclical increase in $[Ca^{2+}]_i$. Continuing cycling of this feedback loop gives rise to sinusoidal oscillations. *DAG*, diacylglycerol; *R*, receptor. (Redrawn and modified from reference [4] with permission of Landes Bioscience.)

Baseline spikes are characterized by rapidly-rising transient increases in $[Ca^{2+}]_i$ from a baseline of $[Ca^{2+}]_i$ that is generally quite close to the resting concentration. In contrast, sinusoidal oscillations more closely resemble true sine-wave oscillations and they are generally of a higher frequency than baseline spikes, >1/min as opposed to frequencies of <1/min for baseline spikes. They also generally appear as symmetrical oscillations superimposed on a sustained concentration of $[Ca^{2+}]_i$ that is somewhat above the prestimulus baseline. Another notable difference between the two types of oscillations is that baseline spikes may, at least in some instances, continue throughout prolonged periods of stimulation, while sinusoidal oscillations tend to diminish with time, generally lasting for only a few minutes. However, the most significant and characteristic distinction between the two types of oscillation is the relationship of the oscillation amplitude and frequency to stimulus strength, or agonist concentration. For baseline spikes, increasing the agonist concentration increases the frequency with little effect on the amplitude, while, for sinusoidal oscillations, increasing the agonist concentration increases the average $[Ca^{2+}]_i$ without affecting the frequency of the oscillations. Furthermore, for baseline spikes, but not for sinusoidal oscillations, the latency before the first $[Ca^{2+}]_i$ spike is inversely related to the agonist concentration. The mechanism underlying the varying frequency of spiking may also be responsible for the varying latency of the first spike, but this is not necessarily so. The persistent, constant amplitude baseline spikes require a *positive feedback* mechanism, sometimes called *feed-forward*, to accelerate the reaction and generate the spikes, followed by either a negative feedback or some capacity limitation, such as full depletion of an intracellular pool, to terminate each spike. However, negative feedback alone is sufficient to explain the behavior of constant-frequency sinusoidal oscillations.

In most instances, baseline spiking will continue for at least a few cycles in the absence of extracellular Ca^{2+} and therefore it is generally agreed that they represent cycles of discharge and reuptake of Ca^{2+} by intracellular stores. Although other models have been proposed, the currently favored mechanism of baseline $[Ca^{2+}]_i$ spiking involves fluctuations in $[Ca^{2+}]_i$ while cellular concentrations of IP_3 are constant (Fig. 22-4). Models along this theme require that Ca^{2+} itself provide both positive and negative regulation of the $[Ca^{2+}]_i$ signal. Important evidence for such regulation would be the demonstration of $[Ca^{2+}]_i$ spiking induced by direct intracellular application of IP_3. This has been demonstrated in oocytes and, in a few cases, in small, mammalian cells by intracellular introduction of IP_3 through whole-cell patch pipette perfusion. These findings suggest that a mechanism operates distally to the production of IP_3 to generate $[Ca^{2+}]_i$ spikes.

As discussed above, biphasic regulation of IP_3-induced Ca^{2+} release by Ca^{2+} is reminiscent of the behavior of the CICR channel from skeletal muscle. In addition, the inhibitory action of Ca^{2+} on IP_3-induced Ca^{2+} release develops more slowly than the activation, with a time constant of about 0.5 seconds. There is obvious similarity between this scheme, which involves a rapid Ca^{2+} activation of release followed by a more slowly developing Ca^{2+} inhibition, and the feed-forward and feedback regulation of action potentials by depolarization. Also as discussed above, changes in ER luminal $[Ca^{2+}]$ may regulate the sensitivity of the IP_3 receptor. Thus, these kinetically distinct modes of regulation of the IP_3 receptor by both cytoplasmic and luminal Ca^{2+} may provide the ingredients for production of regenerative spikes and waves of $[Ca^{2+}]_i$.

Much less attention has been paid to sinusoidal oscillations of intracellular Ca^{2+}, although, strictly speaking, they may represent the only instance in which the term 'oscillations' is applied correctly. These are roughly symmetrical fluctuations usually superimposed on a raised basal level of $[Ca^{2+}]_i$. The most significant characteristic of these oscillations is their constant frequency at different agonist concentrations. These sinusoidal oscillations are considerably simpler than the baseline spike type of $[Ca^{2+}]_i$ oscillation and can be explained simply by a single negative feedback on the $[Ca^{2+}]_i$-signaling mechanism. Although this may not apply in all cases, it appears that the negative feedback responsible for the sinusoidal oscillations is often due to protein kinase C (PKC). The site of negative inhibition appears to be on or proximal to PI-PLC. Activation of PI-PLC increases diacylglycerol (DAG), which in turn activates PKC. The latter feeds back and inhibits PI-PLC (see Ch. 20). This leads to a diminution in DAG production, diminished PKC activity and relief of the inhibition of PI-PLC. Continuous cycling of this delayed feedback loop generates oscillations in PI-PLC activity, which, in the absence of some feed-forward input, gradually damp down to a sustained level under tonic control by the opposing forces of receptor activation and PKC inhibition. It is important to point out that in this particular scheme $[Ca^{2+}]_i$ is simply a passive follower of the oscillating IP_3 production and plays no obvious active role in generating or modulating the oscillations. Thus, this particular type might more appropriately be called 'DAG oscillations' or 'PI-PLC/PKC oscillations' (Fig. 22-4).

Release of intracellular Ca^{2+} may occur from 'calciosomes', a subfraction of the endoplasmic reticulum. Considerable evidence suggests that the major site of calcium sequestration and the source of intracellular calcium for signaling is the ER. In addition to the points already made, subcellular fractionation studies have demonstrated positive correlations of ATP-dependent Ca^{2+} accumulation and of IP_3-mediated Ca^{2+} release with classical enzymatic markers for ER. Calcium uptake into the IP_3-sensitive store is augmented by oxalate; this augmentation is a property generally associated with the ER.

However, there are some inconsistencies with the idea that the IP_3-sensitive calcium store and the ER are entirely coincident. For one, there is really no correlation between

the quantity of ER present in a given cell type and its sensitivity to calcium signaling through IP_3. In contrast to the studies cited above, there are at least an equal number of subcellular fractionation studies in which a separation of IP_3-induced Ca^{2+} release or IP_3 binding from enzymatic markers for the ER has been observed. Perhaps the sarcoplasmic reticulum of muscle presents the best characterized paradigm for an organelle distinct from generic ER and specialized for storing and releasing calcium (see Ch. 43). A biochemical characteristic of sarcoplasmic reticulum that distinguishes it from ER is the presence of high concentrations of a high-capacity, low-affinity calcium-binding protein, calsequestrin. A similar protein, calreticulin, found in nonmuscle cells, is structurally and functionally homologous to calsequestrin. On the basis of such findings, it has been proposed that an organelle specialized for storing and releasing calcium exists and that this organelle is distinct from generic ER. It has been proposed that this organelle be designated 'calciosome' [19].

With the exception of skeletal muscle, perhaps the Purkinje cell of the cerebellum has been most extensively characterized with respect to its Ca^{2+}-releasing and -sequestering organelles. The Purkinje cells of birds are especially amenable to immunocytochemical studies because they contain much higher concentrations of IP_3 receptors than any other cell type and unusually high concentrations of SERCA pumps and ryanodine receptors. In birds, the major calcium-storage protein, calsequestrin, is almost identical to the mammalian molecule. High-affinity antibodies, which facilitate immunocytochemistry, are available. In these bird Purkinje cells, IP_3 receptor distribution in membranes is not linked tightly to SERCA pumps or even to calsequestrin. However, a population of vacuoles positive for calsequestrin as well as for either the IP_3 receptor or the ryanodine receptor was found. These structures may qualify as calciosomes since presumably they are involved in storing as well as releasing calcium ion [20]. It has been suggested that specialized stacks of ER, rich in IP_3 receptor but without substantial quantities of SERCA pumps or calsequestrin, may act as either reservoirs of receptor or buffer of cytoplasmic IP_3. Interestingly, overexpression of IP_3 receptors in fibroblasts leads to the formation of structures similar to the IP_3 receptor-containing ER stacks found in Purkinje cells.

Although the existence of a specialized subcompartment of the ER is widely accepted [20], the term 'calciosome' has not gained universal acceptance. This may be due to the fact that in many systems the distinction between the calciosome and the ER is still not clearly established [21].

Ca^{2+}-signaling events in excitable and nonexcitable cells, although distinct, share some common characteristics. Traditionally, calcium-signaling studies have been divided into two separate categories: studies focusing on excitable cells, such as nerve and muscle, and studies focusing on electrically nonexcitable cells, such as epithelial or blood cells. Although both cell types utilize the release of intracellular Ca^{2+} as one means of generating cytoplasmic calcium signals, excitable cells often accomplish this by CICR while the predominant mechanism for nonexcitable cells involves IP_3 [22] (see also Ch. 20). Also, signaling in both cell types depends to a large degree on plasma membrane Ca^{2+} channels, but the Ca^{2+} channels in the plasma membrane of nonexcitable cells do not appear to be regulated by membrane potential, and their pharmacology is quite distinct from that of the channels in excitable cells. Even so, calcium-signaling mechanisms in excitable and nonexcitable cells may be much more alike than is generally appreciated. A shared paradigm involves coordinated regulation of intracellular calcium ion release and calcium ion entry across plasma membranes [22]. For reasons probably having to do with their distinct functions, interesting distinctions as well as similarities in basic mechanisms have evolved in these two general cell types.

In electrically nonexcitable cells, Ca^{2+} signaling is typically a biphasic process. Neurotransmitters and hormones cause a release of calcium ions to the cytoplasm from an intracellular organelle, and this is followed by entry of calcium ions into the cytoplasm across the plasma membrane. The intracellular-release phase of the calcium signal is attributable to IP_3, while the second phase of the response is attributed to capacitative calcium ion entry, a process of retrograde signaling such that the empty calcium-storage organelle produces a signal for calcium ion entry across the plasma membrane.

In many instances, neurons and other electrically excitable cells also utilize the IP_3-signaling system. For example, there are smooth muscle types which function in the IP_3 mode, the voltage-dependent calcium channel and CICR mode or even combinations of the two. In some instances, when intracellular calcium ions are released by IP_3 in excitable cells, this may be coupled to capacitative calcium ion entry, but there are clear examples where this is not the case. Rather, virtually all excitable cells have plasma membrane Ca^{2+} channels that are activated by membrane depolarization. In addition, excitable cells frequently express another intracellular Ca^{2+} release channel, the ryanodine receptor (discussed above). The physiological activator of the ryanodine receptor is believed to be Ca^{2+} itself: the channel opens when the Ca^{2+} concentration in its vicinity increases rapidly, generating CICR, and this can give rise to the regenerative 'all-or-none' calcium ion release behavior for which muscle and nerve cells are noted. In heart cells, for example, Ca^{2+} signaling is initiated by membrane depolarization, which activates surface membrane voltage-gated Ca^{2+} channels. A rapid entry of calcium ions serves as a 'trigger' for activating the ryanodine receptor and, subsequently, a much larger release of intracellular calcium ions [22].

The common conceptual feature of these two calcium-signaling motifs (Fig. 22-5) is that they provide a tight coordination of calcium ion entry and intracellular

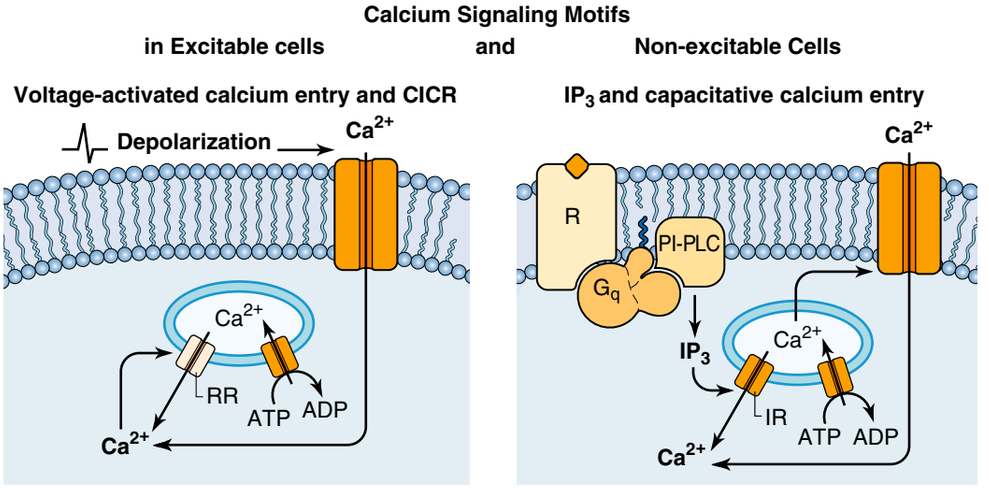

FIGURE 22-5 Motifs of $[Ca^{2+}]_i$ signaling. In electrically excitable cells (**left**), Ca^{2+} may enter when voltage-dependent Ca^{2+} channels are activated by the depolarization associated with action potentials. This Ca^{2+} can cause further release of intracellularly stored Ca^{2+} by activating a Ca^{2+}-induced Ca^{2+} release (*CICR*) mechanism associated with the ryanodine receptor (*RR*) Ca^{2+} channels. In electrically nonexcitable cells (**right**), signaling generally is initiated when an agonist activates a surface membrane receptor (*R*), which, usually, through a G-protein (G_q or G_i), activates phosphoinositide-specific phospholipase C (*PI-PLC*), which degrades phosphatidylinositol 4,5-bisphosphate, releasing the soluble messenger, inositol 1,4,5-trisphosphate (*IP_3*). IP_3 activates an IP_3 receptor (*IR*) and, thus, releases Ca^{2+} from an intracellular organelle to the cytoplasm. The release of Ca^{2+} from the organelle causes a signal to be generated, which activates a plasma membrane Ca^{2+} entry pathway, capacitative Ca^{2+} entry. (Modified with permission from reference [22].)

calcium ion release. They also provide amplification for the calcium signal but in functionally distinct ways. In excitable cells, the process of CICR amplifies the *magnitude* and *spatial distribution* of the momentary calcium signal, assuring, in the case of the heart for example, sufficient Ca^{2+} for catalyzing rapid cross-bridge formation and force development. In nonexcitable cells, the retrograde signaling provided by capacitative calcium ion entry amplifies the duration of the calcium signal, providing sustained, tonic responses of, for example, exocrine gland cells.

These characteristics of excitable and nonexcitable cells are not as distinct as once believed. For example, capacitative calcium ion entry contributes to calcium signaling in a number of excitable cells types [15]. Also, as discussed in the preceding section, the all-or-none regenerative calcium signals that occur in nerves and muscles now are known to occur in nonexcitable cells. Thus, it is interesting that while the term nonexcitable appropriately describes the electrophysiological behavior of their surface membranes, the regenerative intracellular Ca^{2+} spikes that often are exhibited by so-called nonexcitable cells constitute a clear example of excitable behavior of their intracellular milieu. This occurs because the IP_3 receptor functions as a CICR receptor whose sensitivity to Ca^{2+} is regulated by the binding of IP_3 and *vice versa*. Perhaps this is not so surprising, given the considerable homology between the amino acid sequences of the IP_3 and ryanodine receptors. Electrically nonexcitable cells generally contain a single, relatively homogeneous intracellular pool of calcium, which is sensitive to IP_3, while electrically excitable cells may have a more complex arrangement of several intracellular calcium pools, each regulated by a distinct mechanism.

Thus, there may be substantial similarity between Ca^{2+}-signaling systems in excitable and nonexcitable cells. It may be useful for neuroscientists and others traditionally focusing on excitable cells to take note of developments in the rapidly evolving field of calcium signaling and calcium channels in nonexcitable cells [22]. It is especially important to take cognizance of variations in calcium-signaling motifs in studies of the relationship between patterns of gene expression and specific calcium-signaling pathways.

Ca^{2+}-REGULATED PROCESSES

Ca^{2+} is required for acute cellular responses such as contraction or secretion. Sydney Ringer is credited with the first appreciation of the role of calcium as a regulator of cell function. He discovered that frog hearts beat longer *in vitro* if the saline solutions are made from 'hard' water rather than from distilled water, and with this observation the role of Ca^{2+} in excitation-contraction coupling was discovered [23] (see Ch. 43). Subsequently, Ca^{2+} was found to act as a signal for myriad cellular responses. It is a major component of the epithelial cell secretion signaling pathways, including both discharge of proteins and transepithelial secretion of salts and water. In the liver, Ca^{2+} regulates carbohydrate metabolism, including both glycogenolysis and gluconeogenesis (see Chs 31 and 42). In blood cells, various functions including secretion and chemotaxis are subtended by PI-PLC-dependent $[Ca^{2+}]_i$ signaling. Muscle contraction involves interaction of Ca^{2+} with calcium-binding proteins specific to the muscle type: in fast skeletal muscle, this protein is troponin and its binding of Ca^{2+} leads to disinhibition of myosin ATPase

(see Ch. 43). In other muscle types, regulation of the phosphorylation of myosin light chain is mediated by calcium.

In virtually all cases, the initial target of calcium is a specific calcium-binding protein. The most extensively characterized of these, calmodulin [24], was discovered as a regulator of cyclic nucleotide phosphodiesterase [24] (see Ch. 21) and it is now recognized as the most ubiquitous Ca^{2+}-sensing protein, found in all eukaryotic organisms including yeasts. Calmodulin has four binding sites for Ca^{2+} with dissociation constants in the 1–10 µmol/l range. Ca^{2+} binding induces a conformational change that imparts signaling information to a number of different molecules, including protein kinases and phosphatases.

Ca^{2+} also plays a role in more prolonged cellular events such as mitogenesis and apoptosis. Following mitosis, a cell must either commit to re-enter the cell cycle or exit into the quiescent state, G_0, in which a differentiated function is maintained (see Ch. 25). At a later time and with appropriate stimuli, many, but not all, cell types may re-enter the cell cycle to divide further. In circumstances in which a particular cell function is no longer needed, as in the case of the thymus, systemic signals may initiate the complex process of self-digestion and packaging, termed apoptosis [25] (apoptosis is discussed in Ch. 35). Mutations of key genes, the proto-oncogenes that control mitogenesis, can lead to inappropriate cell division or cancers. The mutated genes responsible for these transformations are called oncogenes. It is noteworthy, although perhaps not unexpected, that the vast majority of proto-oncogenes code for proteins involved in signal transduction pathways. One proto-oncogene, *bcl-2*, acts as a suppressor of apoptosis. Consequently, if it is expressed in excess, cancerous growth can result.

Ca^{2+} signaling is believed to play an important role in the regulation of cell growth and differentiation [26] as well as in apoptosis [25] (discussed below). Ca^{2+} is generally required for the activation of mitogenesis and, in many instances, increases in $[Ca^{2+}]_i$ have been associated with the actions of mitogenic agents: calcium ionophores or thapsigargin can stimulate DNA synthesis, especially if combined with a PKC activator such as an active phorbol ester. Although not proven, this action of phorbol esters may explain their well-known tumor-promoting activity.

In addition to being regulated by cytoplasmic Ca^{2+}, there is evidence that mitogenesis may be regulated by intracellular Ca^{2+} stores, perhaps via capacitative calcium ion entry. Interestingly, depletion of intracellular stores by thapsigargin can induce either mitogenesis, a quiescent G_0 state, or apoptosis, depending on the cell lines studied. These differential effects could result from thapsigargin-induced Ca^{2+} signals or from inhibition of protein synthesis due to diminished concentrations of stored Ca^{2+}. The concentration of ER Ca^{2+} plays an important role at several steps in protein synthesis. In instances in which prolonged treatment with thapsigargin induces apoptosis, inhibition of protein synthesis may contribute to this process.

Sustained increases in intracellular Ca^{2+} have various toxic effects on cells [27]. However, in the case of thapsigargin toxicity, it appears that the cells are killed by the specific, genetically programmed process known as apoptosis. When cells die as a result of toxic insult, the process is often one of necrosis. This is a relatively nonspecific process involving cell lysis, the release of cellular contents, which subsequently must be 'cleaned up' by the immune system, involving an inflammatory reaction. However, complex organisms also have the need for cells to die at appropriate times in development and in maintenance of normal organ function. In such instances, a more orderly, noninflammatory process of programmed cell death, or apoptosis, occurs. This involves degradation of nuclear DNA by specific endonucleases, resulting in the characteristic DNA ladders that are diagnostic of apoptosis; cellular shrinkage; and ultimately the packaging of cellular constituents in membrane-delimited structures, known as apoptotic bodies, for disposition by leukocytes [25] (see also Ch. 35).

There is considerable evidence for a role of Ca^{2+} signaling in apoptosis [28], and it is interesting to note that the above mentioned Bcl-2, which acts as a suppressor of apoptosis, has been located in mitochondria and ER, organelles involved in Ca^{2+} homeostasis. In one form of apoptosis, induced by glucocorticosteroids, studies have identified a Ca^{2+} influx associated with lymphoid cell death. In addition, the action of glucocorticoids to induce apoptosis could be mimicked by Ca^{2+} ionophores. Similarly, thapsigargin triggers a full apoptotic response. Chelation of Ca^{2+} by intracellular chelators, extracellular ethyleneglycotetraacetic acid (EGTA) or overexpression of the Ca^{2+}-binding protein calbindin inhibits apoptosis due to glucocorticoids and other agents. Finally, calmodulin antagonists have been reported to disrupt apoptosis in a variety of systems. Together, these data suggest a central role for Ca^{2+} in apoptosis in response to glucocorticoids and other agents [29]. However, there is also evidence that, in addition to changes in cytoplasmic calcium ion serving as a signal or modulator for apoptosis, a fall in the concentration of Ca^{2+} in the ER can signal a full apoptotic response, independently of the associated rise in cytoplasmic Ca^{2+} [30]. It is not clear at this time whether this response proceeds through the same signaling pathway involved in capacitative calcium ion entry.

REFERENCES

1. Tsien, R. Y., Rink, T. J. and Poenie, M. Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium* 6: 145–157, 1985.
2. Miyawaki, A. Fluorescence imaging of physiological activity in complex systems using GFP-based probes. *Curr. Opin. Neurobiol.* 13: 591–596, 2003
3. Miller, R.J. Voltage-sensitive Ca^{2+} channels. *J. Biol. Chem.* 267: 1403–1406, 1992.

4. Putney, J. W. Jr *Capacitative Calcium Entry.* Austin, TX: Landes Bioscience,1997.
5. Berridge, M. J. Discovery of the $InsP_3$–Ca^{2+} pathway. A personal reflection. *Adv. Second Messenger Phosphoprotein Res.* 26: 1–7, 1992.
6. Irvine, R. F. 20 years of $Ins(1,4,5)P_3$, and 40 years before. *Nat. Rev. Mol. Cell Biol.* 4: 586–90, 2003.
7. Brini, M. Ca^{2+} signaling in mitochondria: mechanism and role in physiology and pathology. *Cell Calcium* 34: 399–405, 2003.
8. Echevarria, W., Leite, M. F., Guerra, M. T., Zipfel, W. R. and Nathanson, M. H. Regulation of calcium signals in the nucleus by a nucleoplasmic reticulum. *Nat. Cell Biol.* 5: 440–446, 2003.
9. Furuichi, T., Kohda, K., Miyawaki, A. and Mikoshiba, K. Intracellular channels. *Curr. Biol.* 4: 294–303, 1994.
10. Lee, H. C., Walseth, T. F., Bratt, G. T., Hayes, R. N. and Clapper, D. L. Structural determination of a cyclic metabolite of NAD^+ with intracellular Ca^{2+}-mobilizing activity. *J. Biol. Chem.* 264: 1608–1615, 1989.
11. Rutter, G. A. Calcium signaling: NAADP comes out of the shadow. *Biochem. J.* 373: e3–4, 2003.
12. Brailoiu, E., Patel, S. and Dun, N. J. Modulation of spontaneous transmitter release from the frog neuromuscular junction by interacting intracellular Ca^{2+} stores: critical role for nicotinic acid-adenine dinucleotide phosphate (NAADP). *Biochem. J.* 373: 313–318, 2003.
13. Dunlap, K., Luebke, J. L. and Turner, T. J. Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurol. Sci.*18: 89–98, 1995.
14. Putney, J. W. Jr A model for receptor-regulated calcium entry. *Cell Calcium* 7: 1–12, 1986.
15. Putney, J. W. Jr and Bird, G. S. J. The inositol phosphate-calcium signaling system in non-excitable cells. *Endocr. Rev.* 14: 610–631, 1993.
16. Penner, R., Fasolato, C. and Hoth, M. Calcium influx and its control by calcium release. *Curr. Opin. Neurobiol.* 3: 368–374, 1993.
17. Clapham, D. E. TRP channels as cellular sensors. *Nature* 426: 517–524, 2003.
18. Thomas, A. P., Bird, G. S. J., Hajnoczky, G., Robb-Gaspers, L. D. and Putney, J. W. Jr Spatial and temporal aspects of calcium signaling. *FASEB J.* 10: 1505–1517, 1996.
19. Volpe, P., Krause, K.-H., Hashimoto, S. *et al.* 'Calciosome', a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells? *Proc. Natl Acad. Sci. U.S.A.* 85: 1091–1095, 1988.
20. Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* 74: 595–636, 1994.
21. Rossier, M. F. and Putney, J. W. Jr. The identity of the calcium storing inositol 1,4,5-trisphosphate-sensitive organelle in non-muscle cells: Calciosome, endoplasmic reticulum… or both? *Trends Neurosci.* 14: 30–314, 1991.
22. Putney, J. W. Jr. Excitement about calcium signaling in excitable cells. *Science* 262: 676–678, 1993.
23. Carafoli, E. The calcium-signaling saga: tap water and protein crystals. *Nat. Rev. Mol. Cell Biol.* 4: 326–332, 2003.
24. Klee, C. B. and Newton, D. L. Calmodulin: An overview. In J. R. Parratt (ed.), *Control and Manipulation of Calcium Movement.* New York: Raven Press, 1985, pp. 131–145.
25. Schwartzman, R. A. and Cidlowski, J. A. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr. Rev.* 14: 133–151, 1993.
26. Berridge, M. J. Control of cell division: a unifying hypothesis. *J. Cyclic Nucleotide Res.* 1: 305–320, 1975.
27. Orrenius, S. and Nicotera, P. The calcium ion and cell death. *J. Neural Transm.* 43: 1–11, 1994.
28. Rizzuto, R., Pinton, P., Ferrari, D. *et al* T. Calcium and apoptosis: facts and hypotheses. *Oncogene* 22: 8619–8627, 2003.
29. Distelhorst, C. W. Recent insights into the mechanism of glucocorticosteroid-induced apoptosis. *Cell Death Differ.* 9: 6–19, 2002.
30. Bian, X., Hughes, F. M. Jr, Huang, Y., Cidlowski, J. A. and Putney, J. W. Jr Roles of cytoplasmic Ca^{2+} and intracellular Ca^{2+} stores in induction and suppression of apoptosis in S49 cells. *Am. J. Physiol.* 272: C1241–C1249, 1997.
31. Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. and Putney, J. W. Jr Calcium pools in saponin-permeabilized guinea-pig hepatocytes. *J. Biol. Chem.* 258: 15336–15345, 1983.

CHAPTER

23

Serine and Threonine Phosphorylation

James A. Bibb

Eric J. Nestler

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PROTEIN PHOSPHORYLATION IS OF FUNDAMENTAL IMPORTANCE IN BIOLOGICAL REGULATION

Protein phosphorylation is the major molecular mechanism through which protein function is regulated in response to extracellular stimuli both inside and outside the nervous system. Virtually all types of extracellular signal, including neurotransmitters, hormones, light, neurotrophic factors and cytokines, produce most of their diverse physiological effects by regulating phosphorylation of specific phosphoproteins in their target cells. Roughly one third of all proteins in eukaryotes are phosphorylated and virtually every class of neuronal protein is regulated by phosphorylation. The process is reversible, enabling cells to respond dynamically to a myriad of signals in the environment. Although proteins are covalently modified in many other ways, including ADP-ribosylation, acylation (acetylation, myristoylation, isoprenylation), carboxymethylation, tyrosine sulfation and glycosylation, none of these mechanisms is nearly as widespread and readily subject to regulation by physiological stimuli as is phosphorylation. Thus, protein phosphorylation is by far the most prominent mechanism of neural plasticity, an idea first proposed by Paul Greengard more than 30 years ago. In this chapter, we present an overview of the vital role played by protein phosphorylation in the regulation of neuronal function.

Regulation of protein phosphorylation involves a protein kinase, a protein phosphatase and a substrate protein. These components interact according to the scheme shown in Figure 23-1. A substrate protein is converted from the dephospho form to the phospho form by a protein kinase, and the phospho form is converted back to the dephospho form by a protein phosphatase [1].

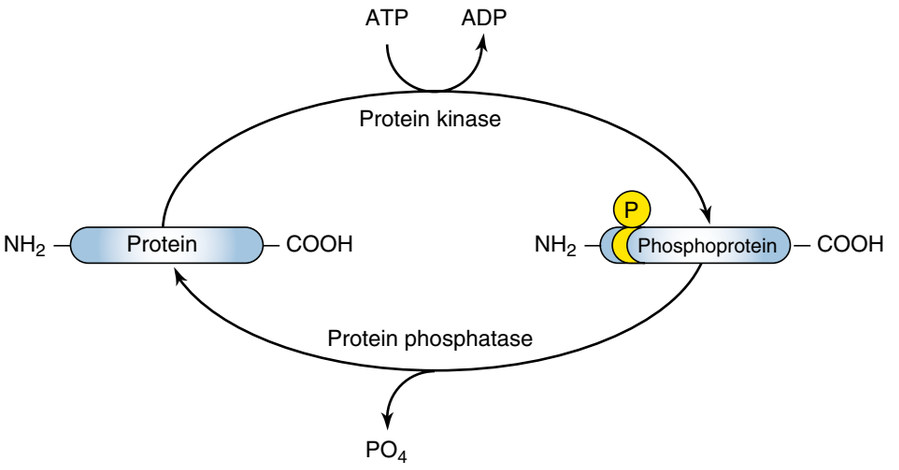

FIGURE 23-1 Schematic diagram of the conversion of a dephosphoprotein to a phosphoprotein by a protein kinase and the reversal of this reaction by a protein phosphatase.

Protein kinases constitute one of the largest single enzyme families and account for 1.7% of all human genes. The protein kinase complement of the human genome, 'the kinome', is made up of 518 protein kinase genes [2]. Figure 23-2 shows a dendrogram constructed by comparisons of the genomic sequences of model organism kinomes, which illustrates the complexity of this diverse family of enzymes. Protein kinases are classified as protein serine–threonine kinases, which phosphorylate substrate proteins on serine or threonine residues, or as protein tyrosine kinases, which phosphorylate substrate proteins on tyrosine residues (discussed in Ch. 24). A small number of protein kinases, as will be seen below, are referred to as 'dual-function' kinases because they phosphorylate substrate proteins on serine, threonine and tyrosine residues. Over 95% of protein phosphorylation occurs on serine residues, 3–4% on threonine residues, and less than 1% on tyrosine residues. In all cases, the kinases catalyze the transfer of the terminal (γ) phosphate group of ATP to the hydroxyl moiety in the respective amino acid residue; Mg^{2+} is required for this reaction. Protein phosphatases catalyze the cleavage of these phosphoester bonds through hydrolysis.

Because phosphate groups are negatively charged, phosphorylation of a protein alters its charge, which can then alter the conformation of the protein and ultimately its functional activity. A change in the state of protein phosphorylation can be achieved physiologically through increases or decreases in the activity of either protein kinases or protein phosphatases. Examples of each of these mechanisms occur in the nervous system, often in concert with one another, to elicit complex temporal patterns of protein phosphorylation.

A general scheme of the diverse pathways through which extracellular signals regulate neuronal function through protein phosphorylation is illustrated in Figure 23-3. Two major mechanisms are involved. In one, extracellular signals, or first messengers, regulate protein kinases or protein phosphatases indirectly by acting on plasmalemma receptors, thereby regulating the intracellular concentration of a second messenger in target neurons [1]. This is the case for first messengers that act through G-protein-coupled receptors, including receptors for many neurotransmitters, hormones, cytokines and sensory stimuli such as visible light and odorants (Ch. 19). It also holds for first messengers that act through receptors containing intrinsic ion channels, such as some glutamate, GABA and acetylcholine receptors (see Ch. 10). Prominent second messengers in the nervous system that directly activate protein kinases include cAMP, cGMP (Ch. 21), Ca^{2+} and diacylglycerol (DAG; see Ch. 20). The next steps in these pathways are the activation of specific classes of protein serine–threonine kinases or protein phosphatases by these second messengers and the subsequent phosphorylation or dephosphorylation of specific substrate proteins, leading, through one or more steps, to specific biological responses.

In the other major mechanism, first messengers directly regulate protein kinase and phosphatase activities by acting on plasma membrane receptors that possess the enzyme activity within their cytoplasmic domains. This is the case for most types of neurotrophic factors as well as many cytokines (Ch. 27). The intrinsic protein kinase and phosphatase activities are stimulated upon ligand binding, which leads to changes in the phosphorylation state of specific substrate proteins and to the generation of specific biological responses.

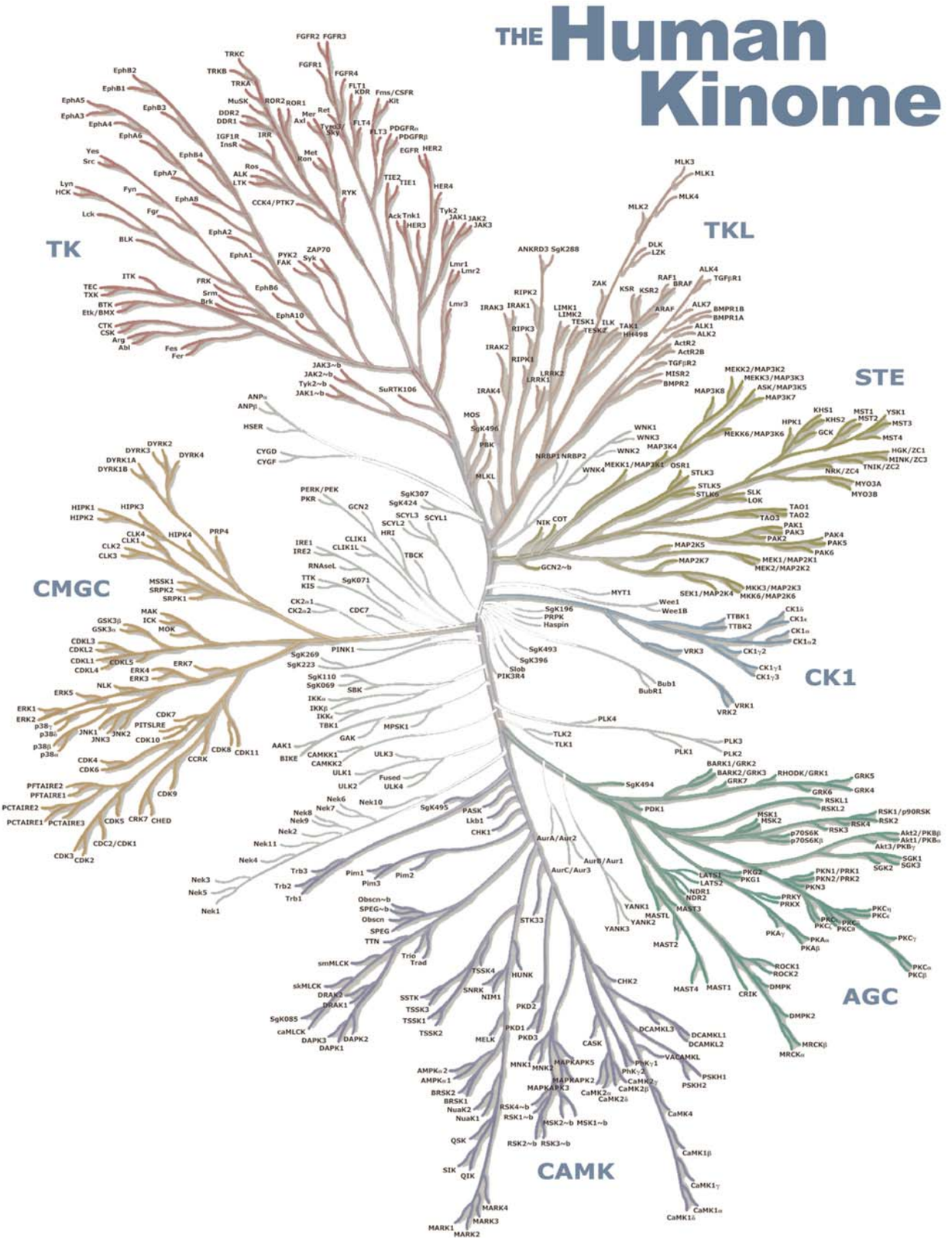

FIGURE 23-2 The human kinome. This dendrogram or phylogenetic tree demonstrating the complexity of the protein kinase superfamily shows the relationships between different protein kinase groups. Group names: AGC includes PKA, PKG, and PKC families; CAMK, Ca^{2+}/calmodulin-dependent protein kinase; CKI, cell kinase1; CMGC includes CDK, MAPK, GSK3, CLK families; TK, tyrosine kinase; TKL, tyrosine-kinase-like; STE are members of the MAPK cascade and include homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases. Related information can be found at www.kinase.com. (Adapted from the original article [2] with permission from *Science* and Michael Melnick of Cell Signaling Technologies – www.cellsignal.com.)

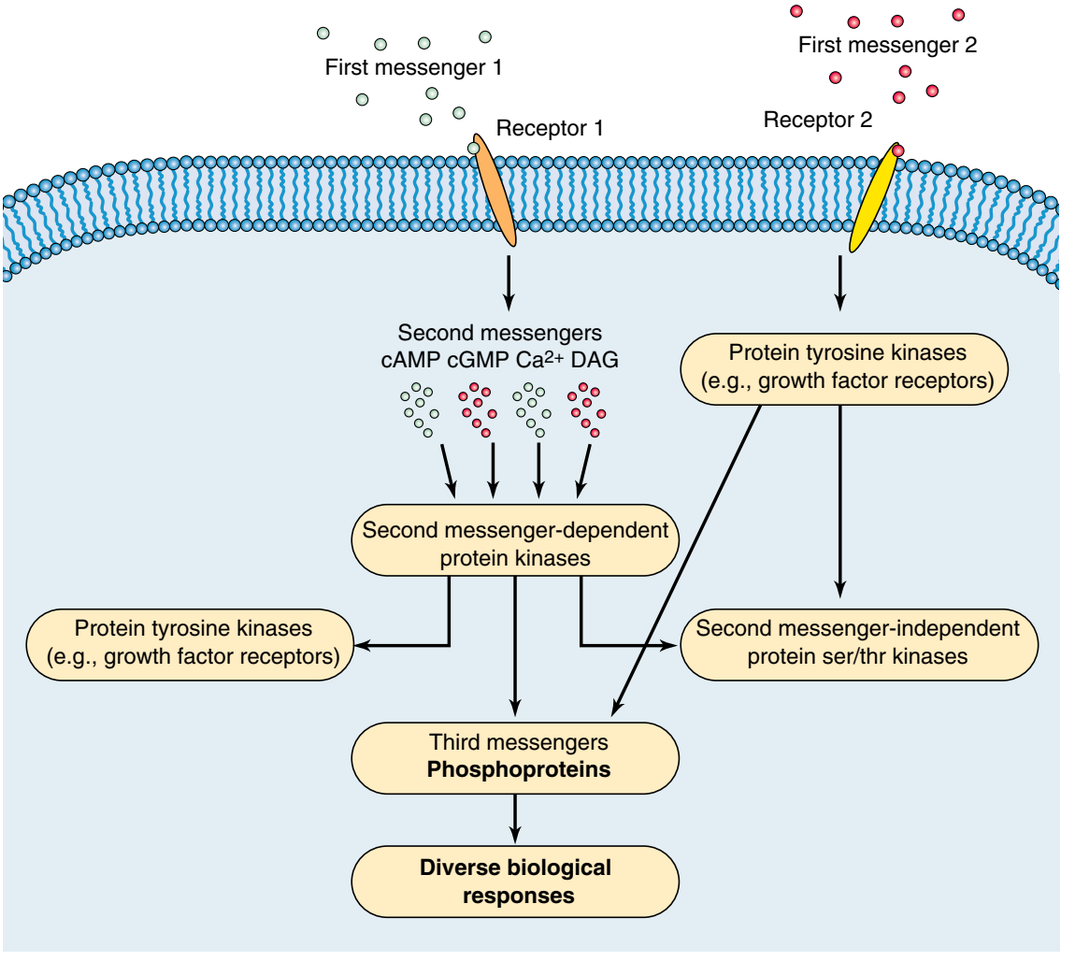

FIGURE 23-3 Signals in the brain. Extracellular signals, termed first messengers, produce specific biological responses in target neurons via a series of intracellular signals, termed second or third messengers. Second messengers in the brain include cAMP, cGMP, Ca^{2+} and diacylglycerol (*DAG*). cAMP and cGMP produce most of their second-messenger actions through the activation of cAMP-dependent and cGMP-dependent protein kinases, respectively. Ca^{2+} exerts many of its second-messenger actions through the activation of Ca^{2+}-dependent protein kinases, as well as through a variety of physiological effectors other than protein kinases. Not illustrated in the figure is the fact that some protein phosphatases can also be regulated directly by second messengers, for example calcineurin, which is activated by Ca^{2+}. The brain also contains a large number of other protein serine–threonine kinases (Table 23-1) that are not activated directly by second-messenger pathways or by protein tyrosine kinases. The brain and other tissues contain two major classes of protein tyrosine kinases. Some of these enzymes are physically associated with plasmalemma receptors, such as most growth factor receptors, and become activated upon ligand binding to the receptors. Others, such as src, are not physically associated with receptors but are influenced indirectly by both second-messenger pathways and receptor protein tyrosine kinases.

PROTEIN SERINE–THREONINE KINASES

Protein kinases differ in their cellular and subcellular distribution, substrate specificity and regulation. These properties determine the functional roles played by the very large number of protein kinases that have been found in mammalian tissues, most of which are known to be expressed in neurons [3]. The major classes of protein serine–threonine kinase in the brain, listed in Table 23-1, are covered in this chapter. The major classes of protein tyrosine kinases in the brain are discussed in Chapter 24. Among the best studied protein kinases in the brain are those activated by the second messengers cAMP, cGMP, Ca^{2+} and DAG [4, 5].

cAMP-dependent protein kinase (protein kinase A; PKA) was first discovered by Edwin Krebs, Edmond Fisher and colleagues and is composed of catalytic and regulatory subunits. The holoenzyme of the kinase, which consists of a tetramer of two catalytic (C) and two regulatory (R) subunits, is inactive. cAMP activates the holoenzyme by binding to the regulatory subunits, thereby causing dissociation of the holoenzyme into a free regulatory subunit dimer and free active catalytic subunits [6].

TABLE 23-1 Major classes of protein serine–threonine kinases

Second-messenger-dependent protein kinases	Protein kinase A (cAMP kinase)
	Protein kinase G (cGMP kinase)
	Ca^{2+}/calmodulin kinases (CaM kinases)
	Protein kinase C
MAP kinases	ERKs
	JNKs or SAPKs
MAP kinase-regulating kinases	MEKs
	SEKs
	Raf
	MEK kinases
Cdks	Cdk1
	Cdk5
CDK-regulating kinases	CAK
	CAK kinase
GRKs	
Others	RSKs
	Cell kinases (CK1)
	IκB kinase (IKK)
	GSK3β

This list is not intended to be comprehensive. The protein kinases listed are present in many cell types in addition to neurons and are included here because of their multiple functions in the nervous system, including regulation of neuron-specific phenomena. Not included are other protein kinases present in diverse tissues, including brain, that play a role in generalized cellular processes, such as intermediary metabolism, and that may not play a role in neuron-specific phenomena. CAK, CDK-activating kinase; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; GRK, G protein receptor kinase; JNK, Jun kinase; MAP kinase, mitogen activated protein kinase; MEK, MAP kinase and ERK kinases; RSK, ribosomal S6 kinase, GSK, glycogen synthase kinase; SAPK, stress-activated protein kinase; SEK, SAPK kinase.

Neurotransmitter receptors that are coupled to G protein complexes that stimulate or inhibit cAMP synthesis have as their target PKA, which is the most important effector for this cyclic nucleotide (see Ch. 21). cAMP phosphodiesterases which hydrolyze this second messenger represent a second mechanism controlling PKA activity. Three isoforms of the C subunit, each of about 40 kDa, and four isoforms of the R subunit, each of 50–55 kDa, have been cloned from mammalian tissues. The three C subunits, designated Cα, Cβ and Cγ, exhibit a very similar and broad substrate specificity, i.e. they phosphorylate a large number of physiological substrate proteins, and can generally be considered isoforms of one another. The four R subunits consist of two forms each of type I and type II proteins. RIIα and RIIβ, but not RIα and RIβ, undergo autophosphorylation, as described below. Most of these R and C subunits of the protein kinase show a wide cellular distribution in the brain.

PKA activity is present throughout the cell, associated with the plasma membrane as well as cytoplasmic and nuclear fractions. The kinase is highly compartmentalized within the cell, in large part via a series of anchoring proteins, termed A kinase anchor proteins (AKAPs) [7]. Several forms of AKAP are known, many of about 75–79 kDa. AKAPs bind specifically with the RIIα and RIIβ subunits of the protein kinase and thereby tether these regulatory subunits and their bound catalytic subunits to specific subcellular sites, for example postsynaptic densities. Postsynaptic densities are specializations in distal dendrites that appose presynaptic nerve terminals and contain some of the neurotransmitter receptors and other proteins required for synaptic transmission. In this way, AKAPs keep the protein kinase in close proximity to the cascade of signal transduction proteins it phosphorylates to regulate synaptic transmission. The important role played by AKAPs under physiological conditions is indicated by experiments in which synthetic polypeptides that disrupt AKAP–RII interactions have been shown to disrupt specific physiological effects of PKA [7].

cGMP-dependent protein kinase (PKG) is a dimer of two identical subunits. Each subunit, with an M_r of ≈75 kDa, contains a regulatory domain, which binds cGMP, and a catalytic domain [8]. As with the cAMP-dependent enzyme, cGMP activates the inactive holoenzyme by binding to the regulatory domain of the molecule; however, unlike the cAMP-dependent enzyme, activation of the cGMP-dependent holoenzyme is not accompanied by dissociation of the subunits. PKG shows a much more limited cellular distribution and substrate specificity than PKA. This reflects the smaller number of second-messenger actions of cGMP in the regulation of cell function. The first messenger, nitric oxide, stimulates cGMP production by directly activating guanylyl cyclases (see Ch. 21). cGMP phosphodiesterases hydrolyze cGMP and are the pharmacological targets of therapeutic agents such as sildenafil used to treat erectile dysfunction [9].

Calcium/calmodulin-dependent protein kinases (CaM kinases; CaMKs) are one of two major classes of calcium-dependent kinases in the nervous system. The brain contains at least six major types of CaMK, each with very different properties. CaMKII is abundant in the CNS and, like PKA, exhibits a broad cellular distribution and substrate specificity and can be considered a 'multifunctional protein kinase' in that it probably mediates many of the second-messenger actions of Ca^{2+} in many types of neuron [10] (Ch. 22). CaMKII contains a regulatory domain that, in the resting state, binds to and inhibits a catalytic domain; this inhibition is relieved when Ca^{2+}/calmodulin binds to the regulatory domain. The enzyme exists under physiological conditions as large multimeric complexes of identical or distinct subunit isoforms of 50–60 kDa. In response to intense stimulation of glutamate receptors, CAMKII is autophoshorylated and becomes active in a Ca^{2+}/calmodulin-independent manner [11] (see Ch. 15). Because of this stimulation-sensor capability, CaMKII, more than any other protein kinase, has been implicated in the synaptic plasticity underlying learning and memory [11] (see Ch. 53).

CaMKs I and IV also appear to play important roles in mediating many of the second-messenger actions of Ca^{2+} in the nervous system, although their substrate specificity remains only partially known. One interesting feature of CaMKs I and IV is that both appear to be activated not only by Ca^{2+}/calmodulin-binding but also upon their phosphorylation by other protein kinases, which have been termed CaMKI kinase and CaMKIV kinase respectively. These CaMK kinases may also be Ca^{2+}/calmodulin-dependent enzymes. CaMKIV kinase has been cloned. Interestingly, this kinase is itself phosphorylated and inhibited by PKA, thereby providing a prominent mechanism by which the cAMP and Ca^{2+} cascades interact, as will be covered in greater detail below.

The remaining three types of CaMK are phosphorylase kinase, myosin light-chain kinase and CaMKIII. These each appear to phosphorylate fewer substrate proteins, and in some cases only one protein, under physiological conditions, and each may therefore mediate relatively fewer actions of Ca^{2+} in the nervous system.

Protein kinase C (PKC) comprises the other major class of Ca^{2+}-dependent protein kinases and is activated by Ca^{2+} in conjunction with DAG and phosphatidylserine (discussed in Ch. 20). Multiple forms of PKC have been cloned, and the brain is known to contain at least seven species of the enzyme. The variant forms of PKC exhibit different cellular distributions in the brain and different regulatory properties. For example, they differ in the relative ability of Ca^{2+} and DAG to activate them: some require both Ca^{2+} and DAG, whereas others can be activated by DAG alone, apparently without an increase in cellular Ca^{2+} concentrations. However, these enzymes show similar substrate specificities and, as a result, are often considered isoforms.

PKC exists under physiological conditions as single polypeptide chains of about 80 kDa. Each polypeptide contains a regulatory domain, which, in the resting state, binds to and inhibits a catalytic domain. This inhibition is relieved when Ca^{2+} and/or DAG binds to the regulatory domain. PKC exhibits a broad substrate specificity and mediates numerous second-messenger functions of Ca^{2+} in target neurons.

Under basal conditions, PKC is predominantly a cytoplasmic protein. Upon activation by Ca^{2+} or DAG, the enzyme associates with the plasma membrane, the site of many of its known physiological substrates, including receptors and ion channels. In fact, the translocation of PKC from the cytoplasm to the membrane has long been used as an experimental measure of enzyme activation. Such translocation has often been assayed by phorbol ester binding; phorbol esters are tumor-promoting agents that selectively bind to and activate PKC. The molecular basis of the translocation of PKC from the cytoplasm to the plasma membrane has been solved. Subsequent to activation, PKC binds with high affinity to a series of membrane-associated proteins, termed receptors for activated C kinase (RACK) [12]. RACKs thereby function by analogy with the AKAPs for PKA to direct or recruit these widely expressed enzymes to subcellular sites where their activity is required.

Diverse actions of extracellular signals are mediated by second-messenger-dependent protein kinases. The intracellular application, by microinjection or transfection of PKA, PKG, CaMKII or PKC into particular types of neuron has been shown to mimic specific physiological responses (regulation of ion channels, neurotransmitter release and gene transcription) to specific first messengers (neurotransmitters or nerve impulses) for those neurons. Where specific inhibitors of the kinases are available, their application has been shown to block the ability of the neurotransmitters to elicit those responses. Taken together, these findings demonstrate that activation of these second-messenger-dependent protein kinases is both a necessary and sufficient step in the sequence of events by which certain first messengers produce some of their physiological effects.

Transgenic methodologies have provided further evidence for the importance of second-messenger-dependent protein kinases in the regulation of brain signal transduction. The best example to date is provided by mice lacking subunits of CaMKII. These animals show deficiencies in a form of synaptic plasticity, long-term potentiation, in the hippocampus as well as abnormal spatial learning, a form of learning dependent on hippocampal function (see also Ch. 53).

The mitogen-activated protein kinase cascade is second-messenger-independent. Although the second-messenger-dependent protein kinases were identified first as playing an important role in neuronal function, we now know that many other types of protein serine–threonine kinase are also essential (Table 23-1). Indeed, one of the most critical discoveries of the 1990s was the delineation of the mitogen-activated protein kinase (MAP kinase or MAPK) cascades.

MAPKs were first described as mitogen-activated protein-kinases and shown to play an important role in cell growth. The same enzymes were described in brain as 'microtubule-associated protein kinases' for their phosphorylation of microtubule-associated proteins and other neuronal cytoskeletal proteins (see Ch. 8). However, the mechanisms by which the activity of the enzymes were controlled by extracellular signals remained a mystery until these mechanisms were elucidated largely through advances in yeast signal transduction and the subsequent identification of homologous signaling proteins in mammalian cells.

The basic scheme for MAPK cascades is shown in Figure 23-4. MAPK is part of a 'phosphorelay' system composed of three sequentially activated kinases [13]. MAPK, inactive under basal conditions, is activated by phosphorylation by another protein kinase, termed

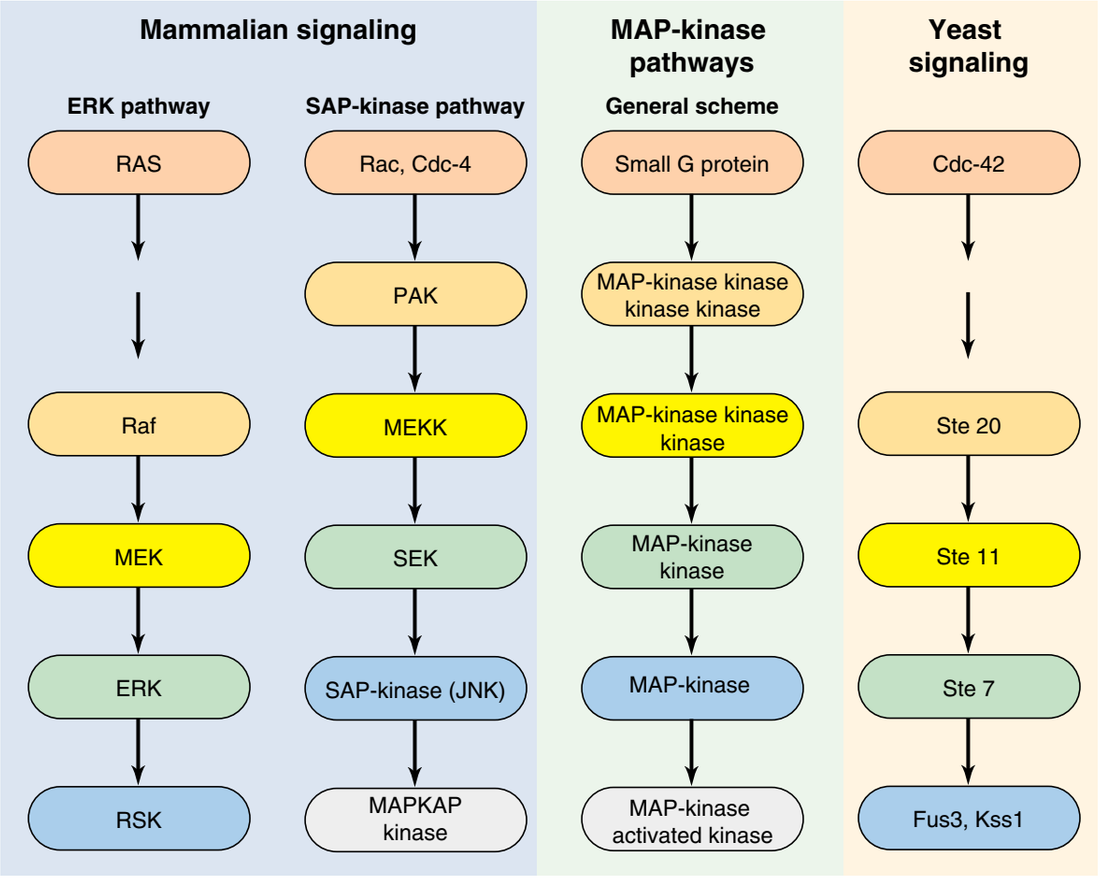

FIGURE 23-4 Schematic diagram of mitogen-activated protein kinase (*MAP-kinase*) pathways. To the **right** is shown the original pathway delineated in yeast. To the **left** are shown the homologous pathways more recently identified in mammalian cells, including brain. *ERK*, extracellular signal-regulated kinase; *JNK*, Jun kinase; *MAPKAP kinase*, MAP-kinase-activated protein kinase; *MEK*, MAPK and ERK kinase; *MEKK*, MEK kinase; *PAK*, p21-activated kinase; *RSK*, ribosomal S6 kinase; *SAP-kinase*, stress-activated protein kinase; *SEK*, SAP-kinase kinase.

MAPK kinase (MAPKK). MAPK kinase itself is activated by phosphorylation by still another protein kinase, termed MAPK kinase kinase (MAPKKK). MAPK kinase kinase is activated upon interaction with a member of the Ras superfamily of small G proteins, which are bound to the plasma membrane (see Ch. 19). The exact mechanism of activation remains unknown, but it is believed that Ras and related proteins, in the activated GTP-bound form, can bind MAPK kinase kinase and thereby draw the kinase to the plasmalemma, where it is activated by as yet unknown factors, perhaps even an additional kinase, MAPK kinase kinase kinase (MAPKKKK). The mechanism governing the activation of Ras and related proteins by extracellular signals is quite complex and involves numerous 'linker' proteins, for example Shc, Grb and Sos, that couple Ras to a variety of plasmalemma-associated growth factor–protein tyrosine kinase receptors (see Chs 20, 24 and 27).

The best characterized family of MAPKs in the brain are the *extracellular signal-regulated protein kinases* (ERKs) that are activated by the neurotrophins and related growth factors (Fig. 23-4) [14, 15]. The MAPK kinases responsible for phosphorylation and activation of the ERKs are referred to as *MAPK and ERK kinases* or MEKs. MEK phosphorylates ERK on a threonine and tyrosine residue, both of which are required for ERK activation. MEK is, therefore, an example of a dual-function protein kinase that can phosphorylate a substrate protein on these two types of residue. The phosphorylated threonine and tyrosine residues in all known forms of ERK are separated by a single amino acid, giving rise to the conserved threonine–amino-acid–tyrosine sequence (T–X–Y). The MAPK kinase kinases responsible for MEK activation include a group of protein serine–threonine kinases termed Raf. Raf, in turn, is activated in part by one of three known forms of Ras, which is activated in response to the binding of many types of growth factors to their receptors. Thus, the neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and related growth factors, such as epidermal growth factor and insulin, bind to plasma membrane receptors that possess intrinsic protein tyrosine kinase activity in their cytoplasmic domains. Activation of this

activity upon growth factor binding leads to Ras activation via a cascade of linker proteins, as mentioned above (see Ch. 27).

The brain contains numerous forms of ERK, MEK and Raf (designated ERK1, 2, 3; MEK1, 2; Raf 1, 2, 3; and so on), which are expressed in distinct populations of neurons. Given that the major activators of the ERK cascade are growth factors, it is not surprising that these enzymes have been implicated in neuronal growth, differentiation and survival. Moreover, it has become apparent that these enzymes also are likely to play a role in synaptic transmission in the adult nervous system.

A second family of MAPKs is referred to as *stress-activated protein kinases* (SAPKs) [3, 14, 15]. This includes JNKs, or Jun kinases, named originally for their phosphorylation of the transcription factor c-Jun. SAPKs were first identified in peripheral tissues on the basis of their activation in response to cellular forms of stress, which include X-ray irradiation and osmotic stress. More recently, they have been demonstrated to be activated in brain by several cytokines as well as by synaptic activity [16]. As shown in Figure 23-3, SAPKs are activated by SAPK kinases (SEKs), which are in turn activated by SEK kinases. The Ras-like small G proteins implicated in SEK kinase activation are Rac and Cdc-42. In this case, it appears that Rac/Cdc-42 triggers activation of SEK kinase by stimulating its phosphorylation by still another protein kinase termed p21-activated kinase (PAK). Thus, PAK can be considered a MAPK kinase kinase kinase, which is analogous to the cascade of protein kinases found in yeast (Fig. 23-4).

A dramatic feature of MAPK cascades is the series of successive protein kinases involved. While the evolutionary basis of such cascades remains unknown, they clearly evolved very early and could provide the basis for explosive amplification of an initiating extracellular signal that acts on very rare plasmalemma receptors. A major challenge in current research is to define the precise mechanisms for the myriad effects of MAPKs by identifying their physiological substrate proteins. Among their best-characterized substrate proteins are transcription factors [3, 14, 15] (see Ch. 26). Phosphorylation of c-Jun by JNK is thought to increase transcriptional activity of c-Jun. Elk-1, also a transcription factor, is phosphorylated and activated by ERK. Upon phosphorylation, Elk-1 complexes with other proteins to form serum response factor, which binds to the serum response element present in the promoters of numerous genes, including c-Fos, another transcription factor (details of transcription are found in Ch. 26).

A third group of MAPKs are p38 kinases, of which there are four subtypes: α, β, γ, and δ. P38 MAPKs are activated by many stimuli including hormones, ligands for G-protein-coupled receptors and stresses [13, 17]. P38 MAPK has been implicated in the pathological changes accompanying inflammatory and apoptotic processes of various cell types, including neurons [17] (see Ch. 35 for discussion of apoptosis). More recently, several additional MAPKs such as ERK5 [18], ERK7 [19] and ERK8 [20] have been identified, which may constitute additional MAPK groups.

Other classes of proteins are also known to be physiological substrates for the MAPKs. Tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamine neurotransmitters, is phosphorylated by ERK, which appears to increase the activity of the enzyme in response to other activating stimuli (see below and Ch. 12). Phospholipase A_2, the first enzyme in the generation of prostaglandin and related metabolites of arachidonic acid, is phosphorylated and activated by ERK (Ch. 33). Cytoskeletal proteins, such as microtubule-associated proteins and tau (see below), are also prominent substrates, although the precise effect phosphorylation has on the physiological activity of these proteins remains poorly understood. Finally, among the substrates for the MAPK are still other protein kinases. Thus, one important effector mechanism for ERK is phosphorylation and activation of ribosomal S6 kinases (RSKs), protein serine–threonine kinases first identified for their phosphorylation and regulation of ribosomal proteins but now known to phosphorylate other types of substrate, including the transcription factor cAMP response element binding protein (CREB) (Ch. 26). An analogous motif is seen for the SAPKs, which also appear to produce some of their biological effects via the phosphorylation and activation of other serine–threonine kinases, such as MAPK-activated protein kinase (MAPKAP).

The brain contains many other types of second-messenger-independent protein kinases. Examples of other second-messenger-independent protein kinases are listed in Table 23-1. Many of these include enzymes that were identified originally in association with a particular substrate protein but shown later to play a more widespread role in brain signal transduction. The functional role of one of these, β-adrenergic receptor kinase (βARK), a type of G protein receptor kinase (GRK), is discussed further below.

Other examples of protein serine–threonine kinases are the cyclin-dependent kinases (CDKs), identified originally for their role in the control of the cell cycle. Such kinases, again identified originally in yeast, are among a large number of proteins termed cell division cycle (Cdc) proteins. Most CDKs are activated through association with cofactors called cyclins and phosphorylation by upstream protein kinases termed CDK-activating kinases (CAKs). An atypical member of the CDK family is CDK5 [21]. CDK5 is highly enriched in neurons and functions during CNS development as well as in fully differentiated adult neurons. Its activity is dependent upon association with the neuron-specific cofactors p35 or p39. CDK5 has been implicated in a broad number of CNS functions, including cortical layer formation, learning and memory, cytoskeletal dynamics, synaptic vesicle recycling, and

regulation of dopamine neurotransmission [22]. It has also been implicated in several neurological and neuropsychiatric disorders such as drug addiction [23, 24], Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis, and is further discussed in the relevant chapters. The list of physiological substrates for CDK5 is growing and includes activators of protein kinases such as MEK1 and inhibitors of protein phosphatases such as inhibitor-1 and DARPP-32 (discussed below).

Another example of a second-messenger-independent serine–threonine kinase is Iκ-kinase (IKK), which regulates the NFκB (nuclear factor κB) transcription factor. This system is classically known to be involved in inflammatory responses and carcinogenesis but in recent years has been implicated in signal transduction mechanisms in the nervous system [25]. Briefly, in response to cytokines or other first messengers, IKK becomes activated and phosphorylates IκB subunits that are bound to NFκB in the cytoplasm. Phosphorylation of IκB triggers its ubiquitination and degradation in proteasomes. This results in the generation of free NFκB, which translocates to the nucleus to regulate gene expression.

Most protein serine–threonine kinases undergo autophosphorylation. The autophosphorylation of most protein kinases is associated with an increase in kinase activity [4, 10]. In some instances, such as with the RII subunit of PKA, autophosphorylation represents a positive feedback mechanism for kinase activation, in this case by enhancing the rate of dissociation of the RII and C subunits. In the case of CaMKII, autophosphorylation causes the catalytic activity of the enzyme to become independent of Ca^{2+} and calmodulin. This means that the enzyme, activated originally in response to elevated cellular Ca^{2+}, remains active after Ca^{2+} concentrations have returned to baseline. By this mechanism, neurotransmitters that activate CaMKII can produce relatively long-lived alterations in neuronal function. In other instances, such as with the receptor-associated protein tyrosine kinases (discussed in Ch. 24), autophosphorylation is an obligatory step in the sequence of molecular events through which those kinases are activated and produce physiological effects.

PROTEIN SERINE–THREONINE PHOSPHATASES

Protein dephosphorylation is catalyzed by phosphohydrolases called protein phosphatases. While the number of protein tyrosine kinases is roughly comparable to the number of protein tyrosine phosphatases, protein serine–threonine kinases vastly outnumber the protein serine–threonine phosphatases, of which about 25 different species are known to exist. This relative under-representation may be accounted for by the alternative diversification of independent regulatory proteins that can control and target a given protein phosphatase [26]. Many first messengers elicit physiological responses in the brain through the specific regulation of protein phosphatases.

The brain contains multiple forms of protein serine–threonine phosphatases. These differ in their regional distribution, substrate specificity and regulation by cellular messengers. The original classification of protein serine–threonine phosphatases as protein phosphatase 1 or 2 (PP1 or PP2) was based on their preferred dephosphorylation of, respectively, the β or α subunit of phosphorylase kinase. PP2 was later subdivided into three subtypes, termed PP2A, PP2B and PP2C, based on differential biochemical and regulatory properties. Each of these enzymes, including several subtypes, has now been cloned along with several additional protein serine–threonine phosphatases [3, 27, 28]. The current classification of these various enzymes, listed in Table 23-2, has retained their historical nomenclature, despite the fact that we now know that such a classification does not follow particular structural or functional properties. For example, PP1 and PP2A are closely related structurally and functionally, whereas PP2C is more distantly related, based on amino acid homology. More recently, newly identified members of the phosphatase family, PP4, PP5 and PP6 have been reported, but little information is yet available regarding their functions or targeting [29, 30].

Protein phosphatase 1. Four subtypes of PP1, derived from three genes, are known. These enzymes, listed in Table 23-2, are highly homologous and exhibit similar substrate specificities; therefore, they can be considered isoforms. However, the proteins exhibit very distinct patterns of distribution in the brain. PP1 dephosphorylates a wide array of substrate proteins. Its catalytic subunit can form complexes with over 50 regulatory subunits in a

TABLE 23-2 Classes of protein serine–threonine phosphatases

Protein phosphatases	Inhibitor proteins
PP1 (α, β, γ_1, γ_2)	Inhibitor 1, inhibitor 2 DARPP-32, NIPP1
PP2A	Inhibitor 1^{2A}, inhibitor 2^{2A}
PP2B (calcineurin)	Immunophilins Cyclosporin A-cyclophilin, FK06-FK506-binding protein
PP2C	
PP4	
PP5	
Others?	
Dual-function phosphatases (VH1 family)	

Cdc, cell division cycle; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; MAPK, mitogen-activated protein kinase; NIPP1, nuclear inhibitor of PP1; PP, protein phosphatase; VH1, vaccinia virus.

mutually exclusive manner [31]. As a result, numerous PP1-dependent cellular functions are controlled by independent mechanisms. Regulation of PP1 by some inhibitor proteins will be discussed below. The molecular structure of PP1 has been solved at the atomic level and has revealed molecular mechanisms for some of these inhibitors [32].

The subcellular localization of PP1 appears to be determined by a series of other proteins, which are now considered to be integral subunits of the enzyme [31]. For example, the M subunit directs PP1 to myofibrils in smooth muscle. In addition, the several known isoforms of PP1 show region-specific expression in the brain. Certain forms, PP1α and PP1γ1, are highly enriched in dendritic spines of certain neurons, which presumably reflects the important role played by the enzyme in synaptic transmission. The subunit responsible for this dendritic localization may be a protein called spinophilin [33, 34]. The cell nucleus also contains high concentrations of PP1, where it is believed to play an important role in the dephosphorylation of transcription factors critical to the control of gene transcription. One PP1 regulatory protein responsible for directing signals to the nucleus ('nuclear targeting'), which was identified by the yeast two-hybrid approach, is called PNUTS (phosphatase 1 nuclear targeting subunit) [35, 36].

Protein phosphatase 2A accounts for as much as 1% of total cellular protein and for the major portion of serine–threonine phosphatase activity in most tissues and cells [37]. It is a multisubunit enzyme composed of three subunits: a catalytic, or C, subunit; a scaffold A subunit, which provides structural support to the C subunit; and a regulatory B subunit, which is in part responsible for PP2A functional specificity. There are two known forms of the C subunit but these are very similar functionally and are considered to be isoforms of each other. In contrast, multiple forms of the B subunit occur, which are expressed differentially in mammalian tissues. Distinct classes of B subunits, termed B, B′, and B″, can bind to the AC complex to form a large array of heterotrimeric complexes. The interactions of the various regulatory B subunits with the AC complex are competitive, and these B subunits can differentially exchange, so that PP2A specificity and location may be controlled through their relative abundance and expression [38]. Some regulatory B subunits are expressed specifically in the brain and are highly enriched in specific regions of the CNS. There is substantial evidence that PP2A activity is regulated by extracellular signals through phosphorylation and other post-translational modifications of PP2A complexes. The relative importance of PP2A in the nervous system and elsewhere may be suggested by the panoply of naturally occurring small molecule inhibitors, some of which will be discussed below.

Protein phosphatase 2B, also called calcineurin, is a Ca^{2+}/calmodulin-activated enzyme composed of two major subunits: the A subunit contains the catalytic activity and is highly homologous to PP1, and the B subunit contains two calmodulin-like domains responsible for binding Ca^{2+}/calmodulin [39]. PP2B is expressed in a region-specific manner in the brain and enriched particularly in striatum. PP2B, like PP1, exhibits broad substrate specificity. Moreover, based on its regulation by Ca^{2+}, it is believed to mediate many physiological responses to neurotransmitters and nerve impulses. PP2B binds with high affinity to AKAPs, which localize the enzyme to subcellular sites also enriched in PKA, such as postsynaptic densities. As will be seen below, this is presumably one molecular basis for the complex interactions found between PP2B, the protein kinase and other regulatory proteins.

The activity of PP2B is regulated by a series of proteins termed immunophilins [28, 40]. These proteins, the prototypical example of which is cyclophilin, were first identified as the targets of potent immunosuppressive drugs such as cyclosporin and FK-506. It is now known that immunophilins bind to PP2B and inhibit its catalytic activity, and immunosuppressive drugs promote this inhibitory action. Both the A and B subunits of PP2B are involved in the binding of immunophilins. The immunosuppressive/immunophilin-mediated reduction in PP2B activity results in loss of the ability of Ca^{2+} to activate T lymphocytes, which mediates the immunosuppressive effects of the drugs. Although first described in the immune system, immunophilins are expressed in the brain in a region-specific manner and have been implicated in the regulation of synaptic transmission [41]. It will be interesting in future studies to further elaborate the functional importance of PP2B–immunophilin interactions in the regulation of neuronal function.

Protein phosphatase 2C, unlike the other serine threonine phosphatases, is a monomeric enzyme for which few targeting proteins have yet been identified. Furthermore, to date, there are no inhibitors of PP2C available. As a result it has been difficult to examine the role of PP2C in intact cells. Although it is encoded by seven different mammalian genes and is expressed in the brain, relatively few neural substrates have been identified. Likely substrates include the autophosphorylation site of CaMKII, the casein kinase I site on DARPP-32 (Ser137) and the PKA site on tyrosine hydroxylase (Ser40) (see discussion of these proteins below). Recently, a role for PP2C in the nervous system has been implicated by the observation that it binds and dephosphorylates one of the metabotrophic glutamate receptors (mGluR3) [42]. PP2C has been found to interact with the cystic fibrosis transmembrane conductance regulator chloride channel as well as the AKT potassium channel in *Arabidopsis*. Clearly more information is needed on the function and regulation of PP2C and its role in the regulation of receptors and channels in the CNS.

Protein serine–threonine phosphatases play a critical role in the control of cell function. This is demonstrated by several naturally occurring toxins that inhibit cellular

protein phosphatase activity. Some of these are potent tumor-promoting agents. Examples of such toxins are okadaic acid and microcystin, which are potent inhibitors of PP1 and PP2A. In addition to demonstrating the critical balance maintained in cells between protein phosphorylation and dephosphorylation, these protein phosphatase inhibitors have been used as tools to elaborate the specific role of protein phosphatases in the nervous system.

Protein phosphatase 1 is regulated by protein phosphatase inhibitor proteins. PP1 is inhibited by at least four such proteins: inhibitor 1, inhibitor 2, dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) and nuclear inhibitor of PP1 (NIPP1) (Table 23-2). Inhibitor 1 is a substrate for PKA; inhibitor 2 is a substrate for glycogen synthase kinase-3; DARPP-32 and NIPP1 are substrates for PKA and casein kinases. DARPP-32 is phosphorylated by additional kinases as well (see below). PKA phosphorylation of inhibitor 1 and DARPP-32 leads to activation of their phosphatase-inhibitory activity, whereas phosphorylation of inhibitor 2 and NIPP1 leads to inactivation of their phosphatase-inhibitory activity. All four inhibitors are low-molecular-weight, acid-soluble, heat-stable proteins and exhibit some homology in their amino acid composition.

Whereas inhibitors 1 and 2 and NIPP1 appear to be widely distributed in mammalian tissues, including brain, DARPP-32 shows a much more restricted distribution. The protein is enriched in discrete populations of neurons in the brain, most prominently those that express D1-dopamine receptors (see Chs 12, 46 and 54). Some neuronal cell types thus appear to contain unique species of phosphatase inhibitor proteins. The critical role played by these proteins in neuronal function is illustrated below.

In contrast to inhibitor 1, DARPP-32 and NIPP1, which regulate signal transduction, the function of inhibitor 2 appears to be different. There is evidence that inhibitor 2 associates with PP1, as the phosphatase is newly synthesized and contributes to the proper folding of the enzyme [40]. Inhibitor 2 can thus be considered a chaperone protein. The inactive PP1–inhibitor 2 complex can then be activated upon phosphorylation of inhibitor 2 by glycogen synthase kinase-3. Whether this process is regulated in neurons in association with synaptic activity remains unknown.

Mitogen-activated protein kinase phosphatases are dual-function protein phosphatases. Just as the MAPK kinases (e.g. MEKs) are unique as dual-functioning kinases in that they phosphorylate MAPKs on threonine and tyrosine residues, there are unique dual-function ing protein phosphatases that reverse the phosphorylation and activation of MAPKs [43]. Such MAPK phosphatases (MKPs) were first identified as a product of vaccinia virus (VH1) and later found in all eukaryotic cells. There are now numerous members of this VH1 family of dual-functioning protein phosphatases. These enzymes are more closely related, in terms of their amino acid sequences, to protein tyrosine phosphatases than to protein serine–threonine phosphatases.

Some members of this family have been shown to mediate the dephosphorylation of MAPKs under physiological conditions. Others dephosphorylate Cdc-2 and related CDKs. However, relatively little is known to date about the regional distribution of these dual-functioning phosphatases in the brain and the specific function these enzymes serve in the regulation of neuronal signal transduction. Considerable interest has focused on one particular MAPK phosphatase, which can be induced very rapidly, at the level of gene transcription, in target cells in response to cellular activation [44].

NEURONAL PHOSPHOPROTEINS

Virtually all types of neuronal protein are regulated by phosphorylation. Examples of neuronal phosphoproteins are given in Table 23-3. Regulation of these proteins by phosphorylation is reviewed in detail elsewhere [4, 10, 45–49]. Phosphorylation of these many types of protein is involved in carrying out or regulating virtually every process in the nervous system. Indeed, it is difficult to identify a neural process, including an example of neural

TABLE 23-3 Examples of proteins regulated by phosphorylation

Enzymes involved in neurotransmitter biosynthesis
Tyrosine hydroxylase
Tryptophan hydroxylase
Neurotransmitter receptors
Adrenergic receptors
Dopamine receptors
Opioid receptors
Glutamate receptors
Many others
Monoamine-reuptake plasma membrane transporters
Monoamine vesicle transporters (VAT)
Ion channels
Voltage-dependent Na^+, K^+, Ca^{2+} channels
Ca^{2+}-dependent potassium channels
Enzymes and other proteins involved in the regulation of second messengers
G proteins
Phospholipases
Adenylyl cyclases
Guanylyl cyclases
Phosphodiesterases
IP_3 receptor
Protein kinases
Autophosphorylated protein kinases (protein kinases phosphorylating themselves)
Protein kinases phosphorylated by other protein kinases (many examples)
Protein phosphatase inhibitors
DARPP-32
Inhibitors 1 and 2

TABLE 23-3 Examples of proteins regulated by phosphorylation—cont'd

Cytoskeletal protein involved in neuronal shape and motility
- Actin
- Tubulin
- Neurofilaments (and other intermediate filament proteins)
- Myosin
- Microtubule-associated proteins
- Actin-binding proteins

Synaptic vesicle proteins involved in neurotransmission
- Synapsins
- SNAP25
- Syntaxin
- Synaptobrevin (VAMP)
- rabphilin
- RIM1
- SNAP
- Synaptotagmin
- Amphihysin
- Dynamin
- Synaptophysin
- Synaptojanin
- Others

Transcription factors
- CREB family members
- Fos and Jun family members
- STATs
- Steroid and thyroid hormone receptors
- NFκB-IKB family
- Serum response factors

Other proteins involved in DNA transcription or mRNA translation
- RNA polymerase
- Topoisomerase
- Histones and nonhistone nuclear proteins
- Ribosomal protein S6
- eIF (eukaryotic initiation factor)
- eEF (eukaryotic elongation factor)
- Other ribosomal proteins

Proteins implicated in neurodegeneration
- APP
- Tau

Miscellaneous
- 14–3-3
- Rhodopsin
- Neural cell adhesion proteins
- MARCKS
- GAP-43

This list is not intended to be comprehensive but to indicate the wide array of neuronal proteins regulated by phosphorylation. Some of the proteins are specific to neurons but most are present in many cell types in addition to neurons and are included because their multiple functions in the nervous system include the regulation of neuron-specific phenomena. Not included are the many phosphoproteins present in diverse tissues, including brain, that play a role in generalized cellular processes, such as intermediary metabolism, and that do not appear to play a role in neuron-specific phenomena. *NMDA,* *N*-methyl-D-aspartate; *CREB,* cAMP response element-binding proteins; *STAT,* signal-transducing activators of transcription; *IP_3,* inositol trisphosphate; *DARPP-32,* dopamine and cAMP-regulated phosphoprotein of 32 kDa; *RIM,* Rab3a interacting molecule; *NFκB,* nuclear factor κB; *BAPP,* amyloid precursor protein; *GAP-43,* growth-associated protein of 43 kDa; *MARCKS,* myristoylated alanine-rich C kinase substrate.

plasticity, in which phosphorylation of specific phosphoproteins is not integrally involved.

In the presynaptic compartment, phosphorylation of enzymes that synthesize neurotransmitters regulates the capacity of a neuron for synaptic transmission. Phosphorylation of ion channels regulates the ability of these channels to open or close in response to the propagation of an action potential, thereby mediating synaptic release of neurotransmitters as well as more general changes in neuronal excitability. Phosphorylation of synaptic-vesicle-associated proteins regulates neurotransmitter release in response to subsequent stimuli. In the postsynaptic compartment, phosphorylation of ion channels controls the membrane potential and excitability of neurons. Phosphorylation of receptors and other signaling proteins regulates the ability of neurotransmitters and other first messengers to produce their physiological effects. Phosphorylation of neuronal cytoskeletal and related proteins regulates axoplasmic transport as well as neuronal growth, shape, survival and elaboration, and retraction of dendritic and axonal processes. Phosphorylation of transcription factors and ribosomal proteins regulates the expression of specific genes in target neurons, the ultimate form of signal transduction and neural plasticity.

Protein phosphorylation is an important mechanism of memory. Phosphorylation of any of the aforementioned types of protein can be viewed as 'molecular memory': a change in the structure and function of a protein that reflects perturbation of a specific signal-transduction pathway in a specific neuronal cell type. Presumably, learning and memory manifested at a behavioral level are established through the accumulation of many types of phosphorylation event that alter the functioning of individual neurons and the neural circuits in which they operate. Short-term memory may involve the phosphorylation of presynaptic or postsynaptic proteins in response to synaptic activity, which would result in transient facilitation or inhibition of synaptic transmission. Long-term memory may involve the phosphorylation of proteins that play a part in the regulation of gene expression, which would result in more permanent modifications of synaptic transmission. For example, long-term potentiation (LTP), one of the most extensively studied models of memory at the cellular level, seems to be initiated through short-term changes in Ca^{2+}-dependent protein phosphorylation and maintained by longer-term changes in gene expression (see Ch. 53 for further discussion of memory).

Neuronal phosphoproteins differ considerably in the number and types of amino acid residues phosphorylated. The complexity of intracellular regulation is underscored by the now numerous and well-established observations of numerous proteins that are phosphorylated on more than one amino acid residue by more than

one type of protein kinase. Depending on the protein, phosphorylation of different residues can lead to similar or opposite changes in the function of that protein. It may take, for example, phosphorylation of multiple nearby residues to produce a change in the charge of the protein sufficient to result in some critical change in function. This appears to be the case for MAPKs, activation of which requires the phosphorylation of nearby threonine and tyrosine residues. In contrast, phosphorylation of one residue may activate a protein, whereas phosphorylation of another might inhibit it; this is the case for CREB, as will be described below. In still other cases, interactions among the sites of phosphorylation of a protein can be more complex: phosphorylation of one residue can influence the ability of the other residues to undergo phosphorylation. Phosphorylation of neuronal proteins by more than one protein kinase can integrate the activities of multiple intracellular pathways to achieve coordinated regulation of cell function.

Phosphorylation of a protein can influence its functional activity in several ways. For many proteins, a change in charge and conformation, due to the addition of phosphate groups to the primary structure, results in alterations in their intrinsic functional activity. For example, the catalytic activity of an enzyme can be switched on or off, or an ion channel can open or close, upon such a change in charge and conformation. For many other proteins, phosphorylation-induced changes in charge and conformation result in alterations in the affinity of the proteins for other molecules. For example, phosphorylation alters the affinity of numerous enzymes for their cofactors and end-product inhibitors, phosphorylation of receptors can alter their affinity for G proteins and phosphorylation of some transcription factors alters their DNA-binding properties.

To illustrate some of the roles played by protein phosphorylation in the regulation of nervous system function, some well characterized neuronal phosphoproteins and some aspects of neurotransmission regulated by phosphorylation in the pre- and postsynaptic compartments are discussed in detail.

Regulation of neurotransmitter synthesis: tyrosine hydroxylase. This protein is the rate-limiting enzyme in the biosynthesis of the catecholamine neurotransmitters dopamine, norepinephrine and epinephrine. Diverse extracellular signals have been shown to stimulate catecholamine biosynthesis *in vivo*, effects mediated at least in part through increases in the catalytic activity of tyrosine hydroxylase (see also Ch. 12). Such changes in the catalytic activity of tyrosine hydroxylase are achieved largely through cAMP-dependent and Ca^{2+}-dependent phosphorylation of the enzyme [50, 51].

Tyrosine hydroxylase is a tetramer of identical 60 kDa subunits. The enzyme is an effective substrate for at least nine different protein kinases (Fig. 23-5). One site, serine 40, of tyrosine hydroxylase is phosphorylated by PKA, PKC, PKG, CaMKII, and MAPKAP kinases 1 and 2. CaMKII and MAPKAP kinases 1 and 2 also phosphorylate a second site, serine 19. Phosphorylation of either site by CaMKII is reported to require the presence of an 'activator' protein, termed 14-3-3. Phosphorylation of tyrosine hydroxylase increases the catalytic activity of the enzyme by increasing its V_{max} and its affinity for its biopterin cofactor and by decreasing the affinity of the enzyme for its end-product inhibitors. Tyrosine hydroxylase also contains additional serine residues that are phosphorylated by other protein kinases. For example, the enzyme is phosphorylated on Ser31 by ERK 1 and 2, which is also thought to increase the catalytic activity of the enzyme. This site has also recently been reported to serve as a substrate for CDK5 [52]. While CDK5 may directly phosphorylate Ser31 of tyrosine hydroxylase, it may also block phosphorylation of this site by ERK1/2 by phosphorylating and inhibiting the ERK-activating kinase MEK. Additionally, phosphorylation of Ser31 of tyrosine hydroxylase in humans may be affected through alternative splicing that results in the expression of isoforms of tyrosine hydroxylase with different amino acid sequences adjacent to Ser31, which alters its ability to undergo phosphorylation. In some cell culture preparations, tyrosine hydroxylase has been observed to be phosphorylated at a fourth site, serine 8, by Cdc2. This is an astounding degree of complex regulation converging on a single enzyme, and efforts are still under way to understand its full physiological significance.

Tyrosine hydroxylase is present in neurons in the CNS and PNS that synthesize catecholamines. The enzyme is also present at high concentrations in cells that are developmentally related to catecholaminergic neurons, such as

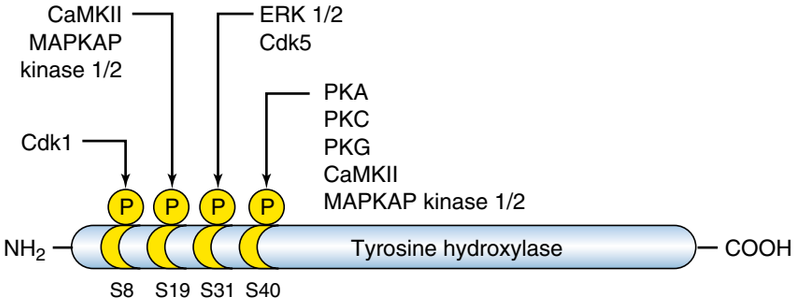

FIGURE 23-5 Schematic diagram of tyrosine hydroxylase with sites of phosphorylation indicated (*yellow*) along with the protein kinases.

adrenal chromaffin cells and pheochromocytoma cells. Tyrosine hydroxylase is predominantly a cytosolic protein and is present in high concentrations in catecholaminergic nerve terminals.

The regulation of phosphorylation of tyrosine hydroxylase is affected by stimuli that increase Ca^{2+} or cAMP concentrations in neurons, including nerve impulse conduction and certain neurotransmitters in well-defined regions of the nervous system, in the adrenal medulla and in cultured pheochromocytoma cells. In addition, tyrosine hydroxylase phosphorylation is stimulated by nerve growth factor in certain cell types, possibly via the activation of ERKs. These changes in the phosphorylation of tyrosine hydroxylase have been shown to correlate with changes in the catalytic activity of the enzyme and in the rate of catecholamine biosynthesis.

Several key questions remain with regard to the regulation of tyrosine hydroxylase by phosphorylation. What is the precise effect of the phosphorylation of each of these serine residues on the catalytic activity of the enzyme? How does the phosphorylation of multiple residues affect enzyme activity? Does the phosphorylation of one residue affect the ability of the others to be phosphorylated? Tyrosine hydroxylase provides a striking example as to how multiple intracellular messengers and protein kinases converge functionally through the phosphorylation of a single substrate protein. Phosphorylation of tyrosine hydroxylase by cAMP-dependent and Ca^{2+}-dependent protein kinases and by MAPK cascades enables a catecholaminergic cell to adjust its rate of neurotransmitter biosynthesis to a host of external stimuli and to changing physiological needs.

Regulation of neurotransmitter receptors: the β-adrenergic receptor. This receptor, of which three subtypes have been cloned, mediates many of the effects of norepinephrine and epinephrine in the brain and peripheral tissues. One of the dramatic features of β-adrenergic receptor function is its rapid desensitization in response to agonist stimulation. It is now known that one important mechanism for this desensitization is phosphorylation of the receptor both by PKA and by a receptor-associated protein kinase, βARK (also called GRK2; Fig. 23-6).

Activation of the β-adrenergic receptor leads, via coupling with G_s, to activation of adenylyl cyclase and increased concentrations of cAMP and of activated PKA. The activated kinase then leads to the many physiological effects of β-adrenergic receptor stimulation via the phosphorylation of numerous substrate proteins. However, among those proteins phosphorylated by the kinase is the receptor itself, which is phosphorylated on several serine residues in its cytoplasmic domains. This phosphorylation reduces subsequent activation of the receptor, and the precise mechanisms underlying this desensitization are becoming increasingly understood. It is now believed that phosphorylation of the receptor triggers its internalization via a dynamin-dependent process from the plasma membrane, rendering it inaccessible to further agonist stimulation.

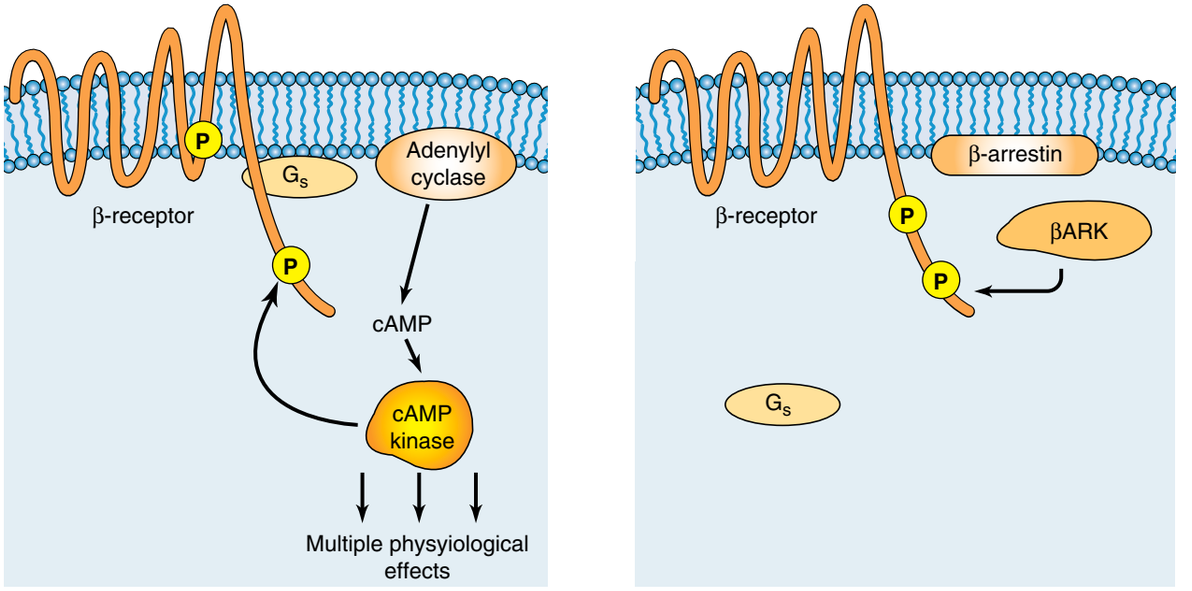

FIGURE 23-6 Schematic diagram illustrating desensitization of the β-adrenergic receptor mediated by receptor phosphorylation. (**A**) Activation of the β-adrenergic receptor by its ligand results, via coupling with G_s, in stimulation of adenylyl cyclase, increased concentrations of cAMP and stimulation of cAMP-dependent protein kinase, which mediates the physiological effects of β-receptor activation through the phosphorylation of numerous cellular proteins. The protein kinase also phosphorylates several serine residues in cytoplasmic domains of the receptor, which results in receptor desensitization. (**B**) Activation of the β-adrenergic receptor also results in a conformational change in the receptor, which renders it an effective substrate for β-adrenergic receptor kinase (β*ARK*). βARK phosphorylates the receptor at distinct serine residues, which leads to the functional 'uncoupling' of the receptor from G_s, thereby resulting in receptor desensitization. This uncoupling requires the action of an additional protein, termed β-arrestin, which interacts preferentially with the phosphorylated receptor to prevent its activation of G_s

Internalized receptor can either be returned to the plasma membrane upon its dephosphorylation or undergo proteolysis during periods of prolonged agonist exposure.

Phosphorylation of the β-adrenergic receptor by PKA can be viewed as a classical example of negative feedback: activation of the receptor stimulates intracellular cascades that feed back to reduce further receptor activation. Phosphorylation of the receptor by PKA could also mediate heterologous desensitization of the receptor: any neurotransmitter–receptor system that works through cAMP would be expected to stimulate β-adrenergic receptor phosphorylation via PKA and lead to receptor desensitization. This is one mechanism by which one neurotransmitter–receptor system can affect another.

The β-adrenergic receptor is also phosphorylated on several distinct serine residues by βARK, a protein serine–threonine kinase that is constitutively active and not regulated by second messengers (Fig. 23-6). Rather, this kinase can phosphorylate the receptor only when the receptor is bound to ligand; i.e. the binding of ligand to the receptor alters the conformation of the receptor such that it is rendered a good substrate for the receptor kinase. Upon phosphorylation by βARK, the receptor is then able to bind an additional protein, called β-arrestin, which in a sense sequesters the receptor and renders it unable to interact further with ligand or G protein. Studies (described in Ch. 19) have shown that the βγ subunits of G proteins are required to bring βARK, predominantly a cytosolic enzyme, into close association with those plasma membrane-associated receptors that are occupied by ligand.

Phosphorylation of the β-adrenergic receptor by βARK, like its phosphorylation by the cAMP-dependent enzyme, represents an example of negative feedback. However, unlike phosphorylation by cAMP-dependent protein kinase, phosphorylation by βARK represents an example of homologous desensitization: only the β-adrenergic receptor would be affected in this process and, moreover, only those receptor molecules occupied by ligand would be affected.

This model of receptor desensitization appears to be a general mechanism by which many G-protein-coupled receptors are desensitized upon persistent ligand binding [53]. Thus, there are at least six forms of βARK-like kinases cloned to date, which are referred to as GRKs. The GRKs are differentially expressed in the nervous system, and each GRK appears to phosphorylate a distinct subset of G-protein-coupled receptors. To date, GRKs have been shown to phosphorylate and desensitize several G-protein-coupled receptors, which, in addition to the β-adrenergic receptor, include α_2-adrenergic receptors and the opioid receptors. In addition, several forms of arrestin are known which are believed to contribute to the specific desensitization of G-protein-coupled receptor function by the various GRKs.

Neurotransmitter release and the synaptic vesicle cycle. Many of the proteins involved in the regulation of neurotransmitter release are regulated by phosphorylation [54, 55]. Neurotransmitter-filled synaptic vesicles congregate in the presynaptic nerve terminal. These vesicles undergo exocytosis when an action potential invades the terminal and triggers the influx of Ca^{2+} via voltage-gated Ca^{2+} channels. The Ca^{2+} signal then triggers the fusion of synaptic vesicles with the plasmalemma and results in neurotransmitter release. Subsequently, the vesicles are recovered by endocytosis and are recycled for subsequent release events (Fig. 23-7). Not surprisingly, mechanisms controlling these processes dramatically affect synaptic plasticity. Also, many of the same processes are involved in synaptogenesis. A large number of protein families have been implicated in the docking, priming and fusion of synaptic vesicles to the nerve terminal plasma membrane, and the molecular details of this complex process are now well established [56]. While most of the proteins involved in this process are phosphorylated by diverse types of protein kinases, the precise physiological role played by each phosphorylation event remains the subject of ongoing research.

For example, the membrane proteins SNAP25 (15 kDa synaptosomal associated protein), syntaxin 1 and synaptobrevin (also known as VAMP) are key mediators of the release process through the formation of a 7S complex. These proteins serve as substrates for various Ca^{2+}-sensitive and -insensitive kinases such as CAMKII (syntaxin 1A, synaptobrevin, SNAP25), PKC (SNAP25) and casein kinase II (syntaxin 1A), but the functions of the individual phosphorylation events await further elucidation. It seems likely that the phosphorylation of these proteins and their interactions with two other soluble phosphoproteins, NSF (N-ethylmaleimide-sensitive fusion protein) and SNAP (soluble NSF attachment protein), contribute to the regulation of vesicle priming and subsequent docking or fusion. Synaptotagmins are Ca^{2+}- and phospholipid-binding synaptic-vesicle phosphoproteins, which interact with SNAP25, synaptobrevin and syntaxin 1A to mediate synaptic vesicle exocytosis. It is probably phosphorylated by casein kinase II and CaMKII. Other synaptic vesicle phosphoproteins include Munc18 (nsec1), RAB3A, rabphilin and synaptophysin, to name a few.

It has recently become evident that some forms of LTP are thought to be expressed by lasting changes in neurotransmitter release, which requires PKA activation. LTP is lost when the genes for the Ca^{2+}/calmodulin-sensitive adenylyl cyclase, as well as regulatory and catalytic subunits of PKA, are knocked out. One synaptic vesicle protein, RIM1, is known to be a PKA substrate and is thought to be a mediator of this process. LTP cannot be detected in hippocampal circuits from RIM1 knockout mice. Loss of RIM1 also leads to the specific loss of presynaptic LTP in cerebellar neuron primary cultures [57]. This latter defect can be rescued by presynaptic expression of wild-type RIM1, but not by a site-directed mutant form of RIM1 in which the PKA site had been changed to alanine so that it could no longer serve as a PKA substrate. The question remains as to whether RIM phosphorylation by PKA is

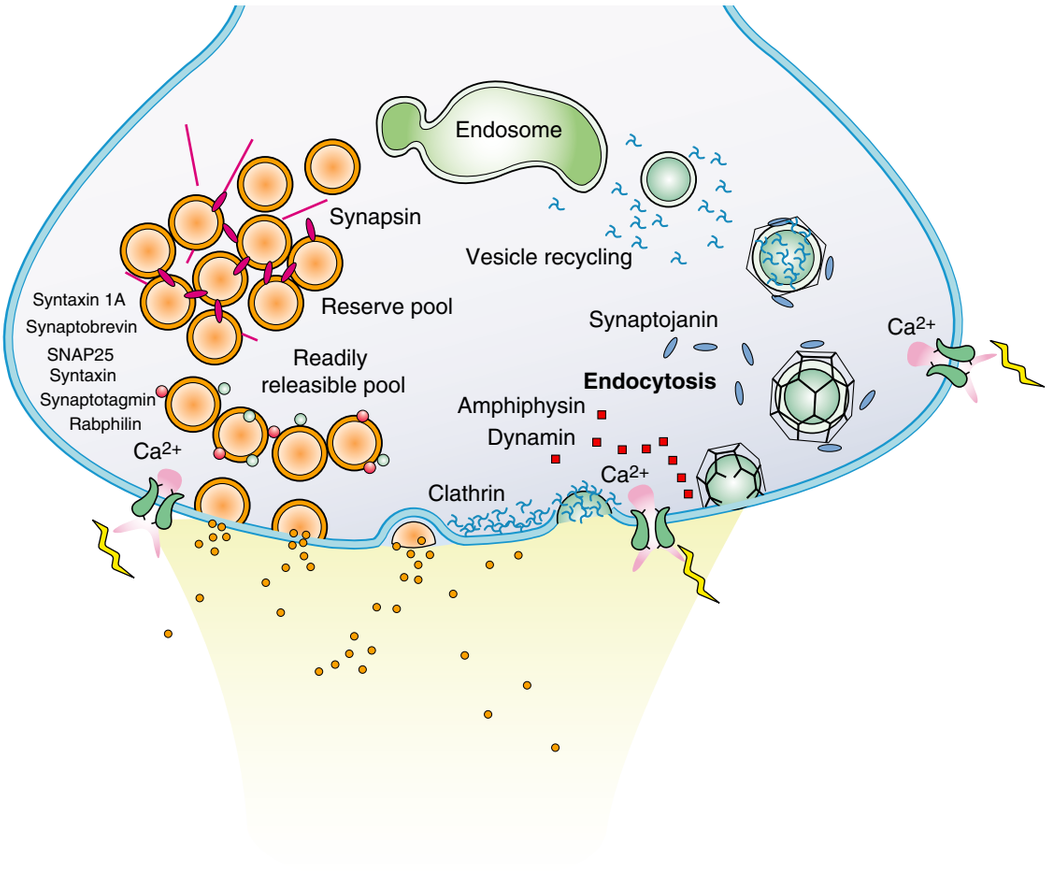

FIGURE 23-7 Schematic diagram of the synaptic vesicle cycle. Neurotransmitter-filled vesicles held in the reserve pool are trafficked to a readily releasable pool where they are docked, primed and fused with the plasmalemma at the synaptic cleft. Also depicted is the clathrin-mediated endocytosis of the fused vesicles, which is followed by their uncoating and recycling via early endosomal fusion and budding of vesicles. This returns the vesicles to the reserve pool. Some of the phosphoproteins which regulate these steps are shown. For a more detailed description of this process and the phosphoproteins involved the reader is directed to the excellent text by Cowen *et al.* [67].

sufficient to induce this LTP, or whether there may be other PKA substrates that cause LTP. In agreement with the role, of RIM1 in synaptic plasticity, knockout mice are also severely deficient in learning and memory [58].

One of the best-characterized phosphoproteins involved in the synaptic vesicle cycle is the peripheral membrane protein synapsin. The synapsin family includes three isoforms, synapsin I, II and III. The precise function subserved by synapsins is unknown, although their phosphorylation by CaMKII may release vesicles from a reserve pool, where they are thought to be anchored to the cytoskeleton via synapsin, allowing them to move to a readily releasable pool poised for membrane fusion and neurotransmitter release. Synapsins also serve as substrates for PKA, MAPK and CDK5. Of course, the voltage-gated Ca^{2+} channels that mediate Ca^{2+} entry into the nerve terminal are substrates for PKA and other kinases, phosphorylation events that robustly regulate the physiological activity of these channels.

In order to maintain sustained responsiveness to repetitive firing and the structural integrity of the synapse, neurotransmitter release (exocytosis) must be tightly coupled to vesicle recovery (endocytosis) [59]. Synaptic vesicle endocytosis is mediated by the structurally unrelated dephosphoin family of phosphoproteins, which includes amphiphysin I, dynamin, synaptojanin, epsin, eps15 and AP180 [60]. These proteins are involved in the clathrin-mediated endocytosis and subsequent uncoating prior to vesicle fusion with synaptic endosomes. All these proteins are coordinately dephosphorylated by calcineurin. Rephosphorylation of the various dephosphins is essential for the maintenance of subsequent cycles of endocytosis. Characterizing the phosphorylation events involving dephosphins is the subject of active study. Amphiphysin, dynamin, eps15, epsin and synaptojanin have each been shown to be substrates for CDK5, and dynamin is also a substrate for PKC.

Postsynaptic signaling pathways and synaptic plasticity. Postsynaptic forms of synaptic plasticity are also heavily regulated by protein phosphorylation. For example, control of protein dephosphorylation catalyzed by PP1 has

been demonstrated to be a key aspect of the signaling pathways underlying LTP and LTD (long-term depression). PP1 activity contributes to the induction of LTD, and inhibition of PP1 has been shown to promote LTP. Numerous studies implicate the regulation of PP1 by inhibitor-1 in the hippocampus and DARPP-32 in the striatum as key mediators of synaptic strength.

In one model of LTP (Fig. 23-8) first suggested by John Lisman [61] involving inhibitor-1, high frequency stimulation causes intense NMDA receptor activation and increased intracellular Ca^{2+} concentrations, which activates Ca^{2+}/calmodulin-dependent forms of adenylyl cyclases (see Ch. 21). Increased cAMP levels activates PKA, which phosphorylates inhibitor-1 at Thr35. As a result, inhibitor-1 is converted into a potent inactivator of PP1. Inhibitor-1-mediated blockade of PP1 activity allows CaMKII to achieve a Ca^{2+}-independent state of activity through its autophosphorylation, as mentioned earlier.

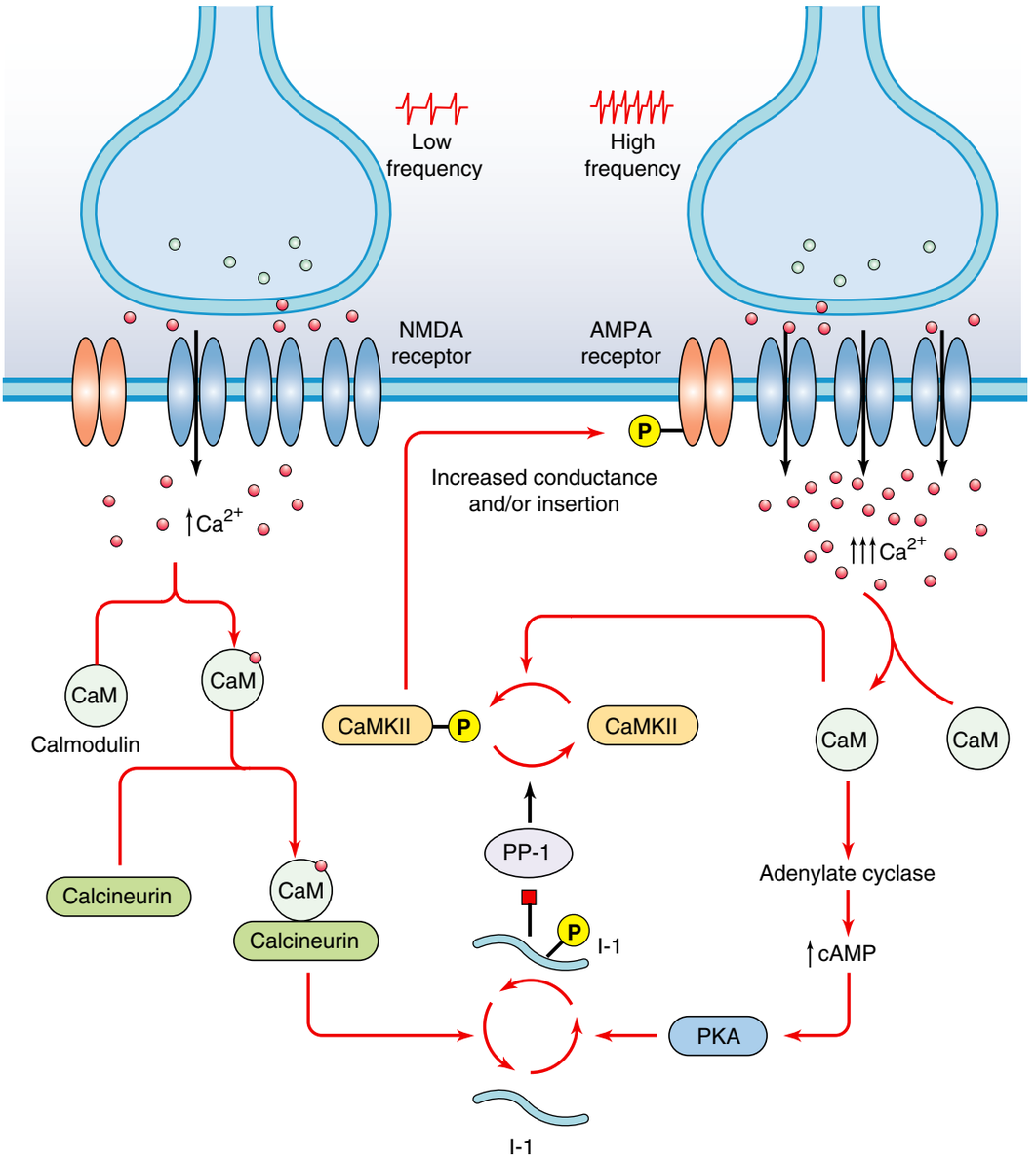

FIGURE 23-8 Schematic diagram of postsynaptic mechanisms of synaptic plasticity. In this model, low-frequency stimulation of postsynaptic neurons results in a mild increase in intracellular Ca^{2+} levels and activation of relatively lower amounts of calmodulin (CaM), which binds with higher affinity to and activates calcineurin, maintaining inhibitor-1 in the dephosphorylated state (**left**). High-frequency activation leads to adequate amounts of activated CaM to activate Ca^{2+}/CaM-dependent adenylyl cyclase and PKA-dependent phosphorylation of inhibitor-1 at Thr35 (**right**). This pathway, in combination with Ca^{2+}/CaM-dependent activation of CaMKII, causes autophosphorylation of CaMKII at Ser286, thereby allowing the kinase to become CaM-independent and contribute to strengthening of the synapse by a number of pathways [24]. (Redrawn with permission from Winder, D. G. and Sweatt, J. D. Roles of serine/threonine phosphatases in hippocampal synaptic plasticity. *Nat. Rev. Neurosci.* 2: 461–474, 2001.)

This, in turn, results in increased levels of phosphorylation of several CaMKII substrates that contribute to increased synaptic strength. These include AMPA glutamate receptors and the transcription factor CREB.

In contrast to LTP, LTD is induced by low-frequency stimulation. This causes the selective activation of PP2B (calcineurin), which maintains inhibitor-1 in its dephosphorylated state. Several studies, including those using inhibitor-1 knockout mice and transgenic mice expressing a constitutively active form of inhibitor-1, demonstrate that the control of PP1 activity by inhibitor-1 in the hippocampus affects neuronal plasticity and learning and memory.

In the dopaminoceptive neurons of the striatum, the inhibitor-1 homolog, DARPP-32, similarly is converted into a PP1 inhibitor when phosphorylated at Thr34 by PKA. However, in the medium spiny neurons of the striatum different neurotransmitter receptors and signaling pathways come into play. In these neurons, activation of D_1 dopamine receptors invokes the G_s-adenylyl cyclase-cAMP-PKA pathway, which causes DARPP-32 to be converted to a PP1 inhibitor. DARPP-32 can also be converted into a PP1 inhibitor through phosphorylation of the same residue by PKG. The numerous pathways that result in increased PKA- or PKG-dependent phosphorylation of DARPP-32 and inhibition of PP1 are opposed by other pathways, such as activation of glutamate receptor channels, which results in increased intracellular Ca^{2+} levels. Under these latter circumstances, increased Ca^{2+} levels activate PP2B, which dephosphorylates Thr34 site on DARPP-32, which converts it back to its PP1-noninhibiting form.

Changes in the phosphorylation state of DARPP-32, through activation of its phosphatase-inhibitory activity, indirectly influence the phosphorylation state of many other proteins and thereby mediate some of the effects of dopamine and of these other first messengers on cell function. The full spectrum of proteins regulated in this way by DARPP-32 phosphorylation has not yet been identified, although voltage-gated Ca^{2+}, Na^+ and K^+ channels, the Na^+,K^+-ATPase, and AMPA and NMDA glutamate receptors represent examples of well characterized target proteins [33]. Regulation of these proteins by DARPP-32 provides a mechanism by which alterations in DARPP-32 phosphorylation lead, under physiological conditions, to changes in the electrical excitability of neurons and to important forms of behavioral plasticity mediated by these cells.

The ability of DARPP-32 and inhibitor-1 to be phosphorylated at their PKA site is affected by phosphorylation at numerous other sites. DARPP-32 is phosphorylated by CDK5 and casein kinases I and II, all of which affect the phosphorylation state at Thr34. Inhibitor-1 also serves as a substrate of CDK5, at a site that is not homologous to the CDK5 site on DARPP-32. Further work is needed to understand the physiological significance of these other phosphorylation reactions.

Regulation of gene transcription: CREB. CREB is a prototypical example of a transcription factor whose physiological activity is regulated by phosphorylation. It is one of a family of proteins that mediates some of the effects of cAMP on gene expression [62]. CREB binds to a specific sequence of DNA, termed a cAMP response element (CRE), in the promoter (regulatory) regions of genes and thereby increases or decreases the rate at which those genes are transcribed (see Ch. 26).

The details of the mechanism by which CREB influences gene expression are becoming increasingly understood (Fig. 23-9) [62, 63]. In the basal, or unstimulated, state, CREB is bound to its CREs but does not alter transcriptional rates under most circumstances. Stimulation of a cell by a variety of first messengers leads to the phosphorylation and activation of CREB, which then leads to the regulation of gene transcription. Such phosphorylation of CREB occurs on a single serine residue, serine 133, and can be mediated by one of several protein kinases.

The detailed mechanisms involved in CREB phosphorylation were first established for the cAMP pathway. A first messenger that increases cAMP concentrations leads to activation of PKA and to translocation of the free catalytic subunit of the protein kinase into the nucleus, where it phosphorylates CREB on serine 133. Such phosphorylation then promotes the binding of CREB to a CREB-binding protein (CBP). CBP, upon binding CREB, interacts directly with the RNA polymerase II complex, which mediates the initiation of transcription. In most cases, such interactions lead to the activation of transcription, although it is possible that the expression of some genes may be repressed.

Stimulation of a cell by first messengers that increase cellular Ca^{2+} concentrations similarly activates CREB (Fig. 23-9). This appears to occur via the phosphorylation of CREB on serine 133 by a CaMK, probably CaMKIV as well as, possibly, CaMKI. It remains to be established whether the activated kinase translocates to the nucleus, by analogy with the catalytic subunit of the cAMP kinase, or whether elevated Ca^{2+} signals enter the nucleus and activate the kinase already there. Interestingly, phosphorylation of CREB on a distinct serine residue, serine 142, by CaMKII appears to inhibit the transcriptional activity of CREB *in vitro*, although whether this inhibitory effect occurs *in vivo* is unknown.

CREB is also phosphorylated on serine 133 by stimulation of growth factor signaling cascades [63]. This occurs via a complex pathway involving MAPK cascades (Fig. 23-9). Thus, as outlined earlier, nerve growth factor and related neurotrophins that act on receptor tyrosine kinases lead to the successive activation of Ras, Raf, MEK and ERK. Activated ERK then phosphorylates and activates a serine–threonine kinase, RSK, particular subtypes of which directly activate CREB via the phosphorylation of serine 133.

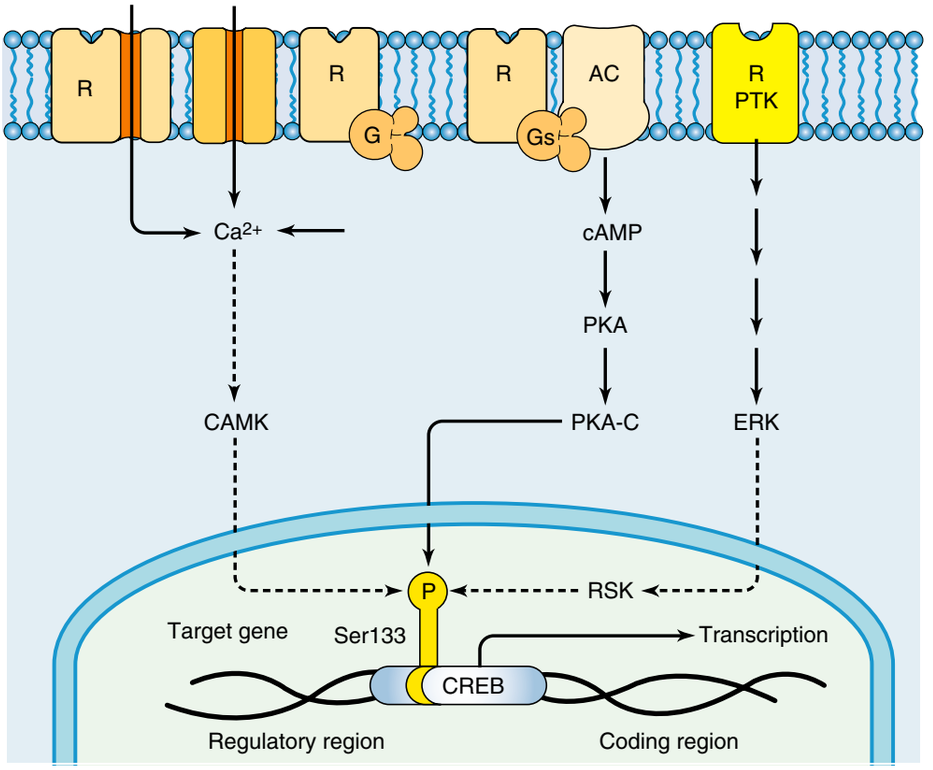

FIGURE 23-9 Schematic diagram of the mechanisms by which diverse types of first messengers converge on the phosphorylation of serine 133 of cAMP response element-binding protein (*CREB*). Some first messengers do this by increasing cAMP concentrations by activation of G-protein-coupled receptors (*R*), G_s, adenylyl cyclase (*AC*) and cAMP-dependent protein kinase (*PKA*), which then phosphorylates CREB on ser 133. Others do so by increasing Ca^{2+} concentrations by activation of ionotropic receptors (*R*) that flux Ca^{2+}, by activation of voltage-gated Ca^{2+} channels through membrane depolarization or by activation of G-protein-coupled receptors that elevate intracellular Ca^{2+} concentrations, for example via the phospholipase C pathway. Increased concentrations of Ca^{2+} then lead to activation of Ca^{2+}/calmodulin-dependent protein kinases (type IV and, possibly, I) (*CaM-K*), which also phosphorylate CREB on ser 133. Still others do so by activating ERK: by activation of receptor-associated protein tyrosine kinases (*R-PTK*) and the mitogen-activated protein kinase cascade (Figs 23-3, 23-4). Activation of extracellular signal-regulated kinase (*ERK*) then phosphorylates and activates ribosomal S6 kinase (*RSK*), which also phosphorylates CREB on ser 133. The step at which the cytoplasmic signal is transduced to the nucleus is well established for PKA: activation of the kinase leads to its dissociation and generation of the free catalytic subunit (*PKA-C*), which then translocates to the nucleus and phosphorylates CREB. The process is less well established for the CaMKs and ERK, as indicated by the *dashed lines*. Phosphorylation of CREB on ser 133 activates its transcriptional activity and leads to changes in the rate of transcription of target genes.

There is evidence that the dephosphorylation of CREB is catalyzed by PP1 and, perhaps, PP2A [28, 40]. Phosphorylation of NIPP-1 (a protein phosphatase inhibitor protein described above) may contribute to the tightly timed pulse of phospho-CREB induced in the nucleus following cell stimulation. According to this scheme, the catalytic subunit of PKA, for example, would rapidly phosphorylate CREB, resulting in a change in transcription and, with a slightly slower time course, phosphorylate and reduce the phosphatase-inhibitory activity of NIPP1. This would release PP1 from its tonic inhibition and result in CREB dephosphorylation.

The number of neural genes known to contain CREs is growing as more and more genes are cloned. Prominent examples are tyrosine hydroxylase, c-*fos*, proenkephalin, prodynorphin, somatostatin and vasoactive intestinal polypeptide (*VIP*). Expression of these genes has been shown to be regulated at their CREs by the phosphorylation of CREB or a CREB-like protein. In fact, for some genes, for example, tyrosine hydroxylase, activation of CREB appears to be the critical transcriptional mechanism controlling both the basal level of gene expression and the induction of expression seen in response to cellular stimulation.

Consideration of CREB highlights the view, mentioned earlier in this chapter, that among the many cellular processes regulated by protein phosphorylation is gene transcription. This view is further underscored by the knowledge that virtually all classes of transcription factor undergo phosphorylation by numerous types of protein kinases. Even transcription factors that are regulated primarily through alterations in their own transcription, such as c-Fos and other immediate early gene transcription factors, are influenced by phosphorylation.

For example, c-Fos is heavily phosphorylated on a series of serine residues in the C-terminal domain of the protein by several types of protein kinases. The likely functional importance of these phosphorylation sites is indicated by the fact that the difference between c-Fos (the normal cellular form of the protein) and v-Fos (the viral oncogene product) is a frameshift mutation in the v-Fos protein, which obliterates the phosphorylated serine residues. It is speculated that the loss of these phosphorylation sites removes one mechanism by which the cell can regulate the protein, thereby leading to cellular transformation.

Cellular signals converge at the level of protein phosphorylation pathways. Individual intracellular messenger pathways, such as cAMP, Ca^{2+} and MAPK pathways, are often drawn as distinct biochemical cascades that operate in parallel in the control of cell function. While this is useful for didactic purposes, it is now well established that these various pathways function as complex webs, with virtually every conceivable type of interaction seen among them.

This can be illustrated by known interactions between the cAMP and Ca^{2+} pathways. A first messenger that initially activates the cAMP pathway would be expected to exert secondary effects on the Ca^{2+} pathway at many levels via phosphorylation by PKA. First, Ca^{2+} channels and the inositol trisphosphate (IP_3) receptor will be phosphorylated by PKA to modulate intracellular concentrations of Ca^{2+}. Second, phospholipase C (PLC) is a substrate for PKA, and its phosphorylation modulates intracellular calcium concentrations, via the generation of IP_3, as well as the activity of PKC, via the generation of DAG, and several types of CAMK. Similarly, the Ca^{2+} pathway exerts potent effects on the cAMP pathway, for example, by activating or inhibiting the various forms of adenylyl cyclase expressed in mammalian tissues (see Ch. 21).

The cAMP and Ca^{2+} pathways also interact at the level of protein kinases and protein phosphatases. This is illustrated by inhibitor-1 and DARPP-32, which are phosphorylated and activated by PKA and then inhibit PP1, which can dephosphorylate numerous substrates for Ca^{2+}-dependent protein kinases. Another example is the physical association between PKA and PP2B (a Ca^{2+}/calmodulin-activated enzyme) via the AKAP-anchoring proteins.

Multiple interactions are also being demonstrated between the traditional second-messenger pathways and the MAPK cascades. Free βγ G protein subunits, generated upon activation of receptors coupled to the G_i family, lead to activation of the ERK pathway. The mechanism by which this occurs, which may involve an interaction between the βγ subunits and Ras or Raf, is a subject of intensive research (see Ch. 19). In addition, increases in cellular Ca^{2+} concentrations lead to stimulation of the ERK pathway, apparently via phosphorylation by CaMKs of proteins, for example Shc and Grb, that link growth factor receptor tyrosine kinases to Ras. Activation of the cAMP pathway exerts the opposite effect in most cells: it results in inhibition of the ERK pathway, possibly via the phosphorylation and inhibition of Raf by PKA.

Another major way by which intracellular messenger pathways interact is via phosphorylation of the same substrate proteins. There are numerous examples of this, some discussed earlier in this chapter. One striking example is provided by CREB, which is phosphorylated on the same single serine residue by PKA, CaMK and RSK, activated by the MAPK cascade.

The above discussion suggests that a single extracellular signal may elicit changes in numerous intracellular signaling cascade, which would raise questions as to how specific biological responses are achieved in a cell. In reality, such specificity is achieved because not all the aforementioned interactions occur in every cell type. Specific interactions depend on the strength and persistence of the original extracellular signal, the subcellular location where the signal acts and the specific subtypes of individual signaling proteins that are expressed in a cell at the relevant subcellular sites. In addition, regulation of intracellular messenger pathways does not depend on the actions of a single extracellular signal but rather on the integration of multiple signals that impinge on cells at any given point in time.

Nevertheless, rather than being generated through a single molecular pathway, most physiological responses should be viewed as the complex product of the coordinated actions of multiple cellular messengers involving multiple molecular pathways. This extraordinary complexity underscores the difficulty in determining the precise molecular basis of a given physiological process. The central role of protein phosphorylation as a regulatory mechanism that mediates the actions of many individual cellular messengers and the interactions among them imbues the study of protein phosphorylation with a unique potential: to provide an experimental framework within which the layers of molecular steps that underlie and regulate cell function may be unraveled.

PROTEIN PHOSPHORYLATION MECHANISMS IN DISEASE

The study of protein phosphorylation has helped to clarify the mechanisms involved in the causes and manifestation of disorders of the nervous system. Two illustrative examples are given here: Alzheimer's disease and opiate addiction.

Abnormal phosphorylation of specific neural proteins may contribute to the development of Alzheimer's disease. Alzheimer's disease is a serious dementing illness of enormous medical and societal importance (see Ch. 47). It involves the degeneration of specific types of neuron in the brain. An invariable feature of Alzheimer's disease is the appearance of amyloid plaques. These plaques contain the Aβ amyloid protein, and there is some

evidence to suggest that the accumulation of Aβ contributes to neuronal degeneration and that mutations in the gene that codes for this protein are involved in a small number of cases of familial Alzheimer's disease.

Aβ is derived from amyloid precursor protein (APP) through proteolytic processing. In normal cells, APP is processed into fragments that are not associated with disease states. It is not yet known why APP is cleaved anomalously to yield Aβ to a greater extent in Alzheimer's disease. However, increasing evidence indicates that signal-transduction pathways involving protein phosphorylation are potent regulators of APP cleavage and can alter the rates of cleavage both at normal sites and at sites yielding putative amyloidogenic fragments [64]. The role of PKC in this process has received the most attention. Activators of PKC, or inhibitors of PP1, such as okadaic acid, dramatically stimulate APP proteolysis, whereas PKC inhibitors diminish cleavage of the protein. The finding that PKC regulates APP processing raises the possibility that agents that regulate this protein kinase, or specific protein phosphatases, might prove to be useful in the clinical management of Alzheimer's disease.

Further support for the potential clinical utility of drugs that influence protein kinases or protein phosphatases in Alzheimer's disease is the increasing evidence that aberrant phosphorylation mechanisms, i.e. excessive phosphorylation of the microtubule-associated protein called tau, contributes to the formation of neurofibrillary tangles, another component of the structural pathology of Alzheimer's disease and the related frontotemporal dementias [64, 65]. Under normal conditions, the most important kinases that phosphorylate tau are CDK5 and GSK3β (glycogen synthase kinase-3 β), both serine–threonine kinases. There is some evidence that other kinases (e.g. MAP kinases) may play a role in the hyperphosphorylation of tau associated with the development of neurofibrillary tangles, although this remains a subject of controversy. Intense research is under way to test whether inhibition of these various kinases may be clinically useful in the treatment of these dementing illnesses.

Upregulation of the cyclic AMP pathway is one mechanism underlying opiate addiction. The mechanisms by which opiates induce tolerance, dependence and withdrawal in specific target neurons has been a major focus of research for many years. The inability to account for prominent aspects of opiate addiction solely on the basis of alterations in endogenous opioid peptides or in opiate receptors has shifted attention to postreceptor mechanisms [66].

The noradrenergic neurons of the locus ceruleus have provided a useful model system for the study of opiate addiction (see Ch. 56). Acutely, opiates inhibit these neurons, in part by inhibiting the cAMP pathway via inhibition of adenylyl cyclase. Chronically, these neurons become tolerant to opiates; that is, their firing rates recover toward normal levels with continued exposure to the drug. Furthermore, they become dependent on opiates; that is, their firing rates increase far above control levels upon removal of the drug. These changes in the electrical excitability of locus ceruleus neurons mediate many of the physical signs and symptoms associated with opiate-withdrawal syndromes.

Increasing evidence indicates that a chronic opiate-induced upregulation of the cAMP pathway, manifested by increased concentrations of adenylyl cyclase, PKA and several phosphoprotein substrates for the protein kinase, contributes to opiate tolerance, dependence and withdrawal exhibited by locus ceruleus neurons [66]. This upregulated cAMP pathway can be viewed as a homeostatic response of the neurons to persistent opiate inhibition of the cells. In the chronic opiate-treated state, the upregulated cAMP pathway helps return neuronal firing rates to control levels, i.e. tolerance. Upon abrupt removal of the opiate via the administration of an opiate receptor antagonist, the upregulated cAMP accounts for part of the withdrawal activation of the cells.

Chronic opiate-induced upregulation of the cAMP pathway appears to be mediated in part by CREB: chronic opiate administration increases CREB expression; mice deficient in CREB show attenuated physical opiate dependence and withdrawal; and selective reductions of CREB in the locus ceruleus prevent upregulation of specific components of the cAMP pathway in response to chronic opiate administration [66]. This latter action is associated with attenuation of the electrical excitability of locus ceruleus neurons and of physical opiate dependence and withdrawal.

Upregulation of the cAMP pathway may be a common mechanism by which a number of neuronal cell types respond to chronic opiates and develop tolerance and dependence (see Ch. 56). There is also evidence that similar mechanisms involving alterations in the cAMP second-messenger and protein phosphorylation pathway may mediate aspects of addiction to other types of drugs of abuse, for example, cocaine and alcohol [66].

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REFERENCES

1. Bradshaw, R. A., Dennis, E. A. *Handbook of Cell Signaling.* New York: Academic Press, 2003.
2. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. The protein kinase complement of the human genome. *Science* 298: 1912–1934, 2002.
3. Hunter, T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80: 225–236, 1995.
4. Nestler, E. J. and Greengard, P. *Protein Phosphorylation in the Nervous System.* New York: Wiley, 1984.

5. Hunter, T. and Sefton, B. M. *Protein Phosphorylation. Part A. Methods in Enzymology*, vol 200. New York: Academic Press, 1991.
6. Taylor, S. S., Buechler, J. A. and Yonemoto, W. cAmp-dependent protein kinase: framework for a diverse family o fregulatory enzymes. *Annu. Rev. Biochem.* 59: 971–1005, 1990.
7. Colledge, M. and Scott, J. D. AKAPs: from structure to function. *Trends Cell Biol.* 9: 216–221, 1999.
8. Wall, M. E., Francis, S. H., Corbin, J. D. *et al.* Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase. *Proc. Natl Acad. Sci. U.S.A.* 100: 2380–2385, 2003.
9. Rosen, R. C. and Kostis, J. B. Overview of phosphodiesterase 5 inhibition in erectile dysfunction. *Am. J. Cardiol.* 92: 9M–18M, 2003.
10. Schulman, H,. Protein phosphorylation in neuronal plasticity and gene expression. *Curr. Opin. Neurobiol.* 5: 375–381, 1995.
11. Fink, C. C. and Meyer, T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr. Opin. Neurobiol.* 12: 293–199, 2002.
12. Mochly-Rosen, D. Localization of protein kinases by anchoring proteins: A theme in signal transduction. *Science* 268: 247–251, 1995.
13. Johnson, G. L. and Lapadat, R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911–1912, 2002.
14. Kortenjann, M. and Shaw, P. The growing family of MAP kinases: regulation and specificity. *Crit. Rev. Oncog.* 6: 99–115, 1995.
15. Block, C. and Wittinghofer, A. Switching to Rac and Rho. *Structure* 3: 1281–1284, 1995.
16. Curtis, J. and Finkbeiner, S. Sending signals from the synapse to the nucleus: possible roles for CaMK, Ras/ERK, and SAPK pathways in the regulation of synaptic plasticity and neuronal growth. *J. Neurosci. Res.* 58: 88–95, 1999.
17. Kim, S.-W., Yu, Y.-M., Piao, C. S., Kim, J.-B. and Lee, J.-K. Inhibition of delayed induction of p38 mitogen-activated protein kinase attenuates kainic acid-induced neuronal loss in the hippocampus. *Brain Res.* 1007: 188–191, 2004.
18. Zhou G, Bao, Z. Q. and Dixon, J. E. Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* 270: 12665–12669, 1995.
19. Abe, M. K., Kahle, K. T., Saelzler, M. P., Orth, K., Dixon, J. E. and Rosner, M. R. ERK7 is an autoactivated member of the MAPK family. *J. Biol. Chem.* 276: 21272–21279, 2001.
20. Abe, M. K., Saelzler, M. P., Espinosa, R. R. *et al.* ERK8, a new member of the mitogen-activated protein kinase family. *J. Biol. Chem.* 277: 16733–16743, 2002.
21. Dhavan, R. and Tsai, L. H. A decade of CDK5. *Nat. Rev. Mol. Cell Biol.* 2: 749–759, 2001.
22. Bibb, J. A., Snyder, G. L., Nishi A. *et al.* Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signaling in neurons. *Nature* 402: 669–671, 1999.
23. Bibb, J. A., Chen, J., Taylor, J. R. *et al.* Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410: 376–380, 2001.
24. Bibb, J. A. Role of Cdk5 in neuronal signaling, plasticity, and drug abuse. *Neurosignals* 12: 191–199, 2003.
25. Greten, D. and Karin, M. The IKK/NF-κB pathway–a target for prevention and treatment of cancer. *Cancer Lett.* 206: 193–199, 2004.
26. Ceulemans, H. and Bollen, M. Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol. Rev.* 84: 1–39, 2004.
27. Barford, D. Protein phosphatases. *Curr. Opin. Struct. Biol.* 5: 728–734, 1995.
28. Wera, S. and Hemmings, B. Serine/threonine protein phosphatases. *Biochem. J.* 311: 17–29, 1995.
29. Cohen, P. T. W. Novel protein serine/threoine phosphatases: variety is the spice of life. *Trends Biochem. Sci.* 27: 59–75, 1997.
30. Price, N. E. and Mumby, M. C. Brain protein serine/threonine phosphatases. *Curr. Opin. Neurobiol.* 9: 336–342, 1999.
31. Cohen, P. T. W. Protein phosphatase 1– targeted in many directions. *J. Cell. Sci.* 115: 241–256, 2002.
32. Goldberg, J., Huang, H. B., Kwon, Y. G., Greengard, P., Nairn, A. C. and Kuriyan, J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* 376: 745–753, 1995.
33. Greengard, P., Allen, P. B. and Nairn, A. C. Beyond the dopamine receptor. the DARPP-32/protein phosphatase-1 cascade. *Neuron* 23: 435–447, 1999.
34. Allen, P. B., Ouimet, C. C. and Greengard, P. Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc. Natl Acad. Sci. U.S.A.* 94: 9956–9961, 1997.
35. Allen, P. B., Kwon, Y. G., Nairn, A. C. and Greengard, P. Isolation and characterization of PNUTS, a putative protein phosphatase 1 nuclear targeting subunit. *J. Biol. Chem.* 273: 4089–4095, 1998.
36. Kim, Y. M., Watanabe, T., Allen, P. B. *et al.* PNUTS, a protein phosphatase 1 (PP1) nuclear targeting subunit. Characterization of its PP1- and RNA-binding domains and regulation by phosphorylation. *J. Biol. Chem.* 278: 3819–3828, 2003.
37. Virshup, D. M. Protein phosphatase 2A: a panoply of enzymes. *Curr. Opin. Cell Biol.* 12: 180–185, 2000.
38. Sontag, E. Protein phosphatase 2A: the Trojan Horse of cellular signaling. *Cell Signal.* 13: 7–16, 2001.
39. Klee, C. B., Ren, H. and Wang, X. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* 273: 13367–13370, 1998.
40. Shenolikar, S. Protein phosphatase regulation by endogenous inhibitors. *Cancer Biol.* 6: 219–227, 1995.
41. Steiner, J. P., Dawson, T. M., Fotuhi, M. and Snyder, S. H. Immunophilin regulation of neurotransmitter release. *Mol. Med.* 2: 325–333, 1996.
42. Flajolet, M., Rakhilin, S., Wang, H. *et al.* Protein phosphatase 2C binds selectively to and dephosphorylates metabotropic glutamate receptor 3. *Proc. Natl Acad. Sci. U.S.A.* 16006–16011, 2003.
43. Keyse, S. M. An emerging family of dual specificity MAP kinase phosphatases. *Biochim. Biophys. Acta* 1265: 152–160, 1995.
44. Nebreda, A. R. Inactivation of MAP kinases. *Trends Biochem. Sci.* 19: 1–2, 1994.
45. Catterall, W. A. Structure and function of voltage-gated ion channels. *Annu. Rev. Biochem.* 64: 493–531, 1995.
46. Palfrey, H. C. and Nairn, A. C. Calcium-dependent regulation of protein synthesis. *Adv. Second Messenger Phosphoprotein Res.* 30: 191–223, 1995.

47. Soderling, T. R. Modulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Neurochem. Int.* 28: 359–361, 1996.
48. Roche, K. W., O'Brien, R. J., Mammen, A. L., Bernhardt, J. and Huganir, R. L. Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* 16: 1179–1188, 1996.
49. Levitan, B. Modulation of ion channels by protein phosphoryaltion and dephosphorylation. *Annu. Rev. Physiol.* 56: 193–212, 1994.
50. Fitzpatrick, P. F. Tetrahydropterin-dependent amino acid hydrolases. *Annu. Rev. Biochem.* 68: 355–381, 1999.
51. Kumer, S. C. and Vrana, K. E. Intricate regulation of tyrosine hydroxylase activity and gene expression. *J. Neurochem.* 67: 443–462, 1996.
52. Kansy, J. W., Daubner, S. C., Nishi, A. *et al.*. Identification of tyrosine hydroxylase as a physiological substrate for Cdk5. *J. Neurochem.* 91: 374–384, 2004.
53. Freedman, N. and Lefkowitz, R. Desensitization of G protein-coupled receptors. *Recent Prog. Horm. Res.* 51: 319–351, 1996.
54. Lin, R. C. and Scheller, R. H. Mechanisms of synaptic vesicle exocytosis. *Annu Rev Cell Dev. Biol.* 16: 19–49, 2000.
55. Turner, K. M., Burgoyne, R. D. and Morgan, A. Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends Neurosci.* 22: 459–464, 1999.
56. Jahn, R., Lang, T. and Sudhof, T. C. Membrane fusion. *Cell* 112: 519–533, 2003.
57. Lonart, G., Schoch, S., Kaeser, P. S., Larkin, C. J., Sudhof, T. C. and Linden, D. J. Phosphorylation of RIM1α by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. *Cell* 115: 49–60, 2003.
58. Powell, C. M., Schoch, S., Monteggia, L. *et al.* The presynaptic active zone protein RIM1α is critical for normal learning and memory. *Neuron* 42: 143–153, 2004.
59. Cremona, O. and De Camilli, P. Synaptic vesicle endocytosis. *Curr. Opin. Neurobiol.* 7: 323–330, 1997.
60. Cousin, M. A. and Robinson, P. J. The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. *Trends Neurosci.* 24: 659–665, 2001.
61. Lisman, J. The CaM kinase II hypothesis for the storage of syaptic memory. *Trends Neurosci.* 17: 406–412, 1994.
62. Frank, D. and Greenberg, M. CREB: A mediator of long-term memory from mollusks to mammals. *Cell* 79: 5–8, 1994.
63. Xing, J., Ginty, D. and Greenberg, M. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* 273: 959–963, 1996.
64. Gandy, S. and Greengard, P. Processing of Alzheimer Ab-amloid precursor protein: cell biology, regulation, and role in Alzheimer disease. *Int. Rev. Neurobiol.* 35: 29–50, 1994.
65. Cruz, J. C., Tseng, H. C., Goldman, J. A., Shih, H. and Tsai, L. H. Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles. *Neuron* 40: 471–483, 2003.
66. Nestler, E. J. and Aghajanian, G. K. Molecular and cellular basis of addiction. *Science* 278: 58–63, 1997.
67. Cowan, M., Sudhof, T. C. and Stevens, C. F. *Synapses.* Baltimore: Johns Hopkins University Press, 2001.

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CHAPTER

24

Tyrosine Phosphorylation

Lit-Fui Lau

Richard Huganir

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TYROSINE PHOSPHORYLATION IN THE NERVOUS SYSTEM

Protein phosphorylation is one of the most important mechanisms in the regulation of cellular function. Proteins can be phosphorylated on serine, threonine or tyrosine residues. Most phosphorylation occurs on serine and threonine, with less than 1% on tyrosine (see Ch. 23). This perhaps accounts for the late discovery of tyrosine phosphorylation, which was found first on polyoma virus middle T antigen in 1979 by Hunter and colleagues [1, 2]. Since then, a plethora of tyrosine-phosphorylated proteins has been discovered. Originally, tyrosine phosphorylation was believed to be involved primarily in regulating cell proliferation, since many oncogene products and growth factor receptors are protein tyrosine kinases (PTKs). However, it has become clear that tyrosine phosphorylation is involved in regulating a variety of cellular processes. In fact, the nervous system contains a large variety of PTKs and protein tyrosine phosphatases (PTPs), and some of these are exclusively expressed in neuronal tissues. Figure 24-1 shows the

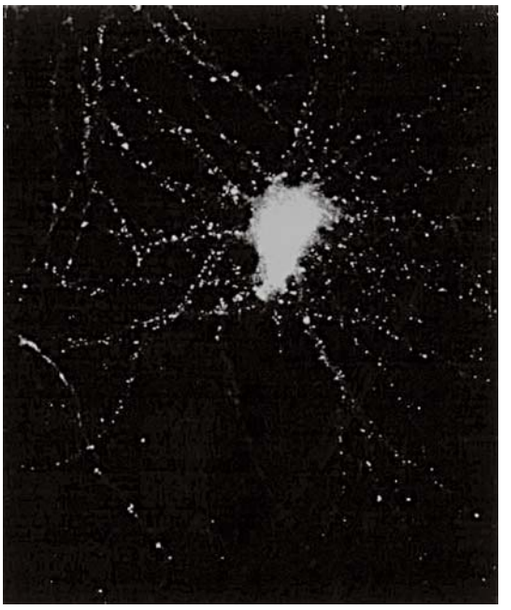

FIGURE 24-1 Phosphotyrosine staining of a hippocampal neuron. A cultured rat hippocampal pyramidal neuron is stained with an antiphosphotyrosine antibody and detected by a secondary antibody conjugated to rhodamine. The staining reflects the presence of tyrosine-phosphorylated proteins throughout the neuron, including the cell body and synaptic regions.

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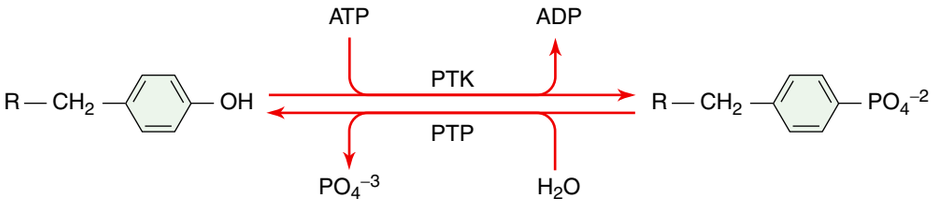

FIGURE 24-2 Tyrosine phosphorylation and dephosphorylation. Protein tyrosine kinases (*PTK*) catalyze the transfer of the γ-phosphate group from ATP to the hydroxyl group of tyrosine residues, whereas protein tyrosine phosphatases (*PTP*) remove the phosphate group from phosphotyrosine. *R*, protein.

immunocytochemical staining of a cultured hippocampal neuron with a phosphotyrosine antibody [3]. It reveals the presence of tyrosine-phosphorylated proteins in the cell body as well as in synapses, suggesting that tyrosine phosphorylation may play a role in neuronal function. Furthermore, many neuronal processes are modulated by inhibitors of PTK or PTP, or by modification of the genes encoding these enzymes.

Tyrosine phosphorylation is regulated by the balance between the activities of PTKs and PTPs (Fig. 24-2). Phosphorylation of a tyrosine residue adds negative charges to its side chain and increases its size. These alterations may consequently trigger structural changes and potential functional modifications in the tyrosine-phosphorylated protein. In addition, the phosphorylated tyrosine and its surrounding amino acids may become a molecular adhesive that interacts with phosphotyrosine binding proteins, thereby changing its subcellular localization and functions.

PROTEIN TYROSINE KINASES

Upon completion of the Human Genome Project, 90 unique genes are identified to encode PTKs [4]. Among these, 58 encode receptor protein tyrosine kinases (RPTKs), which can be categorized into 20 subfamilies (see Fig. 24-6) [5]; and 32 encode nonreceptor protein tyrosine kinases (NRPTKs), which belong to 10 subfamilies (Fig. 24-3) [5]. RPTKs are integral membrane proteins while NRPTKs are intracellular. The catalytic activity of each type of kinase is subject to regulation by extracellular stimuli.

Nonreceptor protein tyrosine kinases contain a catalytic domain, as well as various regulatory domains important for proper functioning of the enzyme. NRPTKs are found in the inner leaflet of the plasma membrane, cytosol, endosomal membranes and nucleus. These include the Src, Jak, Abl, Tec, Ack, Csk, Fak, Fes, Frk and Syk subfamilies (Fig. 24-3). Since a great deal is known about the structure and regulation of the Src family tyrosine kinase, we will use it to illustrate the principles in NRPTK signaling; unique features in other subfamilies will be indicated when appropriate. The catalytic domain of PTKs is about 250 amino acids in length and is by itself a functional enzyme. However, most, if not all, PTKs are larger and contain regulatory domains in addition to the catalytic domain. The regulatory domains restrain the catalytic domain and minimize basal kinase activity in the absence of stimulation. When stimulated, the regulatory domains relieve their restraints on the catalytic domain and allow kinase activation. The regulatory domains also direct the protein to specific subcellular targets or compartments, interact with potential substrates and assemble with other signaling molecules. The structural domains of the Src kinase family are, in order from the N-terminus: the SH4 (Src homology 4), SH3, SH2 and SH1 domains. SH1 is the catalytic domain; SH2 and SH3 are both molecular adhesives important for protein–protein interaction; whereas the SH4 domain, residing at the very N-terminus, is important for membrane attachment. Between the SH4 and the SH3 domains lies a region whose sequence varies considerably among different members of the Src family.

The SH4 domain, at the very N-terminus of the Src family kinases, contains a myristoylation site critical for membrane localization of Src. *N*-myristoylation is catalyzed cotranslationally by *N*-myristoyl transferase. Following removal of the N-terminal methionine, myristate is transferred from myristoyl coenzyme A to the glycine residue at the second position. Although myristoylation is necessary for anchoring Src to membrane, it is insufficient. The basic residues in the SH4 domain in Src and Blk interact with the negatively charged head groups of phospholipids and help to attach these kinases to the membrane. In other Src family members palmitoylation of a cysteine residue in the SH4 domain secures their interaction with membrane. While myristoylation is irreversible, electrostatic interaction and palmitoylation are reversible. For example, Ser17 in Src can be phosphorylated by protein kinase A (PKA). Phosphorylation decreases the local positive charge and consequently dissociates Src from the negatively charged membrane. Similarly, palmitate can be removed from the cysteine residue to release Src family kinases from the membrane to the cytosol. An N-terminal pleckstrin homology (PH) domain in the Tec family tyrosine kinases anchors these kinases to membrane by binding specifically to

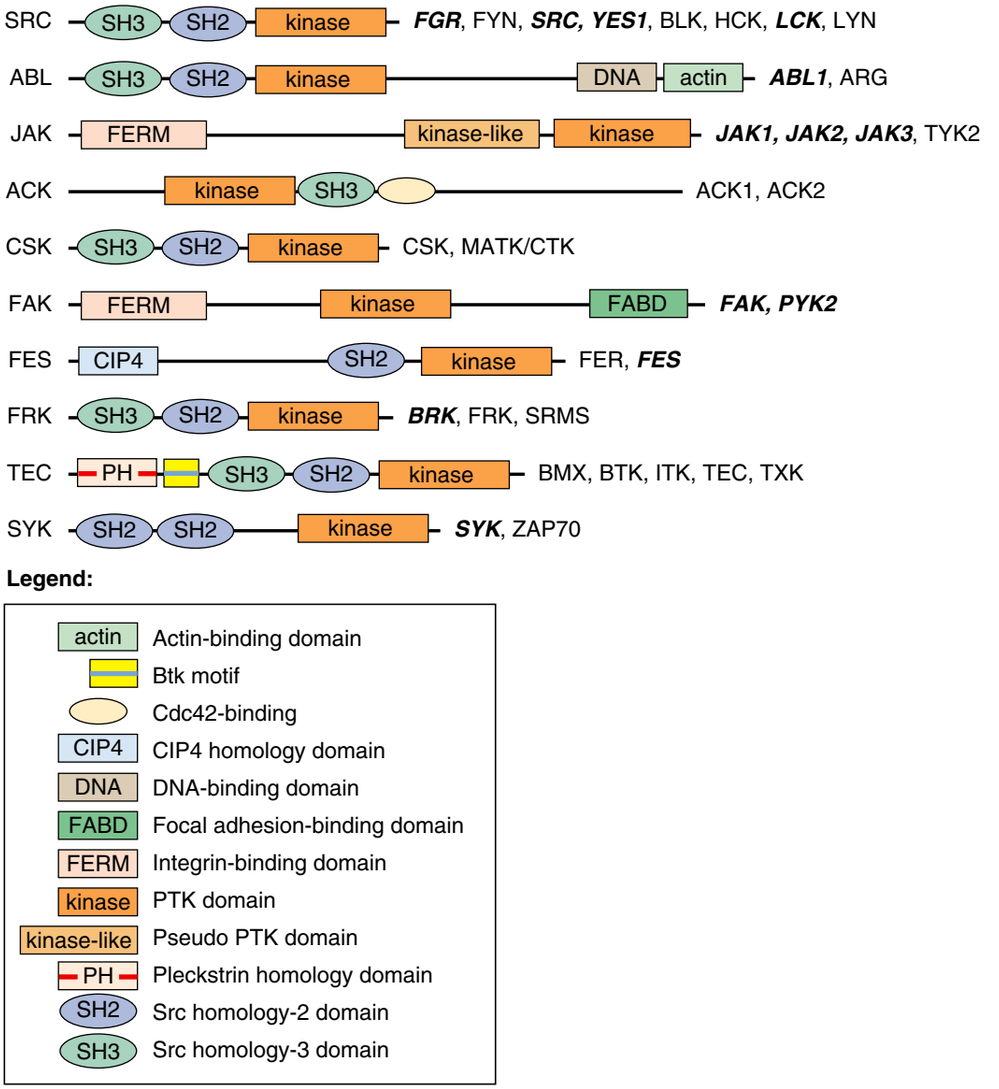

FIGURE 24-3 Nonreceptor protein tyrosine kinase (NRPTK) families. The family members are shown to the right and family name to the left of each NRPTK. Inset indicates the PTK catalytic domain and other domains for the regulation of localization and function. (From reference [5], with permission of *Nature*. © *Nature* 2001.)

phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) in membrane (see Ch. 20).

The unique domain following the SH4 domain from the N-terminus is a region with considerable variation in the amino acid sequences among different Src family members. This unique domain may be involved in protein–protein interactions. For example, the unique region of Lck is linked to CD4 and CD8, while those of Fyn and Lyn may be associated with the T- and B-cell antigen receptors.

The SH3 domain consists of approximately 60 amino acid residues and is C-terminal to the unique domain. Its five β strands form a globular structure whose N- and C-termini are in proximity to each other. SH3 domains mediate both intermolecular and intramolecular interactions, targeting short amino acid sequences that consist of specific proline-rich motifs in a left-handed helix – polyproline type II helix. A typical SH3 domain ligand is composed of two prolines separated by scaffolding residues (XP–X–XP) (reviewed in [6, 7]). It can bind to SH3 domain in either one of two opposite orientations, depending on the position of an additional positively charged residue in the peptide sequence. Type I ligands have a consensus sequence of RPLPPLP. Type II ligands adopt an inverted orientation and have a consensus sequence of ∅PPLPXR, where ∅ represents a hydrophobic amino acid. Small peptide ligands have a low affinity for the SH3 domain (5–50 μmol/l) and low sequence specificity. Interaction between SH3 domains and proline-rich sequences within proteins confers higher affinity and specificity. Despite the presence of consensus SH3 binding sequences, variations from this central theme do exist for SH3 domains from different members of the Src family tyrosine kinases. The neuronal form of Src

contains a six-amino-acid insert within the SH3 domain. This insertion appears to decrease the affinity of the SH3 domain for known ligands since most of the SH3 ligands fail to interact with the neuronal form of Src. This insertion may change the specificity of the Src SH3 domain for neuronal targets and increase its activity by reducing intramolecular interaction.

The SH2 domain is about 100 amino acid residues in length and lies between the SH3 and SH1 domains. This domain interacts with phosphorylated tyrosine residue in a sequence-specific manner; thus this type of protein–protein interaction is directly regulated by tyrosine phosphorylation (reviewed in [8]). SH2 domains contain two binding pockets for their ligands. One of these pockets contains a positively charged arginine residue, which interacts with the negatively charged phosphotyrosine residue. The other pocket interacts with three to five amino acids C-terminal to the phosphotyrosine and plays a major role in determining substrate specificity. However, amino acid residues N-terminal to the phosphotyrosine have also been shown to contribute to SH2 domain binding. The affinity of an SH2 domain for its interacting phosphotyrosine peptide is in the nanomolar range. Removal of the phosphate group from the peptide reduces the affinity for the peptide by about three orders of magnitude. Like the SH3 domain, the N- and C-termini of the SH2 domain are proximal to each other; this juxtaposition allows the formation of a protruding globular structure without significant disruption of the structure of the parent molecule and, importantly, exposes the ligand-binding surface. Compared to SH3 domain ligands, there is less of a consensus peptide sequence among SH2 ligands (Table 24-1). SH3 and SH2 domains are found in a variety of signaling molecules depicted in Figure 24-4.

The SH1 domain is the catalytic domain. The crystal structures of Src-family tyrosine kinases have been solved [9,10]. In general, the catalytic (SH1) domain has an overall bilobal structure, with a small N-terminal lobe and a large C-terminal lobe. The N-lobe contains a glycine-rich sequence, GXGXXGXV, and an invariable downstream lysine for ATP binding. Mutation of this lysine often leads to loss of catalytic activity. The C-lobe contains a glutamate residue that catalyzes transfer of the phosphate group from ATP to tyrosine. ATP fits in the cleft between the two lobes with its γ-phosphate pointing outward while the substrate peptide interacts at the cleft opening. There is considerable flexibility in the relative orientation of these two lobes. The active conformation of the kinase allows the proper alignment of the ATP terminal phosphate with the substrate tyrosine residue. However, the two lobes of an inactive kinase are swung apart so that the ATP terminal phosphate is not aligned with the substrate. Figure 24-5A shows the X-ray crystallographic analysis of hck.

Under physiological conditions, NRPTKs are highly specific in directing tyrosine phosphorylation toward appropriate substrates. This specificity relies on the intrinsic predilection of the catalytic domain towards specific amino acid sequences within protein substrates. In addition, noncatalytic domains, e.g., SH2, SH3 and PH domains of NRPTKs, distribute these kinases to the subcellular region where appropriate substrates are in proximity or abundance, thus favoring phosphorylation of these proteins rather than other substrates.

The regulatory domain is the region C-terminal of the SH1 domain. It contains a tyrosine residue (Y527 in Src; see Glossary for single letter code for amino acids) critical for modulating kinase activity (reviewed in [11]). Under basal conditions, Y527 is phosphorylated by another NRPTK, C-terminal Src kinase (Csk). The phosphorylated Y527 interacts with SH2 domain from the same molecule. This intramolecular interaction favors and is further stabilized by association of Src SH3 domain with the polyproline helix in the linker region between SH1 and SH2 domains. As a result of these interactions, a critical acidic residue is pushed away from the active site, resulting in inhibition of Src kinase activity. Src can be activated by relieving these intramolecular constraints. One of these mechanisms is by dephosphorylation of Y527 and subsequent disruption of the two intramolecular interactions mentioned above. The initial Src kinase activity allows autophosphorylation of Y416 in the activation loop, additional conformational change and maximal enzyme activity subsequently. This model nicely accounts for some earlier observations. For example, v-Src, the tumorigenic form of Src that lacks the negative regulatory domain containing Y527, has very high basal activity. Mutation of Y527 to phenylalanine can increase Src activity. In light of the opposing effects of phosphorylation of Y527 and Y416 on Src kinase activity, different PTPs differentially regulate its enzyme activity. Dephosphorylation of Y527 catalyzed by the leukocyte common antigen (CD45) or an SH2 domain-containing PTP (SHP-2) activates Src while dephosphorylation of Y416 by another phosphatase (SHP-1) inactivates it. Figure 24-5B shows the regulatory mechanisms for Src family tyrosine kinases.

The key in activation of Src family kinases is the disruption of intramolecular interactions and relief of autoinhibition. In addition to dephosphorylation of Y527 by PTPs, the SH2-phosphoY527 bond can be disrupted by competition with a high affinity phosphotyrosine ligand.

TABLE 24–1 Specificity and affinity of SH2, phosphotyrosine binding (PTB) and SH3 domains

Domains	Consensus sequence of ligands	K_D
SH2	$pYR_1R_2R_3$	1–10 nmol/l
PTB	∅ NP*X*pY	25–100 nmol/l
SH3	RPLPPLP, ∅ PPLP*X*R	5–50 μmol/l

The consensus sequences of the ligands interacting with SH2, PTB and SH3 domains are listed with their dissociation constants. Both SH2 and PTB domains bind to phosphotyrosine in a sequence-specific manner. Tyrosine dephosphorylation can reduce the affinity of the SH2 domain to its ligand by 1,000-fold. R_1, R_2 and R_3 represent variable amino acids depending on the specific SH2 domain involved. *X* represents any amino acid. ∅ represents a hydrophobic amino acid; pY, phosphotyrosine.

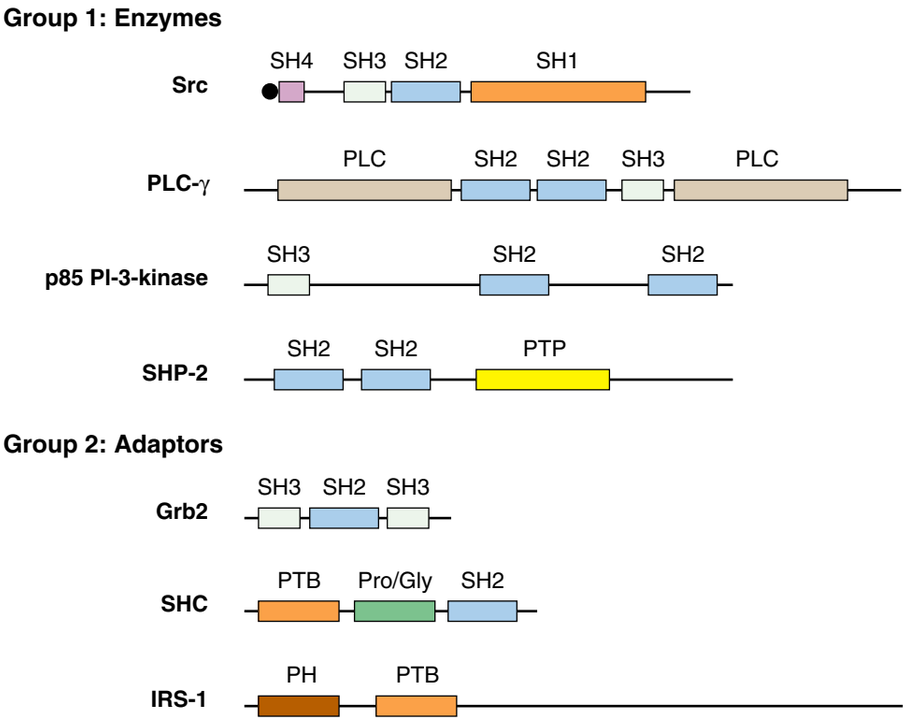

FIGURE 24-4 SH2, SH3 and phosphotyrosine binding (*PTB*) domain-containing signaling proteins. SH2, SH3 and PTB domains are important molecular 'adhesives'. These domains are found in enzymes and play important roles in the regulation of enzyme function. They also are found in proteins lacking any apparent catalytic domain, in which case they may serve as adaptor proteins, assembling signal-transduction complexes. The PTB domain of insulin receptor substrate-1 (*IRS-1*) shares a low degree of homology with the one on SHC. The *black circle* indicates a myristic acid moiety. *PLC*, phospholipase C; *PI3-kinase*, phosphatidylinositol-3-kinase; *PTP*, protein tyrosine phosphatase; *PH*, pleckstrin homology; *SHP*, SH2-containing PTP; *Grb*, growth-factor-receptor-binding protein.

For example, activation of the platelet-derived growth factor receptor (PDGFR) results in autophosphorylation of a number of tyrosine residues, including Y579 in the juxtamembrane region. This phosphotyrosine-containing peptide sequence interacts with the Src SH2 domain and displaces the negative regulatory domain, resulting in Src activation. The displaced Src C-terminal tail is now accessible to PTPs, allowing prolonged activation of Src despite a transient autophosphorylation of the PDGFR. A similar mechanism applies to the activation of Src by autophosphorylated Fak (Fig. 24-5B). Although NRPTKs do not possess an extracellular domain from which they can directly communicate with the extracellular world, they are able to respond to the extracellular stimulation by 'borrowing' extracellular domains from transmembrane proteins that interact with the NRPTKs.

In addition, the phosphotyrosine moiety (Y402) on a proline-rich tyrosine kinase (PYK2, a NRPTK) can apparently compete with phosphoY527 for interaction with SH2 domain on Src and lead to Src activation. PYK2 is a member of the Fak family. It is highly expressed in the nervous system. Its activation depends on both cell adhesion and the presence of calcium or PKC activation. Therefore, certain G-protein-coupled receptors (GPCRs) that release calcium and activate PKC may play a role in the regulation of PYK2 and Src activities (see Ch. 19 for detailed discussion of GPCRs).

Receptor protein tyrosine kinases consist of an extracellular domain, a single transmembrane domain and a cytoplasmic domain. The cytoplasmic domain is composed of a juxtamembrane region, one or two catalytic domains and a C-terminal tail. Like the NRPTKs, the structures outside the catalytic domain are critical for the proper functioning of these enzymes. RPTKs can be classified into at least 20 families [4], including the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), nerve growth factor receptor (NGFR), insulin receptor (INSR) and erythropoietin-producing hepatocellular receptor (EphR) (Fig. 24-6) (see also Ch. 27).

The extracellular domain of RPTKs can be composed of different structural motifs. For instance, the EGFR extracellular domain contains two cysteine-rich regions. The PDGFR extracellular domain consists of five immunoglobulin-like repeats. Other domains found in the extracellular region of RPTKs include fibronectin III repeats,

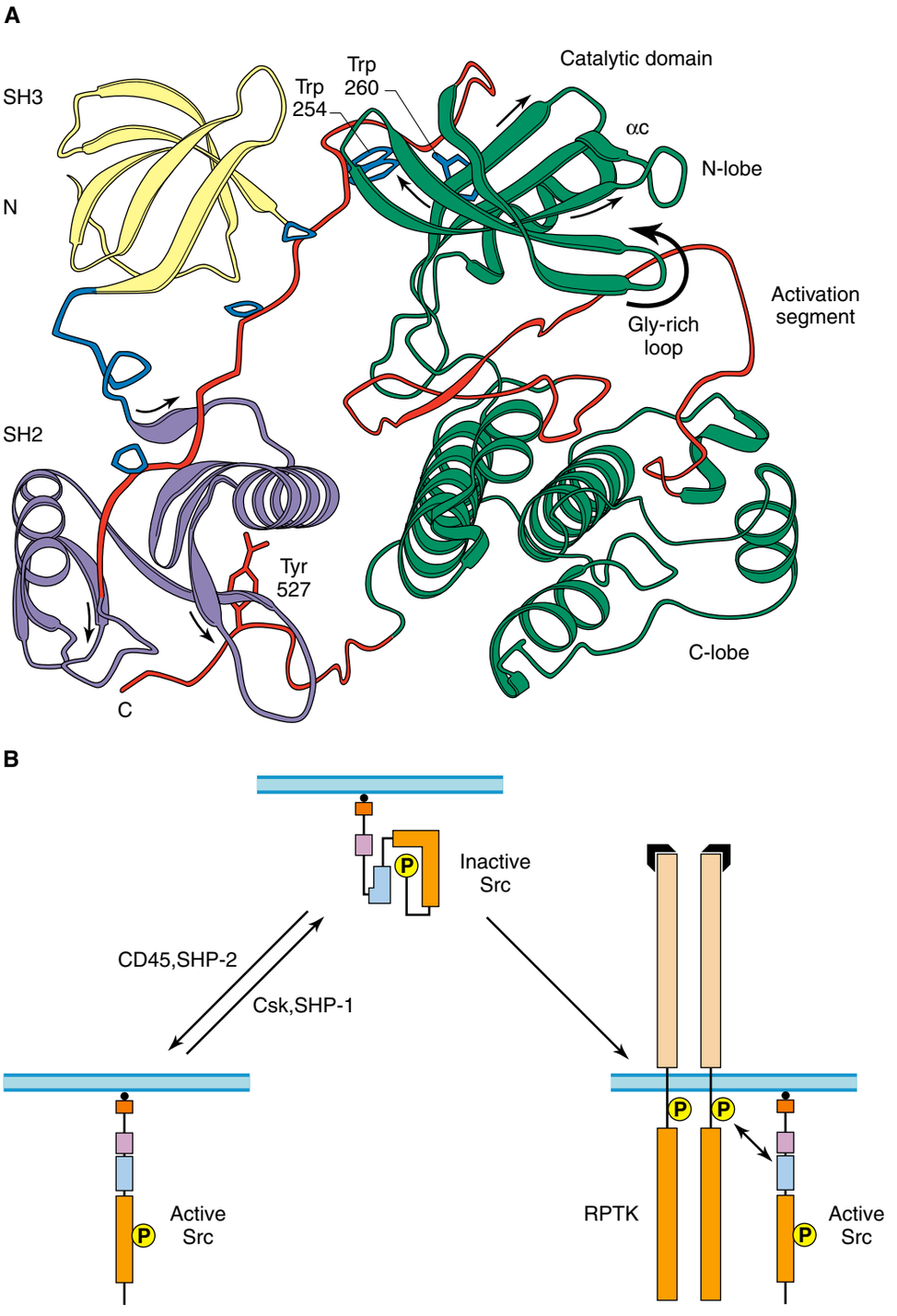

FIGURE 24-5 (**A**) Crystal structure of the Src family kinase hck. The catalytic domain of Src family tyrosine kinases consists of an N-terminal ATP-binding lobe and a C-terminal substrate-binding lobe. There is considerable flexibility in the relative orientation of these two lobes. The active conformation allows the proper alignment of the ATP terminal phosphate, the substrate and a catalytic glutamate residue (Glu310). However, the two lobes of an inactive kinase are swung apart, disrupting proper alignment. (With permission from reference [9].) (**B**) Regulation of Src activity. Src activity can be regulated in a number of ways. In the inactive state, phosphorylated Y527 interacts with the SH2 domain. In addition, the SH3 domain binds the linker region between the SH1 and SH2 domains. These two intramolecular interactions pull the two lobes of the catalytic domain apart and inactivate the kinase. Therefore, dephosphorylation of Y527 by protein tyrosine phosphatases, e.g. CD45 and SHP-2, activates the kinase. Phosphorylation of Y527 by Csk inactivates the kinase. Inactivation is also achieved by dephosphorylating the autophosphorylation site Y416 by SHP-1. Alternatively, Src can be activated by binding to an autophosphorylated receptor protein tyrosine kinase (*RPTK*), such as the platelet-derived growth factor receptor. The RPTK autophosphorylation site binds to the src SH2 domain and thus disrupts the inhibitory intramolecular interactions and activates the kinase. Association of Src with tyrosine-phosphorylated Fak activates the kinase in a similar fashion.

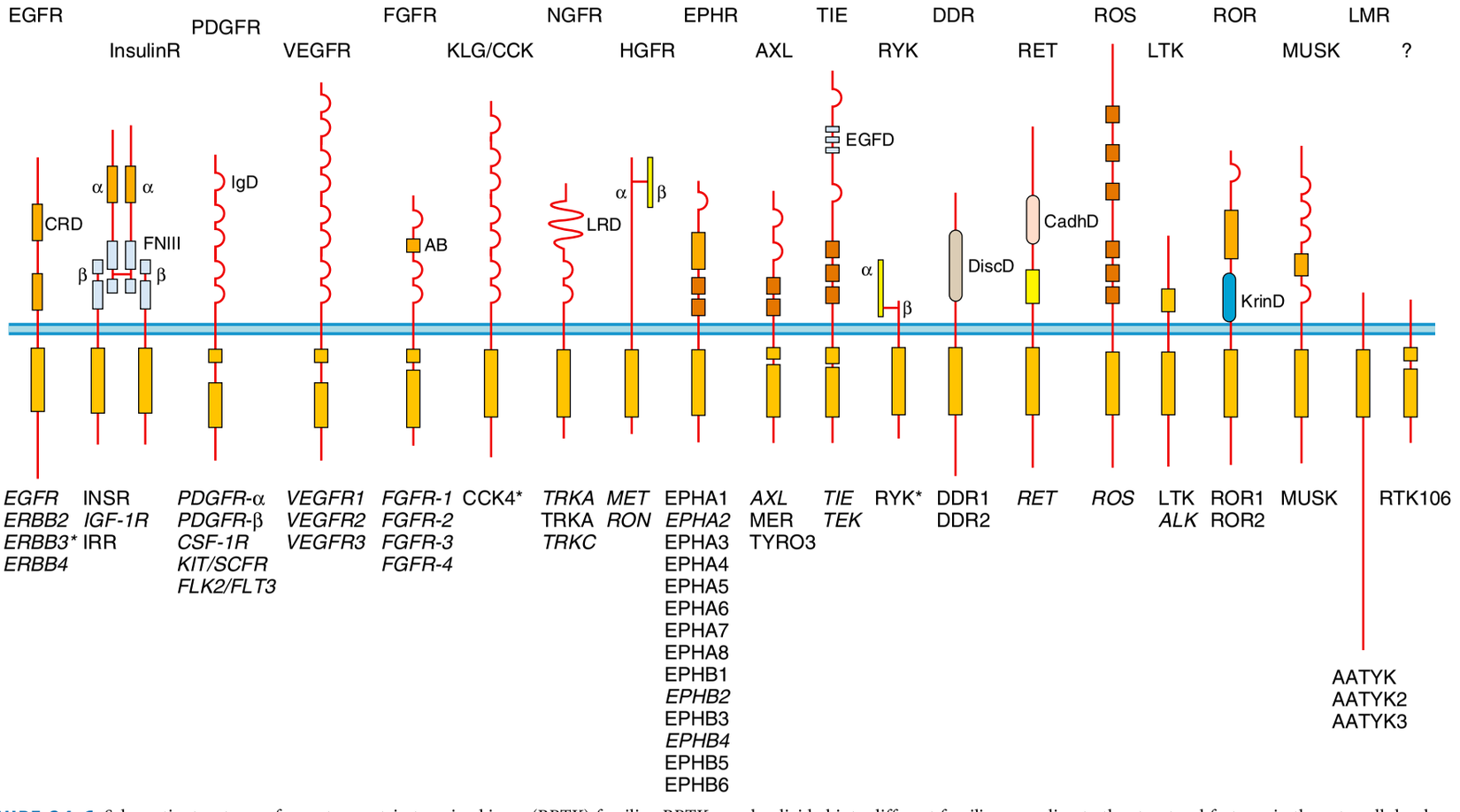

FIGURE 24-6 Schematic structures of receptor protein tyrosine kinase (RPTK) families. RPTKs can be divided into different families according to the structural features in the extracellular domain. Following the extracellular domain are the transmembrane and intracellular domains; the latter contains the catalytic domain. (From reference [5], with permission of *Nature*. © *Nature* 2001.)

kringle domains and leucine-rich motifs. The extracellular domains of members of the insulin receptor subfamily contain disulfide bridges responsible for the formation of a heteroterameric structure ($\alpha_2\beta_2$). The extracellular domains carry the binding sites for RPTK ligands, which range from soluble factors, extracellular matrix proteins, to surface proteins expressed on adjacent cells. The extracellular domain may also be involved in the dimerization of RPTKs, a process critical for the activation of intrinsic tyrosine kinase activity.

The transmembrane domain in the RPTK is a hydrophobic segment of 22–26 amino acids inserted in the cell membrane. It is flanked by a proline-rich region in the N-terminus and a cluster of basic amino acids in the C-terminus. This combination of structures secures the transmembrane domain within the lipid bilayer. There is a low degree of homology in the transmembrane domain, even between two closely related RPTKs, suggesting that the primary sequence contains little information for signal transduction.

The cytoplasmic domain primarily consists of the catalytic domain and various autophosphorylation sites that regulate catalytic function and serve as docking sites for SH2 domain-containing proteins. The protein kinase catalytic domains of RPTKs are highly conserved and similar in structure to those of the NRPTK (see above).

RPTK activation. The activity of RPTKs is normally suppressed in their quiescent state. This suppression is due to the numerous loose and unstructured conformations of the activation loop (A loop) within the catalytic domain; the majority of these conformations interfere with substrate and ATP binding. However, a subset of these conformations is amenable to binding of substrate and ATP, resulting in activation of the RPTKs. Phosphorylation of the tyrosine residue(s) in the A loop shifts the equilibrium towards the active conformation. Because of steric hindrance, PTK catalytic domains appear to be unable to autophosphorylate tyrosine residue(s) in the A loop within the same molecule; rather *trans*-autophosphorylation between two different catalytic domains is necessary for their activation. As a consequence, ligand-induced dimerization is an important step in the activation of RPTKs (Fig. 24-7).

Different RPTKs use different mechanisms to achieve dimerization and *trans*-autophosphorylation. PDGF is a dimer consisting of subunits A and B in different combinations (AA, BB and AB). These dimeric PDGF ligands can physically and directly cross-link two PDGFRs because of their bivalency. In contrast the EGFRs binding to their monomeric ligands may induce a conformational change in the extracellular domain that would subsequently associate with each other. Interestingly, the INSR consists of two catalytic domains already in proximity to each other even in their quiescent state due to their cross-linkage by intermolecular disulfide bonds in the extracellular domains (Fig. 24-6). In this case, the low basal kinase activity in the absence of ligand may be maintained by a suboptimal orientation or distance of the active sites to the A loop. Ligand binding may re-orient or further reduce the distance between the catalytic domains resulting in *trans*-autophosphorylation and activation.

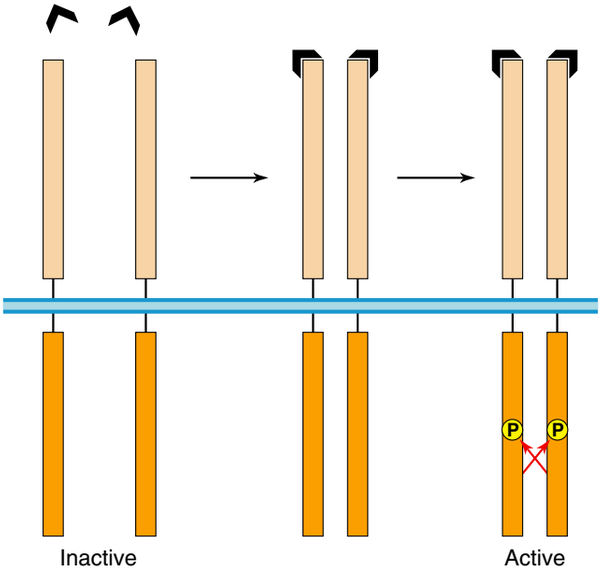

FIGURE 24-7 Activation of receptor protein tyrosine kinase (RPTK) by dimerization and transphosphorylation. Autophosphorylation is a key step in the activation of PTKs. To achieve autophosphorylation, some PTKs dimerize and transphosphorylate each other. Ligand binding induces dimerization of RPTKs so that the catalytic domains are in proximity for transphosphorylation. The autophosphorylated RPTK, thus, is activated. Some nonreceptor protein tyrosine kinases (NRPTKs) can be activated without dimerization (see Fig. 24-5B). However, others, such as Jak/Tyk kinases, are activated by transphosphorylation. Jak/Tyk tyrosine kinases are associated constitutively with transmembrane proteins gp130 and leukemia-inhibitory factor receptor (LIFR)β. Extracellular ciliary neurotrophic factor (CNTF) induces the formation of a quaternary complex consisting of CNTF, LIFRβ, gp130 and another membrane protein known as CNTF receptor α. Consequently, the associated Jak/Tyk are dimerized, allowing transphosphorylation and activation.

RPTK inactivation. Once activated by their ligands, surface RPTKs with their bound ligands are rapidly internalized and degraded. These processes quickly terminate the action of the ligands. The intrinsic tyrosine-kinase activity appears to be important for both internalization and degradation. For example, mutant EGFR without the lysine for ATP binding is neither internalized nor degraded in a mouse fibroblast cell line, NIH3T3 cells. In certain situations, internalized RPTKs continue to mediate signal transduction mechanisms. For instance, in sympathetic neurons, internalized TrkA is transported from the axon terminal to the cell body where it influences signal-transduction cascades that affect gene expression.

Tyrosine phosphorylation of RPTKs. Phosphorylation of specific tyrosine residues on the RPTKs can recruit SH2 domain-containing signaling molecules to the site of action. These signaling molecules include phospholipase C-γ1 (PLC-γ1), phosphatidylinositol-3-kinase (PI-3-kinase), growth factor receptor-binding protein 2 (Grb2), SH2-containing protein (SHC) and Src. Each signaling molecule recognizes a specific tyrosine phosphorylation site on the RPTK, as indicated in Figure 24-8. Recruitment of PLC-γ1 and PI-3-kinase to the membrane brings the enzymes to their substrates in the lipid bilayer. PLC-γ1 is phosphorylated and activated by RPTKs, whereas association of PI-3-kinase with the autophosphorylated RPTKs induces its activity allosterically. PLC-γ1 catalyzes formation of diacylglycerol and inositol trisphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2) (see also Ch. 20). Diacylglycerol activates PKC, while IP_3 triggers calcium release from intracellular stores. PI-3-kinase incorporates a phosphate group in the 3′ position on the inositol ring of phosphatidylinositol phospholipids. One of the products, PI-3,4-P_2, activates Akt kinase and promotes neuronal survival.

Recruitment of Grb2 to the membrane activates the MAPK pathway. Grb2 is an adaptor molecule carrying one SH2 and two SH3 domains (Fig. 24-4). The Grb2 SH2 domain recognizes the tyrosine-phosphorylated moiety on certain RPTKs, such as EGFR (Fig. 24-8), and anchors itself to these membrane-spanning proteins. The Grb2 SH3 domains interact with son of sevenless (SOS), a guanine nucleotide exchange protein. SOS stimulates the release of GDP and subsequent binding of GTP to the membrane-bound, low-molecular-weight, GTP-binding protein Ras (see Ch. 19). GTP-bound Ras interacts with and translocates the serine–threonine protein kinase Raf to the plasma membrane, where Raf becomes activated. Activated Raf phosphorylates MEK, which in turn stimulates MAPK. One of the substrates of MAPK is p90Rsk. Both MAPK and p90Rsk translocate to the nucleus after phosphorylation, where they phosphorylate and activate transcription factors, such as serum-responsive factor (SRF), T-cell-specific transcription factor and cAMP responsive element-binding protein, and, ultimately, alter gene expression.

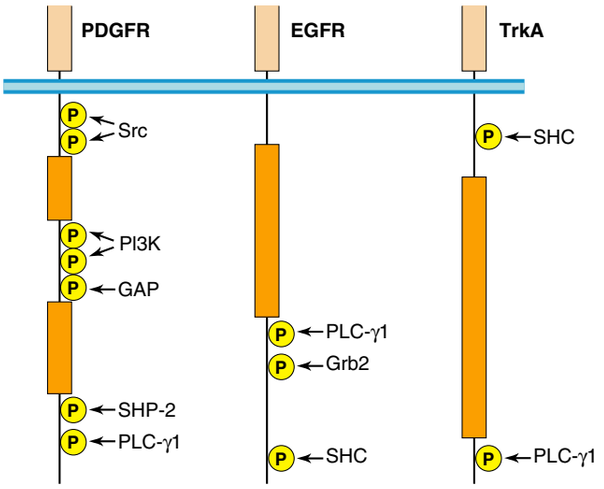

FIGURE 24-8 Recruitment of different signaling molecules to different tyrosine-phosphorylation sites on receptor protein tyrosine kinases (RPTKs). RPTKs usually are autophosphorylated on several different sites. Each tyrosine-phosphorylation site has a unique sequence for interaction with specific signaling molecules containing SH2 or phosphotyrosine-binding (PTB) domains. This provides specificity and diversity for RPTK signaling. *PDGFR*, platelet-derived growth factor receptor; *EGFR*, epidermal growth factor receptor; *PLC*, phospholipase C; *GAP*, GTPase-activating protein; *Grb2*, growth factor receptor binding protein; *PI3K*, phosphatidylinositol-3-kinase; *SHC*, SH2-containing protein; *SHP*, SH-domain containing tyrosine phosphatase.

Some RPTKs cannot interact directly with Grb. They activate the MAPK pathway with the help of an adaptor molecule, such as SHC or insulin receptor substrate-1 (IRS-1). SHC contains a 160-amino-acid phosphotyrosine-binding domain (PTB) that recognizes an autophosphorylation site on some RPTKs. Unlike the SH2 domain, which recognizes the sequence C-terminal of the phosphotyrosine residue, PTB domains recognize amino acid residues upstream of phosphotyrosine. The consensus sequence for interaction with the PTB domain of SHC is ∅NPXpY, where ∅ is a hydrophobic amino acid and X is any amino acid. IRS-1 contains a putative PTB domain that shares a low degree of homology with the PTB domain in SHC. After docking to the phosphotyrosine sites on RPTKs, both IRS-1 and SHC are in turn phosphorylated by their host RPTKs. Tyrosine phosphorylation of these adaptor molecules creates a binding site for Grb2, leading to activation of the MAPK cascade as mentioned above. Similarly, a PTP – SHP-2 – plays a similar adaptor role in mediating the activation of MAPK pathway by RPTKs (see below). RPTKs that interact with a PTB-binding domain-containing adaptor molecule include the nerve growth factor receptor (TrkA), EGFR, INSR and insulin-like growth factor (IGF)-1 receptor. Figure 24-9 shows the role of SHC in RPTK-mediated gene expression.

PROTEIN TYROSINE PHOSPHATASES

The low level of tyrosine phosphorylation in cells is probably attributable to the high specific activity of PTPs, which is about 10–1,000 times that of PTKs. The first PTP purified was PTP1B from human placenta. Based on the sequence of PTP1B, other PTPs were isolated very rapidly using molecular biological techniques, including polymerase chain reaction and low-stringency hybridization. Recent advances in sequencing the human genome have revealed more PTPs; it is estimated that there are in total over 100. PTPs can be divided into those that are specific for phosphotyrosine and those that recognize both phosphotyrosine and

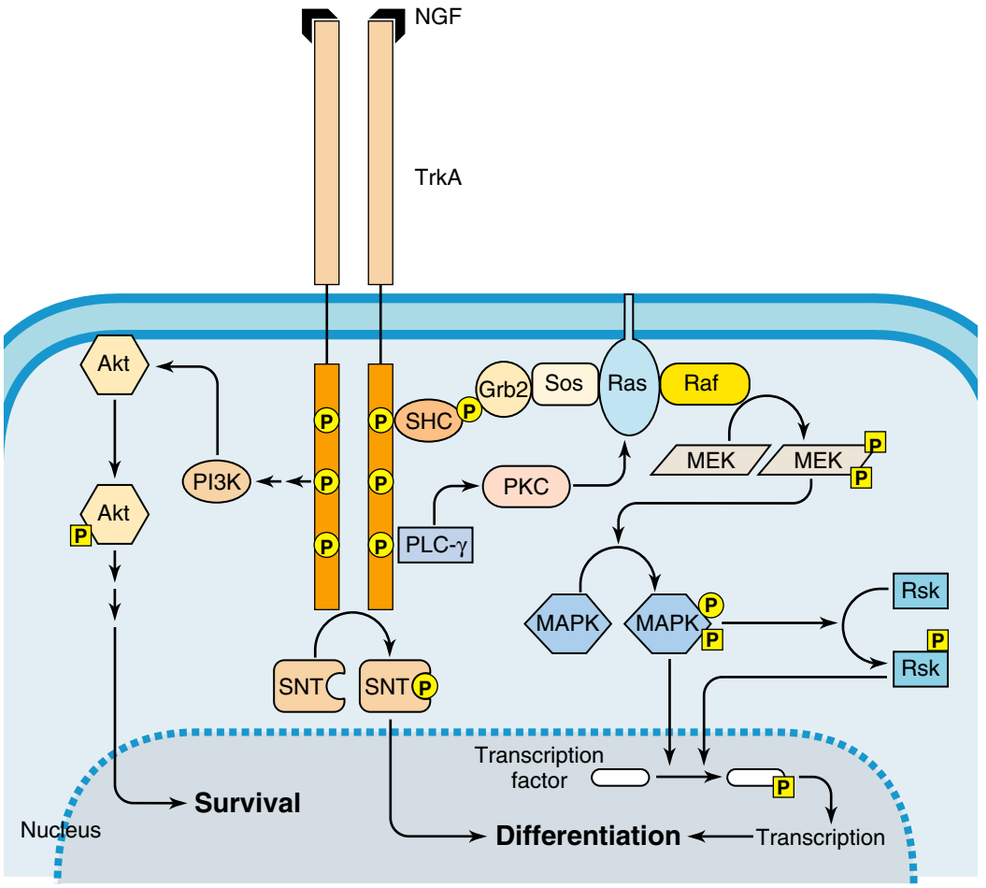

FIGURE 24-9 Signal-transduction mechanisms in nerve growth factor (*NGF*)-mediated neuronal survival and differentiation. NGF binds to TrkA and induces autophosphorylation on three different sites in the intracellular domain. One site interacts with SHC, which eventually leads to the activation of mitogen-activated protein kinase (*MAPK*) and transcription. TrkA also induces tyrosine phosphorylation of *suc*-associated neurotrophic factor-induced target (*SNT*) via a mechanism independent of all known signaling mechanisms. MAPK-induced transcription and SNT tyrosine phosphorylation are believed to be important in neuronal differentiation. TrkA autophosphorylation also activates phosphatidylinositol-3-kinase (*PI3K*) by recruiting it to the membrane. PI3K generates PI-3,4,5-trisphosphate from PI-4,5-bisphosphate. PI-3,4,5-trisphosphate is hydrolyzed to yield PI-3,4-bisphosphate, which activates Akt by translocating it to the membrane. Activated Akt can promote neuronal survival. *Grb2*, growth factor receptor binding protein; *MEK*, MAPK/Erk kinase; *PLC*, phospholipase C; *SHC*, SH2-containing protein; *SOS*, son of sevenless.

phosphoserine/threonine – the dual specificity PTPs (see also Ch. 23). Like PTKs, phosphotyrosine-specific PTPs consist of receptor protein tyrosine phosphatases (RPTPs) and nonreceptor protein tyrosine phosphatases (NRPTPs). Each of these two families of phosphatases can further be divided into subfamilies based on their distinctive structural features (Fig. 24-10).

Nonreceptor protein tyrosine phosphatases are structurally different from serine–threonine phosphatases and contain a cysteine residue in their active sites. The catalytic domains of PTPs are different from those of the serine–threonine phosphatases. The catalytic domains of PTPs are approximately 200–300 amino acids in length and are composed of a central β sheet enclosed by α helices. The highly conserved motif, (I/V)HCXXGXGRS/TG, forms the base of the active-site cleft. The catalytic reaction mechanism is composed of two different steps. First, the nucleophilic thiolate anion of the active-site cysteine residue (Cys215 of PTP1B) attacks the phosphotyrosine on the substrate protein. A thiophosphate intermediate is then formed, accompanied by release of the dephosphorylated product. The second step involves hydrolysis of the thioester bond by an aspartate residue (Asp181 of PTP1B) to regenerate the free cysteine residue and phosphate. A commonly used PTP inhibitor, vanadate, reversibly inhibits PTPs by competing with phosphotyrosine for the active site. On the other hand, pervanadate irreversibly inhibits PTPs by binding to the active site and oxidizing the catalytic cysteine to cysteic acid.

The phosphotyrosine-recognition subdomain confers substrate specificity of PTPs by creating a deep pocket (9 Å) so that only the phosphotyrosine moiety is long enough to reach the cysteine nucleophile located at the base of this pocket; phosphoserine and phosphothreonine

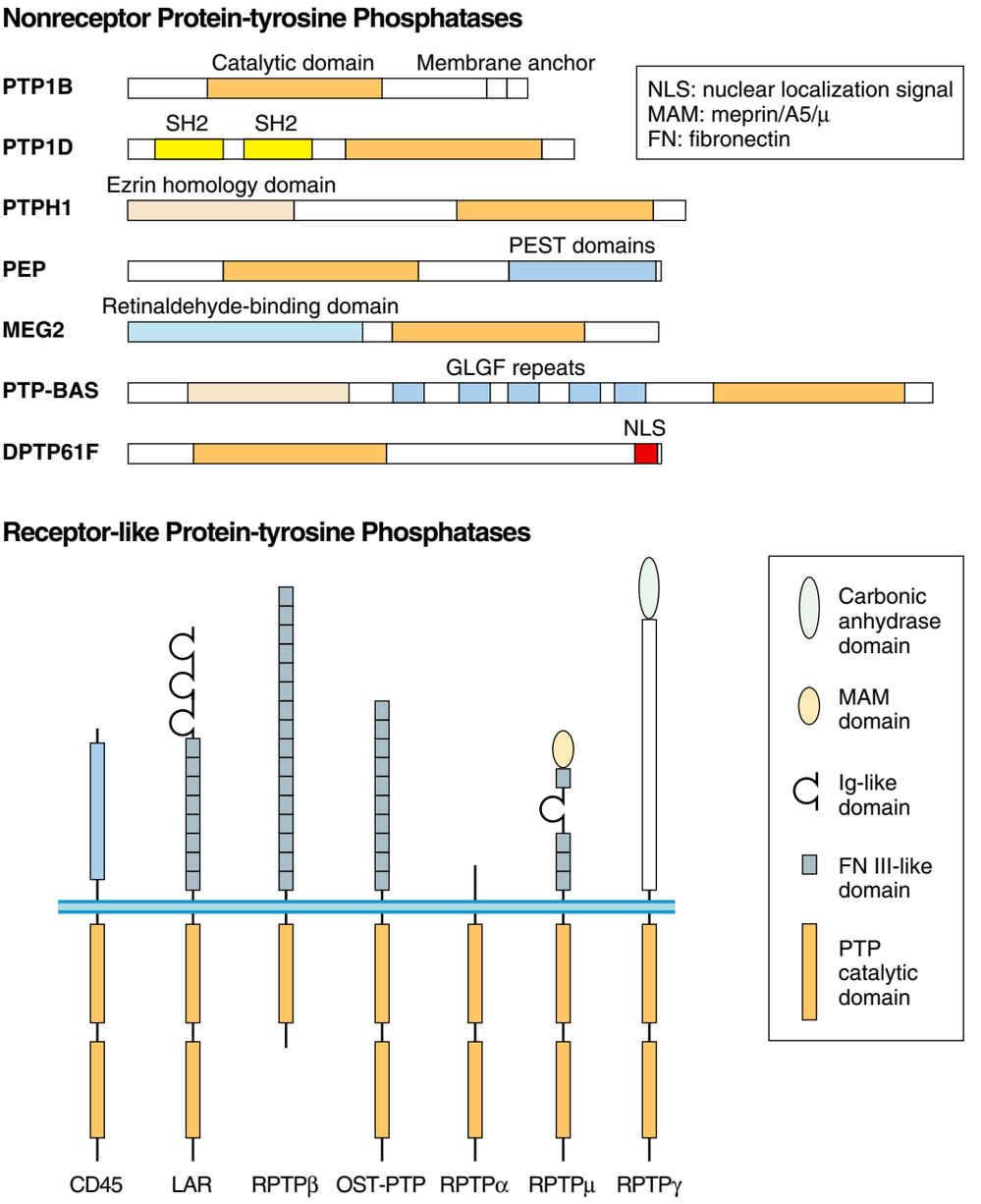

FIGURE 24-10 Schematic structures of nonreceptor protein tyrosine phosphatases (NRPTPs) and receptor protein tyrosine phosphatases (RPTPs). NRPTPs contain a catalytic domain and various regulatory domains. RPTPs are composed of an extracellular domain, a transmembrane domain and an intracellular domain with one or two catalytic domains. Like receptor protein tyrosine kinases, the structural features of the extracellular domains divide the RPTPs into different families. (With permission from reference [12]).

side chains are too short to be dephosphorylated. Dual-specificity PTPs lack the phosphotyrosine-recognition subdomain and have a shallow catalytic-site cleft (6 Å) that is accessible to all three phosphorylated hydroxyamino acids. An example of a dual-specificity protein phosphatase is MAP kinase phosphatase (MKP)-1, which inactivates MAPK by removing phosphate from both the threonine and tyrosine residues in the activation loop.

In addition to their faithfulness to phosphotyrosine, phosphotyrosine-specific NRPTPs appear to have their preferences for substrates. For example, PTP1B prefers EGFR and INSR to many other phosphotyrosine-containing proteins. Such specificity is partly conveyed by a combination of intrinsic properties of the catalytic and regulatory domains that direct the enzyme to specific subcellular compartments where the preferred substrates are enriched. For PTP1B, its hydrophobic C-terminal tail directs this NRPTP to the cytoplasmic face of endoplasmic reticulum. Anchoring PTP1B at this strategic location may ensure that newly synthesized RPTKs are in their

dephosphorylated quiescent state, avoiding unwanted activation before arriving at their final destination in the plasma membrane. Other NRPTPs, SHP-1 and SHP-2, contain SH2 domains that direct the enzyme precisely to specific targets (Fig. 24-4). Despite considerable sequence homology, these two NRPTPs are thought to serve quite different functions. SHP-1 inactivates RPTKs by dephosphorylating autophosphorylation sites while SHP-2 promotes downstream signaling cascades of RPTKs. SHP-2 interacts with the autophosphorylated PDGFR through its SH-2 domain (Fig. 24-8). The activated PDGFR then phosphorylates SHP-2 and creates a binding site for Grb2, eventually resulting in activation of the MAPK pathway.

Regulatory domains that are specific for subcellular targets may have a second function in suppressing the catalytic activity of NRPTPs during the quiescent state. Removal of the C-terminal motif on PTP1B responsible for target specificity is associated with an increase in catalytic activity. Similarly, association of the SH2 domain on SHP-2 with autophosphorylated RPTKs increases its activity by changing the conformation of the enzyme and allowing access of substrates to its active site.

Receptor protein tyrosine phosphatases consist of an extracellular domain, a transmembrane domain and one or two intracellular catalytic domains. RPTPs can be divided into different classes by the structural features of the extracellular domain (Fig. 24-10), which includes the immunoglobulin-like, fibronectin III-like, MAM and carbonic anhydrase domains [12]. The immunoglobulin-like domains contain intramolecular disulfide bonds and a homophilic binding site for cell–cell adhesion molecules, such as neural cell adhesion molecule (NCAM). The fibronectin III-like domains originally were identified in the extracellular matrix protein fibronectin. They consist of conserved hydrophobic residues and may interact with integrins. The MAM domains are named because of their presence in meprins, A5 glycoprotein and PTPμ. These domains contain four conserved cysteine residues. The carbonic anhydrase domains contain only one of the three histidine residues required for catalyzing the hydration of carbon dioxide and are unlikely to be catalytically active. It has been suggested that both the MAM and carbonic anhydrase domains play a role in cell adhesion (Ch. 7).

The catalytic domains of RPTPs are in the intracellular region of the protein. Most RPTP families, except RPTPβ, contain two tandem catalytic domains. The proximal catalytic domain of most RPTPs contains all of the enzymatic activity. The distal catalytic domain appears to be inactive; in some cases, critical catalytic residues are missing. Despite the lack of enzyme activity, the distal catalytic domain may be important for mediating intra- or intermolecular interactions and biological activity of RPTPs. It has been shown that a chimeric CD45 in which the distal catalytic domain is replaced with an equivalent region from LAR becomes deficient in the induction of interleukin-2 secretion and ZAP-70 phosphorylation.

It was originally believed that PTP activity was constitutive and that tyrosine phosphorylation was regulated solely by activating the PTKs. However, it is now clear that PTPs play an active role in the regulation of tyrosine phosphorylation. This was suggested first by the discovery of RPTPs, such as CD45, that have a large extracellular domain reminiscent of that of RPTKs. Their activities are regulated by ligand binding to the extracellular domain. Chimeric studies fusing the intracellular domain of CD45 with the extracellular and transmembrane domains of the EGFR show that the CD45 intracellular catalytic domain is constitutively active. Addition of EGF suppresses the PTP activity of the chimera, suggesting that dimerization may negatively regulate RPTP activity [13]. The mechanism of dimerization-induced inhibition has been revealed by crystallographic studies of a RPTPα fragment consisting of membrane-proximal region and the proximal catalytic domain [14]. These fragments form symmetrical dimers in which the active site of one molecule is blocked by an inhibitory wedge from the membrane-proximal region of the other. Based on this model, the inactive distal catalytic domain may promote tyrosine phosphatase activity of CD45 by competing with and inhibiting homodimerization of the proximal catalytic domain. In summary, activity of RPTPs may be diminished by ligand-induced dimerization, in contrast to activation by dimerization of RPTKs.

ROLE OF TYROSINE PHOSPHORYLATION IN THE NERVOUS SYSTEM

Tyrosine phosphorylation plays a role in virtually every step in the development and functions of a neuron, including survival and differentiation, the extension of axons to their targets and synapse formation and function (Fig. 24-11) (see Chs 25, 27, 28 and 30). Because of the plethora of effects of tyrosine phosphorylation on neuronal function, the following is by no means a comprehensive review but focuses on several examples that demonstrate its significance for the nervous system (Fig. 24-11).

Tyrosine phosphorylation is involved in every stage of neuronal development.

Neuronal survival and differentiation are regulated by many factors including neurotrophins and the Trk family of RPTKs. The prototype neurotrophin, nerve growth factor (NGF), promotes the survival of neurons during a period of programmed cell death in embryonic and early postnatal developmental stages. It is a target-derived neurotrophic factor that modulates the functions of the

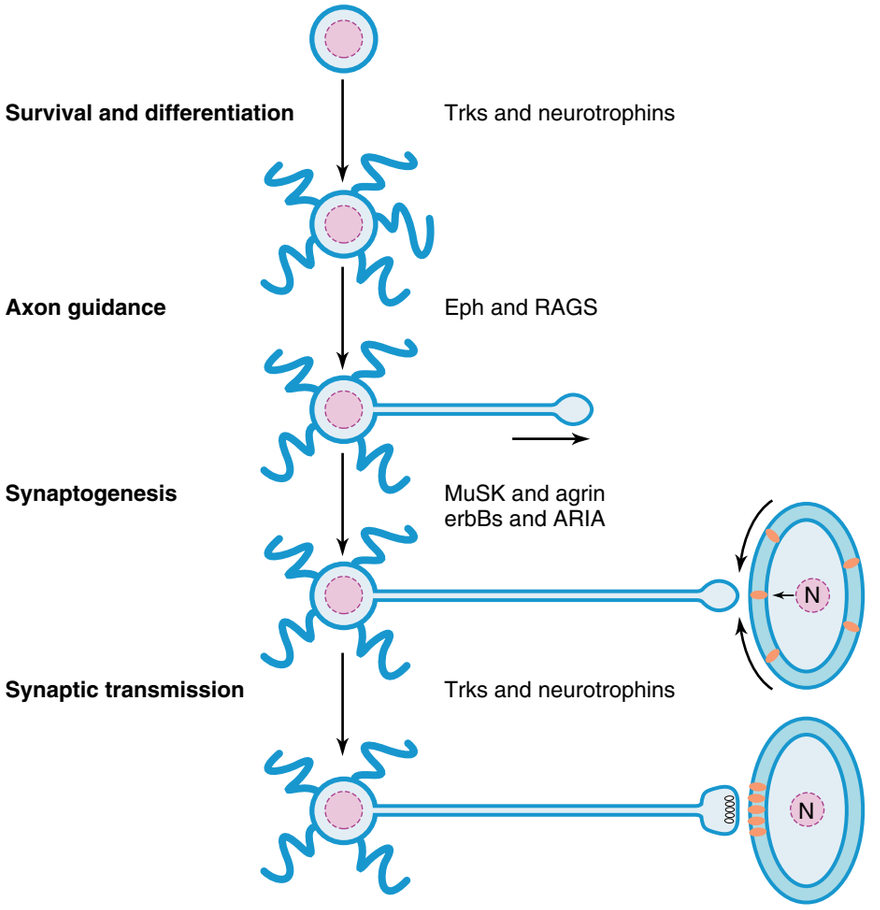

FIGURE 24-11 Examples of the role of protein tyrosine kinases and protein tyrosine phosphatases in the lifetime of a neuron. (*N*). Tyrosine phosphorylation appears to be an important signal-transduction mechanism in every step in the development of a neuron, starting from the survival and differentiation of stem cells, to axon guidance and synaptogenesis and to synaptic transmission at a mature synapse. Examples of each step are given on the right, indicating the receptors and their ligands. *Eph*, erythropoietin-producing hepatocellular factor; *RAGS*, repulsive axon guidance signal, *MuSK*, muscle-specific kinase; *ARIA*, acetylcholine-receptor-inducing activity.

innervating axon terminals as well as gene expression in the distant cell body (Ch. 27).

Although NGF was discovered over half a century ago, its signal transduction mechanism (Fig. 24-9) remained elusive until the identification of a 140 kDa protein, TrkA RPTK, as the NGFR in 1991 [15]. Studies in PC12 cells show that NGF induces autophosphorylation of at least four distinct tyrosine residues on human TrkA. These are Y490 in the juxtamembrane region, Y674, Y675 in the catalytic domain and Y785 in the C-terminal tail. Each phosphotyrosine moiety serves a unique function in the NGF signaling cascade (Fig. 24-8). Y674 and Y675 are the first tyrosine residues phosphorylated and important for subsequent activation of intrinsic kinase activity. Phosphorylation of Y490 and Y785 creates SH2-binding sites for SHC and PLC-γ1, respectively. In addition to SHC, phosphotyrosine 490 is also a binding site for Gab1 and FRS2 (or SNT1). Binding of Gab1 to and subsequent phosphorylation by TrkA recruits PI-3-kinase. Similarly, phosphorylation of FRS2 by TrkA plays a critical role in its subsequent interaction with the Grb2-Sos complex. In summary, association and proximity of signaling molecules to and/or their subsequent phosphorylation by TrkA appear to be an initial and essential part of signal transduction of NGF.

As mentioned above, activation of TrkA by NGF may result in cellular proliferation, differentiation or survival. Exactly how different TrkA-induced signal transduction mechanisms are translated into each one of these functions is incompletely understood. It has been suggested that proliferation and differentiation are mediated by activation of the MAPK cascade while survival is mediated by activation of PI-3-K. The competing actions of neurotrophins on

cellular proliferation versus differentiation largely depend on the duration of MAPK activation: Studies in PC12 cells show that transient activation of the MAPK pathway is associated with a mitogenic response while sustained activation induces differentiation. Interestingly, FRS2 is specifically activated in response to differentiation but not mitogenic factors. Finally, TrkA-induced neuronal survival depends on activation of PI-3-K, generation of 3-phosphoinositides, activation of PDK1 and Akt2, and eventual downregulation of proapoptotic factors (Chs 20 and 35).

Axon guidance. During development, axons often need to navigate through long distances before finding their targets. Through their arduous journey, various extracellular cues serve as signposts in guiding axons through different terrains. Each journey could be divided into shorter stretches for the axons to take one at a time, reaching milestones along the way until they arrive at their final destination. Furthermore, axons may stick together and form fascicles, which can serve as a 'highway' for future traveling axons. Extracellular cues that influence axon guidance can be attractive or repulsive, diffusible or immobilized on the cell surface or the extracellular matrix.

One of the best-studied model systems of axon guidance in vertebrates is perhaps the retinotectal system. Anterior and posterior retinal axons project to the posterior and anterior tectum respectively. Similarly, the dorsal and ventral retinal axons innervate the ventral and dorsal tectum respectively. This reversal of projection is important for the rectification of the upside-down and back-to-front retinal image. Using membrane stripe and growth cone collapse assays, Bonhoeffer and colleagues have identified a 25 kDa protein called repulsive axon guidance signal (RAGS) as an important axon guidance molecule in this system [16]. It is expressed most highly in the posterior tectum and repels the posterior retinal axons from this region. The receptor for RAGS is the Eph RPTK, discovered in the human erythropoietin-producing hepatocellular carcinoma cell line [17]. The ligands for Eph family RPTK are either glycophosphatidylinositol (GPI)-anchored or transmembrane proteins. Their biological activity is lost if they are detached from the membrane. This implies that Eph-mediated axon guidance is limited to short-range contact-dependent activity. Accordingly, the Eph family of receptors can be classified into those (EphA) interacting with GPI-linked ligands (ephrin-A), and those (EphB) with transmembrane ligands (ephrin-B) although crosstalk between these two classes of receptors and ligands has been reported.

Steering of an axon in different directions towards its destination is largely mediated by changes in the cytoskeleton in a structure at the leading edge of an axon known as the growth cone (Chs 8 and 9). The growth cone consists of finger-like protrusions known as filopodia interspaced by web-like structures called lamellipodia. Inside filopodia are organized bundles of actin filaments while a meshwork of actin filaments are found in lamellipodia. Behind the leading edge and into the base of growth cone and the adjacent axon shaft are areas enriched in microtubules. Filopodia and lamellipodia are highly dynamic structures. Attractive extracellular cues favor extension of filopodia by stabilizing and extending the underlying actin filaments while a repulsive cue retracts filopodia by collapsing the actin filaments. The changes in course of a growth cone will subsequently be consolidated by the trailing microtubules.

Mechanisms converting signals from extracellular cues into changes in intracellular cytoskeletal dynamics in the growth cone remain unclear. However, components of this transduction machinery – extracellular cues, corresponding receptors and downstream signaling molecules – are gradually emerging. In addition to the Rho family of small GTPases, others are identified as PTKs, PTPs and their substrates. To date, the extracellular cues for axon guidance can mainly be categorized into four main classes: ephrins, semaphorins, Slits and netrins (reviewed in [18]). Receptors for ephrins (Eph) and some semaphorins (e.g. Met for sema4D) are RPTKs. The Slit receptor family members (Robo) are not RPTKs but are associated with a NRPTK (Abl) and its substrate (Ena), both involved in Robo signaling. Furthermore, Robo itself carries a conserved tyrosine residue, dephosphorylation of which by a RPTP has been suggested to be a prerequisite for Robo to transmit the repulsive signal from Slit. The general involvement of RPTPs in axon guidance has been lucidly demonstrated by genetic manipulation in *Drosophila.*

What is the molecular mechanism linking changes in tyrosine phosphorylation of surface receptors to alterations in the cytoskeletal dynamics in the growth cone – i.e. the steering center of an axon? One of these molecules is Nck, an adaptor molecule consisting of both SH2 and SH3 domains. The SH2 domain of Nck interacts with a phosphotyrosine moiety on activated Eph RPTKs while its SH3 domains recruit PAK and WASP. Since both PAK and WASP regulate reorganization of cytoskeleton, Nck may act as an intermediary between Eph receptor activation and growth cone navigation. Interestingly, the ephrin–eph interaction constitutes a bidirectional communication, i.e., association of this ligand-receptor pair not only activates the eph RPTK but also induces tyrosine phosphorylation of the cytoplasmic domain of the ligand, ephrin, and triggers downstream signal transduction processes. A close homolog of Nck, Grb4, binds to the phosphotyrosine residue on the cytoplasmic tail of ephrin and results in actin depolymerization. Similar to Nck, Grb4 interacts with a number of modulators of cytoskeletal dynamics, including PAK1, Abl-interacting protein-1 (Abi-1) and Cbl-associated protein (CAP).

Tyrosine phosphorylation has a role in the formation of the neuromuscular synapse. For instance, the acetylcholine receptor (AChR) is concentrated at the postsynaptic membrane of the neuromuscular junction at a density of 10,000 receptors/μm^2, which is about three orders of magnitude higher than that of the extrasynaptic region

(Chs 11 and 43). The high concentration of AChRs at the neuromuscular junction allows rapid, reliable and efficient synaptic transmission. The strategic positioning of the AChR right on the crest of junctional folds at the neuromuscular synapse reduces the distance (30–50 nm) for the neurotransmitter to travel before reaching its receptor. Furthermore, the high concentration of AChRs at the neuromuscular junction increases the likelihood that a depolarization is successful in triggering muscle contraction. The significance of this is demonstrated by the pathological condition myasthenia gravis, in which muscle weakness is due to a reduction of the number of AChRs present at the neuromuscular junction. Two mechanisms contribute to the enrichment of AChRs at the neuromuscular junction: (1) clustering of pre-existing, diffusely distributed surface AChRs and (2) local synthesis of receptors by subsynaptic nuclei. Both mechanisms are mediated by tyrosine phosphorylation.

The clustering of pre-existing AChRs on the postsynaptic membrane is triggered by a motor-neuron-derived extracellular matrix protein discovered by McMahan's laboratory in the 1980s called agrin, from the Greek word *ageirein* meaning 'to assemble'. Since then, the agrin hypothesis [19] has gained support from various laboratories and been further elucidated by mice deficient in agrin [20]. These mice are stillborn and immobile, because of the lack of neuronal transmission across the neuromuscular junction where AChR clusters are absent. Nevertheless, small clusters of nonsynaptic AChRs do exist during early stages of embryonic development. These findings suggest that agrin is indispensable to the maturation of these initial AChR clusters and to their alignment with the nerve terminal.

The molar ratio of agrin to AChRs at the neuromuscular junction is between 1:50 and 1:100, suggesting that agrin induces AChR clustering through some intracellular signal transduction mechanisms rather than via any structural constraints. Tyrosine phosphorylation of the AChR has been suggested to play a role in its clustering at the neuromuscular junction. First, agrin induces tyrosine phosphorylation of the AChR prior to clustering of the receptor in muscle. All agents that induce AChR clustering in muscle cells in culture also induce tyrosine phosphorylation of the AChR. These agents include agrin, rapsyn, electrical fields and latex beads. In contrast, PTK inhibitors that inhibit AChR tyrosine phosphorylation also inhibit AChR clustering. Although tyrosine phosphorylation appears to be important to the action of agrin on muscle, a causal relationship between AChR tyrosine phosphorylation and clustering has not been established (see 'Acetylcholine receptors', below).

The putative receptor for agrin is a RPTK known as muscle-specific kinase (MuSK). The extracellular domain of MuSK resembles that of the ROR family of RPTKs, while the kinase domain is similar to that of the Trk neurotrophic receptor (Fig. 24-6). MuSK is expressed at low concentrations in proliferating myoblasts and is induced upon differentiation and fusion. It is downregulated dramatically in mature muscle except at the neuromuscular junction. These properties are consistent with a role of MuSK in muscle development and in the functioning of neuromuscular junctions. MuSK knockout mice show disruption of AChR clusters at the neuromuscular junction [21] similar to that seen in the agrin knockout mice [20], although clusters of nonsynaptic AChR are eliminated. Furthermore, muscle cells from these mutant mice, in culture, are unresponsive to agrin. Expression of MuSK in immature myotubes stimulates agrin binding. These data strongly indicate that MuSK is necessary for the response to agrin. It is postulated that MuSK is only part of the agrin receptor: a myotube-associated specificity component (MASC) is required to form the complete agrin receptor.

In addition to AChRs, agrin recruits and concentrates a host of other proteins to the neuromuscular junction. Among these is the ErbB family of RPTKs. This family contains four known members: ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4. The last three members are expressed in developing muscle and cultured myotubes. Although both AChRs and ErbBs are recruited to the neuromuscular junction by agrin, ErbBs are localized in the troughs of the junctional folds while AChR sits on the tip, suggesting distinct downstream targeting mechanisms. ErbBs at the neuromuscular junction are activated by another motor-neuron-derived factor known as neuregulin. Their activation is responsible for localized transcription of AChR in subsynaptic nuclei. Neuregulin-activated ErbBs are autophosphorylated; the induced phosphotyrosine moiety acts as anchor for an adaptor molecule – SHC – leading to activation of the MAPK pathway and subsequent increased transcription of the AChR gene. In accordance with the significance of tyrosine phosphorylation in this signaling pathway, a PTP, SHP2, has been shown to be a negative regulator.

Tyrosine phosphorylation contributes to the formation of synapses in the central nervous system. This role of tyrosine phosphorylation is well illustrated by studies of neurotrophins – whose signal transduction mechanisms require activation of RPTKs (Ch. 27). The influence of neurotrophins on synapse formation is quite versatile. Neurotrophins affect formation of both excitatory and inhibitory synapses through presynaptic as well as postsynaptic mechanisms. Regarding neurotrophins' role in excitatory synapse formation, it has been shown that addition of exogenous BDNF and NT3 significantly increases the amount of docked synaptic vesicles in cultured hippocampal neurons from E16 embryos. In these neurotrophin-treated cultured neurons functional connectivity can also be ascertained electrophysiologically. Accordingly, transgenic mice overexpressing BDNF experience an increase in synaptic density [22] whereas those deficient in BDNF, TrkB (preferred receptor for BDNF) or TrkC (preferred receptor for NT3) display the opposite [23].

Similar studies have been reported on the potentiation of inhibitory synapse formation by BDNF/TrkB.

Tyrosine phosphorylation plays an important role in synaptic transmission and plasticity. Evidence for this role is that modulators of PTKs and PTPs have been shown to be intimately involved in these synaptic functions. Among the various modulators of PTKs, neurotrophins have been extensively studied in this regard and will be our focus in the following discussion (for details of growth factors, see Ch. 27). BDNF and NT-3 have been shown to potentiate both the spontaneous miniature synaptic response and evoked synaptic transmission in *Xenopus* nerve-muscle cocultures. Neurotrophins have also been reported to augment excitatory synaptic transmission in central synapses. These effects of neurotrophins in the neuromuscular and central synapses are dependent on tyrosine kinase activities since they are inhibited by a tyrosine kinase inhibitor, K-252a. Many effects of neurotrophins on synaptic functions have been attributed to the enhancement of neurotransmitter release; BDNF-induced increase in neurotransmitter release is a result of induced elevation in presynaptic cytosolic calcium. Accordingly, a presynaptic calcium-dependent phenomenon – paired pulse facilitation – is impaired in mice deficient in BDNF.

The significance of tyrosine phosphorylation in synaptic plasticity was perhaps first revealed by the impairment of long-term potentiation (LTP) by tyrosine kinase inhibitors (see Ch. 53 for detailed discussion of memory). Induction of long-term depression (LTD) has subsequently been reported to be susceptible to these inhibitors as well. Genetic knockout experiments have put forth some candidate PTKs involved in these processes. Mutant mice deficient in Fyn exhibit diminished LTP in the hippocampus [24]. Re-introduction of Fyn to the adult brain in Fyn knockout mice restores LTP, suggesting that Fyn plays a direct role in the manifestation of LTP independent of its developmental effects [25]. Conversely, transgenic mice overexpressing Fyn display a lower threshold for the induction of LTP [26]. Mutant mice lacking BDNF or TrkB also exhibit diminished LTP in the CA1 region of the hippocampus [27,28]. Treatment with TrkB-IgG fusion protein, which adsorbs endogenous BDNF, reduces LTP induced in the hippocampus. Furthermore, addition of exogenous BDNF promotes the induction of LTP elicited by paired synaptic stimulation in the dentate gyrus.

The exact mechanisms by which BDNF enhances the induction of LTP remain obscure. Nevertheless, both pre- and postsynaptic mechanisms appear to be possible. As mentioned above, BDNF elevates presynaptic cytosolic calcium level and thus increases vesicular neurotransmitter release. When a postsynaptic neuron is injected with a Trk tyrosine kinase inhibitor (K252a), BDNF-augmented LTP is curtailed; this suggests that a postsynaptic mechanism is adopted by BDNF in the manifestation of LTP. It has been postulated that neurotrophins may act as synaptic morphogens that induce formation of new synaptic contacts. The mechanisms by which Fyn or Src mediate induction of LTP are described in the section below on tyrosine phosphorylation of the NMDA receptors.

Neurotransmitter receptors and voltage-gated ion channels play a pivotal role in modulating synaptic efficacy and plasticity. Many of these receptors and ion channels in both the peripheral and central nervous systems are tyrosine-phosphorylated (reviewed in [29]). Furthermore, tyrosine phosphorylation of these surface-signal-transducing molecules significantly modulates their electrophysiological properties, producing a prominent effect on synaptic transmission and/or plasticity.

Acetylcholine receptors. Because of its enrichment and easy access in the *Torpedo* electric organ, the AChR is one of the best-studied neurotransmitter receptors (Ch. 11). It is located on the postsynaptic side of the mammalian neuromuscular junction. Upon binding acetylcholine, this ligand-gated ion channel depolarizes the sarcolemma and triggers muscle contraction. The AChR is composed of five subunits, $\alpha_2\beta\gamma\delta$ (embryonic) or $\alpha_2\beta\varepsilon\delta$ (adult). Each subunit has four transmembrane domains; both the N- and C-termini are in the extracellular space. Between the third and fourth transmembrane domains is a large cytoplasmic region containing a single conserved tyrosine residue in the cytoplasmic loop of each of the β, γ and δ subunits (Fig. 24-12). In fact, the AChR is highly tyrosine-phosphorylated in intact electric organ and muscle, in contrast to its low tyrosine phosphorylation in cultured rat myotubes, <0.001 mol phosphate/mol subunit; tyrosine phosphorylation of the AChR increases as muscle is innervated during development or in culture. In contrast, denervation results in tyrosine dephosphorylation of the AChR. The motor-neuron-derived agrin and the agrin receptor, MuSK, are responsible for the induction of tyrosine phosphorylation of the AChR.

The physiological functions of the tyrosine-phosphorylated AChR remain unclear. It had been suggested that tyrosine phosphorylation of the AChR β subunit was important for AChR clustering at the neuromuscular junction because of the tight correlation of these two processes. For instance, agrin induced tyrosine phosphorylation of the AChR β subunit and AChR aggregation in the same dose range; the former process preceded the latter. Agrin-induced tyrosine phosphorylation of the β subunit and AChR aggregation were both inhibited by tyrosine kinase inhibitors. Nevertheless, recent evidence shows that tyrosine phosphorylation of the AChR β subunit is neither sufficient nor necessary for AChR clustering. For example, when the extracellular domain of MuSK is replaced by that of TrkC and expressed in myotubes, this chimeric receptor is fully capable of inducing tyrosine phosphorylation of the β subunit of AChR but unable to induce AChR clustering, suggesting that tyrosine phosphorylation of the AChR β subunit is insufficient for clustering. Moreover, mutation of all three cytoplasmic

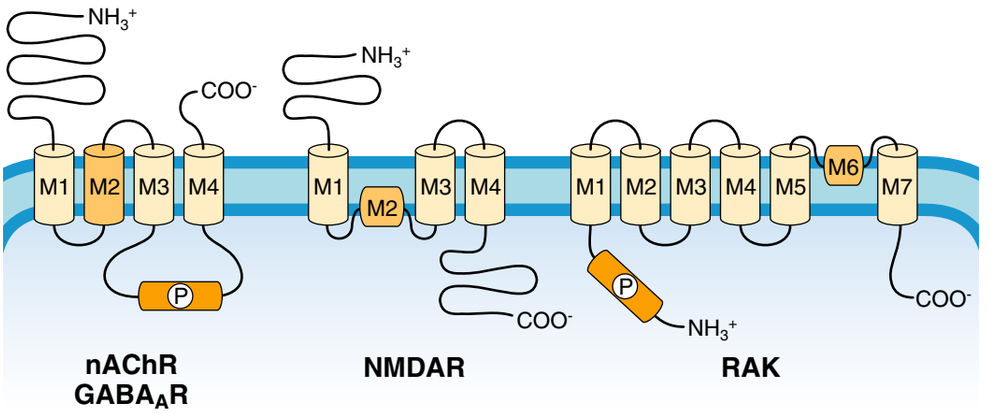

FIGURE 24-12 Transmembrane topology of three ligand-gated ion channel subunits and their potential domains for tyrosine phosphorylation. Both the acetylcholine receptor (*AchR*) and $GABA_A$ receptor (*$GABA_AR$*) subunits have four transmembrane domains with both the N- and C-termini on the outside. Tyrosine phosphorylation sites are present within the large intracellular loop between the third and fourth transmembrane domains. *N*-methyl-D-aspartate (*NMDA*) receptor subunits have a somewhat different topology. They have three transmembrane domains and a membrane-spanning domain. The delayed rectifier potassium channel (*RAK*) has six transmembrane domains and a membrane-spanning domain. Mutagenesis studies have revealed the intracellular N-terminus as the tyrosine-phosphorylation site-bearing region, although other studies have suggested the presence of other tyrosine-phosphorylation sites.

tyrosine residues on the AChR β subunit to phenylalanine does not appear to inhibit AChR aggregation, suggesting that tyrosine phosphorylation of the β subunit is unnecessary for AChR clustering [30]. What, then, is/are the physiological function(s) of AChR tyrosine phosphorylation? First of all, tyrosine phosphorylation of the AChR has been shown to affect the rate of receptor desensitization [31] and may thus regulate receptor sensitivity at the neuromuscular junction. Secondly, since tyrosine-phosphorylated AChR can interact with the SH2 domain on adaptor molecule (e.g. Grb2) and Src family NRPTKs (e.g. Fyn, Fyk), it is likely to play a modulatory role in signal transduction.

***N*-methyl-D-aspartate receptors.** Glutamate is the major excitatory neurotransmitter in the central nervous system (Ch. 15). Its receptors can be divided into three types: AMPA/kainate, NMDA and metabotropic receptors. NMDA receptors are composed of two different types of subunit – NR1 and NR2. They play an important role in the induction of synaptic plasticity and excitotoxicity.

The activity of NMDA receptors can be enhanced by a NRPTK – Src (reviewed in [32]). Application of Src or a Src activating peptide potentiates NMDA receptor activity in cells and inside-out membrane patches. Similar potentiation of NMDA receptor activity can be achieved by the other Src family NRPTK – Fyn. Such increase in NMDA-evoked channel activity is attributed to an increase in probability and duration of channel opening and a decrease in duration of channel closing. Cumulative evidence has suggested that tyrosine phosphorylation of the NR2 subunits regulates NMDA function. For instance, the NR2A and 2B but not NR1 subunits are tyrosine-phosphorylated *in vivo* [3, 33]. NR2A and NR2B can be phosphorylated by Src *in vitro*. Heterologous cells expressing NR1 and NR2A subunits exhibit increased NMDA-induced current in the presence of v-Src, while cells expressing NR1 and other NR2 subunits display no change in NMDA-evoked current. Finally, mutation of tyrosine residues in the cytoplasmic domain of NR2A to phenylalanine abolishes Src-induced NMDA receptor activity.

Both NMDA receptor and Fyn have been implicated in the induction of LTP. Since Src family tyrosine kinases are known to tyrosine phosphorylate and activate NMDA receptor, it is enticing to speculate that Src family kinases and NMDA receptors somehow act in a concerted fashion in the expression of LTP. An axon guidance receptor, EphB2, is expressed in adult hippocampus and interacts with the NR1 subunit of the NMDA receptor. Stimulation of EphB2 by ephrin-B1 activates Src and Fyn with a concomitant increase in tyrosine phosphorylation of the NR2A subunit [34]. Activation of EphB2 by ephrin-B2 has also been shown to induce tyrosine phosphorylation of the NR2B subunit with an accompanying increase in NMDA-receptor-mediated calcium ion influx [35]. The additional calcium ion influx may increase postsynaptic responsiveness. Although *in vivo* experiments show that EphB2 is indispensable for hippocampal LTP, some evidence suggests that its tyrosine kinase domain may be nonessential for this function [34, 36].

It is noteworthy that Src-induced increase in NR1-NR2A receptor activity is promoted by the coexpression of postsynaptic density protein known as PSD-95 [37]. PSD-95 is a scaffolding protein consisting of multiple protein–protein interaction domains – three N-terminal PDZ domains, an SH3 domain and a C-terminal guanylate kinase domain. The first two PDZ domains interact with the NR2 C-terminal tails while the third PDZ domain

binds the SH2 domain of Fyn [37]. The PDZ-SH2 domain interaction is atypical since tyrosine phosphorylation of the PDZ domain is nonessential. In this fashion, PSD-95 serves as an adaptor bringing Fyn and its substrate in proximity of each other.

GABA receptors. GABA is one of the major inhibitory neurotransmitters in the central nervous system (see Ch. 16). The subunits of the $GABA_A$ receptor ($GABA_AR$) consist of α, β, γ, δ, ε, θ and π; subunits found in brain are mainly from the first three classes. Similar to AChR, the $GABA_AR$ complex is believed to be heteropentameric with four transmembrane domains in each subunit. The intracellular region between the third and fourth transmembrane domains contains a number of consensus protein phosphorylation sites (Fig. 24-12). Coexpression of $GABA_AR$ subunits α1, β1 and γ2L with v-Src induces tyrosine phosphorylation on both the β and γ subunits, with a concomitant increase in GABA-mediated whole-cell current [38]. Mutation of two tyrosine residues (Y365/367) on the cytoplasmic domain of the γ subunit abolishes not only the induction of tyrosine phosphorylation but also the functional effects of v-Src, suggesting that direct tyrosine phosphorylation of $GABA_AR$ increases its activity [38]. Tyrosine phosphorylation of the $GABA_AR$ and associated functional facilitation have been observed in neurons as well; the latter has been attributed to an increase in duration and probability of channel opening. Although direct tyrosine phosphorylation of the $GABA_AR$ augments channel activity, activation of some RPTKs appears to indirectly inhibit $GABA_AR$ function. For instance, activation of the PDGFR inhibits $GABA_AR$ in hippocampal neurons as a consequence of activating PLC-γ1, generating IP_3 and elevating intracellular calcium.

In addition to direct enhancement of channel activity, PTKs can indirectly increase GABA-evoked inhibitory current by recruiting intracellular $GABA_AR$ to the surface of postsynaptic membrane. Insulin has been shown to increase surface expression $GABA_AR$ in transfected human embryonic kidney cells. In central neurons insulin rapidly increases the expression of functional postsynaptic $GABA_AR$ in a tyrosine kinase-dependent manner, resulting in an increase in the amplitude of the miniature inhibitory postsynaptic currents.

Voltage-gated ion channels. In addition to ligand-gated ion channels described above, many voltage-gated ion channels appear to be regulated by tyrosine phosphorylation. These include voltage-gated calcium, potassium and sodium ion channels whose function can either be enhanced or impeded by tyrosine phosphorylation. For example, voltage-gated L-type calcium ion channel activity is augmented by tyrosine phosphorylation. Voltage-gated calcium ion channels are composed of α_1, $\alpha_2\delta$ and β subunits. The α_1 subunit forms the pore of the calcium ion-selective channel. It consists of six transmembrane domains with both the N- and C-termini in the cytoplasmic side, carrying a number of potential phosphorylation sites. It is believed that both cytoplasmic ends of the channel impose an inhibitory tone on functional activity; upregulation of channel activity by protein phosphorylation may be attributed to relief of this inhibition. The L-type calcium ion channel is tyrosine-phosphorylated and activated in cerebellar granule neurons in response to stimulation by insulin-like growth factor-1 (IGF-1). This potentiation induced by IGF-1 appears to be mediated by phosphorylation of Y2122 on the α_{1C} subunit via the action of Src.

In contrast to the above example of voltage-gated L-type calcium ion channels, a delayed rectifier potassium ion channel, Kv1.2, serves as an example of downregulation by tyrosine phosphorylation. Kv1.2 coexpressed with the M_1 muscarinic AChR in *Xenopus* oocytes is inhibited by carbachol, a nonmetabolizable analog of acetylcholine. Activation of the M_1 muscarinic receptor releases diacylglycerol and IP_3 from the hydrolysis of PIP_2. Diacylglycerol activates PKC and IP_3 releases calcium from intracellular stores. Possibly by their action on a PYK2-related tyrosine kinase, calcium and PKC eventually lead to tyrosine phosphorylation of Kv1.2 on Y132 in the cytoplasmic domain near the N-terminus, resulting in an impaired functional response. Tyrosine phosphorylation of Kv1.2 and its concomitant reduction in channel activity can be reversed by RPTPα. Similarly, a related potassium channel, Kv1.5, is tyrosine-phosphorylated and suppressed when co-expressed with v-Src in transfected cells. *In vivo* association between hKv1.5 and Src is mediated by proline-rich sequences on the potassium ion channel and Src SH3 domain.

REFERENCES

1. Eckhart, W., Hutchinson, M. A. and Hunter, T. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. *Cell* 18: 925–933, 1979.
2. Hunter, T. and Eckhart, W. The discovery of tyrosine phosphorylation: it's all in the buffer! *Cell* 116: 23, 2004.
3. Lau, L. F. and Huganir, R. L. Differential tyrosine phosphorylation of N-methyl-D-aspartate receptor subunits. *J. Biol. Chem.* 270: 20036–20041, 1995.
4. Robinson, D. R., Wu, Y. M. and Lin, S. F. The protein tyrosine kinase family of the human genome. *Oncogene* 19: 5548–5557, 2000.
5. Blume-Jensen, P. and Hunter, T. Oncogenic kinase signalling. *Nature* 411: 355–365, 2001.
6. Cesareni, G., Panni, S., Nardelli, G. *et al.* Can we infer peptide recognition specificity mediated by SH3 domains? *FEBS Lett.* 513: 38–44, 2002.
7. Macias, M. J., Wiesner, S. and Sudol, M. WW and SH3 domains, two different scaffolds to recognize proline-rich ligands. *FEBS Lett.* 513: 30–37, 2002.
8. Schlessinger, J. and Lemmon, M. A. SH2 and PTB domains in tyrosine kinase signaling. *Sci STKE* 2003: re12, 2003.

9. Sicheri, F., Moarefi, I. and Kuriyan, J. Crystal structure of the Src family tyrosine kinase Hck. *Nature* 385: 602–609, 1997.
10. Xu, W., Harrison, S. C. and Eck, M. J. Three-dimensional structure of the tyrosine kinase c-Src. *Nature* 385: 595–602, 1997.
11. Cole, P. A., Shen, K., Qiao, Y. F. *et al.* Protein tyrosine kinases Src and Csk: a tail's tale. *Curr. Opin. Chem. Biol.* 7: 580–585, 2003.
12. Hunter, T. Tyrosine phosphorylation– past, present and future. *Biochem. Soc. Trans.* 24: 307–327, 1996.
13. Desai, D. M., Sap, J., Schlessinger, J. *et al.* Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell* 73: 541–554, 1993.
14. Majeti, R., Bilwes, A. M., Noel, J. P. *et al.* Dimerization-induced inhibition of receptor protein tyrosine phosphatase function through an inhibitory wedge. *Science* 279: 88–91, 1998.
15. Klein, R., Jing, S. Q., Nanduri, V. *et al.* The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* 65: 189–197, 1991.
16. Drescher, U., Kremoser, C., Handwerker, C. *et al.* In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* 82: 359–370, 1995.
17. Hirai, H., Maru, Y., Hagiwara, K. *et al.* A novel putative tyrosine kinase receptor encoded by the *eph* gene. *Science* 238: 1717–1720, 1987.
18. Dickson, B. J. Molecular mechanisms of axon guidance. *Science* 298: 1959–1964, 2002.
19. McMahan, U. J. The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* 55: 407–418, 1990.
20. Gautam, M., Noakes, P. G., Moscoso, L. *et al.* Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85: 525–535, 1996.
21. DeChiara, T. M., Bowen, D. C., Valenzuela, D. M. *et al.* The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85: 501–512, 1996.
22. Causing, C. G., Gloster, A., Aloyz, R. *et al.* Synaptic innervation density is regulated by neuron-derived bdnf. *Neuron* 18: 257–267, 1997.
23. Martinez, A., Alcantara, S., Borrell, V. *et al.* Trkb and trkc signaling are required for maturation and synaptogenesis of hippocampal connections. *J. Neurosci.* 18: 7336–7350, 1998.
24. Grant, S. G., O'Dell, T. J., Karl, K. A. *et al.* Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science* 258: 1903–1910, 1992.
25. Kojima, N., Wang, J., Mansuy, I. M. *et al.* Rescuing impairment of long-term potentiation in fyn-deficient mice by introducing fyn transgene. *Proc. Natl Acad. Sci. U.S.A.* 94: 4761–4765, 1997.
26. Lu, Y. F., Kojima, N., Tomizawa, K. *et al.* Enhanced synaptic transmission and reduced threshold for LTP induction in fyn-transgenic mice. *Eur. J. Neurosci.* 11: 75–82, 1999.
27. Minichiello, L., Korte, M., Wolfer, D. *et al.* Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* 24: 401–414, 1999.
28. Patterson, S. L., Abel, T., Deuel, T. A. *et al.* Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 16: 1137–1145, 1996.
29. Davis, M. J., Wu, X., Nurkiewicz, T. R. *et al.* Regulation of ion channels by protein tyrosine phosphorylation. *Am. J. Physiol.* 281, H1835–1862, 2001.
30. Meyer, G. and Wallace, B. G. Recruitment of a nicotinic acetylcholine receptor mutant lacking cytoplasmic tyrosine residues in its beta subunit into agrin-induced aggregates. *Mol. Cell. Neurosci.* 11: 324–333, 1998.
31. Hopfield, J. F., Tank, D. W., Greengard, P. *et al.* Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. *Nature* 336: 677–680, 1988.
32. Ali, D. W. and Salter, M. W. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Curr. Opin. Neurobiol.* 11: 336–342, 2001.
33. Moon, I. S., Apperson, M. L. and Kennedy, M. B. The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc. Natl Acad. Sci. U.S.A.* 91: 3954–3958, 1994.
34. Grunwald, I. C., Korte, M., Wolfer, D. *et al.* Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. *Neuron* 32: 1027–1040, 2001.
35. Takasu, M. A., Dalva, M. B., Zigmond, R. E. *et al.* Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. *Science* 295: 491–495, 2002.
36. Henderson, J. T., Georgiou, J., Jia, Z. P. *et al.* The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function. *Neuron* 32: 1041–1056, 2001.
37. Tezuka, T., Umemori, H., Akiyama, T. *et al.* PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. *Proc. Natl Acad. Sci. U. S. A.* 96: 435–440, 1999.
38. Moss, S. J., Gorrie, G. H., Amato, A. *et al.* Modulation of GABAA receptors by tyrosine phosphorylation. *Nature* 377: 344–348, 1995.

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–IV–

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CHAPTER

25

Development

Jean de Vellis

Ellen Carpenter

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FUNDAMENTAL CONCEPTS UNIFYING DEVELOPMENTAL DIVERSITY

Development is the study of the principles and processes that underlie growth and evolution of a biological organism. Most developmental research has been devoted to studying the early periods of intense and extensive transformations that are associated with the unfolding of an organism from a single fertilized egg through embryogenesis to postnatal maturation. During this period, the immense diversity of neural phenotype emerges [1]. What are the mechanisms that regulate the systematic and highly coherent series of events that fashion the vast neural circuitry? In this chapter, we examine the molecular and cellular processes that give rise to the enormous phenotypic diversity of the brain during these developmental periods in neural tissue.

Two fundamental concepts mold our perspective of how the nervous system develops and functions: the dynamic interdependence of genes and the environment and that of neuronal and neuroglial cells. During development, the systematic expression of the genetic blueprint creates and continuously molds the environment at all levels of its hierarchical organization, extending from the level of the single cell to the surroundings of the organism. The genetic machinery, in turn, relies on feedback from the environment to adjust epigenetic mechanisms. This interaction creates a highly integrated, self-referential process that links the genetic information with the multitude of influences existing at all the different layers of the environment [2]. Great progress has been made in the systematic dissection of individual linear pathways of environmental genetic connections. For example, a growth factor and its receptor activate an intracellular signaling pathway to induce the expression of a specific gene (see Chs 26 and 27). In this way, many

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environmental signals and numerous cell processes have been correlated with the appearance of different neural phenotypes.

The presumption behind these studies is that knowledge of these single linear paths of environmental genetic linkage will provide an understanding of the basis of phenotypic diversity. As we have discovered and examined the functions of more and more extracellular factors, membrane receptors and intracellular signaling pathways impinging upon an extensive genetic network functioning in a highly complex, combinatorial fashion, our research perspective is being transformed from a simple linear to a more realistic and integrated parallel model (Fig. 25-1). Multiple families of extracellular factors influence many receptors and intracellular signaling pathways, which exhibit considerable interaction, or 'cross-talk.' In addition, a network of transcription factors is being elucidated (Ch. 26). This network is characterized by extensive inter- and intrafamily interactions contributing to multiple levels of control over the expression and functional activity of transcription factors responsible for differential gene expression. Thus, in contrast to the linear model, neural phenotypic diversity arises from highly integrated networks of intracellular processes linked to environmental signals.

Biological interdependence in neural development is also dramatically displayed in the relationship of neuronal and glial cells [2–6]. The intimate coupling of these two types of cell is a major locus of genetic and epigenetic interaction in determining the functioning and the morphological, chemical and electrical development of the nervous system (see also Ch. 31). Students have traditionally been taught that the neuronal cell represents the structural unit of the nervous system, relegating glia to the status of passive packaging. This narrow perspective has now been replaced by the accumulated evidence for glial cell regulation of nearly every aspect of neuronal development and function, supporting the premise that the fundamental functional unit of the nervous system is the dynamic interaction of neuronal and glial cells.

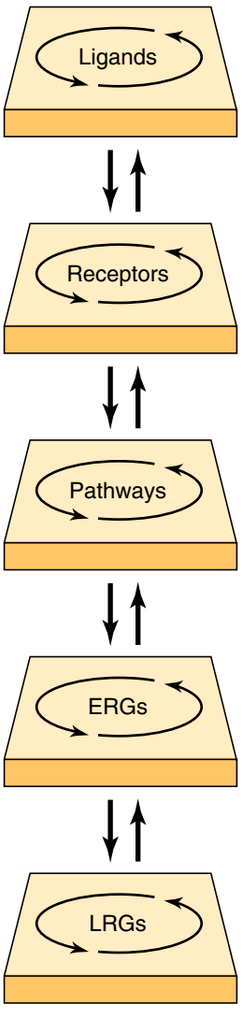

FIGURE 25-1 Hierarchically organized levels of information processing underlying the generation of neural phenotypic diversity. The enormous phenotypic diversity of the nervous system requires a mechanism that can generate increasing degrees of restriction in cell phenotype, such as cell shape and neurotransmitter expression. The original, simple linear perspective of an extracellular factor acting through its receptor to direct the expression of phenotypic characteristics is now being replaced by the recognition that each level of information processing is itself a complex domain of interacting components. In this view, many families of signaling molecules, or factors, bind to a growing number of interacting receptors, with overlapping specificity and component makeup, to activate many different, interacting intracellular signaling pathways, leading to the complex expression pattern of early response genes (*ERGs*). These ERGs, many of which function as transcription factors, in turn interact to regulate the final expression of late response genes (*LRGs*), encoding proteins that are phenotypic determinants. Thus, from this perspective, the developmental fate of each cell is determined by the sum total of the multiple signals and combinatorial intracellular processes.

GENERAL DEVELOPMENT OF THE NERVOUS SYSTEM

During animal development, a single fertilized egg cell divides and differentiates to produce all the cells and tissues of the mature organism. Genetic information contained in the fertilized egg provides all the instructions to produce these tissues. The initial divisions of a fertilized egg produce a blastula. The blastula undergoes gastrulation to produce the three primary germ layers of the developing embryo. During gastrulation, cells migrate inward between the upper and lower layers of the blastula through the primitive streak, resulting in a trilaminar 'sandwich'. The initial point of inward migration is established by the organizer, which in *Xenopus* is the dorsal lip

of the blastopore and which in birds and mammals is the node. The resulting gastrula has three layers, the ectoderm, the endoderm and the mesoderm. The nervous system develops from the ectoderm following an inductive signal from the mesoderm. The initial mesodermal cells condense to form the notochord, which elongates under the primitive streak along the anterior posterior axis of the developing embryo. Signals are released from the notochord, which signals induce the ectoderm to thicken into neural ectoderm in the area immediately overlying the notochord (Fig. 25-2). This neural ectoderm is now committed to develop into neural tissue, as can be demonstrated by transplantation experiments in which neural ectoderm surgically placed into other areas of the developing embryo produces auxiliary neural tissue. In addition, neural ectoderm arising in different places along the anterior–posterior axis of the developing embryo is predestined to develop into specific brain regions. Three proteins secreted by the organizer can induce production of anterior neural plate markers in undifferentiated ectodermal cells. These three proteins, noggin, follistatin and chordin, may establish anterior neural domains by inactivating two neural inhibitors: bone morphogenetic protein (BMP)2 and BMP4. The establishment of posterior domains within the neural plate may stem from signaling by basic fibroblast growth factor (bFGF) or by retinoic acid. Neither of these molecules is by itself sufficient to generate regional posterior identity, but both may mediate signals established by some other mechanism. An additional mechanism used to regionalize the neural plate may be the expression of head-specific genes. Deletion of several of these genes, notably *lim-1* and *otx-I*, deletes all head structures, suggesting a role for these genes in establishing the most anterior portion of a developing animal. Cerberus, a secreted protein expressed in the gastrulating endomesoderm, also has forebrain-inducing activity.

Once the neural plate has been induced by the underlying mesoderm, it begins to differentiate into the neural tube, the primary rudiment of the developing CNS [6]. The center of the neural plate develops a groove in the direction of the anterior posterior axis and the edges of the plate rise up, forming the neural folds (Fig. 25-2). The neural folds fuse together along the dorsal edge of the animal and the neural tube separates from the overlying dorsal ectoderm. During neural tube formation, the adjacent mesoderm is segmented into blocks of tissue called somites. The somites provide precursor cells for axial and appendicular skeletal elements and attached musculature. During neural tube closure, a population of cells separates from the neural folds and migrates into the periphery (Fig. 25-2). These cells are the neural crest (NC), which provides a progenitor population for the developing peripheral nervous system (PNS). The NC migrates away from the neural tube along defined pathways through the anterior half of each adjacent somite and along a dorsal pathway under the ectoderm. NC cells that migrate through the somites populate the dorsal root ganglia as neurons and glia, contribute Schwann cells to the peripheral nerves and provide neurons and glia for the sympathetic ganglia. NC cells that migrate along the dorsal pathway develop into melanocytes.

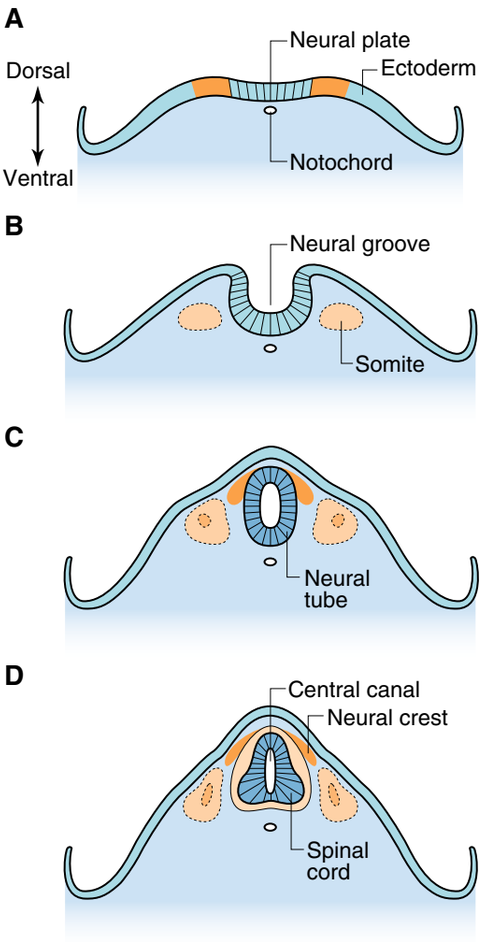

FIGURE 25-2 Four stages in the development of the neural tube. (**A**) After gastrulation, the mesodermally derived notochord induces the overlying ectoderm to form the neural plate. The most lateral portions of the neural plate will give rise to the neural crest (*dark orange areas*). (**B**) The neural plate invaginates to form the neural groove. (**C**) The neural tube fuses at its dorsal margin and separates from the overlying ectoderm. Neural crest cells (*dark orange*) separate from the neural tube and begin to migrate into the periphery. (**D**) At trunk levels, the neural tube forms the spinal cord surrounding a central canal. (Modified from Cowan [6], with permission.)

The neural tube is a pseudostratified epithelium, with cells extending between the apical and basal surfaces of the epithelial wall. The neuroepithelium contains undifferentiated populations of stem cells and radial glia (see Ch. 29). With time, both of these cell types give rise to the three main lineages for neurons, astrocytes and oligodendrocytes. After the final mitotic division, neurons migrate away from the ventricular surface of the neural

tube to form the mantle zone. This outward migration is mostly guided by radial glial fibers, which provide a scaffold extending from the ventricular zone to the surface of the developing brain. The accumulation of postmitotic neuronal cells results in the progressive thickening of the neural tube to produce expansions of the CNS, brain and spinal cord.

DEVELOPMENTAL PROCESSES: ENVIRONMENTAL FORCES MOLDING GENETIC POTENTIAL

Developing cells of the nervous system are embedded in complex fields of mechanical tension, biochemical signals and electrical current. This constantly changing pattern of spatial and temporal information for each cell is created, in large part, by the chemistry of the neural cells themselves and represents the major environmental forces that drive the sequence of developmental processes. The dynamic interaction between these environmental influences and the machinery of neural cells forces the cells to undergo considerable transformation during periods of growth and survival, migration and sorting and morphological and biochemical differentiation [7, 8].

Selective cell survival and proliferation determine the number of each type of nerve cell. A basic tactic in creating complex biological systems is to generate an excess of elements and then, through a set of selective processes, to determine which elements will remain to participate in the final organizational pattern. This strategy is widely used in the construction of the vertebrate nervous system. During development, the interaction between genetic and epigenetic forces is expressed, in part, as the balance between cell survival and cell death [9]. Intimately linked to this balance is the control of cell proliferation (see also apoptosis, Ch. 35).

For invertebrate development, genetic instructions are the prime controller of population size and diversity. Because of the short life-span and the relatively simple nervous system necessary to control the organism in a relatively constant ecological niche, the most reliable and economical approach is to rapidly generate simple, preprogrammed neuronal/glial circuitry. The development of the nematode *Caenorhabditis elegans* is an example of a cell lineage that is generated independently of the environment. Precursor cells undergo cycles of stereotyped divisions, creating a number of restricted, preprogrammed cellular lineages. Cell death appears due to intrinsic instructions that select specific pathways to terminate in senescence [10] (see apoptosis, Ch. 35).

Vertebrate development reflects the other extreme of the genetic/epigenetic continuum: unlike invertebrates, numerous environmental cues are employed to direct lineage decisions, including the decisions for survival, division and death. Prolonged gestation and the plasticity inherent in allowing outside influences to guide developmental decisions permit the construction of large-scale, highly interconnected, activity-dependent circuitries.

Early experiments demonstrated that the presence and size of the target tissue are critical in determining the number and functions of the surviving neuronal cells [9]. Removal of limb buds or transplantation of an extra limb bud results in a predictable decrease or increase, respectively, of the innervating motor and sensory neurons. Each set of neuronal cells exhibits a characteristic degree of target-dependent cell death during development: some groups remain quite stable in number, while others lose up to two-thirds of the cell progeny. What are the factors that influence the balance between cellular survival and death? The nature of the microenvironment of the cell soma and the environment into which the cell process grows are both important. Critical factors produced in the innervated target tissue contribute to the regulation of cell survival: limited amounts of soluble factor(s) released by target cells lead to competition among cells dependent on the factor. Thus, during specific periods of early cell development, trophic factors are necessary for survival (see Ch. 27).

Specific paths of cell migration to final target environments are controlled by gradients of diffusible and substrate-bound neurochemical signals. Migration of cells plays a significant role in brain morphogenesis. Most neurons travel long distances through the complex extracellular terrain of the developing embryo to reach their final position. What mechanisms are used by neurons to move along the path, and what signals are used to guide them? The most common mechanism for cell translocation is a combination of (1) the extension of a cell process and its attachment to the substratum, followed by (2) the pulling of the entire cell toward the point of attachment by means of contractile proteins associated with an intracellular network of microfilaments (Chs 7, 8 and 28).

Directional control of cell movement appears to be of two types: (1) cells moving along other 'guide' cells arranged as scaffolding and (2) cells moving across a multicellular terrain guided by concentration gradients of particular molecules. For example, small molecules diffusing through or attached to the extracellular matrix (see Ch. 2) can alter the behavior of a cell (see Chs 7, 27, 29 and 30). In the complex neuropil of the cerebral cortex, molecular signals undoubtedly permit neurons to distinguish radial glial processes along which they travel from the other neuronal, glial and endothelial surfaces.

NC cell movement is another model of molecular control of cell migration and contrasts with that seen in cortical systems. Since there are no glial cells to create highways for the crest cells, the migration of these cells into the spaces surrounding the neural tube depends on the nature of the extracellular matrix. In order to migrate to specific destinations, a number of signals must be regulated in a

coordinated fashion both temporally and spatially [11]. Nearly all the major components of the matrix – collagen, fibronectin, laminin, proteoglycans and hyaluronic acid – regulate NC cell migration. For example, NC cell migration correlates with the appearance of high amounts of hyaluronic acid.

A variety of substrate- and cell-attached factors influence neural development by regulating adhesion properties of cells (see Ch. 7). Interactions occur directly between cells or between a cell and the extracellular matrix (see Ch. 2). The molecules mediating these interactions have been implicated in regulating the specificity and timing of cell–cell adhesion and the consequences on cell morphology and physiology. Hence, they influence the ability of cells not only to migrate but to sort themselves out and to stabilize spatial relationships considered important for the process of differentiation.

Cell process outgrowth determines the cytoarchitecture and circuitry of the nervous system. Cell process elongation and branching determine the final morphological phenotype of the cell and its participation in the local and global neural circuitries [12, 14]. On average, each of the billions of neuronal cells forms more than 10,000 specific interconnections. This process of morphological differentiation requires the directed growth of a considerable number of cell processes to multiple specific targets, often at great distances from the cell body. Control over the active elongation of a cell process creates the final size and geometry of the neuronal axon and dendritic tree. What are the molecular mechanisms that control the progressive elongation of a cell process? What environmental signals are present to guide the processes through a complex, three-dimensional, multicellular terrain? What are the processes that determine the time and place of branching? Are the molecules implicated in regulating cellular migration involved in controlling neurite outgrowth? In general, many of the same soluble and matrix components that regulate cell proliferation, survival and migration have been shown to also mediate process outgrowth *in vivo* and *in vitro*.

Growth cones are located at the leading edge of a neurite and display two types of motile structure: long spike-like structures called filopodia and thin, broad sheets of membrane called lamellipodia. These delicate structures are important in pathfinding and outgrowth branching (see Ch. 8). Growth cone movement can be influenced by small soluble factors (see Ch. 27). Local concentration gradients of nerve growth factor (NGF) and/or FGF can initiate and direct growth and movement. Specific neurotransmitters, such as serotonin and dopamine, can also alter neurite elongation and growth cone movement. For example, serotonin inhibits neuronal outgrowth of specific subsets of neurons. Inhibition of neurite outgrowth can also be suppressed by electrical activity.

Adhesion molecules [12, 13], either on the cell or in the extracellular matrix (see Chs 2 and 7), may also play an important role in neurite outgrowth, since they mediate neurite–neurite and neurite–glia interactions. The probability of outgrowth initiation, rate of elongation and degree of branching of neurites is strongly influenced by the adhesive quality of the substrate. Adhesion of growth cone structures may stabilize extensions. Growth cones may follow the path of greatest adhesiveness, leading to directional control over neurite outgrowth. The glycoprotein laminin, found in the extracellular matrix and on the surface of Schwann cells, can accelerate neurite outgrowth.

Neuron-glia interactions [14] may also be involved in outgrowth and synapse formation. Sympathetic neurons grown in the absence of Schwann cells extend unbranched, axon-like neurites; in the presence of glial cells, however, process outgrowth is extensively branched and dendrite-like in form. In CNS preparations, this interaction is further characterized by the specificity of the astrocytic environment: glia from local homotypic regions give rise to branched neurites and glia from heterotopic regions induce unbranched neurites.

Intracellular pathways are involved in the regulation of neurite outgrowth. Cyclic AMP (see CREB, Ch. 26) and inositol phospholipids have been implicated as intracellular regulators (see Chs 20 and 21). Work on the control of Ca^{2+} influx has helped to establish a causal relationship with neurite outgrowth: Ca^{2+} influx can regulate both neurite elongation and growth cone movements (see Ch. 22). Studies of invertebrate neurons and isolated growth cones suggest that, in the presence of various agents that inhibit neurite outgrowth, for example serotonin and electrical activity, the amount of free cytoplasmic Ca^{2+} is closely correlated with neurite outgrowth. Further support of Ca^{2+}-mediated control of neurite growth has been obtained from studies under conditions in which neurite outgrowth and Ca^{2+} influx could be directly manipulated. Data suggest that specific levels of Ca^{2+} influx promote normal neurite elongation and growth cone movements.

The basic cytoarchitecture of the cortex is the result of a plastic process of neurite outgrowth, as discussed above, relying on adhesion factors and control of terminal arborization, which can be dependent on neuronal activity (see also Ch. 30). The final lattice-like pattern of complex cortical circuitry and its computational capacity arise from a combination of selective and nonselective process outgrowth and synapse formation events coupled to regressive events, including selective cell death, process elimination and synapse elimination. These events can be activity-dependent or -independent processes. For example, the vertical connections in the cortex arise with little dependence on neuronal activity during development, whereas the horizontal connecting outgrowth is clearly regulated by evoked or spontaneous neural activity. Thus, the formation of cortical clusters is dependent on patterned visual activity; binocular deprivation can eliminate the clustered organization of the horizontal connections.

In the case of cortical cluster organization, selective elimination of collaterals, and not cell death, is the essential molding parameter.

In summary, a number of parameters of outgrowth initiation, elongation, branching and cessation combine to generate axonal or dendritic geometry. These components can be modulated *in vitro* by a variety of soluble and substrate-bound factors, suggesting that, *in vivo,* control over morphological differentiation is multifactorial.

MOLECULAR MECHANISMS OF DEVELOPMENT

Environmental factors control developmental decisions made by cells of each lineage. Diffusible growth factors (see Ch. 27) control the processes of proliferation and differentiation [1, 5, 9]. The discovery of NGF in the early 1950s began an era of remarkable success in developmental neurochemistry. Physiological effects of NGF can be classified as (1) an essential neurotrophic or nourishing influence during early development, resulting in selective neuronal survival; (2) a potent influence on neuronal differentiation; or (3) a strong neurotropic or guiding influence on direction of neurite growth. The main experimental strategy employed over the years to demonstrate the presence of the different activities of NGF has been to either block, via specific antibodies or drugs, or enhance, via addition of exogenous NGF, its actions. The survival and differentiation influences of neurotrophins are not unique. Since the mid-1980s, the existence of many other such factors has been reported (see Ch. 27). The sections of this chapter on cell lineages illustrate the rapidly growing understanding of the importance of growth factors and cytokines in neural cell development.

Genetic networks function in neural development. One of the most fascinating fields of research is the elucidation of the complex network of genetic information that controls development. The immense phenotypic diversity of the nervous system reflects the coordination of the numerous genetic programs that regulate cell-type-specific gene expression. How does a cell become responsive to a given set of environmental signals, encode this information into specific channels of intracellular transduction and regulate differential gene expression? The answer lies, in part, in the complex world of transcriptional regulators (see Ch. 26). It is estimated that 5% of the mammalian genome, about 5,000 genes, encode for transcription factors (Fig. 25-3). Of these, up to half are anticipated to have expression restricted to the nervous system. The large number of transcription factors can be subdivided into families whose members share a conserved amino acid sequence responsible for DNA-binding and dimerization. At present, 12 distinct motifs have been identified. Four classes are illustrated in Figure 25-4. Research demonstrates that members of these and other classes can function as transcriptional regulators exerting control over the developmental processes of proliferation and differentiation [15] (see also Ch. 26).

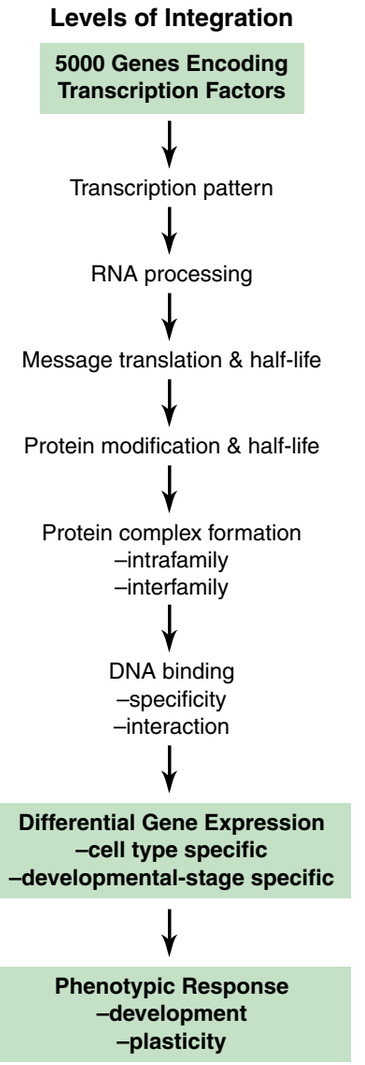

FIGURE 25-3 Levels of integration within the transcription factor network. Of the estimated 5,000 genes in the human genome encoding for transcription factors, perhaps half will exhibit expression specific to the nervous system. This predicted restriction in expression is considered to reflect the need to generate the combinatorial control mechanisms necessary to create the enormous phenotypic diversity unique to neural development. Listed are the various levels of processing whereby complex interactions among many factors and many events are integrated to carry out the critical task of differential gene expression that determines the unique phenotypic response during development and adult plasticity.

There are multiple levels of integration within the transcriptional regulator network. Part of the complexity of control lies in the large number of different factors available to generate cell-type-specific transcriptional regulation. More importantly, control over the expression and functional state of these numerous transcription factors is a source of considerable complexity and opportunity for

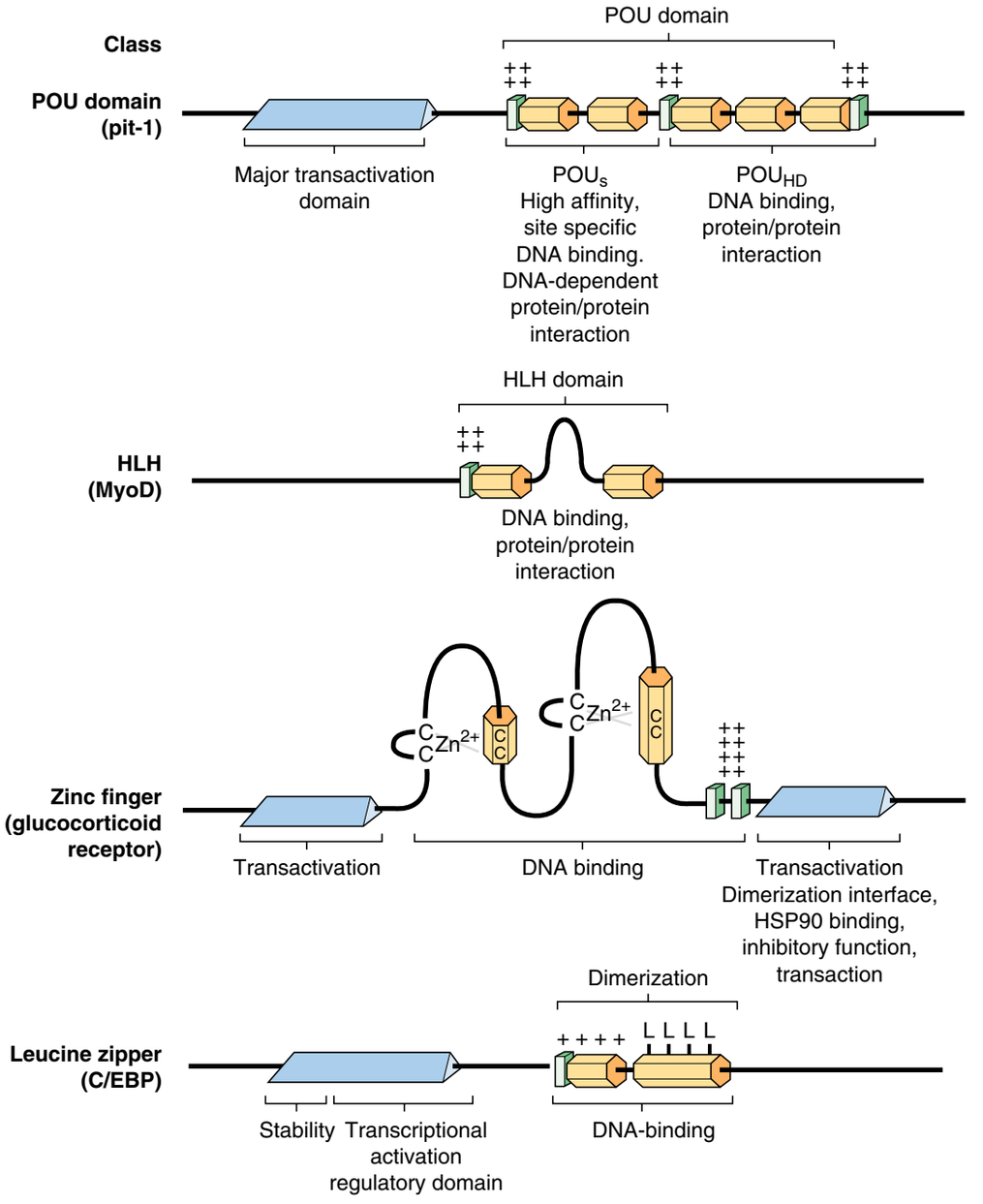

FIGURE 25-4 Major classes of transcription factors. Twelve distinct families of transcription factors have been classified by their DNA-binding motifs. Four examples of these transcription factors implicated in the control of cell proliferation and differentiation are depicted here. Conserved regions unique to each family include regions of basic amino acids functioning as DNA-binding sites (*solid rectangles*) and putative helical regions associated with DNA binding or dimerization (*cylinders*) are shown. Transcriptional activation domains (*solid triangles*) are generally not conserved even within a family, generating considerable diversity of functional transactivation among interacting family members. Each family has at present more than half a dozen members. The classes shown possess a POU domain (pituitary-1, *pit-1*), a basic helix–loop–helix domain (*HLH*, *MyoD*), a nuclear receptor type of zinc-finger domain (glucocorticoid receptors) or the basic region/leucine zipper domain (bZIP; CCAAT enhancer binding proteins, *C/EBP*). (Modified with permission from reference [15].)

combinatorial integration. Figure 25-3 illustrates the many points or levels of control that can be exerted over these developmental regulators. For example, the ability to alter the transcriptional expression of these factors is an initial and key point of control. As shown, other classical points of control, such as translation, are expected to add levels of complexity. Because the activity of most of these transcriptional regulators can be controlled by phosphorylation and dimerization, the number of permutations of interactive modes increases rapidly. Heterodimer formation can determine whether a transcriptional regulator is functionally active or not (discussed in detail in Chapter 26).

Thus, at any point of time in the life of a given cell, the sum total of activity at all levels of integration will be reflected by the nature of the genetic program that is read. This program not only creates the new cell phenotype but also sets the stage for the next round of developmental decisions, in part due to the alteration in the pattern of expression of transcriptional regulators now unleashed to reverberate in the cell physiology.

Early response genes function as development-control signals. How do epigenetic influences participate in decision making? Environmental influences come in the form of growth factors, neurotransmitters and other ligands that are linked to changes in membrane receptors and intracellular signaling pathways, as well as agents or fields capable of altering membrane electrical properties. Most extracellular signals trigger a change in cell physiology that can last for several days. How do these changes come about? In particular, how can environmental factors 'start the ball rolling' and trigger a new wave of genomic activity? Researchers have found that ligand-induced changes result in a very rapid gene expression that is independent of any protein synthesis [16]. This large class of genes can be expressed within minutes of cell activation. Because of the very rapid onset of expression, these genes are referred to as early response genes (ERGs), or primary response genes (Fig. 25-5) (ERGs are discussed further in Ch. 26).

Ligand-activated intracellular signaling pathways induce the transcription of ERGs that encode for four categories of cell proteins: (1) transcription factors, (2) cytoplasm enzymes and structural components, (3) secreted cytokines and (4) membrane proteins. Each category represents developmentally important factors, such as interleukin (IL)-6 and the NGF receptors. Most of the known transcription factors, including members of the families illustrated in Figure 25-4, are ERGs. Therefore, transcriptional regulation of many transcriptional regulators is under tight and rapid control by environmental agents. In addition, since a developing cell is normally exposed to many different signals at a given movement,

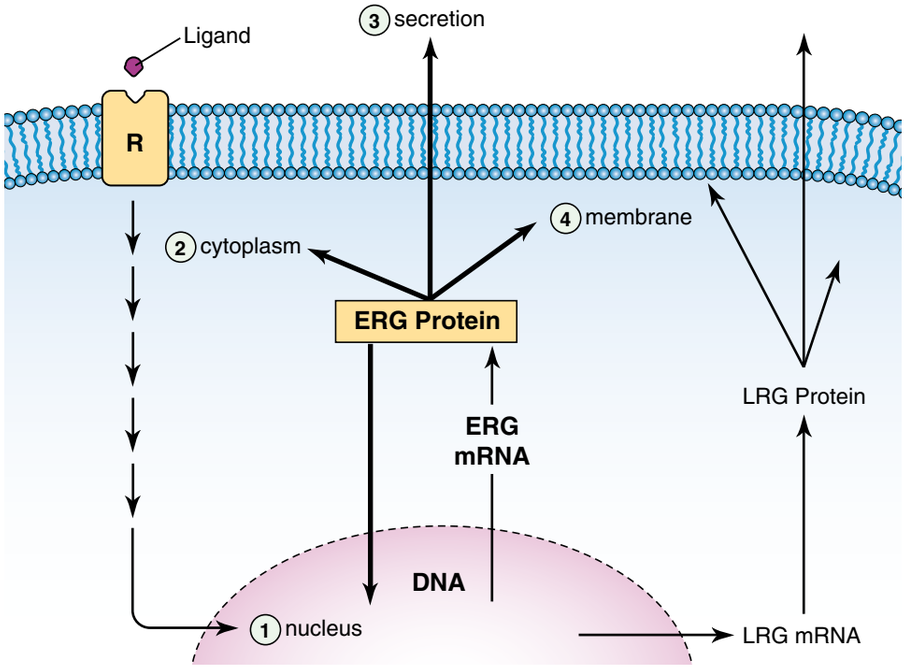

FIGURE 25-5 Early response genes. This figure illustrates the four major classes of early response gene (*ERG*) products serving as either nuclear transcription factors, such as Fos and Jun; cytoplasmic enzymes, such as nitric oxide synthetase and prostaglandin synthetase II or structural components, such as actin; secreted factors, such as interleukin 6 and other cytokines; or membrane receptors such as nerve growth factor receptor. All of these ligand-inducible ERGs are considered to play a role in regulating developmental events. Of particular importance are the ERGs encoding transcription factors, which have the ability to regulate the genomic response of neural cells to extracellular signals. The characteristic rapid and transient induction of this class of ERG mRNAs and proteins represents a self-referral loop of genetic control, whereby genetic information is rapidly expressed only to return to the nucleus and directly participate in the subsequent combinatorial control of late response gene (*LRG*) transcription and phenotypic alterations. Thus, the cell-type-specific, differential control of ERG and LRG expression may be closely coupled. (Modified with permission from reference [36].)

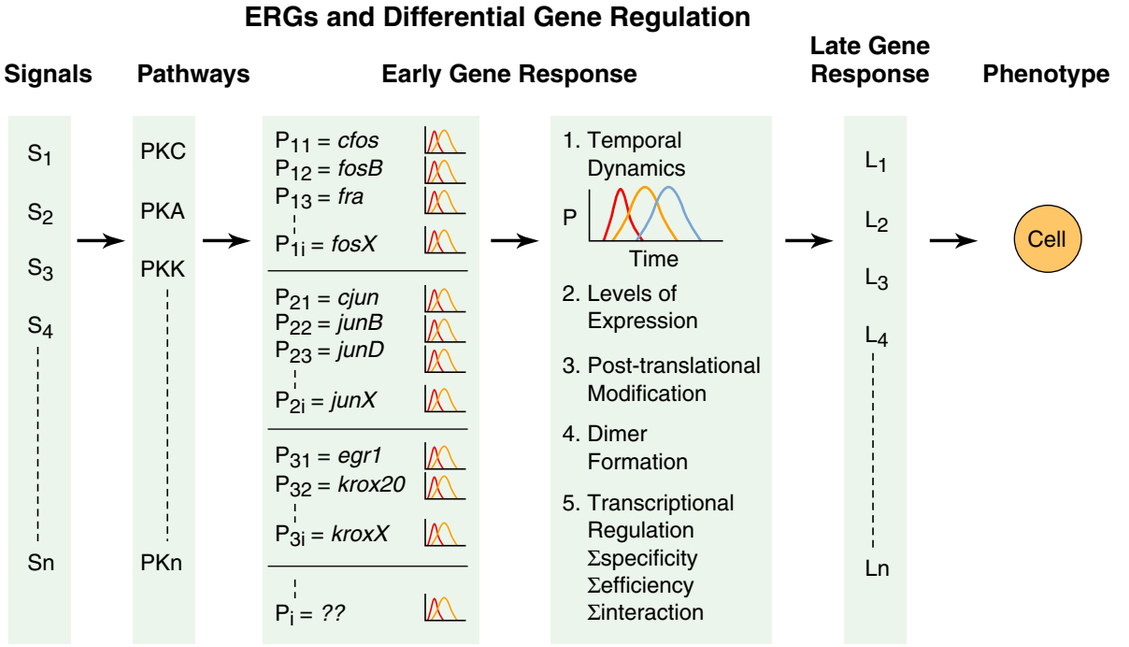

FIGURE 25-6 The early response gene (*ERG*) network and phenotypic diversity. This figure emphasizes the complexity of ERG transcription factor interaction during neural development. The level and kinetics of expression of ERG mRNAs and the subsequent dynamics of interaction among ERG proteins are shown. A wide range of environmental signals (S_1, S_2…*Sn*) can influence a cell by activating intracellular pathways, indicated here by specific protein kinases (*PKs*), such as protein kinases C (*PKC*) and A (*PKA*),…(*PKn*). These kinases are considered to activate target transcription factors capable of inducing the expression of a constellation of ERG families (P_1, P_2…P_i) with family members such as *c-fos* (P_{11}), *fosB* (P_{12}), *fra1* (P_{13})... *fosX* (P_{1i}, hypothetical). The total number of ERGs is equal to $(i) \times (j) = P_{ij}$, estimated to be several hundred. Each pathway induces a largely overlapping subset of ERGs, displaying its own characteristic kinetics and levels of mRNA accumulation, denoted by the small generic graphic symbol inserted to the right of each ERG. The next level of potential complexity is evident in the five main properties summarized for ERG protein synthesis, modification and interaction, which together orchestrate the late gene response (L_1…*Ln*). The concept of combinatorial control suggests that, in addition to the complexity of both ligand-receptor coupling and interaction among signaling pathways responsible for transcriptional activation, the various members of different families of ERG transcription factors extensively interact during the process of transcriptional control of late response genes, exerting a primary role in determining phenotypic diversity.

the ligand-mediated, rapid and transient induction of ERGs represents a tightly controllable, complex mode of encoding environmental information to carry out developmental decisions (Fig. 25-6).

A number of ERG transcription factors have already been shown to play important roles in regulating cell proliferation and differentiation. For example, combinatorial activity of the leucine zipper transcription factors superfamily, including members of the *fos* and *jun* families, is necessary for a cell to respond to a mitogen and enter the cell cycle. Thus, neural phenotypic diversity can be explained in terms of the history of expression of transcriptional regulators, some members changing rapidly (ERGs), some slowly. Together, they exert a combinatorial hold on the genetic programs and physiology of the cell and determine cell fate.

Transcriptional regulator networks function in invertebrate development. Our understanding of the genetic network orchestrating neural development in mammals is rapidly increasing, in large part as a result of the considerable advancement in developmental research of the fly. As part of the genetic network in *Drosophila*, a large number of genes encoding transcription factors and proteins for cell–cell communication help define cell lineages. Most transcription factors perform more than one function during development. For example, many participate during early segmental processes as well as in the later development of the neural, sexual and bristle components. Figure 25-7 illustrates how four major groups of genes interact during development to create the primary body axis and segmentation pattern of the embryo. For example, four maternal-effect proteins, such as bicoid and dorsal, are active in the unfertilized egg. Upon fertilization, these genes establish anterior–posterior concentration gradients that lead to differential effects on the expression of a variety of other genes encoding transcription factors, such as gap genes, which in turn organize the anterior posterior axis of the embryo. Thus, a maternal-effect mutant has no head and two caudal regions. Gap genes, for example, *Krüppel*, help to define the middle segment and to activate the expression of pair-rule genes including *hairy* and *fushi tarazu*, segment polarity genes such as *engrailed* and homeotic genes (see below). Mutations of gap genes produce embryos with deleted middle segments. Homeotic genes, named for the homeobox DNA-binding sequence, specify the identity of each segment. For example, mutation of the *antennapedia* gene

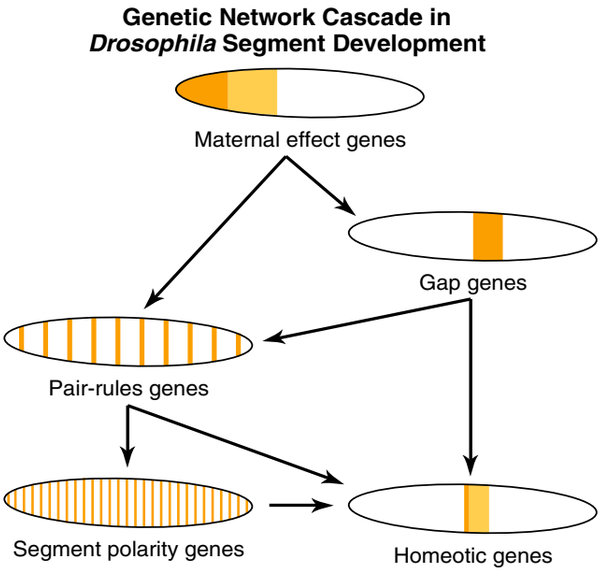

FIGURE 25-7 Genetic network cascade in early *Drosophila* development. Body segmentation in the *Drosophila* embryo results from the sequential and spatially localized expression of specific genes. During early development, maternal-effect genes set up the anterior-posterior orientation of the oblong embryo. Within a very short time, four different groups of genes, depicted here, that encode for transcription factors are expressed and function to regulate not only each other's expression but also many other genes whose products are necessary for proper sequential transformation of linear positional information into a periodic pattern. Note the alternating patterns of gene expression (*orange* vs. *white* areas) that establish boundaries necessary for proper segmentation.

results in the antenna on the head being replaced by a leg! A similar regulatory cascade of transcription factors organizes the dorsal ventral axis (Fig. 25-8).

Our understanding of gene regulation of neural development in *Drosophila* is most complete for the embryonic sensory nervous system [19]. The entire sensory system appears within 5–9 hours of fertilization and is known in detail. Five sets of genes have been described that progressively determine the structure and function of this system. The first set of genes is known as the prepattern genes, described above, which set up the anterior posterior and dorsal ventral body axis and segmentation. Many of these genes contain homeodomains and serve multiple roles during different developmental events; for example, the pair-rule gene *fushi tarazu* later controls the development of the fly CNS. Following the establishment of the body coordinates, proneural gene expression makes cells competent to become neural precursors. All the proneural genes encode helix–loop–helix (HLH) transcriptional regulators. The *achaete–scute* complex (AS-C) is a set of four proneural genes required for sensory organ formation. Mutations lead to no or very few neuronal precursors. Daughterless (da) expression is necessary for sensory organ precursors to appear. Because AS-C and da genes code for basic HLH transcription factors, these two gene products can interact to form heterodimers capable of regulating specific target genes. Two additional HLH proteins interact to expand and further refine the complexity of combinatorial control exerted by these transcription

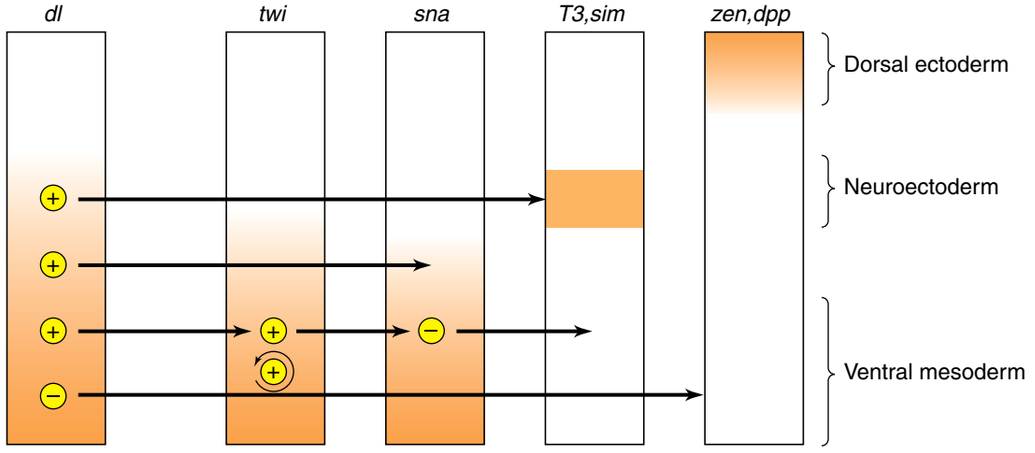

FIGURE 25-8 Dorsal–ventral morphogenesis in *Drosophila*: transcription factor regulatory cascade. The generation of the dorsal–ventral axis is dependent upon a network of transcription factors. The key nuclear morphogen is *dorsal* (*dl*), a member of the *rel/NFκB* family, whose shallow gradient in early embryo nuclei (**left box:** a schematic side view section representation of the embryo, dorsal is at top, ventral at bottom) is based upon a gradient in nuclear translocation: nuclear localization of transcription factors in ventral nuclei (*dark orange*), both nuclear and cytoplasmic localization in lateral regions (*light orange*) and only cytoplasmic localization in dorsal regions (*white*), where it remains inactive. *dl* creates, in turn, the expression gradient of at least six other transcription factors (see **four boxes to right**). *dl* activates the expression of *twist* (*twi*), a helix–loop–helix (HLH) transcription factor and *snail* (*sna*), a zinc-finger transcription factor. The combined expression of *twi* and *sna* leads to induction of the ventral mesoderm. In addition, the combination of *twi*-positive autoregulation (*small circular arrow*) and the ability of *dl* to activate *sna* along with *twi* creates a relatively steep gradient of *sna* expression that creates, in turn, a sharply bound domain of inhibition of *T3,* one of the members of the *AS-C* HLH transcription factor family and *single-minded* (*sim*), an HLH. Thus, the activation of *T3* and *sim* by *dl* (*top long arrow*) is limited to a narrow lateral band of nuclei that become cells of the neuroectoderm that form the nervous system. The dorsal ectoderm cells arise from the control of *zerknült* (*zen*) and *decapentaplegic* (*dpp*), both homeobox transcription factors. Since *dl* exerts a strong inhibitory influence over *zen* and *dpp* (*bottom long arrow*), the gradient of these (**last box**) is the inverse of *dl.*

factors. Hairy (h), named for mutants with ectopic bristles, and extramacrochaete (emc) can interact to form heterodimers with AS-C and da, but since h and emc are HLH proteins lacking functional basic DNA-binding domains, the heterodimers formed are inactive. In this way, h and emc negatively regulate AS-C and da with the resulting loss of neural tissue. Neuronal precursors then express the third set of genes, neurogenic genes, represented by HLH transcription factors such as the enhancer of split (E[spl]) complex and membrane proteins such as mastermind (mam). This set of genes mediates lateral inhibition among cells in proneural cell clusters. Mutations lead to lack of suppression, causing all cells in a cluster to enter the neural lineage. The fourth set are neuronal type-selector genes, controlling the type of sensory neuron that a precursor becomes and, hence, the type of sensory organ that forms. The cut gene product is a homeodomain transcription factor that controls cell-type-specific gene expression and organ type. Finally, cells express cell lineage genes that determine the phenotypic makeup of the cells in the sensory organ. Two genes in particular decide the identity of the final cell phenotypes and, hence, how many neurons and glia will be in each organ. In *numb* mutations, involving loss of a zinc-finger transcription factor, all progeny become socket or hair cells with the loss of neuronal and glial cells. Another gene, *oversensitive*, has the opposite effect: all neurons and glia and no socket or hair cells. Thus, PNS development relies on the sequential expression of HLH, homeobox and zinc-finger classes of transcription factors, which participate in a complex combinatorial network to progressively determine neural cell fate.

Transcriptional regulator networks determine vertebrate development. Vertebrates also rely on networks of transcription regulators to establish body plan and regional identity. However, the remarkably similar mechanisms of genetic and developmental control found in both invertebrates and vertebrates stand in contrast to the timing and form of development in phylogenetically distinct species. Genetic similarities include the existence of highly conserved families of HLH and homeobox transcriptional regulators specifying cell fate. In mammalian development, a genetic regulatory network analogous to that described for *Drosophila* seems to function [17]. In particular, homologous sets of genes are expressed in the rhombomeres of the developing hindbrain, representing a genetic network regulating development of this part of the CNS. Additional studies have implicated other transcriptional regulators in regional pattern formation in more anterior parts of the CNS and in the spinal cord.

The development of the mammalian hindbrain shows strong physical and genetic similarities to invertebrate development. Segmentation is apparent through the transient appearance of rhombomeres, which appear as morphologically distinct 'bumps' in the floor of the fourth ventricle during early development. Rhombomere organization is reiterated through the segmentally repeated

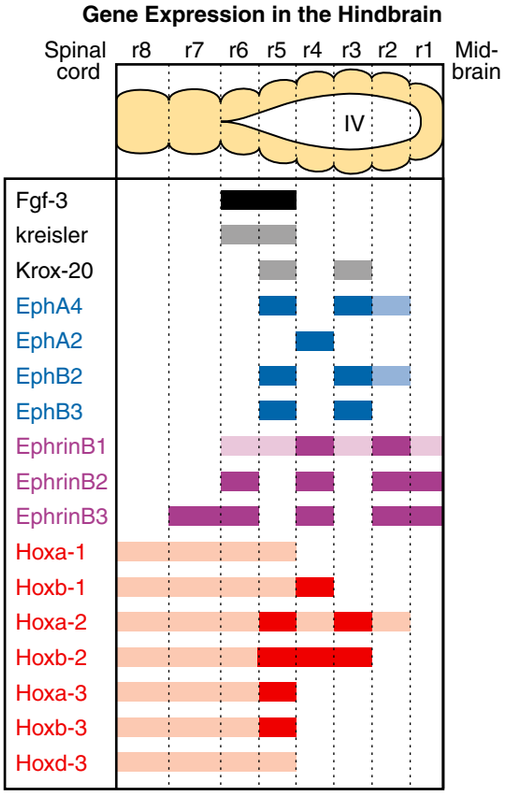

FIGURE 25-9 Segmentally restricted hindbrain gene expression. Vertebrate rhombomeres (*r1–r8*) are analogous to *Drosophila* body segments and are shown in this schematic of the hindbrain and IVth ventricle (IV). The expression pattern of individual genes is indicated below the hindbrain (*black or dark colors*, strong expression; *gray or light colors*, weak expression; *no bar*, no expression). These genes include signaling molecules such as fibroblast growth factor 3 (*Fgf-3*), early-expressed transcription factors such as kreisler and Krox-20, Eph family tyrosine kinase receptors and their ephrin ligands; and the Hox homeobox genes. Note that each of the genes is expressed in a spatially restricted manner that reflects the position of segmental boundaries. These overlapping expression domains create unique combinations of transcription factors within each rhombomere that are considered, in turn, to regulate the phenotype of the cells in each hindbrain region.

reticular formation and branchiomotor neurons found in the hindbrain. A number of genes are expressed in accord with rhombomere boundaries. Figure 25-9 shows the expression pattern of several classes of genes in the vertebrate hindbrain, including early expressed transcription factors such as *kreisler* and *krox-20*, Eph family tyrosine kinase receptors and their ephrin ligands, Hox genes, and genes coding for signaling molecules such as *fgf-3*. Together, these genes may be responsible for creating positional value and establishing segmental identity along the length of the hindbrain [18]. Rhombomeres are cell-lineage-restriction units, similar to the compartments seen in *Drosophila*. Cell mixing across rhombomere boundaries may be inhibited by the complementary expression of Eph receptors and ephrins. Positional value or segmental

identity may be determined by expression of Hox genes. Hox gene expression precedes the formation of the rhombomeres and establishes the location of boundaries between adjacent rhombomeres. Inactivation of Hox genes alters rhombomere identity, suggesting that these genes serve a function akin to that exhibited by homeotic genes in invertebrates. Hox gene expression may be activated by other transcriptional regulators, including *krox-20* and *kreisler*, which are expressed prior to Hox gene expression. Disruption of *krox-20* and *kreisler* activity also disrupts the pattern of Hox gene expression and rhombomere formation, suggesting that these two genes act upstream of the Hox genes. Detection of *krox-20*-dependent enhancers up-stream of Hox genes further supports this hypothesis [19].

The development of other regions of the CNS is not likely to rely on an overt pattern of segmentation such as is seen in the hindbrain, although the genetic mechanisms at play may be shared between vertebrates and invertebrates. Pattern and spatial position in the midbrain may stem from signals provided by the isthmus, a constriction separating the developing midbrain from the hindbrain. Early in development, the midbrain and hindbrain are differentiated from each other by the expression of the transcription factors Otx2 rostral to the isthmus and Gbx2 caudal to this boundary. Two secreted signaling molecules, Fgf8 and Wnt-1 are then expressed at the border between Otx2 and Gbx2 expression. These signaling molecules in turn regulate the expression of the transcription factors engrailed-1 and engrailed-2 in gradients rostral and caudal to the isthmus. Engrailed gene expression is required for the development of mesencephalic structures rostral to the isthmus and for the development of the cerebellum more caudally. Similar interactions are seen in *Drosophila*, with signaling by the Wnt-1 homolog *wingless* required for maintaining *engrailed* expression in neighboring cells.

In the forebrain, a number of transcriptional regulators are expressed in specific longitudinally arrayed domains. These genes include members of the *pax*, *emx*, *otx*, *dlx* and *nkx* homeobox gene families, and other transcription factors such as *bf1* and *tbr-1* [20]. Descriptive expression studies using these genes have suggested that segmentation may act in developing the telencephalon and diencephalon, although the segments identified by gene expression patterns are not restricted lineage units as seen in the hindbrain. For example, GABAergic cortical interneurons arise from the medial and lateral ganglionic eminences, whose territories are initially defined by the expression of *dlx1* and *dlx2*. These cells then migrate tangentially to enter a neocortical domain defined by the expression of *gli3* and *neurogenin1/2* [21]. Inactivation of *dlx1* and *dlx2* does not affect the production of these neurons, but does affect their ability to migrate into the neocortex. The nested expression pattern shown by the *emx* and *otx* genes is similar to the pattern created by Hox gene expression, but the lack of linkage between the four members of this family suggests that a different mechanism is used to establish domains within the forebrain. Some of these genes are expressed in the ventricular zone, suggesting a role in initial patterning of the neuraxis, while others are expressed in the mantle layer suggesting a role in differentiation.

Spinal cord development and regionalization may also be under the control of transcriptional regulators [22]. Regional diversity may be imparted by expression of more distal Hox genes, which continue to be expressed in accord with 'segmental' boundaries within the spinal cord. One inherent difference is that the regional distinctions visible in the spinal cord are imparted by signals from the surrounding mesoderm and not by intrinsic factors within the spinal cord [23]. Thus, external signals may establish the pattern of transcription factor expression along the length of the spinal cord. In addition to patterning along the rostrocaudal axis, the spinal cord, along with all other regions of the developing CNS, is also patterned along the dorsoventral axis. The dorsal–ventral axis of the spinal cord is likely to form through the concurrent action of two signaling systems. A ventralizing signal initially comes from the notochord; transplantation of a second notochord adjacent to the spinal cord results in differentiation of a second set of ventral spinal cord cells. This signal is carried by a molecule called sonic hedgehog, which is secreted by the notochord during early development. The neuroepithelium responds to this signal by developing the floorplate, the most ventral portion of the spinal cord. Once floorplate formation has been induced, the floorplate itself begins to secrete sonic hedgehog in a gradient. The gradient of sonic hedgehog expression in turn establishes distinct progenitor domains within the ventral spinal cord by inducing the expression of homeodomain proteins including Pax, Nkx, Dbx and Irx family members.

These proteins have been divided into two classes based on their differential response to sonic hedgehog signaling. Class I protein expression is repressed by sonic hedgehog, while Class II proteins are activated by sonic hedgehog signaling. Each of these proteins is activated or repressed at a distinct level of sonic hedgehog signaling, allowing each protein to be expressed in a domain that reflects a specific sensitivity to the signal. Expression of these transcription factors then defines five progenitor domains in the ventral spinal cord, each of which generates a specific class of ventral spinal cord neurons. Dorsal spinal cord development relies on a second set of signals emanating from the roof plate, the most dorsal part of the spinal cord. Many factors, including bone morphogenetic proteins, Wnt signaling molecules and fibroblast growth factors, are secreted by the roof plate. Rather than differential responses to a single graded signal as is seen in ventral spinal cord development, the dorsal neural tube appears to be patterned by the combinatorial activity of several different signaling molecules. These signals establish six different classes of progenitor domains that give rise to at least eight different types of neurons.

CELL LINEAGES OF THE NERVOUS SYSTEM

The neural crest lineage is progressively restricted to specific sublineages. The developmental fate of most cells is not fixed initially but becomes progressively restricted by epigenetic and genetic interactions[1, 22]. We have considerable information about the multipotent NC cells and how they become progressively restricted to specific sublineages [11, 24, 25]. NC cells migrate from the neural tube along specific pathways to their peripheral destinations. At these target sites, they differentiate into a diverse number of cell types, including Schwann cells and neuronal cells of the PNS, pigment cells, endocrine cells and cells forming connective tissue of the face and neck (Fig. 25-10).

A basic property of differentiated neuronal cells is the expression of one classical neurotransmitter and several neuropeptides. *In vivo* studies with NC cells first demonstrated that the choice of neurotransmitter phenotype could be altered by the environment. In these studies, NC cells were examined by means of chimera transplantation. Two of the final NC cell phenotypes are sympathetic, mainly adrenergic cells and parasympathetic, cholinergic cells. Presumptive adrenergic neurons transferred to the presumptive cholinergic region of young embryos migrated along the path of vagal NC cells and became cholinergic instead of sympathetic. The nature of the environment could thus switch neurotransmitter phenotype. The inverse experiment also worked: presumptive cholinergic cells become adrenergic when transferred to the adrenergic region. Thus, premigratory NC cells from different axial levels share some common developmental potential and differentiate in a manner appropriate for their final position. These transplantation experiments using heterogeneous cell populations, however, could be interpreted as evidence for selective or instructive processes: selective cell elimination of an inappropriate phenotype or environmental instructions of appropriate cell phenotype.

Environmental factors control lineage decisions of neural crest cells. Several experimental approaches have shown that environmental factors are critical in determining neurotransmitter phenotype by altering existing cell properties and not by selecting different hypothetical subpopulations of NC cells. For example, during normal

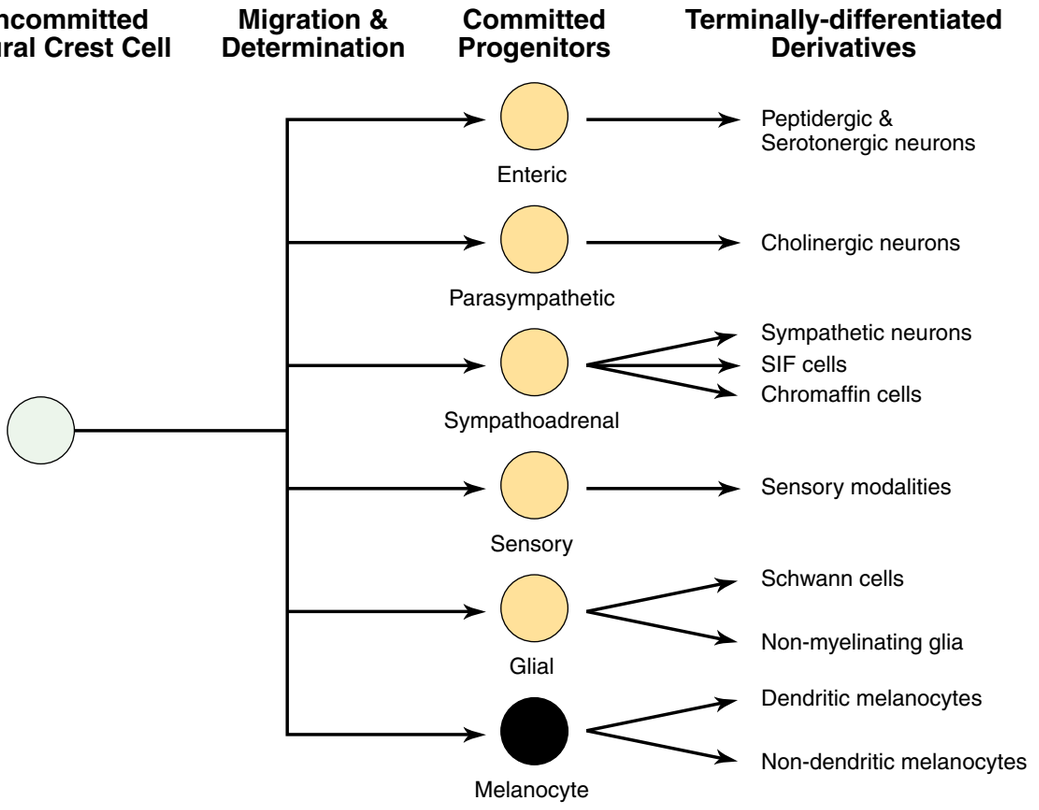

FIGURE 25-10 Neuropoietic model of neural crest cell lineage. Analogous to the process of hemopoiesis, early multipotent neuropoietic stem cells undergo extensive migration along complex pathways to different embryonic environments. Committed progenitor cells, including enteric, parasympathetic and others as listed, generate restricted sublineages under the influence of environmental growth factors. These cell populations expand in number and undergo terminal differentiation to the final adult phenotypes. *SIF*, small, intensely fluorescent cells. (Modified with permission from reference [24].)

postnatal development, the innervation of the sweat glands of the foot pads of cats and rats switches from noradrenergic to cholinergic. Research shows that environmental cues of the target tissue specify both early and late neurotransmitter phenotypes. Cross-innervation studies provide further evidence that the target can retrogradely specify neurotransmitter properties of the neuron that innervates it. Neurons that ordinarily provide noradrenergic innervation of hairy skin become cholinergic and peptidergic when induced to innervate sweat glands. Converse experiments give expected results.

The cellular and molecular mechanisms that specify neurotransmitter phenotype have been described by culture experiments (Fig. 25-11). Polypeptide growth factors such as NGF, bFGF and IL-6 appear to be the primary controllers of neuronal differentiation of the sympathoadrenal progenitor cells. Research findings demonstrate that bFGF promotes the proliferation and initial differentiation of the sympatho-adrenal precursor. For example, bFGF leads to increased neurite outgrowth and upregulation of neuron-specific genes. The survival of these committed neuronal precursor cells, however, ultimately depends upon NGF responsiveness and availability. bFGF can upregulate the low-affinity neurotrophin as receptor ($p75^{NT}$), and cell depolarization induces *trk* genes, which together can produce high-affinity, functional NGF receptors, providing trophic responsiveness.

A number of cytokines appear to control the final stage of differentiation of the sympathetic neuronal lineage. Long ago, it was found that individual sympathetic neurons grown on heart cell monolayers modify their biochemical, pharmacological and electrical properties and shift from adrenergic to cholinergic phenotypes. Growing the neurons in heart-cell-conditioned medium produces similar results, suggesting that non-neuronal cells, for example heart myoblasts or glial cells, release a soluble factor, now termed cholinergic differentiation factor (CDF), also known as leukemia inhibitory factor (LIF), that is responsible for the developmental switch. Ciliary neurotrophic factor (CNTF), belonging to the same cytokine subfamily as IL-6 and LIF, also increases cholinergic and decreases noradrenergic properties. While promoting the cholinergic phenotype, CNTF and LIF also inhibit sympathetic neuroblast proliferation. Thus, non-neuronal cells are a source of many molecules that can influence the choice of neurotransmitter and neuropeptide in cultured sympathetic neurons, suggesting that the spatiotemporal differential expression of a variety of cytokines helps determine the diversity of cell fate.

One more key player is known to participate in sympathoadrenal lineage fate. The presence and concentrations of glucocorticoid strongly influence lineage decisions (see discussions of endocrine regulation of transcription in Chs 26 and 52). Since sympathetic ganglia cells normally grow *in vivo* in the presence of glial cells and become predominantly adrenergic, a second factor was sought in order to explain the discrepancy with the *in vitro* switch studies that showed the appearance of the cholinergic phenotype. Studies showed that physiological levels of glucocorticoid can modulate biochemical differentiation by blocking the shift from adrenergic to cholinergic phenotype. LIF effects can also be blocked by conditions that mimic neuronal activity, such as elevated potassium concentrations. In addition, the concentration of glucocorticoid is an important determinant of the path of the sympathoadrenal precursor: low concentrations generate small, intensely fluorescent (SIF) cells and high concentrations generate chromaffin cells.

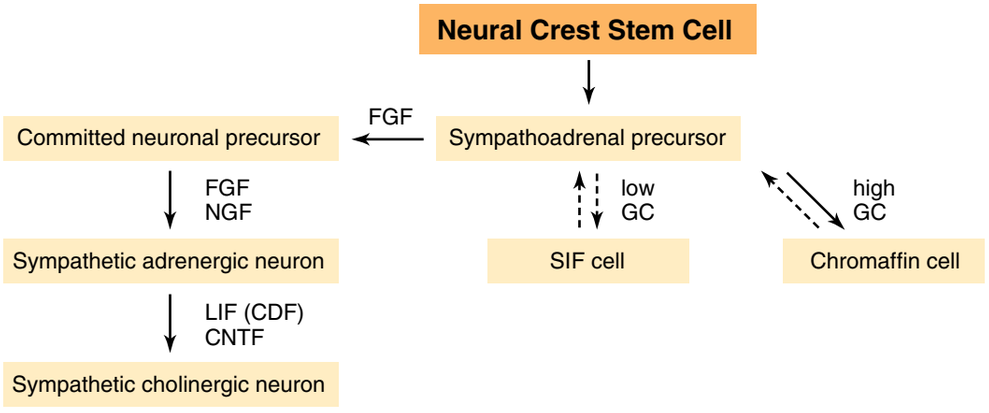

FIGURE 25-11 Growth factor control of neural crest lineage decisions. Regional and temporal differences in environmental factors influence the final phenotype of neural crest progeny. A number of environmental factors influence the committed precursors that give rise to the final four mature phenotypes of the sympathoadrenal lineage. Under conditions of low or high concentrations of glucocorticoids (*GC*), the sympathoadrenal precursors become either small, intensely fluorescent (*SIF*) cells of the sympathetic ganglia or chromaffin cells of the adrenal medulla, respectively. In environments containing high concentrations of fibroblast growth factor (*FGF*) and nerve growth factor (*NGF*) and little or no glucocorticoid, committed neuronal precursors appear, which terminally differentiate into either sympathetic adrenergic neuronal cells or, under the influence of cholinergic differentiation factor (*CDF*), also known as leukemia inhibitory factor (*LIF*), and ciliary neurotrophic factor (*CNTF*) switch neurotransmitter phenotype and become sympathetic cholinergic neuronal cells.

Research has begun to identify the intracellular signaling pathways activated by environmental factors that lead to the induction of developmental control genes in NC cells and their derivatives. For example, developmental restriction of melanogenesis normally occurs before embryonic day 5 in the quail. This process can be altered by treating embryonic day 9 dorsal root ganglion cells with phorbol ester, which activates protein kinase C (PKC) (see Ch. 23). Schwann cell precursors, which normally lack melanogenic activity, undergo a metaplastic transformation into melanocytes when PKC activity is reduced. This suggests that environmental signals capable of altering levels of PKC regulate lineage decisions in NC cells.

Neural crest lineage is transcriptionally regulated. A number of candidate control genes have been characterized that could give rise to progressive restriction in migrating behavior and control over proliferation and differentiation. Several newly discovered genes appear to underlie cell lineage commitment, while other genes appear important as mediators of the ligand-dependent switch of neurotransmitter phenotype in committed neuronal cells. In mammalian development, a gene analogous with the *AS-C* genes in *Drosophila,* which determine whether a cell becomes a neuronal precursor, has been described. The mammalian achaete-scute homolog-1 (*MASH1*) gene is transiently expressed by spatially restricted subsets of early CNS neuroepithelial and PNS NC cells. The precursors of sympathetic and enteric neurons express this bHLH transcription factor just prior to the onset of cell-type-specific gene expression, such as tyrosine hydroxylase (TH), suggesting that *MASH1* is a marker of cells as they enter the sympathoadrenal lineage. It is likely that *MASH1* is an NC control gene determining the commitment step of lineage development. It is expected that *MASH1,* like the activity of *AS-C* genes, will be regulated by a number of other HLH gene families homologous to *da* (E12), *emc* (Id) or *h* (HES) by way of alternative HLH pairing, or heterodimerization, leading to either enhanced or inhibited function in the differentiating mammalian NC cells and CNS.

Once the cells are committed to the sympathoadrenal lineage, a choice of neurotransmitter phenotype must take place. The developmental switch between chromaffin and neuronal phenotype is associated with changes in cell-type-specific gene expression, which require the differential control of NC genetic programs. The molecular mechanism integrating the influences of these two types of environmental signals, peptide growth factors and glucocorticoids, involves, in part, the antagonistic interaction of transcription factors (see Ch. 26). Culture work demonstrates that FGF and NGF are antagonistic to glucocorticoid in their ability to upregulate chromaffin-specific genes. The opposite control is also true. Two classes of transcription factors, Fos, as part of the peptide-inducible AP-1 complex, and glucocorticoid receptors (Fig. 25-4), have been shown to interact in a similar, reciprocal inhibitory fashion. This suggests that, as part of the combinatorial control process, interaction between these different families of transcription factors represents a probable molecular mechanism of reciprocal inhibition exerted by environmental factors.

Glial cell development is critical to nervous system development. Our understanding of glial cell development in the CNS has advanced rapidly since the mid-1980s [1]. The discovery and initial characterization of the oligodendrocyte-type 2 astrocyte (O-2A) cell lineage was the most extensively characterized cell lineage system available to study nervous system development. A variety of cellular and molecular approaches have provided us with an understanding of the environmental signals and the phenotypic responses that are of importance *in vitro.* During the last decade this work has been largely validated *in vivo.* This has led to a rapid increase in our understanding of the key environmental agents that regulate the rate and direction of glial cell differentiation along specific lineage pathways.

The oligodendrocyte and astrocyte lineages have been studied *in vitro*. Oligodendrocytes (OLs) are generated perinatally and pass through a series of cell phenotypes from undifferentiated stem cells to mature myelin-forming cells. This sequential process of maturation of OLs can be reproduced in culture (Fig. 25-12). In the rat, four main stages of development *in vitro* have been delineated. The oligodendrocyte progenitor (OLP) or oligodendroblast, also referred to as the O-2A bipotential progenitor cell, is a proliferating, bipolar cell. These cells, when cultured in chemically defined medium containing 0.5% (low) serum to enhance survival, rapidly differentiate in a relatively synchronous manner into mature oligodendrocytes over a 3–5-day period. In contrast, under high serum conditions or in the presence of CNTF, many cells become positive for glial fibrillary acidic protein (GFAP) and A2B5 and negative for OL markers. These $GFAP^+/A2B5^+$ cells are called type 2 astrocytes (AIIs). This cell type has not been observed *in vivo* and can be considered a plasticity artifact of tissue culture [26]. As bipolar OLPs differentiate to simple multipolar, termed $O4^+$ 'pre-OL' cells, they still proliferate, but at a slower rate than OLPs. When these $O4^+$ cells express the glycolipid galactocerebroside, cyclic nucleotide phosphohydrolase (CNP) and glycerol phosphate dehydrogenase (GPDH) they are called 'immature OLs'. The 'mature OL' phenotype is identified by the appearance of myelin-specific antigens, proteolipid protein (PLP), myelin basic protein (MBP) and myelin-associated glycoprotein (MAG). Thus, immunostaining with glia-specific markers defines six phenotypically distinct cell types *in vitro*, five of which relate to the OL lineage: OLPs, pre-OL cells, immature OL cells, mature OL cells, multipolar AII cells ($A2B5^+/GFAP^+$) and flat AI cells ($A2B5^+/GFAP^+$). This classification scheme can be used to monitor the rate, direction and extent of OL cell differentiation.

Oligodendrocyte lineage *in vivo* resembles that seen *in vitro*. Descriptions of the patterns of gliogenesis in the

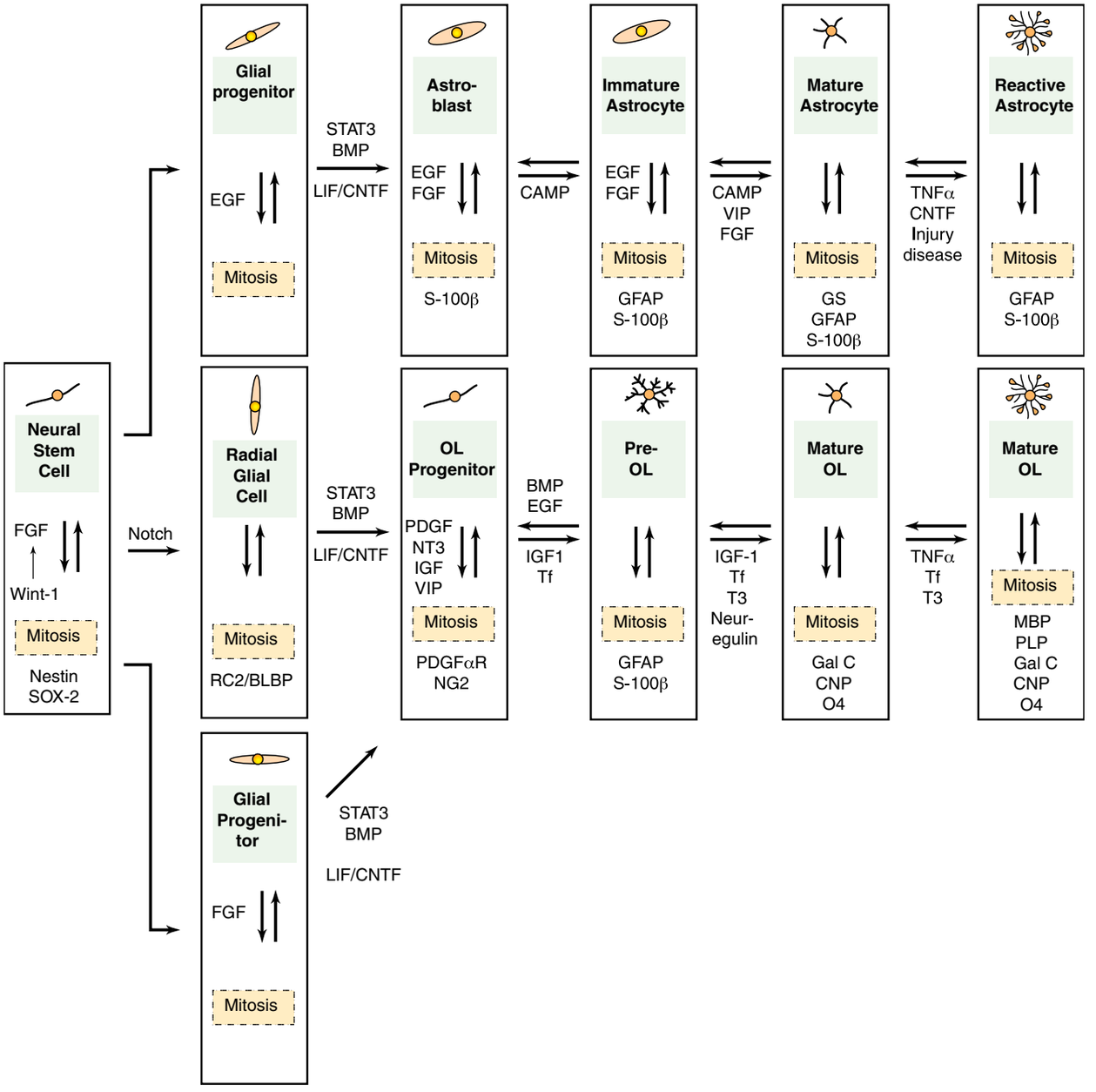

FIGURE 25-12 The oligodendrocyte (*OL*) and astrocyte lineages. The figure illustrates the characteristics that accompany the sequential differentiation within the oligodendrocyte lineage beginning from very early stem cells to the mature phenotypes. This version depicts six cell phenotypes for each lineage based on morphology, proliferative regulation and immunological determinants defined primarily from *in vitro* data. The various growth factors listed have been shown to regulate cell proliferation and/or differentiation. *Dashed arrows* indicate a possible particular direction, though without clear experimental support. The time course of expression of cell-type-specific markers is shown at the bottom. Note that each main stage of lineage development displays not only a different morphology but also a unique antigen profile. Growth factors include basic fibroblast growth factor (*bFGF*), cAMP, ciliary neurotrophic factor (*CNTF*), hydrocortisone (*HC*), insulin-like growth factor (*IGF*), interleukin 2 (*IL-2*), platelet-poor plasma (*PPP*), platelet-derived growth factor (*PDGF*), transferrin (*Tf*), transforming growth factor β(*TGF*β) and triiodothyronine (*T3*). Cell markers (dotted lines indicate unresolved expression pattern): ganglioside (*A2B5*), 2,3-cyclic nucleotide-3-phosphohydrolase (*CNP*), ganglioside (*GD3*), glycerolphosphate dehydrogenase (*GPDH*), galactocerebroside glycolipid (*GC*), ganglioside (*O4*), glutamine synthetase (*GS*), proteolipid protein (*PLP*), myelin basic protein (*MBP*), sulfatide (*SULF*) and vimentin (*VIM*).

forebrain using immunocytochemical probes and lineage tracers closely resemble the *in vitro* data. Similar findings have been reported for the cerebellum and the spinal cord. The ventral subventricular zones (SVZ) covering portions of the lateral ventricles and spinal cord are considered to be the origin of progenitor cells that multiply in the SVZ and as they migrate to and differentiate within the overlying white and gray matter. The separation of OL and AI lineage is considered to occur during embryonic development based on retroviral studies and double labeling, ultrastructural localization and cell morphology. Astrocytes principally originate from radial glia and the SVZ [27]. Immature astrocytes, particularly S100B$^+$ cells, retain the capacity to proliferate and, therefore, do not need to derive from neural stem cells. However, since stem cells are found in many regions of the adult CNS (see Ch. 29), the potential still exists.

A number of glial markers provide static yet overlapping information concerning OL lineage development. Small, proliferating progenitor cells that are positive for ganglioside GD3, NG2 proteoglycan and platelet-derived growth factor (PDGFα) receptor (PDGFR) are observed in the embryonic SVZ. Starting at embryonic day 16 and continuing to 7–10 postnatal days, OLPs proliferate in the SVZ and are similar to the OLPs/O2A cells described *in vitro* above. These cells migrate into the overlying subcortical white matter. By the end of the first postnatal week, the GD3$^+$/PDGFR$^+$ cells diminish in number while O4$^+$ and galactocerebroside$^+$/CNP$^+$/GPDH$^+$ cells rapidly increase in number in the white matter tracts. Thus, early stages of OL differentiation appear to occur in the SVZ, which contains a heterogeneous population of 'progenitor' phenotypes. The subsequent development of O4$^+$ progenitors into galactocerebroside-positive 'immature' OLs and MBP-positive 'mature' OLs occurs in the white and gray matter and not in the SVZ or neighboring subcortical regions.

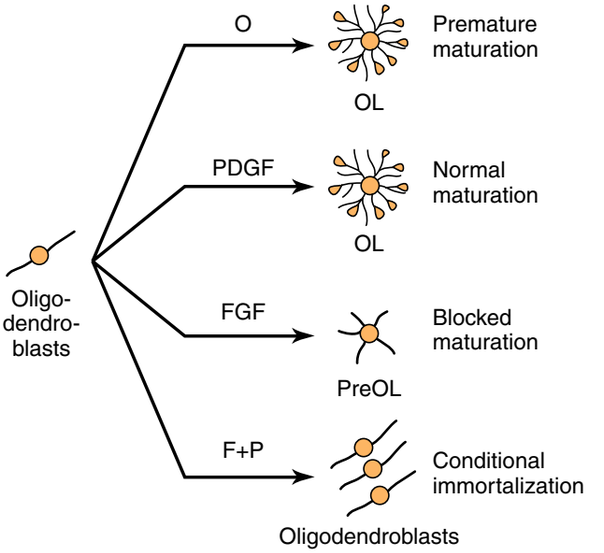

FIGURE 25-13 Growth factor control of oligodendrocyte (*OL*) lineage decisions. *In vitro* studies have demonstrated that the developmental fate of O-2A progenitor cells in culture can be regulated by environmental factors. Progenitor cells cultured in the absence of either basic fibroblast growth factor (*FGF*) or platelet-derived growth factor (*PDGF*) develop to mature OLs but do so on a timetable more rapid than *in vivo*. The addition of PDGF stimulates several rounds of cell division, slowing/delaying the time of development to mature OLs so that it more closely resembles the normal *in vivo* time course. In contrast, FGF stimulates continual proliferation while blocking phenotypic development at the pre-OL (O4$^+$/galactocerebroside glycolipid$^-$) cell stage. Evidence suggests that FGF can lead to downregulation of cell surface galactocerebroside glycolipid and simplification of morphology, indicating possible 'dedifferentiation' or plasticity of the galactocerebroside glycolipid$^+$ cell phenotype. The combination of both factors leads to a stable condition of the immature O-2A phenotype exhibiting continual proliferation. Upon removal of either FGF or both factors, cells leave the state of 'conditional' immortalization and proceed to differentiate normally.

Growth factors regulate oligodendrocyte development. The OL cell lineage culture is an excellent system to study the influence of growth factors on cell lineage development. Each of the four main stages of OL lineage progression can be identified by its characteristic markers and, through the use of growth factors, experiments can be designed to increase or decrease the degree of proliferation and/or block, delay or accelerate maturation of developing precursor cells. Although research shows that OL differentiation is a 'default' pathway, occurring in serum-free medium, in the absence of environmental signals, a number of studies demonstrate that OL development is more complex and is responsive to numerous environmental signals. For example, OL lineage cells continue to proliferate for extended periods *in vivo*, in contrast to *in vitro*. In addition, treatment of OL progenitor cells with growth factors known to be present in the developing CNS yields a complex set of ligand-dependent, phenotypic responses. bFGF, PDGF, NT3, IGF-I [28, 29] and PACAP [30] are key molecular signals controlling OL cell development (Fig. 25-13). In the presence of PDGF, OL progenitor cells are stimulated to divide with a short cell cycle length of 18 hours; are highly motile and bipolar; and differentiate in a synchronous, symmetrical, clonal fashion with a time course similar to that *in vivo*. In the presence of bFGF, however, progenitor cells are stimulated to divide with a longer cell cycle length of 45 hours, become nonmotile pre-OLs with a multipolar shape and are inhibited from expressing galactocerebroside glycolipid, PLP or MBP. Progenitor cells treated with both bFGF and PDGF exhibit a third phenotypic response, remaining motile, bipolar progenitor cells that divide indefinitely and do not differentiate. This ligand-dependent, conditional 'immortalization' can greatly expand the OL progenitor cell population over extended periods of time. Upon removal of bFGF or both mitogens, the progenitor cells differentiate along the OL lineage.

The source of PDGF *in vitro* appears to be AI cells, but *in vivo* PDGF is produced by neurons in the brain and

spinal cord, except in the optic nerve where astrocytes perform this function. From *in situ* hybridization data, the PDGFα receptor is expressed only in galactocerebroside glycolipid/OL lineage cells and not in identifiable astrocytes or neuronal cells. In addition, bFGF appears to upregulate the PDGFα receptor in OL progenitor cells. By altering the responsiveness of these cells to PDGF, bFGF and PDGF together maintain the 'proliferative' state. Members of the transforming growth factor (TGF)β family, such as bone morphogenetic proteins (BMP)2 and 4 inhibit generation of OL and promote astrocyte generation. CNTF and LIF act primarily as survival factors, in contrast to IGF-I, which acts to commit neural stem cells to the OL lineage and then stimulate proliferation, survival and differentiation of OLPs both *in vivo* and *in vitro* [28,29]. The neuropeptide family comprising VIP and PACAP and their receptors was recently shown to play a major role in neural embryogenesis. PACAP stimulates OL proliferation and can delay myelination [30].

Transferrin is an iron carrier protein that acts as a trophic survival factor for neurons, astrocytes and OLs. As the blood–brain barrier becomes established during development, neural cells become dependent on transferrin produced by OLs and choroid plexus epithelial cells (Fig. 25-14). OLs are the major source of transferrin in the CNS. This suggests an important function for OLs in addition to myelination of axonal tracts. Transferrin concentration in CSF is highest at the time of peak myelination. Oligodendrocytes, myelin and several areas of the brain contain higher concentrations of iron than the liver, presumably in the form of ferritin-bound iron. Transferrin seems to play a unique role in OL by regulating the transcription of the *MBP* gene and because its action synergizes with IGF-I to enhance myelin formation and repair [31].

The Schwann cell lineage is also characterized by sequential and overlapping expression of many stage-specific markers. The Schwann cell lineage arises from NC cells. Expression patterns of antigens have allowed the precise delineation of three distinct developmental stages, both *in vivo* and *in vitro,* that give rise to two types of Schwann cells in the adult, myelinating and nonmyelinating cells (Figs 25-15 **and** 25-16). First is the appearance of the Schwann cell precursor, a short-lived population of highly motile cells that depend on two axonal signals, neuroregulin-1 and endothelin, for survival and rapid proliferation. Two transcription factors, Krox -20 and Sox-10, respectively needed for myelination and for the establishment of the Schwann cell lineage, are also required for lineage progression to the second stage, the so called immature Schwann cells [32]. The molecular phenotype

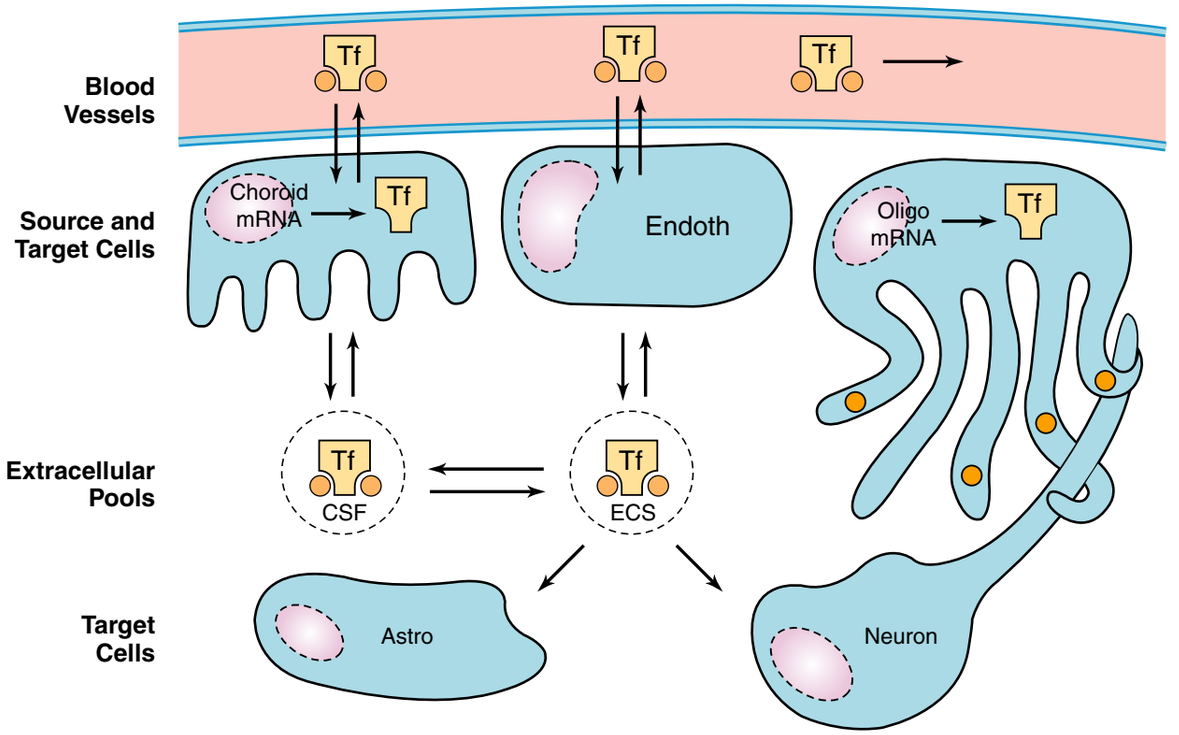

FIGURE 25-14 Regulation of transferrin (*Tf*) in the CNS. Tf is an important molecule regulating the movement and storage of iron in the nervous system. Although blood-borne Tf is exchanged by choroid epithelial cells and the endothelial cells of the capillary beds in the brain, only the oligodendrocytes and choroid cells in the brain can produce Tf *in vivo*. Each Tf molecule can bind two atoms of iron (*dark orange balls*) that in the brain are distributed between the two large pools, the cerebrospinal fluid (*CSF*) and the extracellular space (*ECS*). Astrocytes and neurons are considered to be completely dependent upon these pools and, in turn, upon the oligodendrocytes, choroidal cells and the body for Tf.

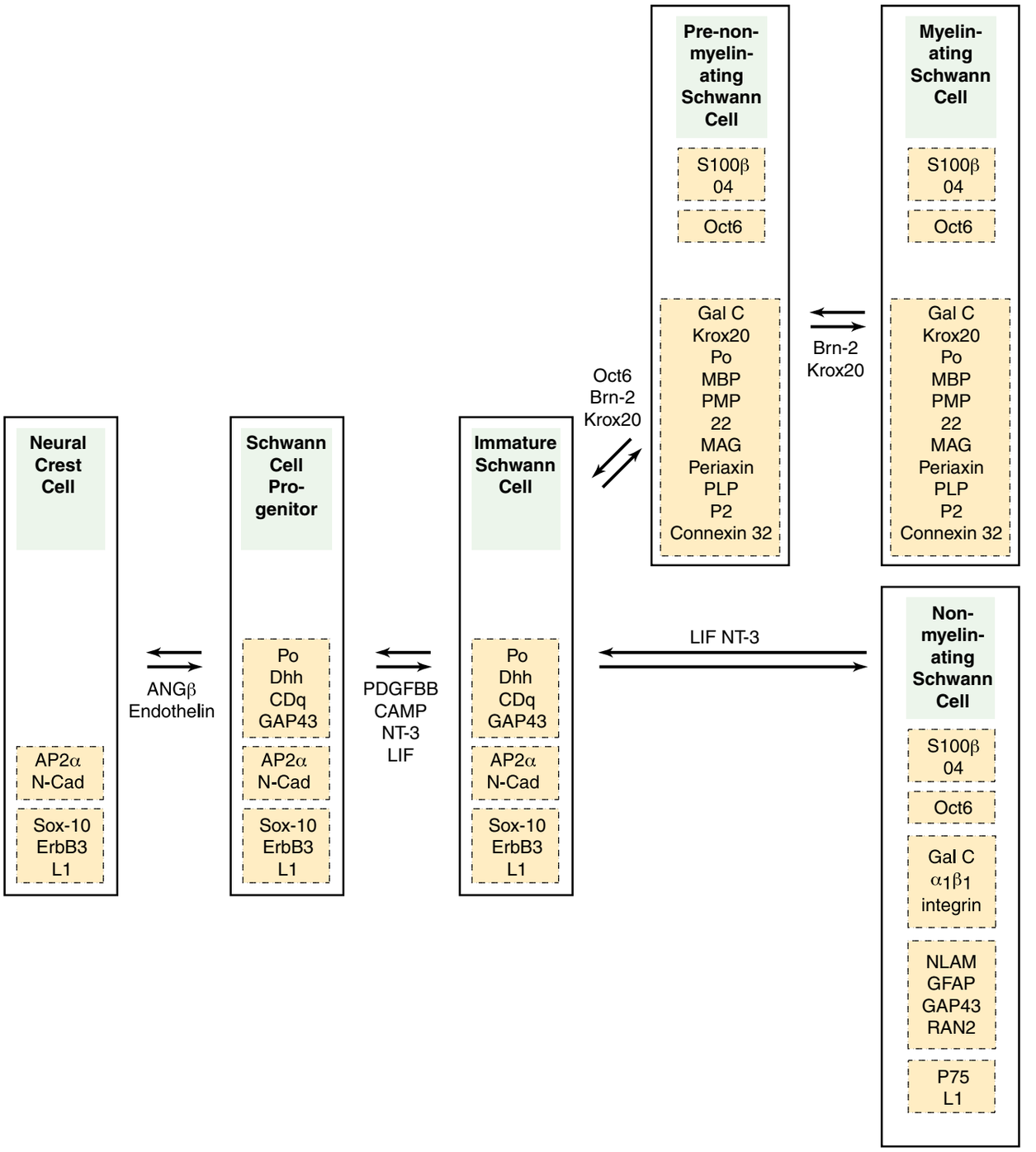

FIGURE 25-15 The Schwann cell lineage. The Schwann cell is the axon-ensheathing cell of the PNS and, thus, displays many properties and developmental processes similar to CNS oligodendrocytes. The figure illustrates the sequential differentiation of Schwann cells from early, uncharacterized neural crest stem cells to the final two mature phenotypes, surrounded by *boxes:* the nonmyelinating and the myelinating Schwann cells of the adult, based on the size of the axons they ensheathe. See Figure 25-16 for the developmental anatomy of these two mature phenotypes. Unlike CNS oligodendrocytes, the vast majority of Schwann cells do not become myelin-producing. This six-stage version of Schwann cell development is based upon cell morphology, proliferative potential and, most importantly, the expression of stage-specific antigens, that is, the appearance and/or disappearance of specific markers, derived from both *in vivo* and *in vitro* data. It appears that upon reaching the nonmyelinating phenotype (**box 4 from left**), cells are fated either to remain in this state and represent the adult population of nonmyelinating cells or to differentiate further, expressing myelin-associated genes. The premyelinating phenotypes then become myelinating cells of the PNS (stage 6).

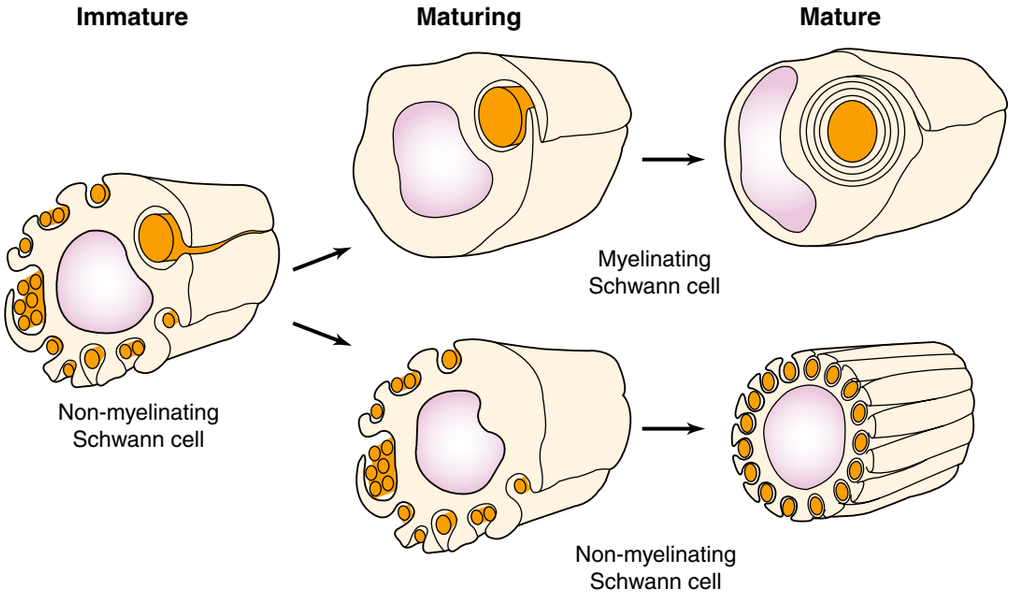

FIGURE 25-16 Schwann cell development and axonal sorting. Immature O4-positive Schwann cells can be found to take one of two paths of differentiation. Initially, both large- and small caliber axons, usually less than 1 μm in diameter, are found embedded in furrows along the surface of a chain of immature Schwann cells. This illustration shows a cross-section through a single cell. During the period of cell proliferation and axonal sorting, large axons that require myelin laminar ensheathment separate, by some undefined process of axonal/Schwann cell interaction, from smaller-caliber axons that require only a simple, nonmyelin ensheathment. In maturing, myelinating Schwann cells, single axons are enveloped by the lip of the trough on the cell surface, one of which elongates to create the myelin wrapping as the number of turns of membrane increase, and the lamellae become more compact as the cytoplasmic content is lost. In maturing, nonmyelinating Schwann cells, no myelin membrane is formed but many small-caliber axons remain completely embedded within separate furrows in each cell. Thus, the adult nerve contains two Schwann cell phenotypes, nomyelinating and myelinating.

of Schwann precursors is complex (Fig. 25-15). It includes markers conserved from NC cells, such as AP-2α and N-cadherin, which are downregulated by the immature Schwann cell stage, and markers from more mature Schwann cell stages, such as Po, the major myelin glycoprotein, Desert Hedgehog, GP 43, PMP22, CD9 and PLP. The appearance of S100, GFAP, O4 and Oct6 define the immature Schwann cell stage. When cells exit this stage, they stop proliferating to give rise to a nonmyelinating Schwann cell type defined by the appearance of galactocerebroside, $\alpha_1\beta_1$ integrin and association with axons. They also retain the immature Schwann cell markers, neural cell adhesion molecule (NCAM), L1 recognition molecule and those mentioned above. Note the curious combination of antigens in the nonmyelinating Schwann cell: O4 and galactocerebroside glycolipid indicative of the CNS OL phenotype, and GFAP and S100 found in CNS astrocytes.

In the PNS, the ensheathing Schwann cells, depending upon the size of the axons with which they are associated, either remain at the nonmyelinating stage (small axons) or begin to produce myelin-associated proteins and myelin-ensheathing large axons (Fig. 25-16). The latter developmental path involves the appearance of proliferative, premyelinating Schwann cells that express myelin genes, such as P_0, *MBP*, *MAG*, P_2, *connexin 32*, *PMP22*, *krox-20* and *periaxin*. These cells then lose GFAP, p75 NTR/NGF receptor, N-CAM, L1, GFAP, Gap 43, RAN-2 and A5E3 while producing myelin membrane as myelinating Schwann cells. Krox is highly expressed in myelinating Schwann cells while Oct6/SCIP is downregulated [32].

cAMP in culture can mimic most of the effects of axons on immature Schwann cells, including expression of early progenitor antigens, suggesting that a cAMP trigger drives most Schwann cell development *in vivo*. For example, cell proliferation by bFGF or PDGF requires the presence of high concentrations of intracellular cAMP. The role of cAMP may be to upregulate mitogen membrane receptors. Thus, in the developing nerve, axon-associated factors elevate cAMP, which is responsible for inducing two key events. The first event is the expression of O4 antigen and the responsiveness to mitogens in immature progenitors and nonmyelinating Schwann cells. The second event is the further differentiation into premyelinating and myelinating Schwann cells, if the suppression of proliferation takes place owing to some secondary permissive signal associated with the ensheathed large-caliber axons.

Of great interest is the expression pattern of suppressed cAMP-inducible POU domain protein (SCIP). SCIP is a member of the POU family of transcription factors, with characteristics of an early response gene, originally defined by the presence of a homology domain between the four transcription factors, Pit-1, Oct-1, Oct-2 and Unc-86. POU proteins are usually considered to function as transcriptional activators of cell-type-specific genes and determine cell fate. In CNS and PNS myelinating cells, SCIP is

expressed at high levels only during a very narrow window of development and acts as a repressor of myelin-specific genes. In Schwann cells, SCIP is expressed in the progenitors but not in the more mature, myelinating Schwann cells (Fig. 25-15). In these proliferating nonmyelinating and premyelinating Schwann cells, SCIP acts on promoters of myelin-associated genes, such as P_0 and *MBP*, as a repressor. Thus, SCIP expression is found in cells with elevated concentrations of cAMP to be correlated with rapid downregulation of the ERG c-Jun transcription factor and the onset of rapid proliferation and repression of myelin genes. In response to denervation, SCIP is rapidly and transiently re-expressed during the period of rapid cell proliferation of premyelinating Schwann cells and its absence is considered to delay the expression of myelin-specific genes.

CONCLUSIONS

Most development focuses on the formation of the complexity of neural tissue during embryonic and postnatal development. These changes provide a basis for later juvenile and adult stages of growth, in which the dynamic interaction between the genes and the environment continues. Although development is now less dramatic, environmental signals continue to produce lasting changes in neural structure and function in the adult nervous system. Genetic epigenetic interaction is now associated with physiological and behavioral learning (Ch. 53). It is likely that the main elements employed during early development that allow a cell to 'learn' to participate in the system are the same elements used during the continual process of learning and corresponding plastic modification of the nervous system across the life-span of the organism. Learning during the life of the organism is correlated with changes at every level of the hierarchy of information flow (Fig. 25-1) leading to the continued expression and refinement of neuronal/glial networks. External and internal events continue to regulate gene expression, and neuronal and glial cells continue to interact metabolically[3]. In its broadest sense, development continues during adulthood [33]. Since neural plasticity can be defined as the long-lasting changes in neural structure and function following environmental perturbation, the term is applicable to adult development. The molecular and cellular basis of adult neural plasticity has focused on environment-induced changes in neurotransmitter metabolism, synaptic structure and function and local circuits. Three experimental models have proved to be most successful in helping to delineate the molecular basis of plasticity: environmental control over catecholamine biosynthesis, long-term potentiation [33] and experience-dependent alterations in brain structure and function [34, 35].

In the future, advances in neuroscience will further our understanding of how the abstract genetic intelligence becomes progressively and continually transformed into the hierarchically organized dynamic networks of information and structure. Throughout the life-span of an organism, environmental perturbation leads to alterations in neural structure and function. Therefore, development can be viewed as a continual process of short- and long-term information storage whereby genetic and epigenetic interaction, at every step of development, is represented in the evolving structural and functional design of the nervous system. The nervous system is always adapting and we are always learning.

REFERENCES

1. Rao, M. R. and Jacobson, M. *Developmental Neurobiology*, 4th edn. New York: Kluwer Academic, 2005.
2. Arenander, A. T. and de Vellis, J. Frontiers of glial physiology. In R. Rosenberg (ed.), *The Clinical Neurosciences*. New York: Churchill Livingstone, 1983, pp. 53–91.
3. Hatton, G. I. and Parpura, V. (eds) *Glia–Neuronal Signaling*. Boston: Kluwer Academic Publishers, 2004.
4. Kettenmann, H. J. and Ransom, B. R. (eds) *Neuroglia*. Oxford: Oxford University Press, 2004.
5. Sun, Y. E., Martinovich, K. and Gee, W. Making and repairing the mammalian brain-signaling toward neurogenesis and gliogenesis. *Semin. Cell Dev. Biol.* 14: 161–168, 2003.
6. Cowan, W. M. The development of the brain. *Sci. Am.* 241: 12–133, 1979.
7. Anderson, D. J. Stem cells and pattern formation in the nervous system: the possible versus the actual. *Neuron* 1: 19–35, 2001.
8. McConnell, S. K. Strategies for the generation of neuronal diversity in the developing central nervous system. *J. Neurosci.* 15: 6987–6998. 1995.
9. Burek, M. J. and Oppenheim, R. W. Programmed cell death in the developing nervous system. *Brain Pathol.* 6: 427–446, 1991.
10. Pucha, G. V. and Johnson, E. M. Men are but worms: neuronal cell death in *C. elegans* and vertebrates. *Cell Death Differ.* 11: 38–48, 2004.
11. LaBonne, C. and Bronner-Fraser, M. Molecular mechanisms of neural crest formation. *Annu. Rev. Cell Dev. Biol.* 15: 81–112, 1999.
12. Redies, C., Treubert-Zimmerman, U. and Luo, J. Cadherins as regulators for the emergence of neural nets from embryonic divisions. *J. Physiol. (Paris)*. 97: 5–15, 2003.
13. Tessier-Lavigne, M. and Goodman, C. S. The molecular biology of axon guidance. *Science* 274: 1123–1133, 1996.
14. Slezak, M. and Pfrieger, F. W. Role of astrocytes in the formation, maturation and maintenance of synapses. In G. I. Hatton and V. Parpura (eds), *Glia–Neuronal Signaling*. Boston: Kluwer Academic Publishers, 2004, pp. 417–435.
15. He, X. and Rosenfeld, M. G. Mechanisms of complex transcriptional regulation: implications for brain development. *Neuron* 7: 183–196, 1991.
16. Arenander, A. T. and Herschman, H. R. Primary response gene expression in the nervous system. In S. Loughlin and J. Fallon (eds), *Neurotrophic Factors*. New York: Academic Press, 1992, pp. 89–128.
17. Veraksa, A., Del Campo, M. and McGinnis, W. Developmental patterning genes and their conserved functions: from model organisms to humans. *Mol. Genet. Metabol.* 69: 85–100, 2000.

18. Lumsden, A. and Krumlauf, R. Patterning the vertebrate neuraxis. *Science* 274: 1109–1115, 1996.
19. Maconochie, M. K., Nonchev, S., Manzanares, M., Marshall, H. and Krumlauf, R. Differences in Krox20-dependent regulation of Hoxa2 and Hoxb2 during hindbrain development. *Dev. Biol.* 223: 468–481, 2001.
20. Rubenstein, J. L. R., Shimamura, K., Martinez, S. and Puelles, L. Regionalization of the prosencephalic neural plate. *Annu. Rev. Neurosci.* 21: 445–477, 1998.
21. Anderson, S. A., Marín, O., Horn, C., Jennings, K. and Rubenstein, J. L. R. Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* 128: 353–363, 2001.
22. Jessell, T. M. Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. *Nat. Rev. Genet.* 1: 20–29, 2000.
23. Ensini, M., Tsuchida, T. M., Belting, H.-G. and Jessell, T. M. The control of rostrocaudal pattern in the developing spinal cord: specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm. *Development* 125: 969–982, 1998.
24. Anderson, D. J. Cellular and molecular biology of neural crest cell lineage determination. *Trends Genet.* 13: 276–280, 1997.
25. Anderson, D. J. Molecular control of cell fate in the neural crest: the sympathoadrenal lineage. *Annu. Rev. Neurosci.* 116: 129–158, 1993.
26. Espinosa de los Monteros, A., Zhang, M. and de Vellis, J. O2A progenitor cells transplanted into the neonatal rat brain develop into oligodendrocytes but not astrocytes *Proc. Natl Acad. Sci. U.S.A.* 90: 50–54, 1993.
27. Levison, S. W., de Vellis, J. and Goldman, J. E. Astrocyte development. In M. S. Rao and M. Jacobson (eds), *Developmental Neurobiology*. New York: Kluwer Academic/Plenum Publishers, 2005, pp. 200–226.
28. Miller, R. and Reynolds, R. J. Oligodendroglial lineage. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. Amsterdam: Elsevier/Academic Press, 2004, pp. 289–310.
29. Hsieh, J., Aimone, J. B., Kaspar, B. K., Kuwabara, T., Nakashima, K. and Gage, F. H. IGF-I instructs multipotent adult progenitor cells to become oligodendrocytes. *J. Cell Biol.* 164: 111–122, 2004.
30. Lee, M., Lelievre, V., Zhao, P. *et al.* PACAP stimulates DNA synthesis but delays maturation of oligodendrocyte progenitors. *J. Neuorosci.* 21: 3849–3859, 2001.
31. Espinosa-Jeffrey, A., Kumar, S., Zhao, P. M. *et al.* Transferrin regulates transcription of the MBP gene and its action synergizes with IGF-I to enhance myelinogenesis in the md rat. *Dev. Neurosci.* 24: 227–241, 2002.
32. Jessen, K. R. and Mirsky, R. Schwann cell development. In Lazzarini, R. A. (ed.), *Myelin Biology and Disorders*. Amsterdam: Elsevier/Academic Press, 2004, pp. 329–370.
33. Kaas, J. H. Plasticity of sensory and motor maps in adult mammals. *Annu. Rev. Neurosci.* 14: 137–167, 1991.
34. Teyler, T. J. and DiScenna, P. Long-term potentiation. *Annu. Rev. Neurosci.* 10: 131–161, 1987.
35. Branchi, I., Francia, N. and Alleva, N. Epigenetic control of neurobehavioral plasticity: role of neurotrophins. *Behav. Pharmacol.* 15: 353–362, 2004
36. Arenander, A. A. and de Vellis, J. Early response gene expression signifying functional coupling of neuroligand receptor systems in astrocytes. In S. Murphy (ed.), *Astrocytes*. New York: Academic Press, 1993, pp. 109–136.

CHAPTER

26

Transcription Factors in the Central Nervous System

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The functioning of the CNS requires regulated communication between neurons that are in contact with one another. Generally, this can be viewed as presynaptic neurons modulating the activity of postsynaptic neurons. While the propagation of action potentials is the most rapid form of neuron–neuron communication, there is a slower form of communication where the neurotransmitters and neuromodulators released into the synaptic cleft from presynaptic axons bind to postsynaptic neuronal membranes, inducing the postsynaptic cell to respond. This postsynaptic response can be a cascade of events, often resulting in regulation of the production of mRNA and proteins within cells. This direct cell–cell communication can be modulated by factors that diffuse over long distances and, consequently, do not require specific secretion into the synaptic cleft, yet these factors can also function in selected cell types to again alter transcription and post-transcriptional events. In this chapter, the regulation of transcription in neurons will be discussed first by presenting some basic background concerning transcription and then by highlighting two important classes of modulators of transcription, namely, those associated with glucocorticoid and cAMP regulation of transcription. Finally, given the complexities of transcriptional regulation, a brief discussion of possible sites for pharmacological intervention in the transcriptional process will be presented.

THE TRANSCRIPTIONAL PROCESS

To understand regulation of transcription, it is necessary to categorize the molecules involved. Transcription is the process by which RNA is made from genomic DNA. DNA is a linear polymer composed of four nucleotides, deoxyadenosine, deoxyguanosine, deoxycytosine and thymidine, which are linked in a linear sequence via phosphate linkages. Genomic DNA is double-stranded, oriented in an antiparallel arrangement such that base pairing of adenosine with thymidine and of guanosine with cytosine will stabilize the structure. The linear sequence of the nucleotides contains sequences known as genes that are the regions of genomic DNA that can be copied into RNA. This arrangement suggests that the functional structure of the DNA sequences giving rise to RNAs is modular, with an RNA-encoding region as well as regulatory regions of sequence both 5′ and 3′ to the transcription

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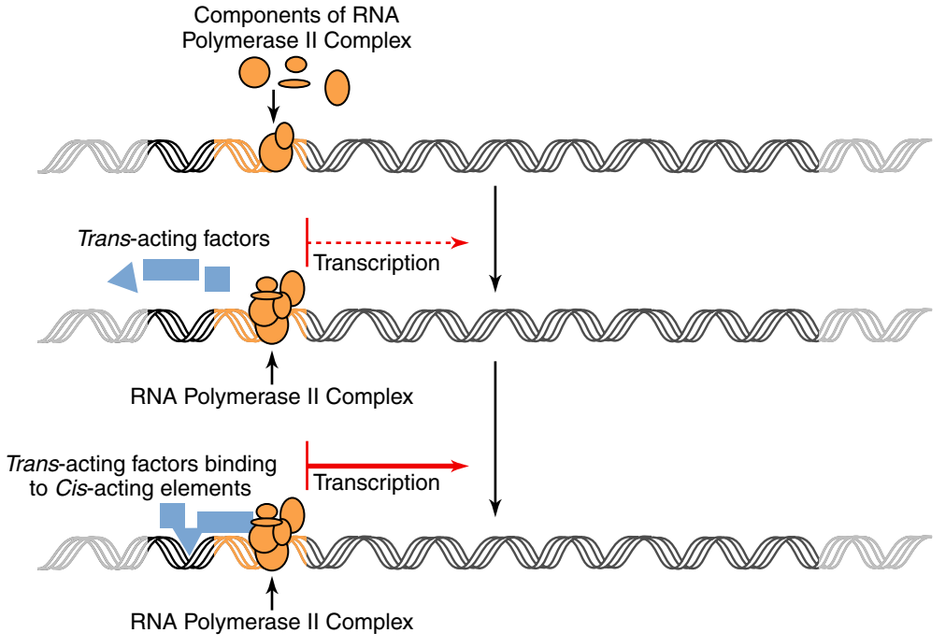

FIGURE 26-1 Formation and regulation of the transcriptional complex. The transcribed region of a gene is indicated in *black*. Immediately to the **right** of this box, on the 5′-side, is an *orange helix*, indicating the TATA box sequence. These are *cis*-regulatory sequences. The transcription complex binds to the TATA box and can initiate transcription when stimulated. Transcription can be regulated by binding of various *trans*-acting factors to the enhancers (*black shapes*) adjacent to the TATA box. The positioning of these sequences permits a direct interaction between *trans*-acting factors and the RNA polymerase II complex.

unit (Fig. 26-1). The transcription process giving rise to mRNA requires the binding of proteins responsible for RNA synthesis to the DNA template immediately 5′ to the start of transcription. Indeed the recent sequencing of multiple genomes has revealed that the transcriptional machinery is largely conserved between different species ranging from yeast to human, reflecting the fundamental nature of the transcriptional process. These studies have revealed good sequence conservation of over 100 proteins known to be involved in the transcriptional process. The protein that synthesizes mRNA is known as RNA polymerase II. RNA polymerase II is a large protein complex composed of multiple subunits. This protein binds to a DNA sequence, which is known as the TATA box because of the linear arrangement of the DNA sequence and its proximity to the start of transcription. However, RNA polymerase II does not bind to the TATA box without the prior association of several other proteins, including transcription factors TFIID, TFIIA and others, with this DNA region [1]. These proteins interact with one another, forming a complex to which RNA polymerase II can bind. This scaffolding of protein interactions at the TATA box forms the transcriptional apparatus. This basic transcriptional complex is probably the same for all genes in all cells of an organism, yet it is clear that transcription of selective genes can be on or off, as well as differentially regulated, in distinct cell types to yield different amounts of mRNA. This process of transcriptional regulation is multifaceted and involves the association of several additional proteins in particular arrangements with the transcriptional complex. The arrangement and identity of transcriptional accessory proteins, also called transcription factors, can be unique for individual genes.

Finally, and perhaps most importantly, transcription is the starting point for a series of biological amplifications necessary for cellular functioning. To illustrate this point, a single gene can be transcribed into multiple mRNAs, in some cases, giving rise to thousands of mRNA molecules (Fig. 26-2). Next, each of these mRNAs may be translated into hundreds of protein molecules, hence providing the quantities required for cellular viability. Cells could not function without this amplification of genetic information. Transcription of selective genes in selective cell types

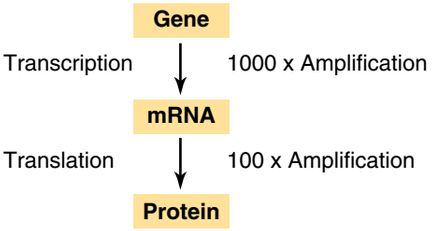

FIGURE 26-2 Gene expression as a biological amplifier. Transcription is an amplification step, giving rise to thousands of mRNA molecules from a single gene. Translation is a second amplification process, producing hundreds of protein molecules from a single mRNA molecule.

and the resultant cell-specific abundances of the transcribed RNAs, provides the opportunity for other biological processes to modulate protein production. The involvement of transcription factors in regulating the first biological amplification step is the subject of the remainder of this chapter

Co-regulators of transcription – histone acetylases modulate chromatin structure. An important feature of a gene's transcriptional response that has been slow to be appreciated is the chromatin structure of the gene. Co-regulators of transcription fall into two classes, co-activators and co-repressors, and are usually proteins that modulate RNA polymerase activity, often at the level of modulating chromatin structure [2]. Eucaryotic DNA exists in the nucleus as a long, double-stranded molecule that wraps around a core protein complex composed of histone proteins. This structure is called a nucleosome. Nucleosomes are packed into a tight bundle that forms the chromatin structure of the gene and genome. Transcription occurs at genes where the promoter is accessible such that RNA polymerase and the modulatory transcription factors can bind. The polymerase moves from the promoter across the gene making RNA as it progresses; this occurs through the activity of the core transcriptional complex displacing the DNA from the nucleosome such that it can be copied into RNA. While the nucleosome doesn't necessarily hinder RNA elongation, the ability of nucleosome to block RNA polymerase binding to the promoter is now well documented. The blocking activity of the nucleosome is mediated in large part through the regulated activity of the histone proteins. Acetylation of the histones tends to facilitate transcription of the gene while deacetylation usually blocks access of the polymerase to the promoter. Often the acetylation state of the promoter-associated histones is modulated by transcription factors that act as histone acetyltransferases. These co-regulators associate with the RNA polymerase core and are often recruited through the binding of transcription factors to the core transcriptional complex.

REGULATION OF TRANSCRIPTION BY TRANSCRIPTION FACTORS

Transcription factors are co-activators or co-repressors that are categorized as *trans*-acting factors because they are regulatory agents that are not part of the regulated gene(s). These *trans*-acting factors regulate gene transcription by binding directly or through an intermediate protein to the gene at a particular DNA sequence, called a *cis*-regulatory region. This *cis*-regulatory region is usually located in the 5′-flanking promoter region of the gene and is composed of a specific nucleotide sequence, for example the cAMP response element (consensus sequence TGACGTCA). There is also a class of *cis*-regulatory elements called enhancers, which can be positioned anywhere in a gene and, consequently, are not restricted to the promoter region, which bind, for example, the glucocorticoid receptor. Binding of the *trans*-acting factor to the *cis*-regulatory region alters the initiation of transcription, probably through a direct interaction of the *trans*-acting factor with the RNA polymerase complex. This binding probably occurs through the secondary and tertiary structures of the genomic DNA–protein complexes. Usually, transcription factors bind as dimers to the DNA, suggesting that there are dimerization sites on transcription factors, as well as DNA-binding sites. There are several types of transcription factors, which are grouped by virtue of sequence similarities in their protein interaction and/or DNA-binding domains (Fig. 26-3). Similarities in protein–protein interaction sites suggest that monomers of different transcription factors can interact to form heterodimers. An individual heterodimer may bind to multiple *cis*-acting elements or, alternatively, interact with differing affinity for the same *cis*-acting element as compared to the homodimer. Indeed, these types of protein interaction do occur and provide a major source of regulatory complexity.

A particular *cis*-acting element may be present in multiple genes so that activation of a single transcription factor has potential for altering the expression of multiple target genes. Furthermore, an individual transcription factor may increase transcription of one gene while decreasing the transcription of another gene. This difference is partly due to the positioning of the *cis*-acting element relative to the start of transcription as well as to the identity of the protein partners in the heterodimer complex. It is important to note that, with the increase in sequence information being generated by the Human Genome Project, *cis*-acting elements can be found in genes, suggesting potential gene regulatory mechanisms without any biological information. While *cis*-acting element sequence identification in a gene is indeed predictive and necessary, this is often not sufficient to fully characterize the transcriptional regulation of that gene. *cis*-acting elements for a particular gene are commonly nonfunctional in all tissues and, even more often, silent in one tissue and active in another because of the differential distribution of transcription factors.

Technology that has hastened the study of transcription. Promoter characterization by filter binding assays and UV-crosslinking and various genetic assays such as knockout and regulated overexpression cellular and animal models have been invaluable in assessing translational regulatory mechanisms. However, the cellular complexity of the CNS makes use of these standard biochemical analysis problematic. Recently two methodologies have been developed that facilitate a more specific analysis of gene transcriptional mechanisms in the CNS and other tissues.

The chromatin immuno-precipitation assay (ChIP) is a procedure that is employed to determine which genes are

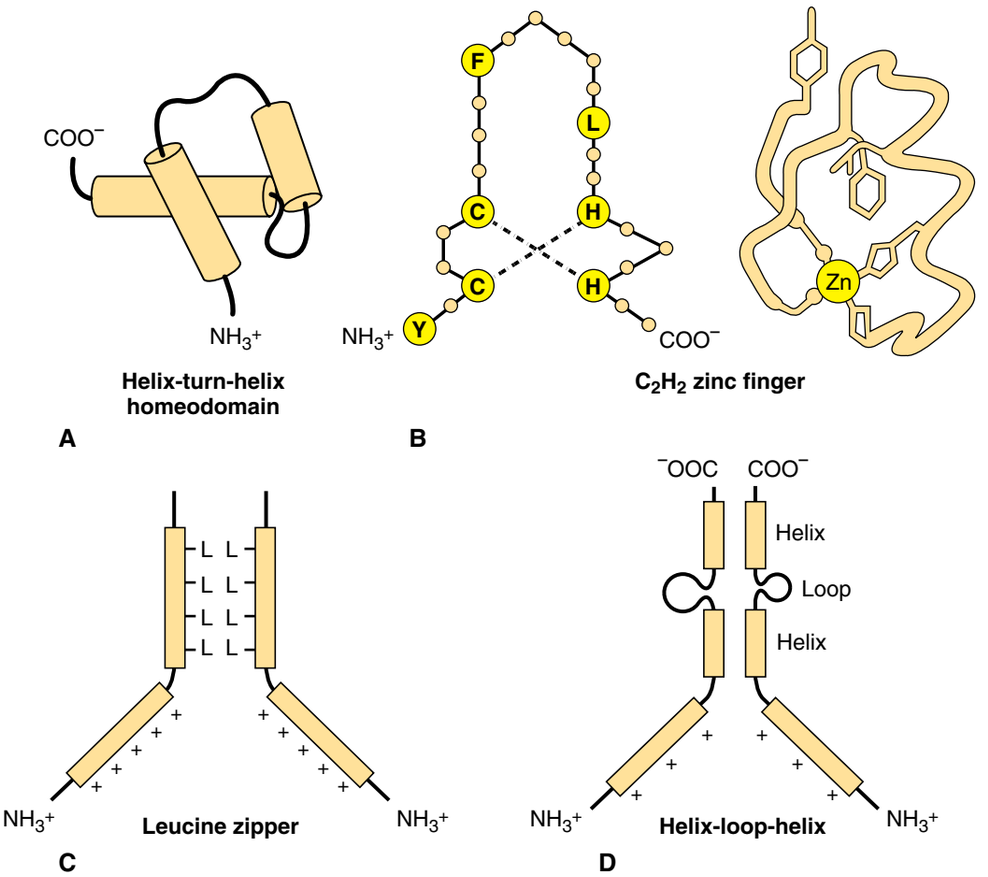

FIGURE 26-3 Structure of transcription factors from different families.

being transcriptionally regulated by a particular transcription factor, co-activator or co-repressor or any protein that affects gene activity. Biochemical analysis of transcription requires the isolation of cellular lysates and, when the cell is lysed, charged proteins, such as many transcription factors, often bind indiscriminately to DNA. The ChIP assay has the advantage of providing *in vivo* targets for analysis (Fig. 26-4). The assay works by first cross-linking DNA-binding proteins to chromatin DNA *in vivo*, using formaldehyde. This cross-linking freezes the protein of interest to the DNA to which it is bound. The chromatin is then isolated and sheared to small fragments. Antibodies to the protein of interest (e.g. transcription factor) are used to immunoprecipitate the specific protein and any DNA that is cross-linked to the protein. The immunoprecipitated protein-bound DNA is then amplified by polymerase chain reaction (PCR) and sequenced to identify the genes to which the protein was bound. The disadvantage is that the specificity of the reaction can be limited by the number of proteins cross-linked to a particular protein–DNA complex. Nonspecific results can easily be generated so great attention must be paid to secondary screens to confirm the ChIP-generated data.

Traditionally the analysis of genes that are regulated by particular transcription factors has proceeded one candidate gene at a time. With the advent of microarrays it is now possible to assess the abundance of thousands of mRNAs simultaneously. Microarrays have been used to determine the target genes for various transcription factors through the specific activation or repression of the biological activity of the transcription factor followed by RNA isolation, probe generation and microarray screening (Fig. 26-4). Since transcription can be a very rapid event, effort is made to isolate the RNA quickly after experimental manipulation of the experimental system. While such data can be quite useful, various cautionary notes must be kept in mind when assessing these data. The static RNA abundances assessed using this procedure reflects a balance between the transcriptional activity of the gene and the degradation of the RNA product. Little is known about the regulation of RNA degradation but, presuming that it can be a regulated and rapid event, then microarray data will inform the investigator of genes that are transcription- and degradation-regulated. Large-scale microarray analysis has been referred to as genomics and the resultant data as the transcriptome, i.e. the transcribed

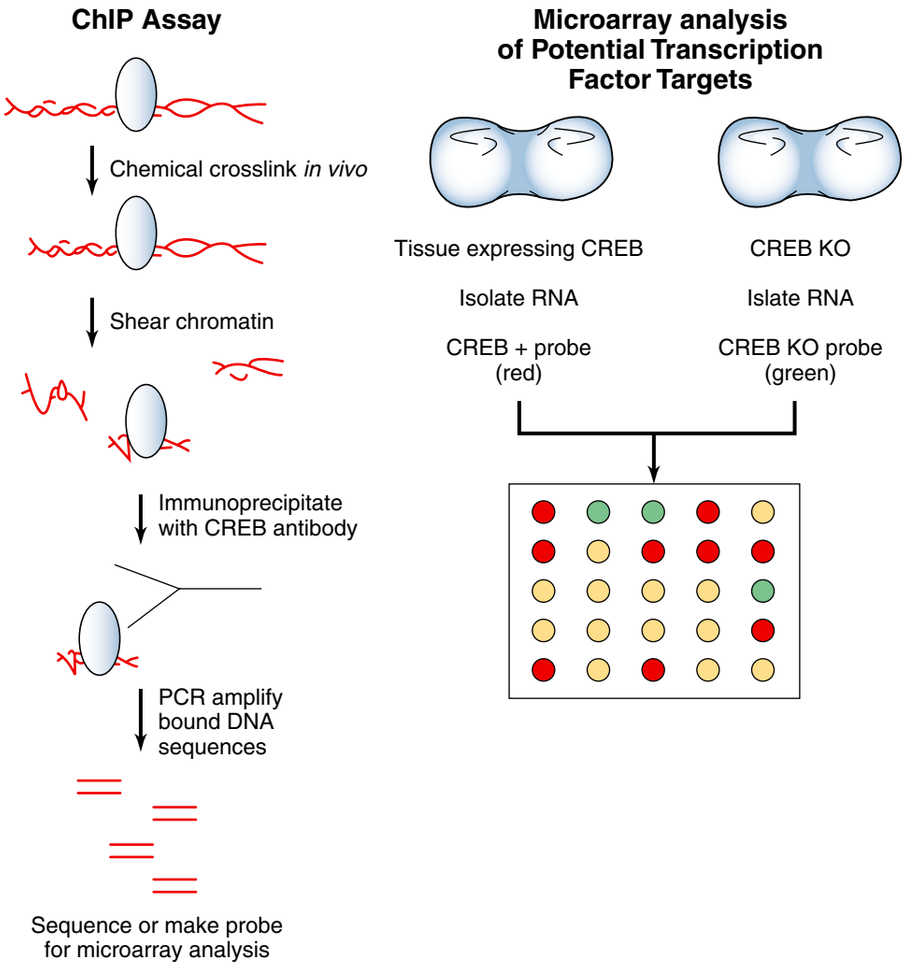

FIGURE 26-4 ChIP and microarray analysis of transcription. The chromatin immunoprecipitation assay is depicted in (**A**). Each of the steps leading to characterization of the DNA sequence associated with selected transcription factors is illustrated for the cAMP response element-binding protein (*CREB*) transcription factor. (**B**) A schematic of one method for selecting putative CREB-responsive genes. Brain tissues from a normal mouse and a CREB knockout (*CREB KO*) mouse are dissected, RNA is isolated and probes are made from each tissue with the $CREB^+$ probe giving rise to red fluorescence while the KO probe gives rise to green fluorescence. When simultaneously hybridized to an array containing thousands of immobilized clones, any RNA that is more abundant in CREB-containing tissue would be *red*, any RNA that is less abundant in CREB-containing tissue (knockout mouse) would be *green* and those RNAs that are present in nearly equal abundances appear as the *yellow* overlapped signal. The *red* fluorescing clones correspond to candidate genes whose transcription is stimulated by CREB while those that are *green* represent candidate genes whose transcription is inhibited by CREB. PCR, polymerase chain reaction.

genes in a particular cell or tissue. The regulated transcriptome for any particular type of stimulation would produce a subset of regulated genes that are good candidates for being associated with the function of particular transcription factors.

GLUCOCORTICOID AND MINERALOCORTICOID RECEPTORS AS TRANSCRIPTION FACTORS

The steroid hormones known as mineralocorticoids and glucocorticoids are synthesized in the adrenal cortex of mammals [3]. The physiological mineralocorticoid is aldosterone, and it is involved in regulating unidirectional Na^+ transport across the epithelium. The physiological glucocorticoid in most mammalian species is cortisol; however, in rats and mice, it is corticosterone (CORT). Initially, glucocorticoids were characterized by their ability to stimulate glycogen deposition and by their release into the circulation in response to stress. Glucocorticoids regulate a wide range of responses, including aspects of immunosuppression and inflammation (see Ch. 49). Glucocorticoids are released in response to increases in circulating adrenocorticotrophic hormones.

Two distinct classes of mineralocorticoid-binding sites were first described in the rat kidney. High-affinity cytosolic aldosterone-binding sites are termed mineralocorticoid receptors [4]. Lower-affinity aldosterone-binding sites

are termed glucocorticoid receptors. Glucocorticoid receptors are the same as dexamethasone-binding receptors [5].

While the affinity of mineralocorticoid receptors for aldosterone is higher than that for CORT, circulating concentrations of CORT are several orders of magnitude higher than aldosterone. CORT is effectively blocked from binding to the mineralocorticoid receptors by plasma proteins such as transcortin, which bind preferentially to CORT. Another mechanism that serves to alter the balance of CORT and aldosterone binding to receptors is the activity of 11β-hydroxysteroid dehydrogenase, which, in the rat kidney, rapidly oxidizes CORT to inactive metabolites. This facilitates binding of aldosterone to mineralocorticoid receptors in the presence of high concentrations of CORT [6].

Corticosteroid receptors regulate transcription in the nervous system. Intracellular binding sites selective for [^{3}H]-CORT were first identified in various brain regions, in particular the hippocampus, of adrenalectomized rats. A similar autoradiographic pattern was obtained using [^{3}H]-aldosterone as a ligand, suggesting that these receptors were mineralocorticoid receptors. The [^{3}H]-dexamethasone-binding pattern shows selective differences in the pattern of binding compared to the CORT and aldosterone patterns, suggesting the existence of multiple corticosteroid receptors. [^{3}H]-CORT binding performed in the presence of unlabeled dexamethasone or aldosterone reveals that CORT binds to at least two receptor types in the brain.

Two high-affinity intracellular corticosteroid receptors have been characterized and are distinguished on the basis of binding properties, amino acid sequence, neuroanatomical distribution and physiological function. The type I corticosteroid receptor, or mineralocorticoid receptor, is localized in various brain regions, including the septum and hippocampus [7]. The type I receptor binds CORT and aldosterone with high affinity, ≈0.5 nmol/l. The relative steroid binding affinity of the type I receptor for CORT is greater than or equal to that for aldosterone, which is greater than that for dexamethasone. The type I receptor is present in all subregions of the hippocampus, i.e. in the CA1, CA2, CA3 and the granular cells of the dentate gyrus (Fig. 26-5).

The type II corticosteroid receptor, also called the glucocorticoid receptor, is widely distributed in the brain and exhibits high-affinity binding to dexamethasone. The type II receptor binds with tenfold lower affinity to corticosterone, 5 nmol/l, as compared with the type I receptor. The relative binding affinities for steroid interaction with the glucocorticoid receptor are such that dexamethasone binding is greater than corticosterone binding, which is much greater than aldosterone binding. Glucocorticoids are present at high concentrations in all regions of the hippocampus, with the exception of the CA3 region, where concentrations are exceedingly low [8].

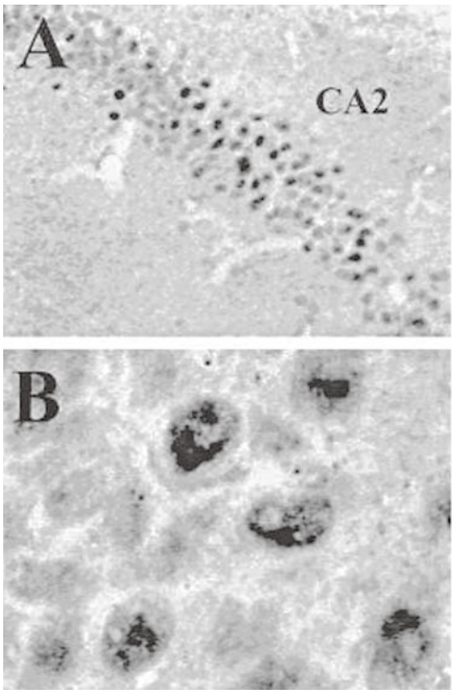

FIGURE 26-5 Immunohistochemical localization of type I corticosteroid receptor (mineralocorticoid receptor) in the rat hippocampus. (**A**) Mineralocorticoid immunoreactivity is concentrated in pyramidal cell fields of the cornu ammonis (*CA2*). (**B**) High-power photomicrograph shows that steroid-bound mineralocorticoid receptors are primarily localized to neuronal cell nuclei. (Courtesy of Dr James P. Herman, Department of Anatomy and Neurobiology, University of Kentucky.)

Mineralocorticoid and glucocorticoid receptors can coexist in the same neurons, as evident from their demonstrated colocalization in the CA1 region of the hippocampus [9]. These CA1 neurons are exquisitely sensitive to the whole range of glucocorticoid concentrations. Because of their high affinity for corticosteroids, the type I receptors are approximately 80% occupied, suggesting that the number of type I receptors is likely to be the rate-limiting factor in mineralocorticoid receptor functioning. However, glucocorticoid receptor functions are most dependent on the extent of glucocorticoid receptor occupancy and are likely to be regulated by the concentration of the available steroid. The presence of multiple binding sites for corticosteroids and their differing concentrations in different cell types forms the molecular basis for their differential actions in the rat brain [10].

The mechanisms of corticosteroid receptor regulation of transcription have been elucidated. Both type I and type II corticosteroid receptors are members of a superfamily of ligand-activated transcription factors defined by protein sequence similarity. Included in this superfamily are various other steroid receptors, such as the estrogen receptor, as well as members of the retinoic acid receptor

family and thyroid hormone receptors. The ligand-activated transcription factor superfamily is estimated to contain several hundred members. Mineralocorticoid and glucocorticoid receptors are each composed of four distinct protein regions, including an N-terminal region, a DNA-binding domain, a nuclear localization signal and a C-terminal hormone-binding region. The N-terminal region is associated with activation of transcription through an as yet unknown mechanism. The DNA-binding domain is a charged protein sequence, known as a zinc-finger. In this case, there are two fingers, each composed of the sequence Cys-X2-Cys-X13-Cys-X2-Cys. The C-terminal region binds glucocorticoids, which, when bound, initiate the conformational change of the receptor which facilitates its translocation.

In the cytoplasm, the mineralocorticoid and glucocorticoid receptors are associated with a large multiprotein complex that contains the heat shock proteins (HSP) HSP70 and HSP90 (Fig. 26-6). This complex maintains unbound corticosteroid receptors in a ligand-accessible but inactive protein conformation. Binding of ligand causes dissociation of receptors from the heat shock proteins, followed by translocation of the activated receptors to the nucleus. In the nucleus, ligand-bound corticosteroid receptors bind to a *cis*-acting element called glucocorticoid response element (GRE). The GRE is a 15-base sequence, TGGTACAAATGTTCT, and is also called an enhancer element because it generally functions to enhance transcription. The GRE can be positioned anywhere within the gene, not just in the promoter region. Modulation of corticosteroid-responsive genes occurs through interactions of the type I or II receptors, bound to the enhancer sequence, with the transcriptional complex.

Ligand-bound corticosteroid receptors have been shown to interact to form heterodimers with other transcription factors, such as the *jun* protein. Such interactions are responsible for transactivation of the *cis*-regulatory sites known as AP-1 sites and for the glucocorticoid-mediated suppression of transcription, such as that seen in the pro-opiomelanocortin gene. A number of such specific protein interactions have been reported; these interactions and their locations relative to other transcription factors transform a ubiquitous steroid hormone signal into a tissue-specific, graded cellular response.

More recently, the protein–protein coimmunoprecipitation assay coupled with the ChIP assay has highlighted particular genes whose promoters bind to steroid receptors and whose transcription can be modulated by these receptor-associated proteins [11, 12]. Among these co-activators are various acetyltransferases such as CBP and p300 and the ATP-coupled chromatin remodeling SWI/SNF complex, as well as the protein methylase CARM-1 [13]. Each of these proteins has the capacity to modulate chromatin structure through post-translational modification of the chromatin. Co-repressors of steroid receptor-mediated gene transcription have also been

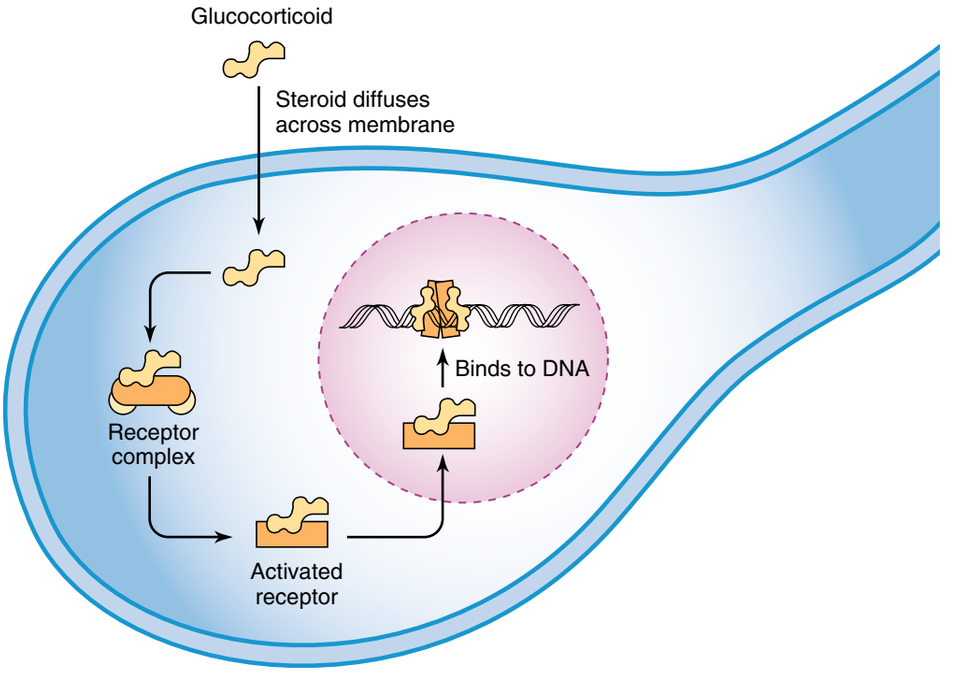

FIGURE 26-6 Activation of glucocorticoid receptors. Glucocorticoids diffuse across the plasma membrane and bind to the glucocorticoid receptor. Upon glucocorticoid receptor binding to the steroid, the receptor undergoes a conformational change which permits it to dissociate from the chaperone heat shock proteins (*light orange circles*). The activated glucocorticoid receptor translocates across the nuclear membrane and binds as a homo- or heterodimer to glucocorticoid response element sequences to regulate gene transcription.

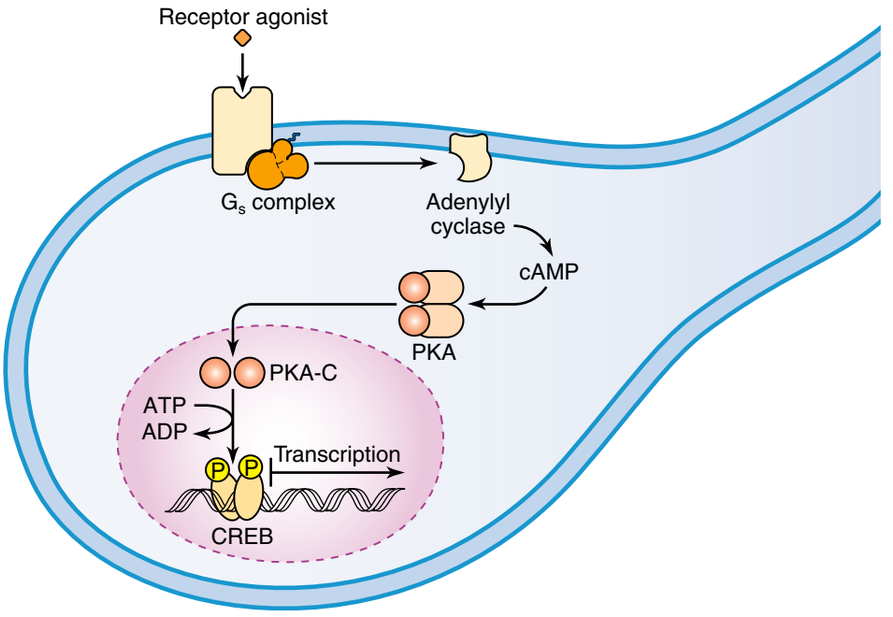

FIGURE 26-7 Activation of cAMP-regulated genes. When agonists bind to G-protein-coupled receptors that activate dissociation of G_s from the G_s complex, G_s will interact with the enzyme adenylyl cyclase, resulting in synthesis and increase of intracellular cAMP. cAMP binds to the regulatory subunits (*orange ovals*) of protein kinase A (*PKA*). This binding facilitates the dissociation of the catalytic subunits (*gray circles*) from the PKA complex, which then translocate across the nuclear membrane and phosphorylate cAMP response element-binding protein (*CREB*) and CREB-related proteins. Phosphorylated CREB increases gene transcription (*right arrow*).

characterized using similar methodologies [12]. At least some steroid receptor co-repressors can function through chromatin deacetylation through the mediation of Sin3 and histone deacetylase. The characterization of co-regulators of gene expression promises to highlight mechanisms for the coordinate regulation of multiple genes by a specific modulator as well as mechanisms for cell specific responses to regulatory signals.

A compendium of genes that are good candidates for being regulated by glucocorticoids has been generated using microarray technology. For example, the glucocorticoid-regulated abundances of RNAs encoded by various genes have been characterized in lymphoid cells during apoptosis. In support of the selectivity of the glucocorticoid responsiveness of these genes, data were generated showing that many of these genes are nonresponsive to glucocorticoids when glucocorticoid receptors are blocked by a glucocorticoid receptor antagonist. Data such as these highlighting potential multiple targets of steroid hormone receptor regulation may provide therapeutic targets to modulate unwanted 'side effects' of glucocorticoid receptor therapeutics.

CYCLIC AMP REGULATION OF TRANSCRIPTION

Cyclic AMP controls phosphorylation of the cAMP response element binding protein. Activation of a transcription factor known as the cAMP response element binding protein (CREB) is the final step in a signal-transduction pathway that is initiated by the binding of a specific class of cell-surface receptors [14] (Fig. 26-7). Binding of a ligand to a G_s protein-coupled receptor, such as the D_1 dopamine receptor or the 5-hydroxytryptamine-1 receptor, liberates G_s protein from the G-protein complex (see Ch. 20). Subsequently, G_s activates adenylyl cyclase, which in turn stimulates cAMP production. The increase in intracellular cAMP induces the dissociation of protein kinase A (PKA) catalytic subunits from their regulatory subunits (see Ch. 22). The catalytic subunits move into the nucleus, where they phosphorylate a number of proteins, including the CREB transcription factor. This phosphorylation event activates CREB protein and transcription is stimulated [15]. The genes which are activated by CREB are those that have the *cis*-acting palindromic CRE consensus sequence (TGACGTCA) in the promoter region. This is distinct from the enhancer activity of the previously discussed GREs, which can function at sites distinct from the promoter region of a given gene. CREB protein appears to be ubiquitously expressed in the brain, with high concentrations in the hippocampus (Fig. 26-8) and cortex. As seen in Figure 26-8, it is apparent that much of the CREB immunoreactivity is present in the nucleus of CA1 pyramidal cells.

The critical feature of CREB protein activation is phosphorylation, which is required for CREB-mediated stimulation of transcription [16]. PKA phosphorylates CREB on a serine positioned at amino acid 133 in the CREB protein sequence. How phosphorylation activates

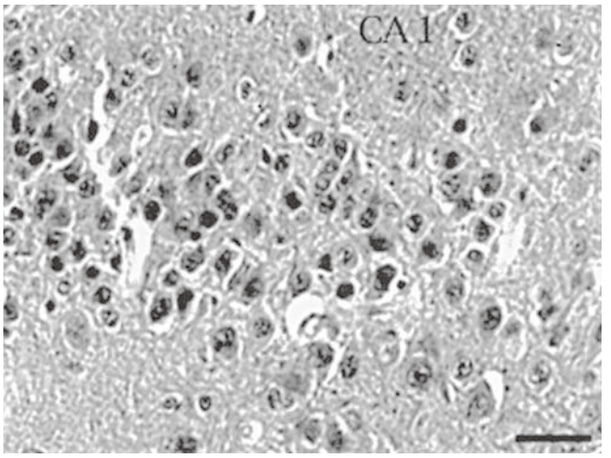

FIGURE 26-8 Immunohistochemical localization of cAMP response element-binding protein (*CREB*) in rat hippocampal neurons. Using a polyclonal antibody which recognizes both CREB and phospho-CREB protein, it is apparent that CREB protein is enriched in the nucleus of pyramidal cells of the CA1 region of the hippocampus. Scale bar is 30 μm. (Courtesy of Dr Stephen Ginsberg, Department of Pathology, University of Pennsylvania.)

CREB-mediated transcription is unclear. Some investigators believe that phospho-CREB has a slightly higher affinity for CRE binding and others believe that transcription factor interaction with the transcriptional complex is stimulated by phosphorylation. The time course for CREB stimulation of transcription is relatively rapid, peaking at approximately 30 minutes after cAMP stimulation, followed by a gradual decrease to basal levels of activation over the course of several hours. This decrease in activation occurs through the activity of protein phosphatase 1 and other phosphatases, which remove the phosphate group from CREB protein. It is important to note that there is increasing evidence that CREB phosphorylation can be induced by factors other than an increase in intracellular cAMP concentrations. The relative contribution of these other stimulators, including an increase in intracellular Ca^{2+}, to the transcriptional activation exhibited by phospho-CREB is under active investigation.

Numerous studies have shown that once CREB is phosphorylated it recruits the histone acetyltransferase CBP to activate gene transcription and that this activation can be ameliorated through the activity of histone deacetylase [17]. While this consensus model does appear to account for the bulk of CREB-mediated transcription, more recent data using a constitutively active CREB mutant and microarray analysis of neuronal gene expression have highlighted the plethora of CREB-regulated genes [18, 19]. Examination of a subset of these genes upon CREB activation in the presence of a histone deacetylase inhibitor has show that not only are the RNA abundances for some of the CREB-responsive genes enhanced, a subset of the genes is inhibited by this treatment. ChIP assays have shown that the differential roles of histone deacetylases in CREB-mediated transcription are evident at the level of preinitiation complex recruitment. These data suggest a complexity of CREB-responsive gene expression that probably involves several co-regulators beyond the simple acetyltransferase mediators.

In large part, the modulation of CREB-regulated genes represents the intracellular balance of active kinases and phosphatases, which is a reflection of cellular activation. This is one of the major differences between CREB and glucocorticoid-receptor-regulated transcription: CREB is an integrator of intracellular homeostasis, while the glucocorticoid receptor integrates whole-body homeostasis.

The cAMP response element binding protein is a member of a family containing interacting proteins. After the mRNA encoding CREB protein was cloned and sequenced, several other members of the CREB family of transcription factors were identified. Using homology screens, the PCR and interaction screening assays, a protein called cAMP response element modulator (CREM) was isolated. CREM can both stimulate and inhibit cAMP-mediated gene transcription. Transcriptional inhibition can occur as a result of generation of modified CREM proteins because of alternative splicing of the CREM mRNA (Fig. 26-9). Among these different proteins are the α, β and γ forms of CREM, which lack the glutamine-rich region present in the activator forms of both CREM and CREB that is required for stimulation of transcription [20]. Inhibition occurs due to heterodimer formation between this inactivating form of CREM and either CREM or CREB [21]. The alternatively spliced forms of CREM, as well as those recently discovered for CREB, which can also act as transcriptional inhibitors, occur in a tissue- and cell-type-specific manner. Additionally, recent data have shown that CREB can interact with other leucine-zipper-containing transcription factors to produce heterodimers that can interact with *cis*-acting elements distinct from the CRE, as previously discussed with regard to the corticosteroid receptors [22]. These findings provide a mechanism for generation of tissue- and cell-type differences in cAMP responsiveness [23].

The function of the cAMP response element binding protein has been modeled in transgenic organisms. The observation that serotonin stimulates learning and memory in the *Aplysia* system with the subsequent discovery that cAMP will mimic this effect suggested that cAMP was involved in this physiological process (see Ch. 50). Recently, direct evidence for the involvement of CREB in learning and memory was presented in the *Drosophila* system (see below). *Drosophila* as an experimental system has several advantages over mammalian systems, including the ease of generation of mutant as well as transgenic flies.

The role of CREB in learning and memory was first anticipated by screening *Drosophila* mutants in a learning and memory task in which flies were behaviorally tested

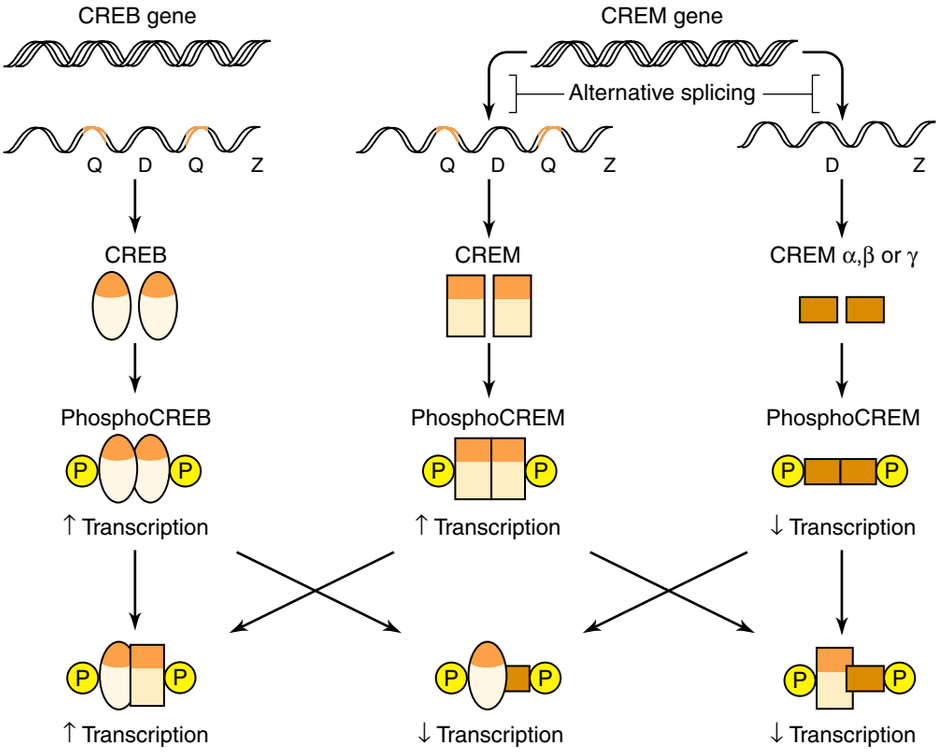

FIGURE 26-9 Mechanism for generation of activators and repressors of cAMP-stimulated transcription. The cAMP response element binding protein (*CREB*) and the cAMP response element modulator (*CREM*) genes can give rise to alternatively spliced mRNAs, which give rise to distinct protein products. While CREB and CREM can stimulate CRE-mediated gene expression, generation of the α, β or γ forms of CREM will produce proteins that can heterodimerize with activated CREB and CREM subunits, inhibiting their stimulatory capacity. The symbols used to highlight functional regions of CREB and CREM protein are *Q* for the polyglutamine tract, which stimulates transcription; *D* for the DNA-binding region; and *Z* for the leucine zipper region, which is involved in dimerization.

in an odorant-association task, where an odorant was paired with an electric shock. Flies with 'normal' phenotype learn that the odorant is associated with an electric shock and will learn to avoid the odorant. Two fly mutants were discovered that could not learn this association: *dunce* and *rutabaga*. The *dunce* mutant was deficient in cAMP phosphodiesterase, which breaks down cAMP, and the *rutabaga* mutant had a mutation in adenylyl cyclase, which synthesizes cAMP. These data combined with the prior *Aplysia* work suggested that CREB was involved in acquisition of learning and memory [24].

More recently, with the same odorant-association behavioral test, the specific role of CREB in learning and memory was assessed by using a dominant-negative CREB (dCREB) protein expressed in transgenic flies [25]. This dCREB complexes with normal CREB through a functional leucine zipper region, yet inhibits the transcriptional activation mediated by normal CREB protein. The experiment was designed so that dCREB was controlled by a heat shock promoter and could be activated in the adult animal. This experimental paradigm allows for the assessment of the role of CREB in the adult animal by using heat shock to induce dCREB expression rather than by examining the behavioral consequences of CREB deficiency during development. Heat shock does not, in and of itself, alter learning and memory [26]. Data from these experiments show that the form of memory requiring protein synthesis, called long-term memory (see Ch. 50), was inhibited by expression of dCREB. This inhibition was not observed when a mutant dCREB, in which the leucine zipper was inactivated by site-directed mutagenesis, was expressed in flies. This leucine zipper mutant precludes dCREB dimerization with normal CREB. These data show that the relative concentrations of activating and inhibiting CREB monomers are critical to the development of long-term memory.

The dissection of CREB involvement in various physiological or behavioral paradigms in the mammalian system has been much slower than in *Drosophila*. In the mammalian system, targeted gene disruption has been used in attempts to inhibit CREB function. In one set of experiments, the exon of the gene containing the initiator methionine was deleted in hopes of blocking CREB production. However, the targeting of this region facilitated an alternative splicing event so that a previously undiscovered spliced variant mRNA of the CREB gene was produced and was, in fact, upregulated. In the other targeted disruption experiments, the DNA-binding region

was disrupted and, indeed, CREB protein production was inhibited. Unfortunately, this disruption resulted in perinatal lethality, so no adult animals could be generated. The problem with the design of these experiments is that targeted disruption of CREB production would result in a developmental deficit, making it difficult to attribute any particular modification of behavior in the adult to a direct deficiency of CREB.

What is needed to directly assess the involvement of CREB in adult mammalian neurons is the production of a conditional knockout mouse in which the knockout could be activated in the adult animal in a tissue-specific manner. This mutant could be similar to the production of a dominant-negative mutant, as was done in the *Drosophila* system, or potentially through induction of genetic recombination in adult neurons using an inducible system similar to that being used to produce conditional knockouts for other genes (see Ch. 40). Such a model system would be very useful in understanding the potential involvement of CREB in long-term memory in the mammalian system as well as in other physiological conditions associated with CREB functioning, such as opiate tolerance and withdrawal (see Ch. 53) or aspects of behavioral aggressiveness (see Ch. 49).

TRANSCRIPTION AS A TARGET FOR DRUG DEVELOPMENT

It should be clear from this brief discussion of transcription factors that disruption of transcription factor function may have dire consequences for neuronal functioning. Because of their pivotal role in the regulation of multiple genes, they are potential targets for pharmacological intervention in the control of disease processes [27]. Indeed, the multigenic regulatory role of transcription factors suggests one mechanism by which multigenic diseases can exhibit multiple physiological characteristics. The combinatorial nature of transcription factor activity implies that, should the activity of a transcription factor be modulated then multiple downstream genes will likewise be regulated. This suggests that the problem with development of drugs targeted to transcription factors is that there are several hundred estimated *trans*-acting factors in the nucleus, many of which can heterodimerize to produce distinct transcriptional responses. The strategies being investigated to modulate transcription factor function focus on antisense knockout of expression and modulators targeted toward post-translational modifiers of transcription factors. Such modifiers include protein kinases and phosphatases.

Often, it is desirable to block expression of particular genes that are activated by transcription factors. The antisense knockout experiments are directed toward this end and require the addition of an antisense oligonucleotide, which will anneal to the *cis*-acting regulatory element for a particular transcription factor in a specific gene. The hope is that a triple helical structure will form around this oligonucleotide-binding site, inhibiting the expression of the downstream gene.

This same strategy can be attempted at the mRNA level to inhibit the translation of individual mRNAs. These experiments are almost never 100% efficient; hence, the problem becomes how much neutralization is necessary to elicit the desired effect. If the antisense oligonucleotide is targeted toward the mRNA for a particular transcription factor or splice form of a transcription factor, then it may be possible to knock down or reduce the expression of that particular protein. The result of such a knockdown will be a new balance of transcription factor subunits in the cell, probably resulting in alteration of the relative amounts of particular homo- and heterodimers.

Finally, development of drugs targeted toward modification of kinases and phosphatases required for activation or inhibition of particular transcription factors is a promising therapeutic approach. The problem with this paradigm is the specificity of the kinases and phosphatases, since they will often act enzymatically on multiple proteins (see Chs 24 and 25). Furthermore, such drugs often lack specificity and may interact with multiple kinases or phosphatases.

Clearly, transcription is a critical regulatory nexus in neuronal function. More information is being generated about the biology of transcription factors, including how many transcription factor genes exist, which proteins dimerize, identification of the *cis*-acting elements to which they bind and how transcription is modulated by these proteins. From these studies, significant insight into the mechanisms of transcriptional responses to the local environment and to pharmacological agents in the normal and abnormal nervous system will be gained.

REFERENCES

1. Hori, R. and Carey, M. The role of activators in assembly of RNA polymerase II transcription complexes. *Curr. Opin. Gen. Dev.* 4: 236–244, 1994.
2. Natoli, G. Little things that count in transcriptional regulation. *Cell* 118 :406–408, 2004.
3. Funder, J. Adrenal steroids: new answers, new questions *Science* 237: 236–237, 1987.
4. Arriza, J. L., Weinberger, C., Cerelli, G. *et al.* Cloning of the human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor. *Science* 237: 268–275, 1987.
5. Hollenberg, S., Weinberger, C., Ong, E. *et al.* Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* 318: 635–641, 1985.
6. Reul, J. and deKloet, E. Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. *Endocrinology* 117: 2505–2511, 1985.
7. Arriza, J., Simerly, R., Swanson, L. and Evans, R. The neuronal mineralocorticoid receptor as mediator of glucocorticoid response. *Neuron* 1: 887–896, 1988.

8. Chao, H., Choo, P. and McEwen, B. Glucocorticoid and mineralocorticoid receptor mRNA expression in rat brain. *Neuroendocrinology* 50: 365–371, 1989.
9. DeKloet, E., Wallach, G. and McEwen, B. Differences in corticosterone and dexamethasone binding in rat brain and pituitary. *Endocrinology* 96: 598–609, 1975.
10. Herman, J., Patel, P., Akil, H. and Watson, S. Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. *Mol. Endocrinol.* 3: 1886–1894, 1989.
11. Nagaich, A. K., Walker, D. A., Wolford, R. and Hager, G. L. Rapid periodic binding and displacement of the glucocorticoid receptor during chromatin remodelling. *Mol. Cell* 14: 163–174, 2004.
12. McKenna, N. and O'Malley, B. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 108: 465–474, 2002.
13. Glass, C. and Rosenfeld, M. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* 14: 121–141, 2000.
14. Habener, J. Cyclic AMP response element binding proteins: A cornucopia of transcription factors. *Mol. Endocrinol.* 4: 1087–1094, 1990.
15. Montminy, M., Gonzalez, G. and Yamamoto, K. Regulation of cAMP-inducible genes by CREB. *Trends Neurosci.* 13: 184–188, 1990.
16. Yamamoto, K., Gonzalez, G., Biggs, W. and Montminy, M. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* 334: 494–498, 1988.
17. Banniste, A. J. and Kouzarides, T. The CBP co-activator is a histone acetyltransferase. *Nature* 384: 641–643, 1996.
18. Fass, D., Butler, J. and Goodman, R. Deacetylase activity is required for camp activation of a subset of CREB target genes. *J. Biol. Chem.* 278: 43014–43019, 2003.
19. Cha-Molstad, H., Keller, D., Yochum, G. *et al.* Cell-type-specific binding of the transcription factor CREB to the cAMO-response element. in *Proc. Natl Acad. Sci. U.S.A.* 101: 13572–13577, 2004.
20. DeGroot, R. and Sassone-Corsi, P. Hormonal control of gene expression: Multiplicity and versatility of cyclic adenosine 3′,5′-monophosphate-responsive nuclear regulators. *Mol. Endocrinol.* 8: 145–153, 1993.
21. Foulkes, N. and Sassone-Corsi, P. More is better: activators and repressors from the same gene. *Cell* 68: 411–414, 1992.
22. De Cesare, D., Fimia, G. and Sassone-Corsi, P. Signaling routes to CREM and CREB: plasticity in transcriptional activation. *Trends Biochem Sci.* 7: 281–285, 1999.
23. Hai, T. and Curran, T. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl Acad. Sci. U.S.A.* 88: 3720–3724, 1991.
24. Tully, T., Preat, T. Boynton, S. and Del Vecchio, M. Genetic dissection of consolidated memory. *Drosophila Cell* 79: 59–67, 1994.
25. Yin, J., Del Vecchio, M., Zhou, H. and Tully, T. CREB as a memory modulator: Induced expression of a dCREB2 activator isoform enhances long-term memor. *Drosophila Cell* 81: 107–115, 1995.
26. Yin, J., Wallach, J., Del Vecchio, M. *et al.* Induction of a dominant negative CREB transgene specifically blocks long-term memory. *Drosophila Cell* 79: 49–58, 1994.
27. Darnell, J. Transcription factors as targets for cancer therapy. *Nature Rev. Cancer* 2: 740–749, 2002.

CHAPTER

27

Growth Factors

Gary E. Landreth

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GROWTH FACTORS ARE ESSENTIAL FOR NERVOUS SYSTEM DEVELOPMENT AND FUNCTION

Growth factors have been defined as proteins that stimulate cellular proliferation and promote cellular survival. The number of molecules considered to have growth factor activity has been substantially expanded with the discovery of the diverse and complex roles these molecules play both in the developing animal and in the adult. Historically, the first growth factor to be identified was nerve growth factor (NGF) by Rita Levi-Montalcini [1]. This molecule has been termed a neurotrophic factor, since its actions are largely restricted to the nervous system. NGF has now been shown to have an extraordinarily wide array of activities and has forced a substantial reevaluation of how growth factors are defined. The nervous system exhibits remarkable cellular heterogeneity and one of the major challenges is to elucidate how a relatively small number of growth factors act to direct the development of the nervous system and sustain these cells in the mature animal [2]. Some growth factors act only during restricted periods during development while others function throughout the life of the animal. In the past, attempts have been made to distinguish between 'neurotrophic factors' and growth factors; however, this has not proved to be a useful distinction given our current appreciation of the broad range of actions of these molecules.

At very early developmental stages, growth factors play critical roles in determining cell fate throughout the organism. Stem cell populations that give rise to the nervous system are influenced by growth factors that direct the cell toward either a neuronal or glial fate (see Ch. 29). Subsequent decisions on the phenotypic commitment to become a specific cell type and subclass are also governed by the combinatorial actions of several growth factors.

The nervous system is subject to a unique set of constraints during development. Many more neurons are generated during development than are required in the mature nervous system. Importantly, after their last mitotic division neurons are unable to re-enter the cell cycle and can no longer proliferate. As development proceeds there is a period during which as many as half of all neurons die, a process known as normal or programmed cell death that occurs by apoptosis of the cell (see Ch. 35).

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Programmed cell death typically occurs at the time that the axonal processes of the innervating neurons arrive at and invade their peripheral target tissues. The selective survival of only a fraction of the initial number of neurons is accomplished by the competition of the innervating processes for a limited amount of trophic factor elaborated by the target tissue. Those neurons that fail to obtain the target-derived trophic factor then die. This concept forms the basis of what is now known as the 'neurotrophic factor hypothesis'. This mechanism can largely account for the tailoring of the number of neurons that connect with their targets in the periphery to the size of the target field, resulting in a stable neuronal population whose size is essentially determined at birth. Trophic factors are then continuously supplied by the target to those neurons that establish functional connections. The trophic factor is internalized at the nerve ending and then retrogradely transported to the cell body (Fig. 27-1). While much of the data supporting this hypothesis has been gathered through the study of the peripheral nervous system, it is clear that analogous mechanisms also operate in the central nervous system [3].

Trophic factors can also be supplied through less specific mechanisms. Growth factors are synthesized by a wide variety of cell types, including neurons and glial cells. The factors are secreted into the extracellular milieu where they diffuse and then act in a paracrine fashion on other cells (Fig. 27-1). Indeed, there is evidence that this type of paracrine support is necessary to sustain neurons as they extend their processes over long distances in the developing nervous system [2]. An analogous process, autocrine stimulation, occurs when a cell synthesizes and secretes a growth factor to which the cell itself is responsive. In this case, the cell provides its own trophic support.

Individual neurons and glial cells are responsive to a number of different growth factors. Growth factors play both unique and overlapping roles in the development and sustenance of these cells. Perhaps the most dramatic example of this is the elaborate array of trophic factors that have evolved to support spinal motor neurons. There are presently at least 15 different factors that are known to influence the survival of these cells [4].

Recently, it has been appreciated that some of these growth factors serve a much wider role. For example, nerve growth factor has been shown to acutely regulate aspects of neurotransmitter synthesis and release, as well as mediating both synaptic plasticity and the stabilization of synaptic contacts. It is likely that in subsequent years we will discover additional novel actions of these molecules.

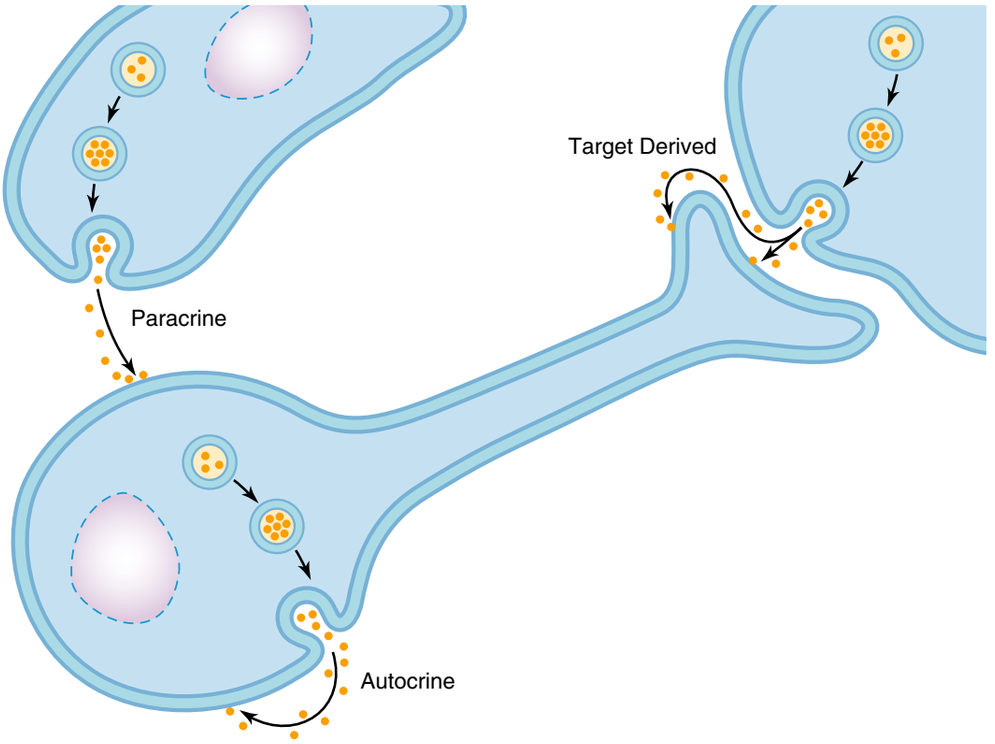

FIGURE 27-1 Mechanisms of growth factor/trophic factor support. Growth factors can be provided to cells through autocrine mechanisms in which the cell secretes growth factors to which itself responds while paracrine support is mediated by secretion of factors which then act upon neighboring cells. Target-derived support of neurons is mediated by the growth of fibers into their target tissues. The target tissue synthesizes the growth factor and provides it to the innervating neurons.

Cells respond to growth factors as a consequence of the binding of the factors to specific cell-surface receptors. The association of the growth factors with their receptors leads to activation of extraordinarily complex signal transduction pathways, leading to biochemical changes in the cells that mediate cellular survival as well as the acquisition and maintenance of the cellular phenotype. The responsiveness of a cell to any particular growth factor is dependent upon expression of specific receptors on the plasma membrane. The regulated expression of such receptors is a well documented feature of development and temporally regulates the sensitivity of the cells to growth factors in their environment. It is noteworthy that the receptors for many of the growth factors are themselves protein kinases whose enzymatic activity is stimulated upon association with their specific ligand, thereby initiating intracellular signaling events [5, 6]. Other growth factor receptors also employ protein kinases to initiate their action but do so through the incorporation of these enzymes within large signaling complexes that are assembled subsequent to growth factor binding. The specificity of growth factor action is governed by the intrinsic structure of the receptor; the extracellular domain imparts ligand binding specificity while the intracellular domain specifies the intracellular signaling pathways that are activated upon ligand binding.

Growth factors activate multiple signal transduction pathways required for survival and differentiation. The signal transduction pathways that mediate the actions of growth factors have been examined in detail. Multiple signaling pathways are activated in parallel following receptor binding that result in cellular survival and direct the acquisition and maintenance of the cellular phenotype [5, 6]. The principal signaling pathway that is responsible or sustaining for cellular viability involves the activation of phosphotidylinositol 3-kinase (PI3K) [7]. The binding of growth factors to their receptors result in the formation of large receptor-associated complexes comprised of a diverse array of signaling elements on the inner face of the plasma membrane. PI3K is recruited to the receptor and is then enzymatically activated. The enzymatic product of PI3K is phosphatidylinositol trisphosphate, which is a bioactive lipid species that is responsible for the formation of stable signaling complexes and the activation of the serine/threonine kinase Akt. Akt activation, in turn, stimulates a signaling cascade that results in the synthesis of proteins that protect the cell from death, inactivates proapoptotic signaling proteins and stimulates the basic metabolism of the cell, such as protein synthesis. The activation of a second major signaling pathway, the MAP kinases or extracellular signal regulated kinases (ERKs) is a common effect of cellular stimulation by all growth factors. ERK activation is linked to a large number of growth factor actions. In neurons, ERK activity is required for the initiation and extension of neuritic processes and the morphological differentiation of these cells [8]. The individual growth factors elicit specific effects and the signaling events that subserve these actions are the subject of intense investigation. A more detailed description of intracellular signaling pathways can be found in Chapters 23 and 24.

CLASSES OF GROWTH FACTORS ACTING IN THE NERVOUS SYSTEM

There are several major classes of growth factors that act within the nervous system (Table 27-1). Some of these, such as the neurotrophins, act almost exclusively in the nervous system, while others, for example fibroblast growth factors and insulin-like growth factor I, act on a number of cell types throughout the body in addition to the nervous system. Unexpectedly, it has been discovered that a subset of growth factors whose actions where thought to be restricted to the immune system have important actions on both neurons and glial cells. The determination of which molecules can legitimately be termed growth factors has thus become less clear. There are a growing number of factors that act to regulate

TABLE 27-1 Growth factors acting in the nervous system

Growth factor	Receptor
Neurotrophins	
Nerve growth factor	TrkA
Brain-derived neurotrophic factor	TrkB
Neurotrophin 3	TrkC
Neurotrophin 4/5	TrkB
Neurokines	
Ciliary neurotrophic factor	CNTFRα, LIFRβ + gp130
Leukemia inhibitory factor	LIFRβ, gp130
IL6	IL6Rα, gp130
Cardiotrophin 1	LIFRβ, gp130 + ?
Fibroblast growth factors	
FGF-1 (acidic FGF)	FGFR1–4
FGF-2 (basic FGF)	FGFR1–3
Transforming growth factor β superfamily	
Transforming growth factors β	TGFβRI and TGFβRII
Bone morphogenetic factors	BMPR I and BMPRII
Glial-derived neurotrophic factor family	
Glial-derived growth factor	c-Ret, GDNFRα-1
Neurturin	c-Ret, GDNFRα-2
Artemin	c-Ret, GDNFRα-3
Persephin	c-Ret, GDNFRα-4
Epidermal growth factor superfamily	
Epidermal growth factor	EGFR
Transforming growth factor α	EGFR
Neuregulins [GGF, ARIA, SMDF, etc.)	ErbB2, ErbB3, ErbB4
Other growth factors	
Platelet derived growth factor	PDGFRα and β
Insulin-like growth factor I	IGFR-I
Hepatocyte growth factor	c-Met
Macrophage-stimulating protein	c-Ron

TABLE 27-2 Neurotrophin targets

	Peripheral	Central
Nerve growth factor	Sympathetic neurons Sensory neurons:dorsal root ganglia	Basal forebrain cholinergic neurons Striatal cholinergic neurons Purkinje cells
Brain-derived neurotrophic factor	Sensory neurons: nodose dorsal root neurons	Spinal motor neurons Basal forebrain cholinergic neurons Substantia nigra dopaminergic neurons Facial motor neurons Retinal ganglion cells
Neurotrophin 3	Sympathetic neurons Sensory neurons	Basal forebrain cholinergic neurons Locus ceruleus neurons
Neurotrophin 4/5	Sympathetic neurons Sensory neurons: nodose dorsal root ganglia	Basal forebrain cholinergic neurons Locus coeruleus neurons Motor neurons Retinal ganglion cells

specific aspects of development and are unlikely to play significant roles or have currently unappreciated roles in the nervous system of the mature animal.

The neurotrophins comprise a family of highly related molecules that act to support the survival and phenotypic specificity of select subsets of neurons. The neurotrophins (Table 27-2) are small highly basic proteins of approximately 13 kDa that dimerize to form the biologically active species [5]. The neurotrophins have a highly conserved structure. This family includes five distinct members: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5) and neurotrophin 6 (NT6). NT6 is found only in fish and will not be discussed further.

The neurotrophins interact with two distinct cell surface receptor species [5, 6, 9] (Fig. 27-2). The neurotrophins bind to the Trk family of receptors, which serve as the principal signal transducer for this class of growth factors. The Trk receptors comprise a small, highly related family of molecules that possess an extracellular ligand binding domain that selectively interacts with the individual neurotrophin species. Trk A specifically binds NGF, TrkB interacts with BDNF and NT4/5, and TrkC preferentially binds NT3. Importantly, the Trk receptors have an intracellular tyrosine kinase domain that is activated upon neurotrophin binding. The kinase domains of the Trk family members are highly conserved and the Trks differ mainly in the structure of their extracellular domains. Trk receptor expression is limited to neurons and the

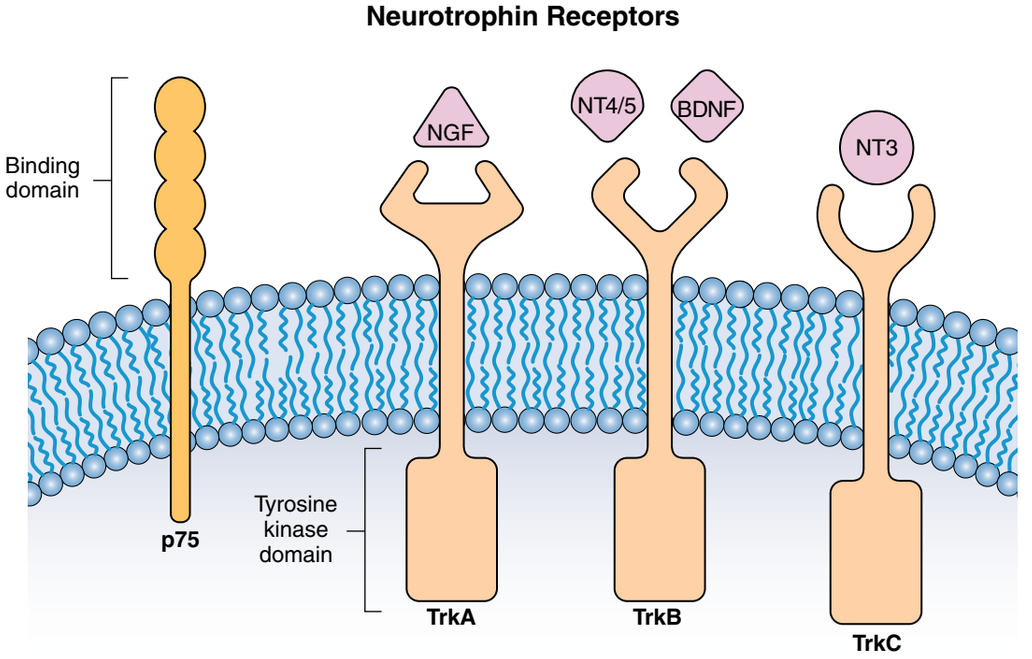

FIGURE 27-2 Neurotrophin receptors. Neurotrophin family members bind specifically to cognate Trk receptors. The low affinity neurotrophin receptor, p75, promiscuously binds all neurotrophins. *BDNF*, brain-derived neurotrophic factor; *NGF*, nerve growth factor; *NT*, neurotrophin.

responsiveness of subpopulations of neurons to the neurotrophins is due to the restricted and selective expression of the individual Trk receptor species. The neurotrophins also bind to another receptor, termed p75 or the low affinity neurotrophin receptor. p75 is promiscuous and binds all mature neurotrophin species with low affinity. It has recently been shown to also bind to the longer unprocessed precursor forms of NGF. p75 can interact with the Trks to participate in a heteromeric receptor complex to transduce neurotrophin signals. It has recently been discovered that p75 binds the immature pro-forms of the neurotrophins with high affinity, and this interaction may play a role in nervous system responses to injury. The p75 receptor has also been shown to be a co-receptor for other cell surface receptors. It is clear that the p75 receptor plays more diverse and complex roles that previously appreciated and that its actions are not restricted to neurotrophin biology [10].

The specific actions of the individual neurotrophins have been the subject of intense interest (Table 27-2). It is now evident, through analysis of animals in which the individual neurotrophin genes or their receptors have been knocked out, that the family members have unique actions acting exclusively to support some neuronal subpopulations [11]. However, in some neuronal populations, the action of several of the neurotrophins overlap. For example, in the peripheral sensory ganglia, individual neurons are responsive to more than one neurotrophin.

Nerve growth factor is the prototypic member of the neurotrophin family [9]. It was discovered on the basis of its ability to stimulate the dramatic outgrowth of processes from sympathetic ganglia. NGF is absolutely required for the survival of sympathetic and a subset of sensory neurons during development. NGF is synthesized within the tissues that receive innervation by these neurons and sustains those neurons whose processes extend into these tissues during the period when programmed cell death occurs. The ability of the target organs to provide trophic support underlies the neurotrophic hypothesis, and much of the action of NGF and other neurotrophins can be explained on this basis. NGF is required for sympathetic neuron survival not only during development but also throughout their life. Sensory neurons are also dependent on NGF during development but become independent of this factor for their survival in the mature animal. It should be pointed out that NGF regulates cell number not by supporting the proliferation of neuronal precursors but by permitting the selective survival of neurons that are protected from programmed cell death. NGF has not been shown to directly stimulate cell division of neurons or their precursors. NGF, like other classical growth factors, acts to stimulate cellular metabolism by positively regulating a wide array of biosynthetic processes. It promotes the morphological differentiation of its target cells, promoting extensive outgrowth of axonal and dendritic processes. Indeed, there is evidence that NGF not only acts in a permissive fashion to facilitate the morphological and biochemical differentiation of the neurons and process outgrowth but is also instructive and plays a role in specifying the phenotype of the neuron [9].

NGF also has actions within the CNS, although it is not particularly abundant in the CNS. Its synthesis appears to be largely restricted to the hippocampus and neocortex, and even in these regions it is present at relatively low concentrations relative to the other neurotrophins. The most prominent population of NGF-responsive neurons expressing TrkA are the basal forebrain cholinergic neurons. The principal projections of these neurons are to the hippocampus and cortex, which conforms with the concept that NGF acts as a target-derived trophic factor in the CNS, just as it does in the peripheral nervous system (PNS). NGF also acts on a subpopulation of cholinergic neurons within the striatum. These interneurons express the NGF receptor, TrkA, and respond to NGF. However, they do not appear to rely entirely on NGF for their survival, and the specific actions of NGF on this neuronal population have not been clearly defined. NGF may also have autocrine actions in the CNS, as some neuronal populations have been identified that express both TrkA and NGF.

NGF has effects on the physiological responses of mature neurons. NGF acts as a target-derived trophic factor for pain neurons, which innervate peripheral tissues such as the skin. Inflammation of these peripheral tissues leads to local elevation of NGF synthesis and abundance. Elevated concentartions of NGF are responsible for the enhanced sensitivity to pain that accompanies inflammation. This is due to the ability of NGF to lower the sensory threshold of the pain fibers, leading to hyperalgesia. Nocioceptive sensory neurons mediating pain sensation are entirely dependent upon NGF for their survival as these cells are selectively lost in animal in which either the NGF or TrkA genes have been knocked out. These animals are insensitive to pain and live only a few weeks.

The receptor for NGF is TrkA, a 140 kDa cell surface protein that specifically binds NGF, but not other neurotrophins [5, 6, 9]. TrkA is expressed on the neuronal cell body and on neuronal processes. In its action as a target-derived trophic factor, NGF is secreted within the target organ and it then binds to TrkA receptors present on the growing neuronal process or synapse. The NGF-TrkA complex is then internalized and subsequently translocated to the cell body by retrograde axonal transport. In those cells that respond to NGF through autocrine or paracrine mechanisms, the growth factor can bind to any of the widely distributed TrkA molecules on the neuronal membrane.

Brain-derived neurotrophic factor was identified on the basis of its ability to stimulate process outgrowth from peripheral sensory neurons. It was isolated from brain and, upon analysis of its structure, discovered to be highly homologous to NGF [3]. BDNF has a different spectrum

of targets from NGF and acts on both peripheral sensory ganglionic neurons and parasympathetic neurons of the nodose ganglia, but not sympathetic neurons. In the CNS, BDNF supports the survival and process outgrowth of basal forebrain cholinergic neurons, dopaminergic neurons in the striatum, retinal ganglion cells and some motor neurons. Analysis of mice in which the BDNF gene was knocked out revealed that the vesibulocochlear neurons were completely lost, accounting for the inability of these mice to maintain their balance. BDNF knockout mice die within 1–2 weeks of birth. Analysis of the specific actions of BDNF is significantly complicated because NT4/5 also binds and activates the TrkB receptor. Interestingly, animals in which the *trkB* gene has been inactivated die within 1–2 days of birth, but the basis of this is not understood given the modest phenotype of the NT4/5 knockout mice.

The discovery that sensory neurons themselves synthesize BDNF has led to the suggestion that its secretion from these neurons results in both autocrine and paracrine actions. It is thought that, during development, some neurons, for example sensory neurons of the dorsal root ganglia, may rely on target-derived factors while, during later periods, the neurons synthesize growth factors that support themselves through autocrine stimulation and from cells in their immediate environment. These mechanisms serve to lessen the dependency on targets for trophic support. This view is supported by the finding that axotomy, or target removal, has much diminished effect in older animals. In the PNS, neurotrophins, including BDNF, are also synthesized by glial cells. Glial cells proliferate at later stages of development, largely after all neurons have been born and have passed through the period of normal cell death. Thus, glia are likely to play a role in providing trophic support in the mature nervous system through paracrine mechanisms.

Neurotrophin 3 is the most abundantly distributed of the neurotrophins and appears to have a wider range of action than other members of this family, particularly in the CNS. It is expressed throughout the CNS. In the mature brain, NT3 is found at high levels in the cortex, hippocampus, thalamus and cerebellum. NT3 also acts on subpopulations of spinal motor neurons and on cochlear neurons. It exerts its actions principally through binding to another member of the Trk family, TrkC.

In the periphery, NT3 uniquely supports proprioceptive neurons in sensory ganglia. These neurons and their peripheral targets, the muscle sensory organs, are lost in NT3 knockout mice. Mice in which the NT3 receptor TrkC has been knocked out do not live long and have abnormal movements as a consequence of their loss of proprioception.

NT3 plays an earlier role in development than other neurotrophin family members. It sustains progenitor cells of the neural crest, which then give rise to sympathetic and sensory ganglia, as well as a number of other structures. It is also likely that NT3 functions to regulate the period of time these precursor cells remain mitotically active and, thus, it acts indirectly to regulate cell number. In NT3 knockout mice, approximately 70% of neurons within the sensory ganglia are lost, indicating an absolute requirement for NT3 for the survival of neuronal subpopulations. Analysis of the pattern of NT3 expression has shown that it is highly expressed during periods of rapid process extension in regions adjacent to the developing ganglionic neurons. It is therefore likely that these neurons are supported during this period principally through autocrine and paracrine mechanisms. As the cells differentiate and extend processes to their target, NT3 expression is found in these end organs. Importantly, there is a coincident dramatic reduction in NT3 synthesis in the regions within and adjacent to the developing ganglia, rendering the neurons wholly dependent on target-derived trophic support.

NT3 is also critical for glial development. There is good evidence that NT3 acts to stimulate the proliferation of oligodendrocyte precursor cells, probably in concert with platelet derived growth factor (PDGF) (see below).

Neurotrophin 4/5 is not as well characterized as other members of the neurotrophin family. Much of what is known is derived from analysis of NT4/5 and TrkB knockout mice. Elucidating the actions of NT4/5 is complicated by virtue of the fact that both NT4/5 and BDNF exert their effects via the TrkB receptor. It appears that NT4/5 functions largely overlap with those of other neurotrophin family members, particularly BDNF. NT4/5 knockout mice are essentially normal, in contrast to BDNF knockout mice, which do not live long. NT4/5 is likely to have unique actions on a subpopulation of neurons in the nodose and geniculate ganglia, which are not supported by BDNF. Like BDNF, NT4/5 acts on sensory neurons and retinal ganglion cells, supporting their survival.

NT4/5 is expressed in muscle and this neurotrophin can support facial motor neurons as well as other populations of motor neurons. Interestingly, NT4/5 expression is regulated by the activity of the muscle and this neurotrophin stimulates axonal sprouting, suggesting that it acts as a muscle-derived trophic factor for motor neurons.

Neurotrophins influence neurotransmission and synaptic plasticity. The discovery that the neurotrophins have acute effects in the mature nervous system and can modulate synaptic efficiency has forced a substantial reevaluation of the role of these molecules in modulating the dynamic behavior of the nervous system [12].

Direct application of neurotrophins to the brain of animals results in the rapid initiation of dramatic seizure activity, reflecting the coordinated discharge of large populations of neurons. This effect is the result of neurotrophin-stimulated release of neurotransmitters and enhanced synaptic efficiency. BDNF and NT3 cause

an increase in neurotransmitter release from presynaptic nerve terminals. An analogous effect in the PNS is the ability of NGF to enhance painful sensations. Thus, neurotrophins can act over short intervals to regulate synaptic transmission, a role not previously attributed to these molecules.

Synaptic activity can also provoke the release of neurotrophins and acutely regulate the synthesis of these factors [13]. It is likely that the normal physiological levels of neurotrophins in the CNS are regulated by ongoing neuronal activity in the brain. There are a number of examples showing that the synthesis of neurotrophins, most prominently BDNF, is stimulated following neurotransmitter release. In an extreme example, induction of seizure activity in the brain results in the stimulation of NGF and BDNF synthesis, but not NT3. A growing body of evidence suggests that there is a intricate interplay between the ability of the neurotrophins to regulate synaptic activity in the brain and the effect of such activity on neurotrophin synthesis and secretion. The enhanced synaptic stimulation of neurons then positively regulates the synthesis of neurotrophins. Insights into these events have come from examination of complex phenomena like long term potentiation in the hippocampus using neurotrophin knockout mice.

The ability of the neurotrophins to regulate the structural organization of the peripheral nervous system during development is well documented. The capacity of these molecules to affect the anatomical organization of the CNS is less well studied. Perhaps the most striking example of the ability of neurotrophins to affect neuronal connectivity in the CNS is the development and maturation of visual cortex. In this system, the axons of lateral geniculate neurons normally invade visual cortex and have a broad and diffuse projection pattern. However, as development proceeds the axonal projection fields of these neurons become progressively smaller and more refined, eventually becoming restricted to innervate defined linear columns of cortical neurons, termed *ocular dominance columns*, that are sensitive to the input from a single eye. Neurotrophins regulate the process by which the restriction and segregation of axonal projections occur. Administration of neurotrophins can block the formation of dominance columns by inhibiting the loss of synapses within the broad projection area. These data suggest that normally the visual neurons may compete for a limited supply of neurotrophins elaborated by the cortical neurons and the neurotrophins may act to stabilize synaptic contacts. Thus, neurotrophins appear to play a critical role in the activity-dependent development of cortical connectivity and function.

Neurotrophic cytokines are a small group of cytokine-like molecules that act in the nervous system. The terms neurotrophic cytokines, neurokines or neuropoietins have been applied to a small group of molecules which are most highly related to cytokines and share both structural similarity and employ common effector mechanisms in their target cells [14]. These factors include ciliary neurotrophic factor (CNTF), interleukin 6 (IL-6), leukemia inhibitory factor (LIF), cardiotrophin 1 (CT-1), and oncostatin-M. Some of these molecules act as *bona fide* growth factors while others appear to have more restricted roles and dictate the differentiated phenotype of subpopulations of neurons. Recently, this class of molecules has been shown to regulate the proliferation and differentiation of neural stem cells, both during development and in the mature nervous system.

The cell surface receptors for the neurotrophic cytokines are multisubunit complexes that employ a common receptor subunit, gp130 (Fig. 27-3). gp130 forms a receptor complex, with itself or the LIFβ receptor. In the case of CNTF and IL-6 (and possibly CT-1) the ligand binds to an additional receptor alpha subunit, which imparts ligand-specific binding properties to the receptor, forming an active trimeric receptor complex. The receptor complex transduces signals through its interaction with cytoplasmic tyrosine kinases of the JAK family. Ligand binding to the receptor leads to activation of their intrinsic tyrosine kinase activity of the receptor-associated JAKs, leading to the stimulation of diverse intracellular signaling pathways.

Ciliary neurotrophic factor was discovered through its ability to sustain parasympathetic neurons of the ciliary ganglia. It exhibits an extremely broad range of activity, acting on a number of neuronal populations [15]. CNTF promotes the survival of parasympathetic ganglionic neurons as well as sensory and sympathetic neurons in the PNS. In the CNS, CNTF acts on neurons in the hippocampus, as well as both cholinergic and GABAergic neurons in the basal forebrain. CNTF, like the neurotrophins, can regulate neurotransmitter release and directly affect ion channel function.

Substantial interest in the biological actions of CNTF has arisen as a result of its actions on motor neurons. CNTF is synthesized in muscle and can be actively taken up by motor neurons and then retrogradely transported to the cell body. Application of CNTF to the nerve after injury prevents motor neuron death and can partially reverse the progressive loss of motor function in animal models of neuromuscular disease. On this basis, CNTF appears to act as a target-derived trophic factor for these neurons. However, this view is complicated by the fact that CNTF does not possess a signal sequence which is necessary for its secretion and it is not known how it is released from its sites of synthesis, primarily in muscle and glial cells in both the CNS and PNS. Of particular interest, injury to the nervous system results in the induction of CNTF synthesis by astrocytes and Schwann cells, suggesting it plays a role in the response to traumatic injury. In addition to its actions on neurons, it appears that CNTF acts to promote the differentiation of glial precursor cells into astrocytes and myelin-forming oligodendrocytes.

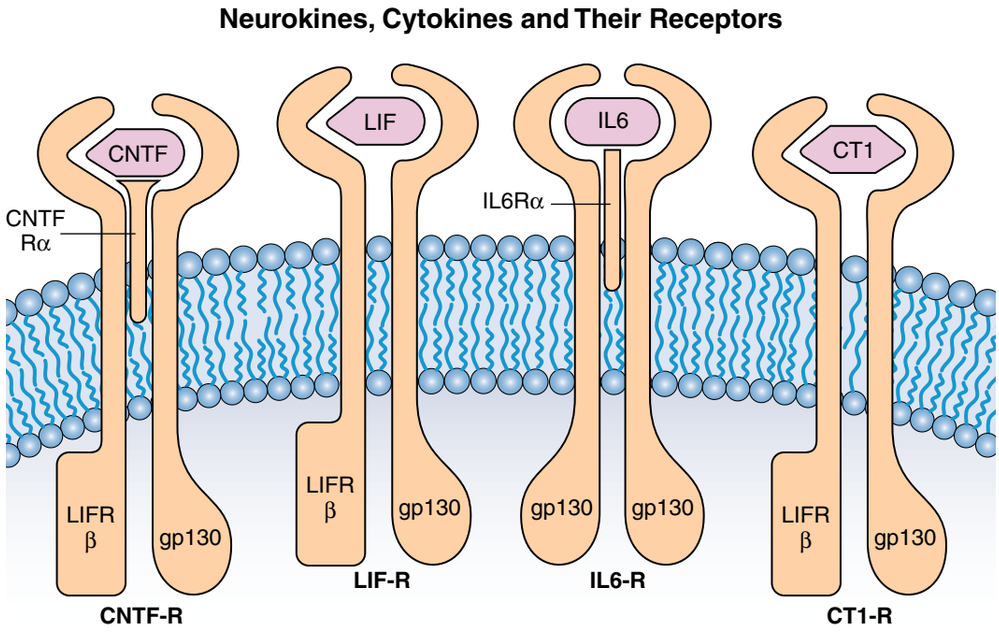

FIGURE 27-3 Neurotrophic cytokines and their receptors. Neurotrophic cytokines are related to IL6 and bind to cell surface receptor complexes that share a common structural organization. The four ligands interchangeably employ two distinct receptor subunits, leukemia inhibitory factor receptor β (*LIF-Rβ*) and gp130, and some employ a ligand-specific α subunit. *CNTF-R*, ciliary neurotrophic factor; *CT-1R*, cardiotrophin 1 receptor; *IL6-R*, interleukin-6 receptor.

In humans and mice, mutations in the CNTF gene have no obvious effect, suggesting that other molecules can mediate all of the significant physiological actions of CNTF. Indeed, about 2% of the Japanese population do not have an active CNTF gene. However, knockout of the CNTF receptor gene has much more dramatic effects with significant loss of all motor neurons. These data suggest that there must be other as yet undiscovered factors that utilize the CNTF receptor and play a more critical role in development than CNTF itself.

Leukemia inhibitory factor (LIF) has well described actions in the immune system. The recognition that this factor also acts in the nervous system followed the discovery that it acts on cultured sympathetic neurons to direct a change in neurotransmitter expression from a noradrenergic to a cholinergic phenotype and regulates the expression of neuropeptide transmitters in these cells. LIF exhibits a similar activity toward spinal motor neurons through its ability to stimulate the biosynthesis of acetylcholine. LIF also functions as a trophic factor for peripheral sensory neurons and spinal motor neurons *in vitro*, supporting their survival. LIF is a trophic factor for oligodendrocytes and promotes astrocytic survival and differentiation. It has recently been recognized that it plays an important role in neurogenesis in the adult nervous system by promoting the proliferation of neural stem cells. LIF has functionally critical roles in responses to nervous system injury, promoting neuronal survival, injury-induced neuropeptide synthesis and astrocyte differentiation following damage to the mature nervous system.

Interleukin 6 (IL-6) actions in the nervous system are not well studied, nor is this cytokine particularly abundant. IL-6 has been shown to sustain cultured hippocampal neurons as well as basal forebrain cholinergic and mesencephalic catecholaminergic neurons. IL-6 is likely to be synthesized in the CNS principally by astrocytes, although microglia are also a source for this cytokine. Microglial-derived IL-6 inhibits neural stem cell generation. IL-6 is synthesized by neurons and has recently been shown to be an important regulator of neurogenesis in the adult nervous system. IL-6 has been shown to promote the differentiation of neural stem cells into astrocytes while inhibiting them from acquiring a neuronal phenotype in the developing cortex.

Cardiotrophin 1 acts as a survival factor for spinal motor neurons. CT-1 is synthesized by both skeletal muscle and myotubes and has been shown to be secreted from the latter, suggesting that it acts as a target-derived trophic factor. Indeed, in CT-1 knockout mice there is a loss of motor neurons in the spinal cord and brainstem [16]. CT-1 also promotes the survival of dopaminergic neurons and ciliary neurons.

Other Cytokines including interleukins 1β, 2, 3, 4, 5, 7, 9, 11 and 12 have been reported to exhibit neurotrophic activities [17]. The concentrations of these cytokines in

nervous tissue are not high and their effects are not particularly well documented. For many of the cytokines, their significance in nervous system development and function is not clear. These molecules play central roles in immune system function and many of their described actions follow injury to the nervous system and in disease states. In this regard, the microglia are the principal immune effector cell in the brain and function as macrophages. As such, the microglia are the likely sources of many of the cytokines that have been reported to be present in the CNS. Astrocytes also synthesize a number of cytokines and are responsive to some of these molecules.

The fibroblast growth factors comprise a gene family of nine members that share substantial sequence homology and have diverse effects in the nervous system. Fibroblast growth factors (FGFs) were initially identified on the basis of their ability to stimulate the proliferation of fibroblasts, it was subsequently been found that they have an extremely broad distribution in the body and act on a host of different cell types. FGFs stimulate the proliferation of neurons in the developing nervous system and glial cells throughout the life of the organism. FGFs act on a large number of neuronal populations and all glial cell subtypes [18]. In the mature nervous system only two members of this family, FGF-1 (also known as acidic FGF) and FGF-2 (also known as basic FGF), are present at significant levels. In the adult brain the FGFs represent the major mitogenic species. They are present at significantly higher levels than the neurotrophins, FGF-1 levels are estimated to be approximately 500-fold and FGF-2 up to 50 times greater than that of NGF. FGF-1 is found to be expressed at high levels in ganglionic sensory neurons in the PNS. In the mature CNS, FGF-1 is detected at highest levels in motor, basal forebrain cholinergic and substantia nigra neurons, and in neurons of other subcortical nuclei. FGF-2 is expressed abundantly by astrocytes, although hippocampal pyramidal cells have also been shown to express this growth factor. FGF2 has trophic effects on most neurons derived from the CNS. There is evidence that the FGFs may play a role in facilitating axonal regeneration in the PNS and provide trophic support to neurons following trauma or injury.

The FGFs stimulate the proliferation of mesodermally and ectodermally-derived cells and play central roles in mammalian development. Members of the FGF family are expressed in the embryonic period and are required for several critical events in neural development and specifically for neural induction. FGF-8 is necessary for positional identity required for axial specification and patterning of limb development. FGF-2 stimulates the proliferation of multipotential stem cells that subsequently give rise to neurons of the cortex and other brain regions.

The complexity of FGF action is compounded by the existence of at least four receptors for FGF (FGFR1–4). Three FGFRs are expressed in the CNS where they exist as multiple alternatively spliced products. The FGF receptors are all ligand-activated tyrosine kinases and comprise a distinct subfamily of the receptor tyrosine kinases. The interaction of the various FGFs with the four FGF receptors and their multiple splice products is bewilderingly complex and is incompletely understood. The FGFR1 receptors appear to be expressed exclusively in neurons, while FGFR2 and FGFR3 are expressed principally by glial cells. Interestingly, neurons of the substantia nigra and some motor neurons appear to express both FGF-1 and its receptor FGFR1, suggesting that FGF-1 may act in an autocrine fashion to support these cells. FGFR1 and FGFR2 appear to play a role in development as both receptors are expressed at a time prior to that of their ligands, FGF-1 and FGF-2. These data support the view that other FGF family members are the functionally relevant species during embryogenesis. FGFR1 is expressed in the primitive neuroepithelium. A novel aspect of FGF biology is the ability of these growth factors to bind to cell surface proteoglycans, specifically heparan sulfate proteoglycans. Indeed, it appears that these proteoglycans can act as low affinity receptors for the FGFs. It is thought that FGFs bind to the proteoglycans that effectively immobilize them and induces or stabilizes an active conformation, facilitating binding to the FGFR.

Transforming growth factors β are the prototypic members of a large family of related factors that have a diverse roles both in development and in the mature animal. There are over 50 members of this extended family that have been assigned to five distinct subfamilies [19]. The actions of most transforming growth factor β (TFGβ) superfamily members are best described in other organ systems. However, the TGFβ-related factors have important roles in the nervous system where they are thought to act principally through autocrine and paracrine mechanisms. This family of growth factors employs a unique receptor complex involving two distinct subunits, both of which are serine/threonine kinases (see Ch. 23). The ligand first binds to the type II subunit and this dimeric complex then associates with the type I subunit. The hetero-oligomeric receptor complex then phosphorylates downstream signaling transduction elements, which then initiate intracellular signaling events leading to the activation of a specific group of transcription factors, termed SMADs.

TGFβ subfamily in mammals comprises three subfamily members, $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$. $TGF\beta_1$ is expressed principally by glial cells and is not present in significant amounts in the mature nervous system. $TGF\beta_1$ is thought to function principally following injury to the nervous system, where its expression is dramatically induced in microglial cells [20]. It is synthesized, albeit at lower levels, by astrocytes. In some neurons TFGβ1 is a component of the response to neurodegeneration or trauma and its synthesis and secretion are elevated in these settings.

The synthesis of TGFβ$_1$ following injury in the nervous system is consistent with its effects on inflammatory responses in other organ systems. Although this factor is not particularly well studied in the developing nervous system, it has been reported to play an instructive role in specifying cellular phenotype.

TGFβ$_2$ and TGFβ$_3$ are widely expressed in both the peripheral and central nervous systems [19]. Early in development TGFβ$_2$ is found associated with developing fiber tracts and later in development it is found in astrocytes. TGFβ$_3$ acts as a mitogen for amacine cells in the developing retina. In the adult TGFβ$_2$ and TGFβ$_3$ are found in many neuronal populations and in both astrocytes and Schwann cells.

The TGFβ family has diverse effects that are likely to be cell-type-specific and it is presently impossible to make general statements about their functions. It has been documented that TGFβ$_2$, in contrast to many growth factors, can inhibit cellular proliferation and is growth inhibitory towards neurons. It has antimitotic effects on astrocytes and also acts to arrest the proliferation of oligodendrocytes and subsequently promotes their differentiation. There is now evidence that the TGFβs act principally to modulate that actions of other growth factors. For example, TGFβ enhances the survival promoting properties of CNTF, FGF and the neurotrophins on ciliary ganglionic neurons. Whereas TGFβ$_2$ and TGFβ$_3$, acting alone, have also been reported to exhibit frank negative effects on the survival of these neurons. Similarly, TGFβs augment the actions of the glial-derived neurotrophic factor (GDNF). TGFβ$_2$ and TGFβ$_3$ act as survival and trophic factors for dopaminergic neurons in the striatum and midbrain in concert with GDNF, both during development and in the mature animal. These factors are expressed principally within the projection fields of these neurons, thus these findings have been interpreted to support the view that members of the TGFβ family act as target-derived trophic factors for dopaminergic neurons.

Bone morphogenetic protein subfamily comprises a TGFβ subfamily of factors that are most highly related to the *Drosophila* decapentaplegic gene product, a critical regulator of morphogenesis and axial specification. These factors, as their name indicates, were first identified on the basis of their effect on bone formation. The focus of most studies of these factors has been on their role in inductive events in development. These factors have important roles early in development of the nervous system. BMP2 has been shown to act to influence the differentiation of multipotential cells of the neural crest into a neuronal phenotype. The BMPs also have significant roles as trophic factors. BMP4 acts in the cerebellum to promote the differentiation of both neurons and astrocytes. BMP6 and 7 have been shown to enhance cerebellar granule cell survival. Sensory neurons in the PNS are responsive to BMPs produced in target tissues and serve to regulate their differentiation. There is a growing literature suggesting that these BMP family members may act in concert with other factors to regulate neuronal differentiation and survival.

The glial-derived neurotrophic factor family represents a newly recognized family of target-derived neurotrophic factors. The GDNF-family ligands (GFLs) include GDNF, neurturin, artemin and persephin [21]. These factors are synthesized by a number of tissues and support the survival of the innervating neurons [22]. The GFLs exist as homodimers and share general structural features with the TGFβ family but have little sequence homology.

GFLs bind to a related family of four cell surface receptors termed GDNF receptor-α (GFRα), which are attached to the membrane through a glycosyl phosphatidylinoisitol anchor (Fig. 27-4). The individual GFLs bind specifically to GFRα species; GDNF binds to GFRα-1, neurturin to GFRα-2, artemin to GFRα-3 and persephin to GFRα-4. The ligand-bound GFRα receptors then interact with c-Ret, a receptor tyrosine kinase. The formation of the GFRα-Ret complex results in activation of the tyrosine kinase activity of Ret and stimulation of downstream signaling pathways [23].

Glial-derived neurotrophic factor as its name suggests, is synthesized by glial cells; in the CNS by astrocytes and in the PNS by Schwann cells. GDNF was first characterized as a trophic factor which supported the survival and differentiation of dopaminergic cells of the midbrain and striatum. It is this latter cell population that is selectively lost in Parkinson's disease. GDNF has been shown to selectively support these cells after physical or chemical insults, suggesting this factor may have utility as a therapeutic agent in this disease. GDNF acts as a target-derived trophic factor as it is synthesized in areas receiving dopaminergic innervation and the factor is retrogradely transported to the cell bodies of the dopaminergic neurons.

In the periphery, GDNF acts as a trophic factor for both sympathetic and parasympathetic neurons as well as several distinct subpopulations of sensory neurons. Consistent with its role as a target-derived trophic factor, it is highly expressed in a variety of tissues receiving sympathetic and sensory innervation. GDNF is essential for the development of enteric neurons and thus the innervation of the gut. Importantly, GDNF is a potent trophic factor for spinal motor neurons and can rescue these cells from programmed cell death during development, as well as prevent their death following injury. GDNF is synthesized by skeletal muscle and is retrogradely transported suggesting that it acts as a target-derived trophic factor for the innervating spinal motor neurons.

Neurturin (NRTN) plays a critical role in supporting parasympathic neurons innervating peripheral target tissues. NRTN is required for the survival and function of enteric neurons and a subset of ganglionic sensory neurons, particularly those mediating temperature sensation.

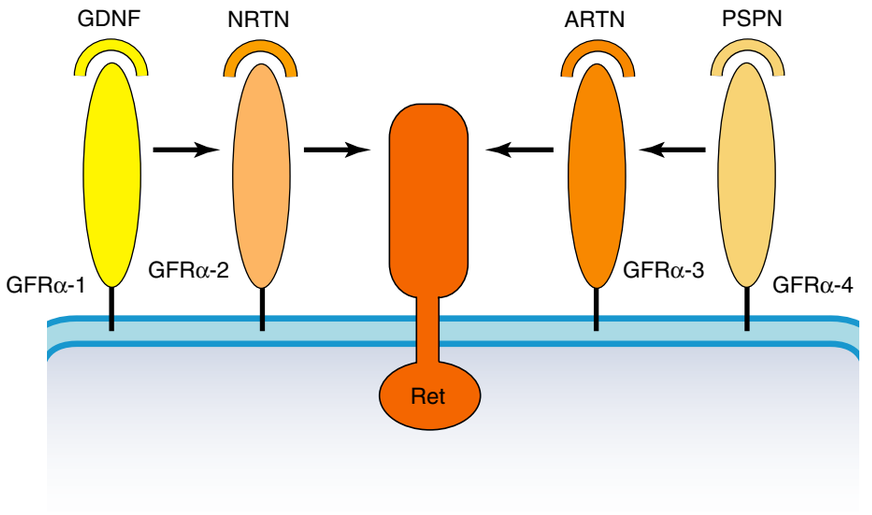

FIGURE 27-4 Glial-derived neurotrophic factors and their receptors. The glial-derived neurotrophic factors, GDNF, neurturin, artemin and persephin bind to specific GFRα subunits. The GFRα-ligand complex then associates the Ret and activates its intrinsic tyrosine kinase activity, stimulating downstream signal transduction pathways. *ARTN*, artemin; *GDNF*, glial-derived neurotrophic factor; *GFRα*, glial-derived neurotrophic factor receptor alpha.; *NTRN*, neurturin; *PNSN*, persephin.

The most dramatic phenotype in NRTN knockout mice is a breathing defect due to loss of about half of the neurons in the petrosal ganglia. NRTN can support both dopaminergic neurons and spinal motor neurons *in vitro*.

Artemin The biological actions of artemin (ARTN) have been elucidated largely through examination of $GFR\alpha_3$ knockout animals. These animals exhibit defects in the proliferation, migration and axonal outgrowth of superior cervical ganglia neurons. Loss of ARTN function results in the loss of most neurons in this ganglia in older animals. ARTN is expressed along blood vessels and it has been postulated to act in a chemotactic manner to guide sympathetic fibers along blood vessels. ARTN is reported to reduce pain following nerve injury, although the basis of this effect is unknown.

Persephin (PSPN) has been shown to promote the survival of basal forebrain neurons including both cholinergic neurons. Moreover, it supports midbrain dopaminergic neurons. PSPN is expressed in the striatum, consistent with its role as a target-derived trophic factor for these cells.

Epidermal growth factor and related factors have a diverse range of actions in the nervous system.

Epidermal growth factor (EGF), is the most prominent member of a family of growth factors and was originally identified by its mitogenic actions on ectodermally derived cell types. In the nervous system, EGF's best documented actions are related to neural stem cell survival and proliferation. Some populations of neural precursor cells respond to EGF, allowing their expansion and transition to a multipotent stem cell. EGF acts in concert with FGF2 to support proliferation and the initial commitment of neural stem cells to neuronal and glial lineages. EGF is not likely to play a major role in the mature nervous system. A structurally related growth factor, *transforming growth factor* α (TGFα), is widely expressed throughout the developing embryo including the nervous system. TGFα binds to and activates EGF receptors and may be the physiologically relevant ligand for these receptors during development. Little is known about the specific actions of TGFα in the nervous system, but it is likely to act principally through autocrine or paracrine mechanisms.

Neuregulins are a large and unique family of growth and differentiation factors that arise from the alternative splicing of mRNAs derived from the NRG1 gene and possess an EGF-like motif [24, 25]. There are three additional neuregulin genes whose functions are unknown. There are currently about a dozen recognized neuregulin isoforms derived from the NRG1 gene and include molecules originally termed glial growth factor (GGF), heregulin, neu differentiation factor (NDF), sensory and motor-neuron-derived factor (SMDF) and acetylcholine-receptor-inducing activity (ARIA). These molecules are important intermediates subserving neuronal–glial interactions. Neuregulins are both membrane bound and secreted proteins. The secreted forms have been shown to act by both paracrine and autocrine mechanisms. The membrane bound isoforms are responsible for direct cellular interactions leading to contract-dependent neuregulin signaling, for example the axonal membrane-dependent proliferation of Schwann cells.

Neuregulins bind to a family of receptor tyrosine kinases that are homologous to the EGF receptor, termed

ErbB (or HER in the human). There are four ErbB receptors that form homo- or heterodimers in various combinations upon ligand binding. Specific NRG isoforms preferentially interact with different ErbB dimers. The ErbB receptors are ligand-activated tyrosine kinases structurally similar to the EGF receptor.

Neuregulins are highly expressed in the nervous system by neuroblasts, cortical neurons, peripheral sensory ganglionic cells and spinal motor neurons as well as myelin-forming glia.

In mice in which the NRG1 gene has been inactivated, Schwann cells and cranial ganglia fail to develop. Neuregulins have been shown to be critical in mediating the differentiation of neural crest cells into glial phenotypes and the subsequent proliferation of Schwann cell precursors. Neuregulins have similar effects in the CNS and promote oligodendrocyte precursor mitogenesis.

Neuregulins are likely to act principally in a paracrine manner on adjacent glial cells and on non-neuronal targets tissues innervated by neuregulin-expressing neurons (such as muscle).

The neuregulins also act on Schwann cells to stimulate motility and migratory activity. It is postulated that this interaction provides a mechanism by which neurons can influence glial metabolism and behavior, which are important both in development and for peripheral nerve regeneration.

An important insight into the function of neuregulins was gained by the observation that they are synthesized by motor neurons and are released at the neuromuscular junction where they interact with the erbB receptors expressed on the muscle cell membrane. Interestingly, in this context the neuregulins were first detected as an activity that stimulated the expression and aggregation of acetylcholine receptors at sites of nerve contact and was dubbed acetylcholine receptor inducing activity, or ARIA. Neuregulin/ARIA acts as a synaptic signal that regulates muscle gene expression, most prominently the acetylcholine receptor genes.

A number of other growth factors act in the nervous system.

Platelet-derived growth factor (PDGF) is synthesized by neurons and astrocytes throughout the central nervous system and by Schwann cells in the PNS. These various cell types also express PDGF receptors, and it is thought that this growth factor acts in an autocrine and paracrine fashion. The best described action of PDGF is on oligodendrocyte progenitor cells stimulating the proliferation of these cells and subsequently promoting their differentiation into mature oligodendrocytes.

Insulin-like growth factor I (IGF-I) has a well recognized role as a trophic and survival factor for nervous system cells in tissue culture [26]. However, its specific functions in the developing and mature nervous system have been difficult to define largely because its actions as a trophic factor overlap extensively those of other growth factors. IGF I is expressed in nervous tissue only late in development and at highest levels in neurons of the olfactory bulb, hippocampus, thalamus, cerebellum and retina. IGF I and insulin enter the brain from the systemic circulation, thus it is unclear to what degree the endogenously synthesized proteins contribute to the levels of IGF-I and insulin in the brain. IGF-I and insulin sustain many nervous-system-derived cell types in tissue culture; however, the amount of insulin required to elicit biological effects in most of these cells is quite high, suggesting that many of the reported effects of insulin on cells of the nervous system *in vitro* are mediated through insulin acting on the IGF I receptor. The IGF I receptor possesses a ligand-stimulated tyrosine kinase domain whose structure is quite similar to that of the insulin receptor. The IGF I receptor is ubiquitously expressed throughout the nervous system and is found at higher levels in the developing brain than in the adult, in contrast to IGF I expression. *In vitro* studies have shown that IGF-I is likely to act principally through autocrine or paracrine mechanisms to promote the proliferation of neuronal and glial precursors and facilitate their subsequent differentiation and survival. Interestingly, mice overexpressing of IGF-I possess larger brains owing to larger numbers of neurons. Conversely, inactivation of the IGF-I gene or its receptor in mice and humans results in reduced brain size, diminished numbers of neurons and hypomyelination, suggesting that IGF-I plays an important role in neuronal proliferation and maintenance. Recent studies have demonstrated that IGF-I stimulates the proliferation of neural progenitor cells in the hippocampus and drives the induction of a neuronal phenotype from progenitor cells in adult mice. These effects may underlie the dramatic effects of IGF-I on overall brain size.

IGF I has recently been the focus of considerable interest due to its actions on motor neurons. It can prevent normal motor neuron cell death during development, reduce the loss of these cells following nerve injury and enhance axonal regeneration. In the adult, injection of IGF I results in sprouting of motor neuron terminals and increases the size of the neuromuscular junction. These and other studies suggest potential therapeutic applications of IGF I in several neurological diseases including amyotrophic lateral sclerosis and peripheral neuropathies.

Hepatocyte growth factor (HGF), also known as scatter factor, has been shown to support the survival of sympathetic neuroblasts and subpopulations of spinal motor neurons. In the former case this is apparently an autocrine effect since these cells have been shown to synthesize HGF.

HGF serves as an attractant for developing spinal motor neurons and thalamic axons *in vitro*. HGF was shown to protect cerebellar granule neurons against excitotoxicity.

Macrophage stimulating protein (MSP) is structurally related to HGF and signals through the Ron tyrosine

kinase receptor. MSP is a target-derived neurotrophic factor for subsets of sensory and sympathetic neurons at different times during their development. Moreover, MSP appears to act on a restricted subset of motor neurons innervating the tongue.

GROWTH FACTORS ACT COMBINATORIALLY AND SEQUENTIALLY TO REGULATE NERVOUS SYSTEM DEVELOPMENT

One of the primary outcomes of the investigation of growth factor action has been the recognition that there are a relatively small number of growth factors that must act in concert to orchestrate the survival and differentiation of a diverse population of neuronal and non-neuronal cell types. The foregoing review has detailed the broad spectrum of actions of some classes of growth factor while other growth factors have more restricted roles. It is apparent that most cells are dependent upon more than one growth factor through embryogenesis and in the mature nervous system [3]. One of the most significant outcomes from analysis of neurotrophin (and their receptors) knockout mice is that these mice show very modest phenotypic changes in the CNS. This reflects the extensive interconnectivity of neurons in the brain, providing a wide range of cellular contacts from which trophic support may arise. In the PNS, the phenotypes are more dramatic, with significant cell loss, indicating the more restricted source of trophic factors available to these cells. There is ample evidence that both neurons and glial cells express receptors for a number of growth factors that allow the cells to respond to multiple sources of trophic support. Moreover, responsiveness to several factors also serves to broaden cellular sensitivity to changes in the environment during development and in the mature nervous system and may subserve a dynamic response to changing rates of activity or to injury. Also, trophic support for neurons is delivered at different cellular loci. For example, adult sensory neurons express multiple members of the Trk family; however, NGF appears to be delivered to the neurons through axonal transport, while BDNF and other factors are supplied by paracrine mechanisms to the cell body.

Motor neurons provide a compelling example of how a single class of neurons can be supported by an extraordinarily wide range of factors. More than 15 growth factors have been shown *in vitro* or *in vivo* to sustain these cells. Some of these are derived from the muscle target, while others are elaborated by ensheathing Schwann cells and by cells resident within the spinal cord. It is likely that motor neurons rely upon multiple factors for their survival and different subpopulations of motor neurons may exhibit unique combinations of trophic factor dependence [4].

Detailed study of the actions of growth factors has revealed that they act in concert to mediate developmental events and, further, that they act sequentially [15]. The ability of a neuron to respond to a given trophic factor is frequently dependent on its developmental stage. The complexity of trophic factor dependence exhibited by some populations of neurons is a consequence of both the developmentally regulated expression of growth factor receptors and the spatiotemporal pattern of growth factor expression. For example, pluripotent neural crest cells giving rise to sensory neurons are dependent upon NT3 for proliferation during early periods in development. The cells lose their responsiveness to NT3 and become NGF-dependent during the period when they establish their contacts with their peripheral targets [2]. Similarly, neuroblasts of the cortex and hippocampus are stimulated to proliferate in response to FGF-2, and subsequently, they become responsive to NT3 that then promotes their survival and differentiation. These examples illustrate the serial dependence of neurons upon different factors for mitotic expansion, acquisition of a differentiated phenotype and their sustenance in the mature nervous system.

One of the most significant conclusions to be derived from the study of growth factors in the nervous system is that we surely have discovered only a fraction of the growth/trophic factors that are responsible for the generation of the cellular complexity and the specific and extensive interconnections between populations of cells. In the last few years, largely through the power of molecular biology and molecular genetics, we have identified a number of novel factors. This process of discovery will undoubtedly continue at an accelerating pace.

REFERENCES

1. Levi-Montalcini, R. and Angeletti, P. U. Nerve growth factor. *Physiol. Rev.* 48: 534–569, 1968.
2. Davies, A. M. Regulation of neuronal survival and death by extracellular signals during development. *EMBO J.* 22: 2537–2545, 2003.
3. Lewin, G. R. and Barde, Y. A. Physiology of the neurotrophins. *Annu. Rev. Neurosci.* 19: 289–317, 1996.
4. Oppenheim, R. W. Neurotrophic survival molecules for motoneurons: an embarrassment of riches. *Neuron* 17: 195–7, 1996.
5. Segal, R. A. Selectivity in neurotrophin signaling: theme and variations. *Annu. Rev. Neurosci.* 26: 299–330, 2003.
6. Huang, E. J. and L. F. Reichardt, Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* 72: 609–642, 2003.
7. Brunet, A., Datta, S. R. and Greenberg, M. E. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr. Opin. Neurobiol.* 11: 297–305, 2001.
8. Markus, A., Patel, T. D. and Snider, W. D. Neurotrophic factors and axonal growth. *Curr. Opin. Neurobiol.*, 12: 523–531, 2002.

9. Sofroniew, M. V., Howe, C. L. and Mobley, W. C. Nerve growth factor signaling, neuroprotection, and neural repair. *Annu. Rev. Neurosci.* 24: 1217–1281, 2001.
10. Barker, P. A. p75NTR is positively promiscuous: novel partners and new insights. *Neuron* 42: 529–533, 2004.
11. Snider, W. D. Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* 77: 627–638, 1994.
12. Schinder, A. F. and Poo, M. The neurotrophin hypothesis for synaptic plasticity. *Trends Neurosci.* 23: 639–645, 2000.
13. Lu, B. BDNF and activity-dependent synaptic modulation. *Learn. Mem.* 10: 86–98, 2003.
14. Taga, T. and Kishimoto, T. Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* 15: 797–819, 1997.
15. Ip, N. Y. and Yancopoulos, G. D. The neurotrophins and CNTF: two families of collaborative neurotrophic factors. *Annu. Rev. Neurosci.* 19: 491–515, 1996.
16. Oppenheim, R. W., Wiese, S. and Prevette, D. *et al.* Cardiotrophin-1, a muscle-derived cytokine, is required for the survival of subpopulations of developing motoneurons. *J. Neurosci.* 21: 1283–91, 2001.
17. Wang, J., Asensio, V. C. and Campbell, I. L. Cytokines and chemokines as mediators of protection and injury in the central nervous system assessed in transgenic mice. *Curr. Top. Microbiol. Immunol.* 265: 23–48, 2002.
18. Dono, R. Fibroblast growth factors as regulators of central nervous system development and function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284: R867–R881, 2003.
19. Krieglstein, K., Strelau, J., Schober, A., Sullivan, A. and Unsicker, K. TGF-beta and the regulation of neuron survival and death. *J. Physiol. Paris* 96: 25–30, 2002.
20. Pratt, B. M. and McPherson, J. M. TGF-beta in the central nervous system: potential roles in ischemic injury and neurodegenerative diseases. *Cytokine Growth Factor Rev.* 8: 267–292, 1997.
21. Airaksinen, M. S. and Saarma, M. The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* 3: 383–394, 2002.
22. Baloh, R. H., Enomoto, H., Johnson, E. M. Jr and Milbrandt, J. The GDNF family ligands and receptors – implications for neural development. *Curr. Opin. Neurobiol.* 10: 103–110, 2000.
23. Sariola, H. and Saarma, M. Novel functions and signalling pathways for GDNF. *J. Cell Sci.* 116: 3855–3862, 2003.
24. Falls, D. L. Neuregulins: functions, forms, and signaling strategies. *Exp. Cell Res.* 284: 14–30, 2003.
25. Buonanno, A. and Fischbach, G. D. Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr. Opin. Neurobiol.* 11: 287–296, 2001.
26. Anderson, M. F., Aberg, M. A., Nilsson, M. and Eriksson, P. S. Insulin-like growth factor-I and neurogenesis in the adult mammalian brain. *Brain Res. Dev. Brain Res.* 134: 115–122, 2002.

CHAPTER

28

Axonal Transport

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NEURONAL ORGANELLES IN MOTION

The axon comprises a major portion of the total volume and surface area in most neurons and may extend several thousand cell body diameters. Since the genetic material and nearly all the protein synthesis machinery are localized to the cell body, a supply line is maintained to provide structural and functional materials to sites all along the length of the axon. Insights as to how neurons accomplish this task can be obtained by real-time imaging of living axons with video-enhanced light microscopy [1] (Fig. 28-1).

Such video images reveal an array of organelles moving down the axon toward the nerve terminal (anterograde direction) as well as returning to the cell body (retrograde direction). The movements create patterns as engrossing as the ant farms of our childhood and initially appear as chaotic. Some organelles glide smoothly, while others move in fits and starts. On closer examination, an underlying order emerges: the organelles moving in the anterograde direction are typically fainter and smaller but more numerous than those moving in the retrograde direction, and all organelles appear to travel along gently curving fibrils. Occasionally, two organelles are seen to travel in opposite directions along the same fibril, appearing destined for a head-on collision but seeming to pass through each other; other organelles hop from one fibril to another. The images imply, and other studies confirm, that the

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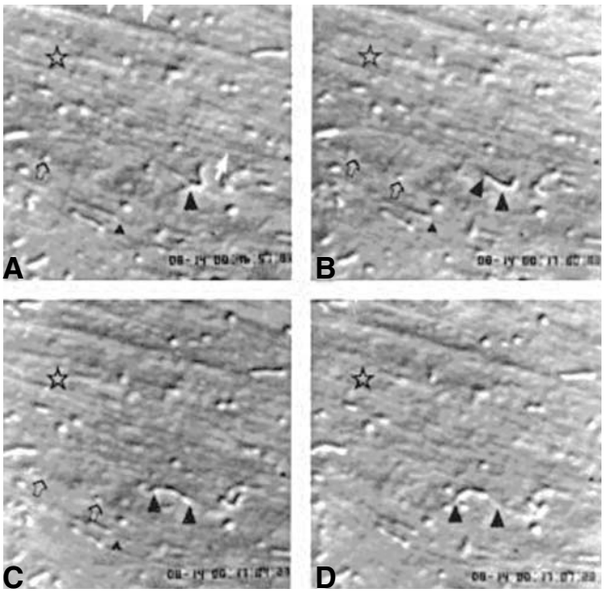

FIGURE 28-1 Sequential video images of fast axonal transport in isolated axoplasm from the squid giant axon. In this preparation, anterograde axonal transport proceeds in the direction from upper left to lower right (from 10 o'clock toward 4 o'clock). The field of view in these stills is approximately 20 µm, and the images were recorded in real time on videotape. The large, sausage-shaped structures (*filled triangles*) are mitochondria. Medium-sized particles (open arrows) most often move in the retrograde (right to left) direction. Most structures of this size are lysosomal or prelysosomal organelles. The majority of moving particles in these images are faint and moving rapidly (2 µm/s), so they are difficult to catch in still images; however, in the region above the star, a number of these organelles can be visualized in each panel. The entire field contains faint parallel striations (such as those indicated by the white arrows in (**A**)) that correspond to the cytoskeleton of the axoplasm, primarily microtubules. The movement of membranous organelles is along these structures, although organelles can occasionally be seen to switch tracks as they move (see the mitochondrion indicated by *large triangles*). (With permission from Brady, S. T., Lasek, R. J. and Allen, R. D. Fast axonal transport in extruded axoplasm from squid giant axon. *Science* 218: 1129–1131, 1982.)

organelles represent membrane-bound packets of materials *en route* to a variety of intraneuronal destinations. Unseen in these images because their movements and changes occur orders of magnitude more slowly, structural elements of the axon, the cytoskeleton, are equally dynamic.

The life cycle of these organelles, their kinetics and molecular cargo, the molecular motors driving their transport and the substrates along which these movements track constitute interrelated aspects of what is broadly termed axonal transport. A primary aim of this chapter is to provide an understanding of this form of intraneuronal traffic. Achieving this goal requires an appreciation of the dynamics and structure of the relevant neuronal components and structures. Studies of how cellular structures and components move from where they are synthesized to where they are utilized comprise an area of intensive research in cellular and molecular neurobiology. To encompass this topic, we examine how our concepts of axonal transport evolved to our present understanding of this complex and dynamic field.

DISCOVERY AND CONCEPTUAL DEVELOPMENT OF FAST AND SLOW AXONAL TRANSPORT

The size and extent of many neurons presents a special set of challenges. Since protein synthesis for the entire neuron takes place in the cell body, which may represent only 0.1% of the total cell volume, growth and maintenance of neuronal processes requires timely, efficient delivery of material to axonal and dendritic domains. The idea that materials must be transferred from cell body to axon was suggested by Ramon y Cajal and other pioneers during the early part of this century. For many years, the existence of such transport processes could only be inferred.

The first experimental evidence for axonal transport resulted from studies on peripheral nerve regeneration, which were stimulated by the desire to improve treatment of limb injuries sustained during World War II. In the classic work of Weiss and Hiscoe [2], surgical constriction of a sciatic nerve branch led to morphological changes in the nerve that directly implicated the cell body as the source of materials for axon regrowth. After several weeks, the axon appeared swollen proximal to the constriction but shriveled on the distal side. Following removal of the constriction, a bolus of accumulated axoplasm slowly moved down the nerve at 1–2 mm/day, very nearly the rate observed for outgrowth of a regenerating nerve. Weiss and Hiscoe concluded that the cell body supplies a bulk flow of material to the axon. This view dominated the field for two decades, but the characteristics of this slow 'flow' of material did not seem adequate to explain some aspects of nerve growth and function.

Cell biologists subsequently provided convincing arguments for the necessity of this intracellular transport. Neuronal protein synthesis was almost completely restricted to the cytoplasm surrounding the nucleus (translational cytoplasm, which includes polysomes, rough endoplasmic reticulum and the Golgi complex) and ribosomes were undetectable in the axon [3]. If proteins cannot be synthesized in the axon, then materials necessary for axonal function have to be supplied by transport from the cell body. Thus, axonal transport must be a normal, ongoing process in neurons. By the mid-1960s, the use of radioactive tracers confirmed the existence of a slow 'bulk flow' component of transport. Using autoradiography, Droz and Leblond [4] elegantly showed that systemically injected ^{3}H-amino acids were incorporated into nerve cell proteins and transported along the sciatic nerve as a wavefront of radioactivity. These methods demonstrated that newly synthesized proteins were transported,

TABLE 28-1 Major rate components of axonal transports

Rate component	Rate (mm/day)	Structures and composition
Fast transport		
Anterograde	200–400	Small vesiculotubular structures, neurotransmitters, membrane proteins and lipids
Mitochondria	50–100	Mitochondria
Retrograde	200–300	Lysosomal vesicles and enzymes
Slow transport		
SCb	2–8	Microfilaments, metabolic enzymes, clathrin complex
SCa	0.2–1	Neurofilaments and microtubules

but some responses of the neuron occurred too rapidly to be readily explained solely by a slow 'flow'.

Shortly thereafter radiolabeling and histochemical studies demonstrated that faster rates of transport occur [5]. Unlike slow transport, the faster components move material bidirectionally, both toward and away from the cell body. Both endogenous proteins and exogenously applied labels were detected moving at fast transport rates. These findings expanded the concept that axonal transport materials move in both anterograde and retrograde directions and that rates vary by as much as three orders of magnitude (Table 28-1).

At first, emphasis was placed on the characterization of fast and slow axonal transport. The kinetics of axonal transport were analyzed by injection of radiolabeled amino acids into the vitreous of the eye or the dorsal root ganglia (to 'label' sensory neurons) or ventral spinal cord (motor neurons). In the case of fast transport, a wavefront of labeled protein was detected traveling away from the cell body at 250–400 mm/day in mammals. Using this same approach, slow transport rates were shown to approximate 1 mm/day. Rates for fast transport were also determined by measuring the amount of a transported substance, such as acetylcholinesterase or norepinephrine, accumulating at a nerve constriction over a few hours, well before bulk accumulation of axoplasm is detectable. These two approaches for studying axonal transport – locating a radiolabeled wavefront by analysis of successive nerve segments and monitoring the accumulation of materials at a constriction with time (Fig. 28-2) – generated considerable information on the kinetics and on the metabolic and ionic requirements of axonal transport [5]. Such findings formed the basis for more detailed characterization of the axonally transported materials and for studies of the underlying molecular mechanisms.

Fast and slow components of axonal transport differ in both their constituents and their rates. Fast transport is bidirectional: many proteins that are distributed by fast

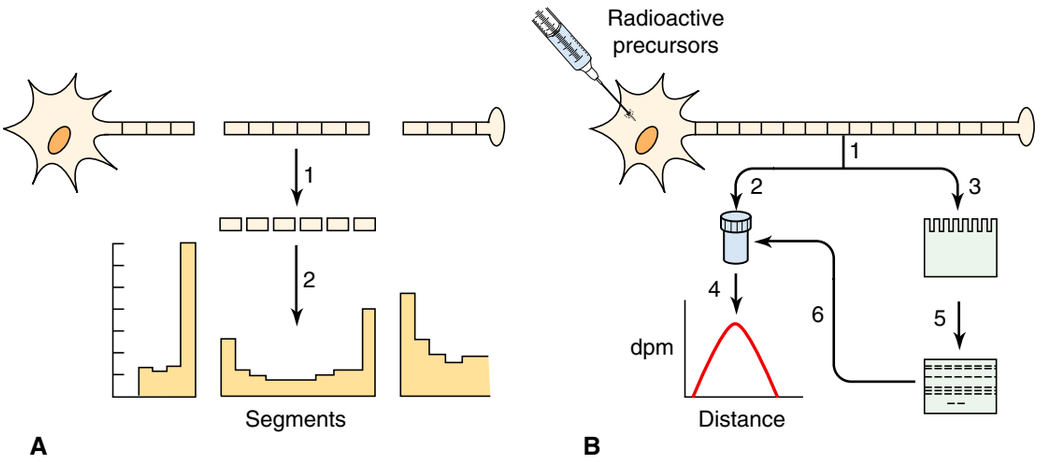

FIGURE 28-2 Schematic diagram of two common methods for analyzing the component rates of axonal transport. (**A**) Accumulation of transported material can be studied at a focal block of axonal transport caused by a cut, a crush, a cold block or a ligature. This approach is a variation of that employed by Weiss and Hiscoe and has been used often in studies of fast axonal transport. In this example, two cuts have been made in order to detect both anterograde and retrograde transport. After time for accumulation at the ends, the nerve segments are cut into uniform segments for analysis (*step 1*). Each segment is analyzed either for radioactivity in labeled nerves or for enzyme activity, and the rate of accumulation is estimated (*step 2*). (**B**) With segmental analysis, the nerve must be pulse-labeled, usually with radioactive precursors. After an appropriate injection-sacrifice interval to label the rate component of interest, the nerve is also cut into segments (*step 1*). In some cases, only a single segment is used as a 'window' onto the transport process. Each segment is analyzed both by counting the radioactivity in an aliquot (*step 2*) and by gel electrophoresis (*step 3*), where each lane corresponds to a different segment. The amount of radioactivity in different polypeptides can be visualized with fluorography (*step 5*) and individual bands cut out of the gel (*step 6*) for analysis by liquid scintillation counting. The distribution of either total radioactivity or radioactivity associated with a specific polypeptide can then be plotted (*step 4*); *dpm*, disintegrations per minute. (Adapted with permission from Brady, S. T. Axonal transport methods and applications. In A. Boulton and G. Baker (eds), *Neuromethods*, vol. 1: *General Neurochemical Techniques*. Clifton, NJ: Humana Press, 1986, pp. 419–476.)

anterograde transport also return in the retrograde direction. In contrast, proteins transported at slow rates are degraded when they reach their destination and are not detected in the retrograde component. Biochemical fractionation studies have shown that proteins moved by fast anterograde and retrograde transport are predominantly membrane-associated, whereas proteins moved in the slow axonal transport component are recovered in soluble fractions or in cytoskeletal pellets.

When labeled polypeptides traveling down the axon are analyzed by SDS polyacrylamide gel electrophoresis, materials traveling in the axon can be grouped into five distinct rate components [6]. Each rate component is characterized by a unique set of polypeptides moving coherently down the axon (Fig. 28-3). As specific polypeptides associated with each rate class were identified, most were seen to move only within a single rate component. Moreover, proteins that have common functions or interact with each other tend to be moved together. These observations led to a new view of axonal transport, the structural hypothesis [7]. This model can be stated simply: proteins and other molecules move down the axon as component parts of discrete subcellular structures rather than as individual molecules (Table 28-1).

The structural hypothesis, which was formulated in response to observations that axonal transport rate components move as discrete waves, each with a characteristic rate and a distinctive composition, can explain the coherent transport of functionally related proteins and is consistent with the relatively small numbers of motor molecules in neurons. The only assumption is that the number of elements that can interact with transport motor complexes is limited, and this requires appropriate packaging of the transported material. Different rate components result from packaging of transported material into different, cytologically identifiable, structures. In fact, the faster rates reflect the transport of proteins preassembled as membranous organelles, including vesicles and mitochondria, or of proteins contained in the lumen of these organelles (Fig. 28-4) whereas the slower rate components contain the cytoskeletal proteins. Thus tubulin and MAPs move as microtubules, and neurofilaments move as neurofilaments. Cytoplasmic proteins that are not integral components of a cytoskeletal element may be linked in some manner to those structures (Fig. 28-5). While disputes regarding the size and composition of the transported package for cytoskeletal and cytoplasmic proteins (i.e. polymer, oligomer, macromolecular complex, etc.) continue, the idea that complexes of proteins, rather than individual polypeptides, are moved has gained general currency.

Although five distinct major rate components have been identified, the original categories of fast and slow transport remain useful. All membrane-associated proteins move in one of the fast rate components, while cytoplasmic proteins move as part of the slow components. Current studies indicate that the various organelles transported anterogradely are moved along the axon by one or more motor molecules (see below). The differing rates of fast anterograde transport appear to result from the varying sizes of organelles, with increased drag on larger structures resulting in a slower net movement (Fig. 28-1). Less is known about molecular mechanisms of slow transport.

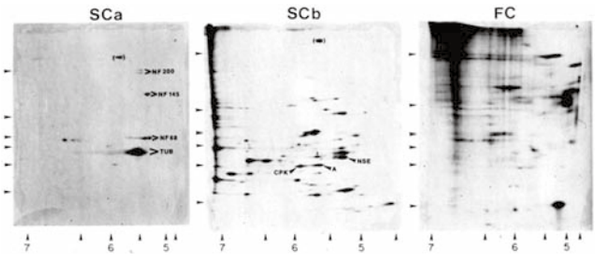

FIGURE 28-3 Two-dimensional fluorographs showing the 35S methionine-labeled polypeptides in the three major anterograde rate components of axonal transport: *SCa*, slow component a; *SCb*, slow component b; *FC*, fast component. Note that rate component not only has a characteristic rate but a characteristic polypeptide composition. The discovery that each rate component has a different polypeptide composition led to the structural hypothesis. (With permission from Tytell, M. *et al. Science* 214: 179–181, 1981 [6]; illustration provided by Dr. Michael Tytell.)

Features of fast axonal transport demonstrated by biochemical and pharmacological approaches are apparent from video images. Video microscopy of axoplasm, as described briefly at the beginning of this chapter, directly confirmed the bidirectionality of fast transport, which had been inferred from the accumulation of radiolabeled materials on both sides of a crush, and established that the populations of organelles moving in each direction are different. Inhibition of transport by agents that disrupt microtubules (MTs) is consistent with movement of organelles along fibrils identified as MTs by correlated video and electron microscopy. Video microscopy also reveals that organelle movement can continue in an apparently normal fashion in axons isolated from their cell bodies and divested of a plasma membrane. The implication is that transport must be driven by local energy-generating mechanisms, as predicted from observations that application of a cold block or metabolic poison (dinitrophenol or cyanide) to a discrete region of a nerve inhibits transport locally [5].

FAST AXONAL TRANSPORT

Newly synthesized membrane and secretory proteins destined for the axon travel by fast anterograde transport. However, not all membrane-bounded organelles (MBOs) are destined for the axon. As a result, the first stage of transport must be synthesis, sorting and packaging of organelles (see Ch. 9). Once assembled, the organelle must then be committed to the transport

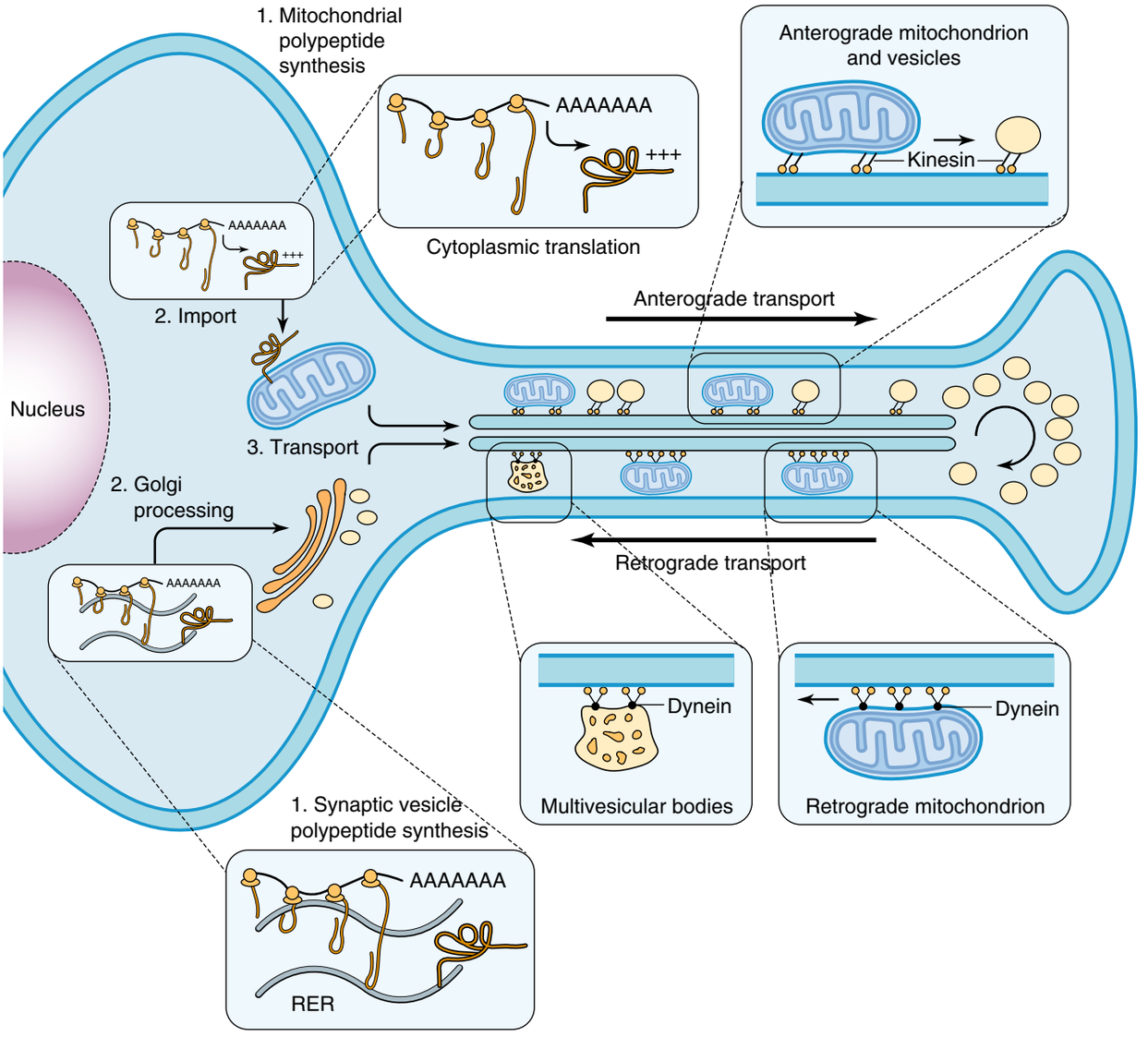

FIGURE 28-4 Schematic illustration of the movement of membrane-associated material in fast axonal transport. Fast axonal transport represents the movement of membrane-bounded organelles along axonal microtubules in both the anterograde and retrograde directions. Two major classes of membrane-bounded organelles that are synthesized and packaged by different pathways are depicted. Synaptic vesicle polypeptides are translated on endoplasmic reticulum-bound ribosomes at which time membrane proteins become properly oriented within the lipid bilayer and secretory polypeptides enter into the lumen of the endoplasmic reticulum. These polypeptides are further processed within the Golgi apparatus where the appropriate post-translational modifications and sorting of polypeptides destined for the axon occur. Once these polypeptides are packaged into vesicular organelles and the appropriate motor molecules are present, the organelles are transported down the axon utilizing axonal microtubules as 'tracks' at rates of 200–400 mm/day. Movement in the anterograde direction is thought to be mediated by the molecular motor kinesin, while the force necessary to move retrograde organelles is thought to be generated by cytoplasmic dynein. Unlike the synthesis of vesicular polypeptides, mitochondrial polypeptides that are supplied by the host cell are synthesized on cytoplasmic ribosomes and contain a targeting sequence that directs the polypeptides to the mitochondria. Following assembly and the association of motor molecules, the mitochondria move down the axon at rates of 50–100 mm/day. Mitochondria can also be detected moving back toward the cell body in the retrograde direction. The morphology of retrogradely transported mitochondria is distinctly different from that of mitochondria moving in the anterograde direction and is believed to represent degenerating organelles that are not metabolically active.

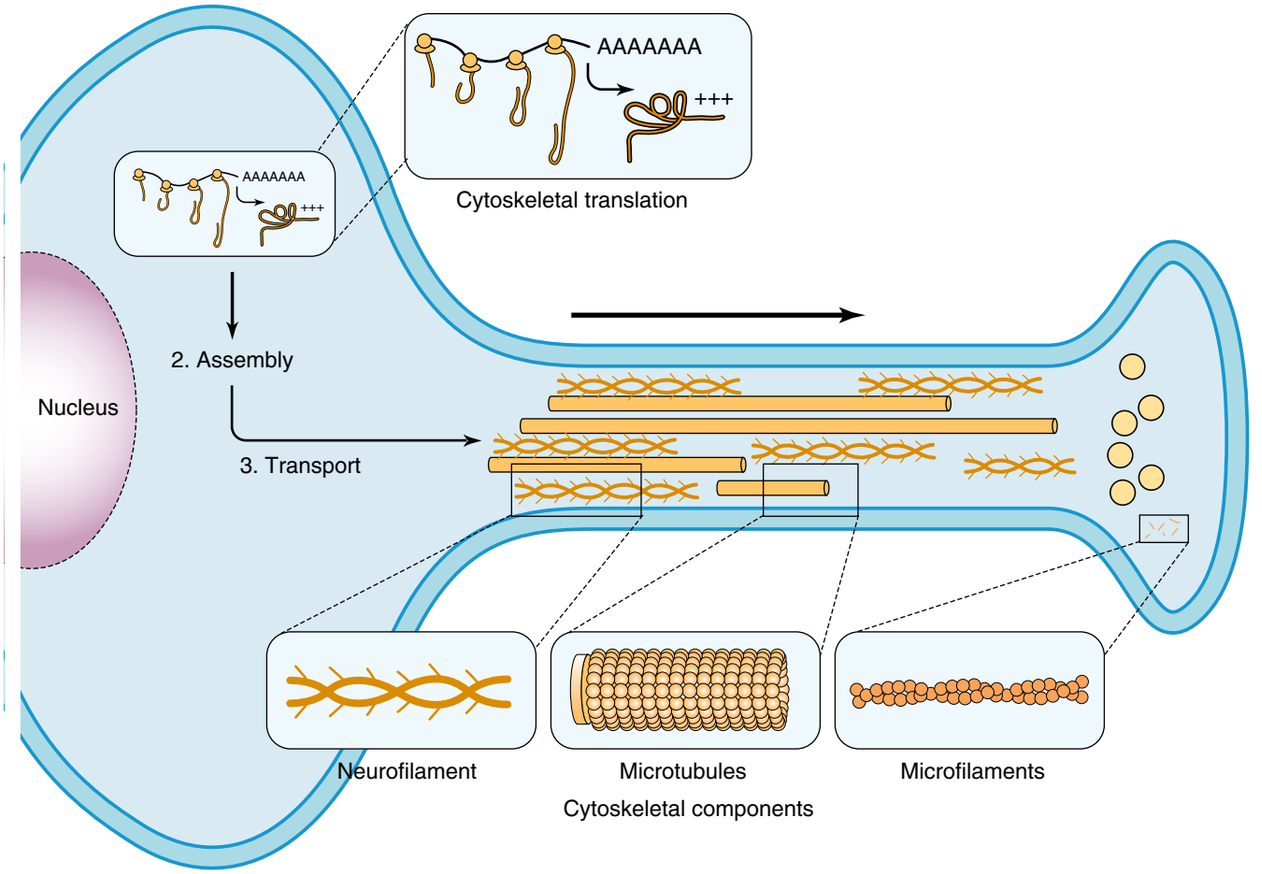

FIGURE 28-5 Schematic illustration of the movement of cytoskeletal elements in slow axonal transport. Slow axonal transport represents the movement of cytoplasmic constituents including cytoskeletal elements and soluble enzymes of intermediary metabolism at rates of 0.2–2 mm/day which are at least two orders of magnitude slower than those observed in fast axonal transport. As proposed in the structural hypothesis and supported by experimental evidence, cytoskeletal components are believed to be transported down the axon in their polymeric forms, not as individual subunit polypeptides. Cytoskeletal polypeptides are translated on cytoplasmic polysomes and then are assembled into polymers prior to transport down the axon in the anterograde direction. In contrast to fast axonal transport, no constituents of slow transport appear to be transported in the retrograde direction. Although the polypeptide composition of slow axonal transport has been extensively characterized, the motor molecule(s) responsible for the movement of these cytoplasmic constituents has not yet been identified.

machinery and moved down the axon. Finally, organelles must be targeted and delivered to specific domains in the axon (presynaptic terminals, axolemma, nodes of Ranvier, etc.). Axonal constituents include integral membrane proteins, secretory products, membrane phospholipids, cholesterol and gangliosides. As predicted by the structural hypothesis and as apparent in video microscopy, rapid transport is achieved after packaging materials into MBOs (Figs 28-4, 28-6). Clearly, an understanding of how MBOs are formed in the cell body and routed to the fast-transport system in axons is essential.

Passage through the Golgi apparatus is obligatory for most proteins destined for fast transport. In all cell types, secretory and integral membrane proteins are synthesized on polysomes bound to the endoplasmic reticulum. Secretory proteins enter the lumen of the reticulum, whereas membrane proteins become oriented within the membrane bilayer. In contrast, components of the cytoskeleton and enzymes of intermediary metabolism are synthesized on so-called free polysomes, which are actually associated with the cytoskeleton. As reviewed in Chapter 9, fast-transported proteins leave the endoplasmic reticulum in association with transfer vesicles that bud off and undergo Ca^{2+}-dependent fusion with the Golgi apparatus. Newly formed membrane-associated proteins must be transferred to the Golgi apparatus for processing and post-translational modification, including glycosylation, sulfation and proteolytic cleavage, as well as for sorting [8].

Drug studies demonstrated a requirement that most proteins destined for fast axonal transport traverse the Golgi stacks, where membrane proteins are post-translationally modified, sorted and packaged [9] (Fig. 28-7). This suggests that proteins in fast axonal transport must either pass through the Golgi complex or associate with

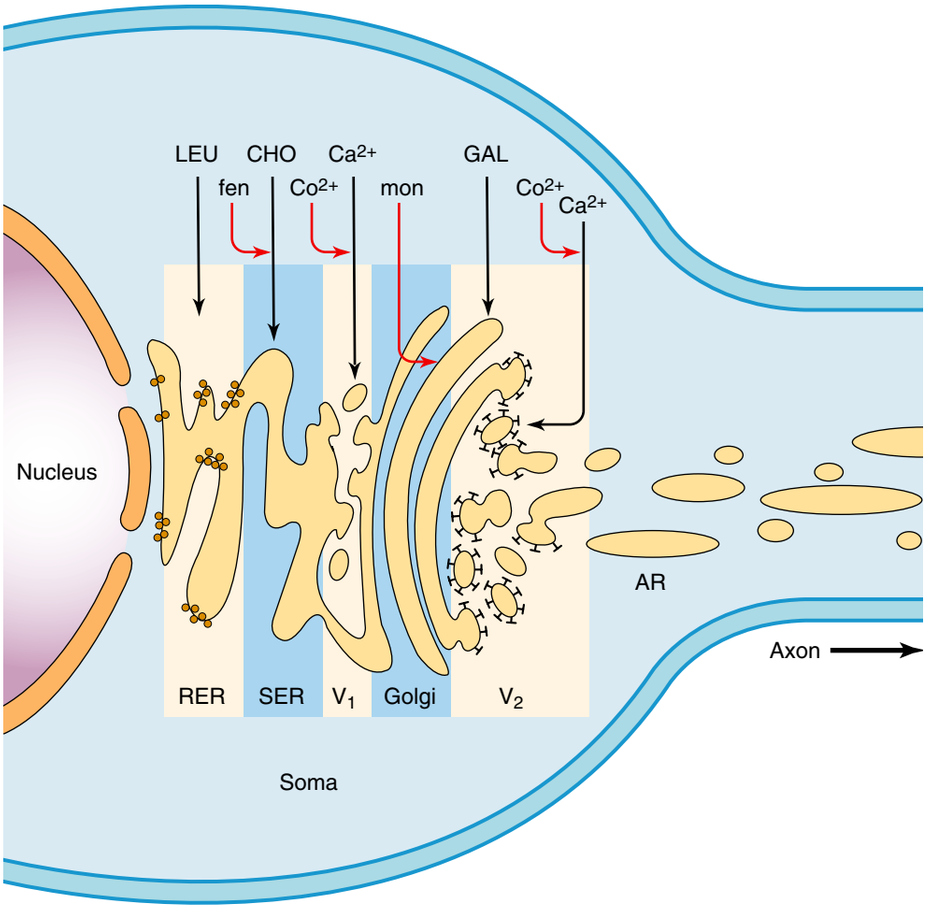

FIGURE 28-6 Summary of pharmacological evidence indicating that newly synthesized membrane and secretory proteins in neurons reach the axons by a pathway similar to that utilized for intracellular transport in non-neuronal cells. Incorporation sites are indicated for several precursors of materials in fast axonal transport: leucine (*LEU*) for proteins, choline (*CHO*) for phospholipids and galactose (*GAL*) for glycoproteins and glycolipids. Sites of action for several inhibitors are also indicated, including fenfluramine (*fen*), monensin (*mon*) and Co^{2+}. One possible site for Ca^{2+}-mediated vesicle fusion is at the transition from rough endoplasmic reticulum (*RER*) and smooth endoplasmic reticulum (*SER*) to the Golgi apparatus via one type of transition vesicle (*V1*). The subsequent budding of vesicles (*V2*) off the Golgi apparatus, presumably mediated by clathrin-coated vesicles, is a second site for Ca^{2+} involvement. The microtubule guides for the axonal tubulovesicular structures in transport and the axoplasmic reticulum (*AR*) are not shown.

proteins that do. Clathrin-coated vesicles mediate transfer from the Golgi apparatus to fast axonal transport system [10] (Fig. 28-6). Coated vesicles, however, are rarely observed in axons, and clathrin, the major coat protein, is primarily a slow transport protein [3]. Thus, Golgi-derived coated vesicles shed their coats prior to undergoing fast transport and travel down the axon either as individual uncoated vesicles or other membrane-bounded structures (Fig. 28-6).

Transport of MBOs to their various destinations is typically mediated by MTs and motor molecules. Membrane and secretory proteins become associated with membranes either during or immediately following their synthesis, and then maintain this association throughout their lifetime in the cell. For example, inhibiting synthesis of either protein or phospholipid leads to a proportional decrease in the amount of both protein and phospholipid in fast-transport, whereas application of these inhibitors to axons has no effect on transport. This suggests that fast axonal transport depends on *de novo* synthesis and assembly of membrane components.

Anterograde transport moves synaptic vesicles, axolemmal precursors and mitochondria down the axon. Fast anterograde transport represents movement of MBOs along MTs away from the cell body at rates ranging in mammals from 200–400 mm/day or 2–5 μm/second [11]. Anterograde transport provides newly synthesized components essential for neuronal membrane function and maintenance. Ultrastructural studies have demonstrated that the material moving in fast anterograde transport includes many small vesicles and tubulovesicular structures as well as mitochondria and dense-core vesicles [12, 13]. Material in fast anterograde transport

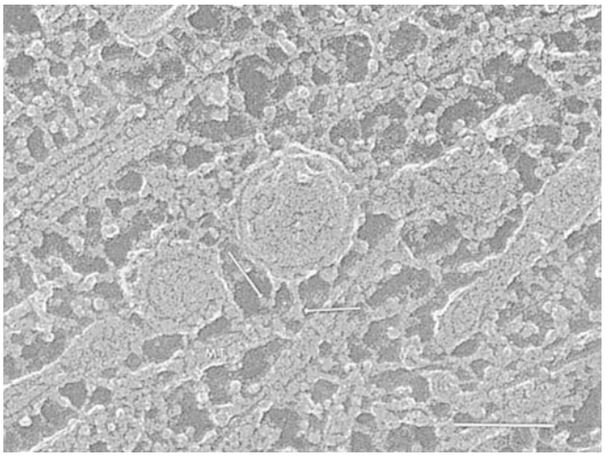

FIGURE 28-7 Axonally transported vesicles and the axonal cytoskeleton in longitudinal section. Quick-freeze, deep etch electron micrograph of a region of rat spinal cord neurite rich in membrane-bounded organelles and microtubules. Arrows point to rod-shaped structures that appear as cross bridges between organelles and microtubules. The bar at the lower right indicates 100 nm. (From Hirokawa *et al.*, Cell 56: 867–878, 1989 [47].)

is needed for supply and turnover of intracellular membrane compartments (i.e. mitochondria and endoplasmic reticulum), secretory products and proteins required for the maintenance of axonal metabolism. The net rate appears to be largely determined by size with the smallest MBOs in almost constant motion, while mitochondria and larger structures frequently pause giving a lower average rate [14].

A variety of materials move in fast anterograde transport including membrane-associated enzymes, neurotransmitters, neuropeptides and membrane lipids. Most are synthesized in the cell body and transported intact, but some processing events occur in transit. For example, neuropeptides may be generated by proteolytic degradation of propeptides. This biochemical heterogeneity extends to the MBOs themselves. The population of small organelles is particularly varied in function and composition. Some correspond to synaptic vesicle precursors and contain neurotransmitters and associated proteins, while others may contain channel proteins or other materials destined for the axolemma. Biochemical and morphological studies have provided a description of the materials transported in fast transport but are not as well suited for identifying the underlying molecular mechanisms involved in translocation.

Video microscopy allows study of molecular mechanisms through direct observation of organelle movements while precise control of experimental conditions is maintained. Fast axonal transport continues unabated in isolated axoplasm from giant axons of the squid *Loligo pealeii* for hours [14]. Video microscopy applied to isolated axoplasm permits a more rigorous dissection of the molecular mechanisms for fast axonal transport through biochemical and pharmacological approaches, because isolated axoplasm has no plasma membrane or other permeability barriers. Such studies have extended earlier observations on properties of fast anterograde transport and demonstrated the existence of new motor proteins that are responsible for axoplasmic transport of MBOs (see below).

Retrograde transport returns trophic factors, exogenous material and old membrane constituents to the cell body. MBOs moving in retrograde transport are structurally heterogeneous and, on average, larger than the structures observed in anterograde transport, which are commonly tubulovesicular [12, 13]. Multivesicular or multilamellar bodies are common in retrograde transport and these are thought to represent materials to be delivered to lysosomes in the cell body. The larger size of these retrograde vectors affects the rate of transport by increasing drag due to interactions with cytoplasmic structures [14].

Both morphological and biochemical studies indicate that MBOs returning in retrograde transport differ from those in anterograde transport. Repackaging of membrane components apparently accompanies turnaround or conversion from anterograde to retrograde transport. The mechanisms of repackaging are incompletely understood, but certain protease inhibitors and neurotoxic agents will inhibit turnaround without affecting either anterograde or retrograde movement. Experiments involving the use of specific protease inhibitors implicate a thiol protease, but the identity of the responsible enzyme is unknown [15, 16]. Consistent with this proposal, protease treatment of purified synaptic vesicles affects directionality of their movements in axoplasm and presynaptic terminals. Exogenously injected fluorescent synaptic vesicles normally move in the anterograde direction, but protease pretreatment of these vesicles results in retrograde transport [17].

Uptake of exogenous materials by endocytosis in distal regions of the axon results in the return of trophic substances and growth factors to the cell body [18]. These factors assure survival of the neuron and modulate neuronal gene expression. Changes in the return of trophic substances play critical roles during development and regeneration of neurites. Retrograde transport of exogenous substances also provides a pathway for viral agents to enter the CNS. Once retrogradely transported material reaches the cell body, the cargo may be delivered to the lysosomal system for degradation, to nuclear compartments for regulation of gene expression, or to the Golgi complex for repackaging.

Molecular sorting mechanisms ensure delivery of proteins to discrete membrane compartments. While pathways by which selected membrane-associated proteins are delivered to the correct destination have been established in general terms, many intriguing questions

regarding the selection process itself remain. How is it that certain membrane proteins remain in the cell body (for example, glycosyltransferases of the Golgi) while others are packaged for delivery to the axon? Among transported proteins, how do some reach the axolemma (i.e. sodium and potassium channels) while others travel the length of the axon to the nerve terminal (a presynaptic receptor or synaptic vesicle) or enter the synaptic cleft (a secreted neuropeptide)? Finally, how are organelles such as the synaptic vesicle directed toward axons and presynaptic terminals but not into dendritic arbors? This question becomes particularly compelling for dorsal root ganglion sensory neurons, where the central branch of its single axon has presynaptic terminals while the peripheral branch of that same axon has none.

The answers to these questions remain incomplete, but some mechanisms have begun to emerge. Some information comes from studies on polarized epithelial cells where the identity of destination signals to deliver newly synthesized proteins selectively to basolateral or apical membranes can be assayed. These mechanisms are relevant to the neuron, because viral proteins that normally go to epithelial basolateral membranes end up in neuronal dendritic compartments, while those targeted to apical compartments may be moved into the axon [19]. However, the underlying mechanisms appear to be complex. Signals may be 'added on', as post-translational modifications including glycosylation, acylation or phosphorylation or 'built in', in the form of discrete amino acid sequences. Both mechanisms appear to operate in cells. For example, addition of mannose-6-phosphate to proteins directs them to lysosomes, while amino acid sequences have been identified that direct proteins into the nucleus or into mitochondria. In general, the targeting signals are likely to direct proteins to specific organelles, whereas other mechanisms direct organelles to appropriate final destinations.

Specific membrane components must be delivered to their sites of utilization and not left at inappropriate sites [3]. Synaptic vesicles and other materials needed for neurotransmitter release should go to presynaptic terminals because they serve no function in an axon or cell body. The problem is compounded because many presynaptic terminals are not at the end of an axon. Often, numerous terminals occur sequentially along a single axon, making en passant contacts with multiple targets. Thus, synaptic vesicles cannot merely move to the end of axonal MTs. Targeting of synaptic vesicles thus becomes a more complex problem. Similar complexities arise with membrane proteins destined for the axolemma or a nodal membrane.

One proposed mechanism for targeting of organelles to terminals might have general implications. The synapsin family of phosphoproteins [20], which is concentrated in the presynaptic terminal, may be involved in targeting synaptic vesicles. Dephosphorylated synapsin binds tightly to both synaptic vesicles and actin microfilaments (MFs), while phosphorylation releases both of them. Dephosphorylated synapsin inhibits axonal transport of MBOs in isolated axoplasm, while phosphorylated synapsin at similar concentrations has no effect [21]. When a synaptic vesicle passes through a region rich in dephosphorylated synapsin, it may be cross-linked to the available MF matrix by synapsin. Such cross-linked vesicles would be removed from fast axonal transport and are effectively targeted to a synapsin- and MF-rich domain, the presynaptic terminal.

More recent studies have begun to map additional pathways for regulation of fast axonal transport based on selective, localized activation of kinase and phosphatase activities within neurons. Some of these phosphotransferase activities have been shown to target specific molecular motors and to regulate some aspect of their functional activities [22]. For example, activation of the GSK-3 kinase in axons leads to phosphorylation of the motor protein kinesin (see below) and this event results in detachment of kinesin from its transported cargo [23]. Other phosphotransferases and phosphotransferase-associated proteins appear to modulate fast axonal transport indirectly, by regulating the activities of enzymes, which in turn modify molecular motors. In this way, molecular pathways leading to the regulation of fast axonal transport have started to be identified [24].

Finally, this section has focused almost entirely on axonal transport, but dendritic transport also occurs [25]. Since dendrites usually include postsynaptic regions while most axons terminate in presynaptic elements, the dendritic and axonal transport each receive a number of unique proteins. An added level of complexity for intraneuronal transport phenomena is the intriguing observation that mRNA is routed into dendrites where it is implicated in local protein synthesis at postsynaptic sites, but ribosomal components and mRNA are largely excluded from axonal domains [26]. Regulation of protein synthesis in dendritic compartments is an important mechanism is synaptic plasticity [27, 28]. The importance of dendritic mRNA transport and local protein synthesis is underscored by the demonstration that the mutation associated with Fragile X syndrome affects a protein important for transport and localization of mRNA in dendrites [27, 29]. Similar processes of mRNA transport have been described in glial cells [30].

SLOW AXONAL TRANSPORT

Cytoplasmic and cytoskeletal elements move coherently at slow transport rates. Two major rate components have been described for slow axonal transport, representing movement of cytoplasmic constituents including cytoskeletal elements and soluble enzymes of intermediary metabolism [3]. Cytoplasmic and cytoskeletal elements in axonal transport move with rates at least two orders of magnitude slower than fast transport.

In favorable systems, the coherent movement of neurofilaments and microtubule proteins provides strong evidence for the structural hypothesis. Striking evidence was provided by pulse-labeling experiments in which NF proteins moved over periods of weeks as a bell-shaped wave with little or no trailing of NF protein. Similarly, coordinated transport of tubulin and MAPs makes sense only if MTs are being moved, since MAPs do not interact with unpolymerized tubulin [31].

Slow component a (SCa) comprises largely the cytoskeletal proteins that form NFs and MTs. Rates of transport for SCa proteins in mammalian nerve range from 0.2–0.5 mm/day in optic axons to1 mm/day in motor neurons of the sciatic nerve, and can be even slower in poikilotherms such as goldfish. Although the polypeptide composition of SCa is relatively simple, the relative contribution of SCa to slow transport varies considerably. For large axons (e.g. alpha motor neurons in the sciatic nerve), SCa is a large fraction of the total protein in slow transport, while the amount of material in SCa is relatively reduced for smaller axons (i.e. optic axons) [32]. The amount and phosphorylation state of SCa protein in axons is the major determinant of axonal diameter.

Slow component b (SCb) represents a complex and heterogeneous rate component, including hundreds of distinct polypeptides ranging from cytoskeletal proteins such as actin (and tubulin in some nerves, see [32]) to soluble enzymes of intermediary metabolism (such as the glycolytic enzymes). The structural correlate of SCb is not as easily identified as the MTs and NFs of SCa. Actin is presumed to form MFs, but actin represents only 5–10% of the protein in SCb and a significant fraction of axonally transport actin is deposited in the membrane cytoskeleton of the axon. Most proteins in SCb may be assembled into labile aggregates that can interact transiently with the cytoskeleton. One possibility is that scaffolding polypeptides such as HSC70, a constitutively expressed member of the heat shock family, may serve to organize these complexes during transport [3].

Although these two rate components can be identified in all nerves examined to date, the rates and the precise compositions varies among nerve populations. For example, SCa and SCb are readily resolved as discrete waves moving down optic axons, but differences in rate are smaller in the motor axons of sciatic nerve so the two peaks overlap. Moreover, virtually all tubulin moves as a single peak in SCa in optic axons, but significant amounts of tubulin move at both SCa and SCb rates in sciatic motor axons [32]. In each nerve, certain polypeptides may be used to define the kinetics for a given slow component of axonal transport. For SCa, those signature polypeptides are the NF triplet proteins, while actin, clathrin and calmodulin serve a similar role for SCb.

Axonal growth and regeneration are limited by rates of slow axonal transport. The rate of axonal growth during development and regeneration of a nerve is roughly equivalent to the rate of SCb in that neuron [3]. This suggests that critical roles are played by the slow axonal transport in growth and regeneration. During development, SCb proteins are prominent and relatively little NF protein is detectable. Tubulin can be detected moving at both SCb and SCa rates. Once an appropriate target is reached and synaptogenesis begins, there is upregulation of NF protein synthesis and a gradual slowing of slow transport.

Axonal regeneration involves a complex set of cell body and axonal responses to a lesion. Downregulation of NF triplet and upregulation of specific tubulin isotypes are hallmarks of cell body responses to a lesion. In CNS neurons that fail to regenerate, changes in NF and tubulin expression are reduced or absent. Since changes in protein expression do not alter axonal cytoskeletal composition until after a regenerating growth cone has formed and extended for some distance, such changes in expression do not affect neurite growth, but may reflect activation of a cellular program for neurite growth. Axonal MTs must be disassembled as part of reorganizing the cytoskeleton for growth, and then reassembled for neurite extension. During axonal growth or regeneration specific tubulin genes are upregulated; during development and in regeneration MAPs are differentially expressed. Finally, in growth and regeneration there are characteristic changes in the axonal transport of tubulin with an increase in the fraction of tubulin moving at SCb rates.

Properties of slow transport suggest molecular mechanisms. Information about mechanisms of slow axonal transport is relatively limited. They are energy-dependent and require an intact axonal cytoskeleton. Indirect evidence suggests that MTs play a critical role, because transport of NFs can be pharmacologically uncoupled from MT transport without eliminating slow transport [33]. In contrast, all agents that disrupt MTs appear to block slow transport of all components. While this does not rule out a role for the MF cytoskeleton in slow transport movements, MTs appear to provide motive force for other elements of the cytoskeleton.

The macroscopic rates measured by radiolabel experiments should not be taken to reflect maximum rates of the motors involved. As with mitochondrial transport, the net rate of slow component proteins reflects both the rate of actual movement and the fraction of a time interval that a structure is moving. The elongate shape of cytoskeletal structures and their potential for many interactions means that net displacements are discontinuous. If a structure is moving at a speed of 2 μm/s, but on average only moves at that rate for one second out of every 100 seconds, then the average rate for the structure will translate to a net rate of only 0.02 μm/s [31].

Recently, methods for direct visualization of fluorescently tagged MTs and NFs in cultured neurons have been developed [34]. In such studies, relatively short MT or NF segments can be seen to move as rapidly as MBOs moving

in fast axonal transport, although they move much less frequently. Integration of these infrequent rapid movements of MTs and NFs over time gives a net rate of approximately 1–2 mm/day instead of the 200–400 mm/day seen for MBOs in mammalian nerve. Other studies permitted visualization of microtubules nucleated at the microtubule organizing center and being translocated toward the cell periphery. The combination of studies of axonal transport using radiolabels and direct observations of individual MTs or NFs with video microscopy provides strong experimental evidence that MTs and NFs can and do move in the axon as intact cytoskeletal structures. As discussed below, there are still questions about the specific motors and mechanisms underlying these movements.

As with membrane proteins, cytoplasmic and cytoskeletal proteins are differentially distributed in neurons and glia. Progress has been made toward identification of targeting mechanisms and some general principles have begun to emerge. Since cytoplasmic constituents move only in the anterograde direction, a key mechanism for targeting of cytoplasmic and cytoskeletal proteins appears to be differential metabolism [3]. Concentration of actin and other proteins in presynaptic terminals can be explained by slower turnover in the presynaptic terminal relative to NF proteins and tubulin. Proteins with slow degradative rates in the terminal accumulate and reach a higher steady-state concentration. Alteration of degradation rates for a protein can change the rate of accumulation for that protein. For example, some protease inhibitors cause the appearance of neurofilament rings in affected presynaptic terminals [35].

Although slow axonal transport of cytoskeletal proteins has received the most attention, all other cytoplasmic proteins must be delivered to distal regions of the neuron as well. Many of these are classical proteins of the 'cytosol' that become solubilized during biochemical fractionation. These include the enzymes of glycolysis and regulatory proteins such as calmodulin and HSC70 [3]. However, in pulse chase radiolabel studies, soluble proteins move down the axon as regularly and systematically as cytoskeletal proteins. This coherent transport of hundreds of different polypeptides indicates a higher level of organization of cytoplasmic proteins than has been traditionally assumed. Such organization is probably necessary to facilitate interactions with motor proteins and targeting mechanisms and to assure a reliable delivery of all required proteins to the axon.

MOLECULAR MOTORS: KINESIN, DYNEIN AND MYOSIN

Prior to 1985, the only molecular motors characterized in vertebrate cells were muscle myosins and flagellar dyneins. Myosins had been purified from nervous tissue, but no clear functions were established. Given evidence that fast axonal transport was microtubule-based, many investigators looked for dynein in the cytoplasm with equivocal success. Moreover, the biochemical properties of fast transport were inconsistent with both myosin and dynein [11, 14]. The pharmacology and biochemistry of fast axonal transport created a picture of organelle transport distinct from muscle contraction or flagellar beating.

The characteristic properties of different molecular motors aided in their identification. One striking difference between fast axonal transport and myosin- or dynein-based motility emerged from studies with ATP analogs. Adenylyl-imidodiphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, is a weak competitive inhibitor of both myosin and dynein. However, when AMP-PNP is perfused into axoplasm, bidirectional transport stops within minutes [1, 36]. Both anterograde and retrograde moving organelles freeze in place on microtubules, and 'pearls on a string' structures became apparent. Inhibition of fast axonal transport by AMP-PNP indicated that fast axonal transport involved a new class of motors and suggested that this new motor should have a high affinity for microtubules in the presence of AMP-PNP. The polypeptide composition of this new motor molecule was soon defined and it was christened kinesin [37, 38]. This discovery raised the possibility of other novel motor molecules and soon additional classes of molecular motors began to be identified. The proliferation of motor types has transformed our understanding of cellular motility.

All three classes of molecular motor proteins are now known to be large protein families with diverse cellular functions [39]. Both the kinesin family (reviewed in [39]) and the myosin family [40, 41] have been defined and proteins grouped into subfamilies. Finally, the elusive cytoplasmic version of dynein was identified and a multigene family of flagellar and cytoplasmic dyneins defined [42]. Members of a given motor protein family share significant homology in their motor domains with the defining member (kinesin, dynein or myosin), but they also contain unique protein domains that are specialized for interaction with different cargoes or differential regulation [43]. This large number of motor proteins may reflect the number of cellular functions that require force generation or movement, ranging from mitosis to morphogenesis to transport of vesicles. Here we focus on motor proteins that are thought to be important for axonal transport or neuronal function, starting with kinesin.

Kinesins mediate anterograde transport in a variety of organisms and tissues. Since its discovery, much has been learned about the biochemical, pharmacological and molecular properties of kinesin [44, 45]. Kinesin is the most abundant member of the kinesin family in vertebrates and is widely distributed in neuronal and non-neuronal cells. The holoenzyme is a heterotetramer comprising two heavy chains (115–130 kDa) and two light

chains (62–70 kDa). Structural studies have shown that kinesin is a rod-shaped protein approximately 80 nm in length, with two globular heads connected to a fan-like tail by a long stalk. High-resolution electron microscopic immunolocalization of kinesin subunits and molecular genetic studies both indicate that kinesin heavy chains are arranged in parallel with their amino terminals forming the heads and much of the stalk. The kinesin heavy chain heads comprise the motor domains, containing both ATP and MT binding motifs. This motor domain is the most highly conserved region within the kinesin family. Binding of kinesin to MTs is stabilized by AMP-PNP and this property remains a hallmark of kinesins. Kinesin light chains localize to the fan-like tail and may also contribute to part of the stalk. The α-helical coil-coiled domains that are present in both heavy and light chains form the stalk itself. Kinesin light chains appear to be unique to conventional kinesin but are highly conserved across species. Light chains are thought to be involved in organelle binding and may also play a role in targeting of kinesin isoforms to different types of MBO [46].

Considerable evidence implicates kinesin as a motor molecule for fast axonal transport. Kinesin is an MT-activated ATPase with minimal basal activity. MTs will glide across kinesin-coated glass surfaces with motor movement toward the MT-plus end. Since axonal MTs have a uniform polarity with their plus ends distal, the directionality of kinesin is consistent with an anterograde transport motor. Immunofluorescence and electron microscopy showed that kinesin is associated with MBOs that are, in turn, associated with MTs [47, 48] (Fig. 28-7). While these properties of kinesin are consistent with a role in axonal transport of MBOs, they are insufficient to prove the hypothesis that kinesin is the fast axonal transport motor. Such proof came from inhibition of kinesin function by antibodies against both heavy and light chains of kinesin. Since kinesin light chains are only associated with conventional kinesin, the ability of kinesin light chain antibodies to inhibit transport is compelling evidence that kinesin is involved in fast axonal transport. Finally, reduction of kinesin levels using antisense oligonucleotides and gene deletion also implicates kinesin in axonal transport processes [46].

In neurons and non-neuronal cells, kinesin is associated with a variety of MBOs, ranging from synaptic vesicles to mitochondria to lysosomes. In addition to its role in fast axonal transport and related phenomena in non-neuronal cells, kinesin appears to be involved in constitutive cycling of membranes between the Golgi and endoplasmic reticulum. However, kinesin is not associated with all cellular membranes. For example, the nucleus, membranes of the Golgi complex and the plasma membrane all appear to lack kinesin. Kinesin interactions with membranes are thought to involve the light chains and carboxyl termini of heavy chains. However, neither this selectivity nor the molecular basis for binding of kinesin and other motors to membranes is well understood.

Cloning and immunochemical studies of kinesin subunits have demonstrated that multiple isoforms of kinesin heavy and light chains occur in brain. Three kinesin heavy chain genes are expressed in mammals, including a ubiquitous kinesin heavy chain gene expressed in all tissues and a neuronal specific heavy chain gene that is expressed only in brain [49]. At least two different genes exist for the kinesin light chains as well and differential splicing for one of these light chain genes generates three different isoforms [50]. These are differentially expressed in tissues. Heterogeneity in kinesin heavy and light chains may regulate the transport of different MBO types and ensure that organelles reach their correct destinations in the axon. Radiolabeled, specific isoforms of the kinesin heavy chains move down the axon at different net rates that correlate with different MBO types, such as synaptic vesicles and mitochondria [51]. These observations suggest that different kinesin isoforms exist to transport different types of organelle.

Multiple members of the kinesin superfamily are expressed in the nervous system. Kinesin has been purified and then cloned from many species including *Drosophila*, squid, sea urchin, chicken, rat and human. Both heavy and light chains of conventional kinesin are highly conserved throughout. However, once the sequence of the kinesin motor domain was available, related proteins with homology only in the motor domain began to be identified. Kinesin related proteins (KRPs) were first identified in yeast and fungal mutants with defective cell division, but many others are now known [39], with more than 40 different genes expressed in mouse and human [52]. A careful analysis of kinesin superfamily sequences from many species led to the definition of a standardized kinesin nomenclature for 14 defined families of kinesins [53]; conventional kinesin is now known as kinesin-1.

Motor proteins of the large kinesin family all have well conserved motor domains, but KRPs are highly variable in sequence and structure. Even the position of the motor domain varies. Kinesin-1 and many other family members have amino terminal motor domains, but other KRPs have motor domains at their carboxyl termini and some have centrally located motor domains. This variation in structure has functional significance. To date, all tested amino and central motor domain proteins have been found to move toward the MT^{+} end, while carboxyl motor domain proteins move toward the MT^{-} end. Many KRPs are known only from their sequences and expression, but a few have been examined for function. Many KRPs are involved in various steps of cell division, but precise cellular functions are still being defined for many of motors.

Systematic cloning strategies based on the conserved motor domain sequences have identified a remarkable number of KRPs expressed in brain. Members of several KRP families expressed in brain have been implicated in forms of MBO transport. Kinesin-2 family members have been implicated in assembly and maintenance of cilia and

flagella and mutations in these motors can lead to sensory defects and polycystic kidney disease [54]. Kinesin-2 motors are heterotrimers with two related KRP motor subunits and a larger accessory subunit. Kinesin-3 family members were proposed as a synaptic vesicle motor because kinesin-3 mutants in the nematode *Caenorhabditis elegans* had defects in synaptic vesicle localization. Subsequently, two distinct kinesin-3 family members were cloned from mouse brain and their distributions examined: one (KIF1A) was neuron-specific and appeared to be associated with synaptic vesicles, whereas the other (KIF1B) was ubiquitously expressed and reported to be associated with mitochondria. Since kinesin has also been localized on mitochondria, some MBOs may have multiple motor types or there may be subsets of MBOs with specific motors. Various other kinesin family members expressed in nervous tissue have been implicated in the transport of other MBO classes [55]. The extent to which these kinesins reflect unique transport mechanisms rather than functional redundancy within the kinesin family is not known.

Curiously, functions proposed for some brain KRPs [55] are very different from functions proposed for similar or identical KRPs in no-neuronal cells. For example, members of the kinesin-13 family have been implicated in both mitotic spindle function and in axonal membrane transport. Similarly, a mouse kinesin-4 was reported to associate with unidentified MBOs in neurites, but its chicken homolog bound to chromosomal DNA and mediates chromosome movements in the mitotic spindle. Finally, a kinesin-6 was originally found to have a role in mitotic spindle function, but members of the kinesin-6 family were also implicated in the transport of MTs into dendrites [56].

Although the last family of motor proteins to be discovered, the kinesins have proved to be remarkably diverse. So far, there are at least 14 distinct subfamilies in the kinesin family and more are likely to emerge, all with homology in their motor domain [53]. Within a subfamily, however, the more extensive sequence similarities are presumed to reflect related functions. At present, many questions remain about the function of these various motors in the nervous system.

Cytoplasmic dyneins may have multiple roles in the neuron. The identification of kinesin as a plus-end directed microtubule motor suggested that it is involved in anterograde transport but left the identity of the retrograde motor an open question. Since flagellar dynein was known to be a minus-end-directed motor, interest in cytoplasmic dyneins was renewed. Identification of the cytoplasmic form of dynein in nervous tissue came as an indirect result of the discovery of kinesin.

Although dynein binding to MTs is not stabilized by AMP-PNP, both cytoplasmic dynein and kinesin associate with microtubules in nucleotide-depleted extracts and both are released by addition of ATP. Early studies with ATP-free MT extracts showed that they are substantially enriched in a minor high-molecular-weight microtubule-associated protein that was called MAP1c. Biochemical analysis showed that MAP1c was not in fact related to the well defined microtubule-associated proteins MAP1a and MAP1b but instead was closely related to flagellar dynein heavy chains. This discovery led to purification and characterization of brain cytoplasmic dynein [57]. Like flagellar dyneins, cytoplasmic dynein is a high-molecular-weight protein complex comprising two heavy chains and multiple light and intermediate chains that form a complex of more than 1,200 kDa.

As with the kinesins, dynein heavy chains are a multigene family with multiple flagellar and cytoplasmic dynein genes [42]. The 530 kDa dynein heavy chain contains the ATPase activity and MT binding domains of dynein. There may be 10–15 dynein heavy chain genes in an organism, but the large size of the dynein heavy chain primary sequence slowed genetic analyses. At present, dynein genes are grouped as members of either flagellar or cytoplasmic dynein subfamilies. The three intermediate (74 kDa), four light intermediate (55 kDa) and a variable number of light chains present in dyneins may also have flagellar and cytoplasmic forms.

The two or more cytoplasmic dynein heavy chain genes could be involved in different cellular functions, but much dynein functional diversity may be due to its many associated polypeptides [58]. The intermediate and light chains of cytoplasmic dynein are thought to be important both for regulation and for interactions with other cellular structures. In addition, a second protein complex known as dynactin copurifies with cytoplasmic dynein under some conditions [59]. The dynactin complex is similar in size to dynein and contains 10 subunits that include $p150^{Glued}$, dynamitin, an actin related protein, and two actin capping polypeptides among others. The $p150^{Glued}$ polypeptide interacts with both dynein intermediate chains and the actin related subunits. Dynamitin may play a role in the binding of cytoplasmic dynein to different types of cargo. Finally, the actin-related protein (Arp1) forms a short filament that may include actin as well as the capping proteins. This short filament may interact with both $p150^{Glued}$ and components of the membrane cytoskeleton such as spectrin. Dynactin may mediate cytoplasmic dynein binding to selected cargoes including the Golgi complex and the membrane cytoskeleton. The wide range of functions associated with cytoplasmic dynein is matched by its complexity and its ability to interact with accessory factors. Additional proposed functions include a role in mitosis and in anchoring and localizing the Golgi complex.

A number of studies have implicated cytoplasmic dynein as playing a role in retrograde axonal transport [11, 39]. *In vitro* motility studies demonstrate that cytoplasmic dynein generates force towards the minus ends of MTs consistent with a retrograde motor. Dynein immunoreactivities have been associated with MBOs and

cytoplasmic dynein accumulates on the distal side of a nerve ligation coincident with retrogradely transported MBOs. Finally, retrograde transport has been reported to be more sensitive than anterograde transport to UV-vanadate treatment. Since exposure of dynein to UV irradiation in the presence of vanadate and ADP cleaves the dynein heavy chain, this has been a signature of dyneins although other ATP binding proteins may be affected as well.

In the nervous system, the most frequent role proposed for dynein is a motor for retrograde axonal transport, but its properties are also consistent with a motor for slow axonal transport [11]. Consistent with this possibility, studies on the axonal transport of radiolabeled cytoplasmic dynein indicated that most cytoplasmic dynein and dynactin moved with SCb [60]. The ability of dynactin to interact with both cytoplasmic dynein and the membrane cytoskeleton suggests a model in which dynactin links dynein to the membrane cytoskeleton, providing an anchor for dynein-mediated movement of axonal microtubules [61]. Some role for the membrane cytoskeleton in the mechanisms of slow axonal transport is likely, since neurons require interaction with a solid substrate for neurite growth. Taken together, these studies suggest that cytoplasmic dynein has a wide variety of functions in the nervous system from anchoring the Golgi to retrograde transport to transport of MTs. Cytoplasmic dynein appears to have adapted to fulfill many cellular functions that require minus-end-directed MT movements.

Different classes of myosin are important for neuronal function. Myosins are remarkably diverse in structure and function. To date, 15 subfamilies of myosin have been defined by sequence homologies [41]. The brain is an abundant source of nonmuscle myosins and one of the earliest studied. Despite their abundance and variety, the roles of myosins in neural tissues have only recently begun to be defined [40].

Myosin II is in the same subfamily as the myosins in muscle thick filaments and it forms large, two-headed myosins with two light chains per heavy chain. Although myosin II is abundantly expressed in brain, little is known about its function in the nervous system. In other nonmuscle cells, myosin II has been implicated in many types of cellular contractility and may serve a similar function in developing neurons. However, myosin II remains abundant in the mature nervous system, where examples of cell contractility are less common.

The second myosin type identified in nervous tissue was the myosin I family. It was first described in protists and subsequently purified from brain. Myosin I is a single-headed myosin with a short tail that uses calmodulin as a light chain [41]. In many cell types it has been implicated in both endocytosis and exocytosis, so it may play an important role in delivery and recycling of receptors. Myosin I is enriched in microvilli and may also be involved in some aspects of growth cone motility along with myosins from other subfamilies. In both cases, it may link MF bundles to the plasma membrane through a membrane-binding domain. Recently, the myosin I family has been implicated in mechanotransduction by the stereocilia of hair cells in the inner ear and vestibular apparatus. A myosin I isoform, myosin Iβ, has been localized to the tips of stereocilia where it appears to mediate sensory adaptation by opening and closing the stretch-activated calcium channel (see Chapter 51).

Two other myosin types have been implicated in hearing and vestibular function [62]. The defect in the Snell's *waltzer* mouse was found to be a mutation in a myosin VI gene that produces degeneration of the cochlea and vestibular apparatus. Myosin VI is localized to the cuticular plate of the hair cell under stereocilia. Similarly, mutations in a myosin VII gene are responsible for the *shaker-1* mouse and several human genetic deafness disorders. This myosin, myosin VIIa, is found in a band near the base of the stereocilia distinct from distributions of myosin Iβ and myosin VI.

Another myosin type that plays a role in nervous tissue is myosin V [41]. Of the myosins identified in brain, myosin I and V are the strongest candidates to act as an organelle motor and myosin V has been reported in association with vesicles purified from squid axoplasm. Myosin V is the product of the mouse *dilute* locus. Mice carrying the mutant *dilute* allele show defects in pigment granule movement that results in a dilution of the coat color. These mice also exhibit complex neurological defects that may be due to altered endoplasmic reticulum localization in dendrites. In addition, an interaction between kinesin and myosin V has also been reported. Curiously, a form of myosin V found in yeast complements mutation in a KRP gene, suggesting an interaction between these two motor molecules. Finally, there is evidence that myosin V plays a role in growth cone motility where it is enriched in filopodia.

Matching motors to physiological functions may be difficult. The three classes of motors are similar in their biochemical and pharmacological sensitivities in many respects [11]. However, some hallmark features can be used to identify a motor. In the case of kinesin, the most distinctive characteristic is stabilization of binding to MTs by AMP-PNP. The affinities of myosin for MFs and dynein for MTs are weakened by treatment with either ATP or AMP-PNP. As a result, if a process is frozen in place by AMP-PNP, then kinesins are likely to be involved. If kinesin is not involved in a process that requires MTs then dyneins are likely to be involved. Similarly, processes requiring MFs suggest that myosins are required. In the case of fast axonal transport, we know that MTs are required and this process is completely inhibited by AMP-PNP, thus implicating the kinesin family. The development of new pharmacological and immunochemical probes specific for different motors will facilitate future studies.

Although many motor proteins are found in nervous tissue, there are few instances in which we fully understand their cellular functions. The proliferation of different motor molecules and the existence of numerous isoforms raises the possibility that some physiological activities require multiple motors. There may be cases in which motors serve a redundant role to ensure that the physiological activity is maintained in the event of a loss of one motor protein. Finally, the existence of so many different types of motor molecule suggests that novel physiological activities requiring molecular motors may be as yet unrecognized.

AXONAL TRANSPORT AND NEUROPATHOLOGY

Inhibition of axonal transport leads rapidly to loss of function in the distal axon and 'dying back' neuropathies are frequently associated with chronic exposure to neurotoxins that inhibit axonal transport. Some of these neurotoxins may act directly on the motor molecules that underlie axonal transport. For example, the neurotoxicant acrylamide has been found to inhibit kinesin function directly. Recently, more direct evidence has emerged directly implicating fast axonal transport defects in neurodegeneration [63, 64]. For example, a loss-of-function mutation in the kinesin I member KIF5A results in a hereditary form of spastic paraplegia. Remarkably, in many cases, mutant proteins are expressed in many neuronal and non-neuronal cells, but specific neuronal populations suffer. For example, genetic analysis showed that mutations in both the ubiquitously expressed dynein heavy chain and $p150^{Glued}$ can lead to motoneuron deficits in mammals [65, 66]. Other disease-related proteins may deregulate fast axonal transport indirectly by affecting the delivery of materials by transport through effects on energy metabolism or by blocking conversion from anterograde to retrograde transport.

A variety of other neurological diseases are also thought to involve axonal transport [45]. For example, motor neuron diseases like amyotrophic lateral sclerosis (ALS) appear to interfere with both fast and slow axonal transport. Analysis of axonal transport in an animal model of ALS documented deficits in both the transport of NFs by slow transport and the delivery of mitochondria to the axon by fast transport [67]. While ALS-like diseases may be caused by a number of different pathogenic mechanisms, all forms feature accumulations of NFs and a dying back neuropathy in motor neurons characteristic of axonal transport deficits [68]. Similar arguments can be made for a variety of neurodegenerative diseases including polyglutamine expansion diseases [64] and Alzheimer's disease [69]. Even when the primary pathogenic pathway has only indirect effects on transport, any cellular event that compromises the timely and efficient delivery of material by axonal transport processes might result in some form of neuronal degeneration.

CONCLUSION

The functional architecture of neurons is comprised of many specializations in cytoskeletal and membranous components. Each of these specializations is dynamic, constantly changing and being renewed at a rate determined by the local environment and cellular metabolism. The processes of axonal transport represent the key to neuronal dynamics. Recent advances have provided important insights into the molecular mechanisms underlying axonal transport, although many questions remain. Continued exploration of these phenomena will improve our understanding of neuronal dynamics in development, regeneration and neuropathology.

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REFERENCES

1. Brady, S. T., Lasek, R. J. and Allen, R. D. Fast axonal transport in extruded axoplasm from squid giant axon. *Cell Mot.* 3 (Video Supplement), 1983.
2. Weiss, P. and Hiscoe, H. Experiments in the mechanism of nerve growth. *J. Exp. Zool.* 107: 315–395, 1948.
3. Brady, S. T. Axonal dynamics and regeneration. In A. Gorio (ed.), *Neuroregeneration.* New York: Raven Press, 1993, pp. 7–36.
4. Droz, B. and Leblond, C. P. Migration of proteins along the axons of the sciatic nerve. *Science* 137: 1047–1048, 1962.
5. Grafstein, B. and Forman, D. S. Intracellular transport in neurons. *Physiol. Rev.* 60: 1167–1283, 1980.
6. Tytell, M., Black, M. M., Garner, J. A. and Lasek, R. J. Axonal transport: Each of the major rate components consist of distinct macromolecular complexes. *Science* 214: 179–181, 1981.
7. Lasek, R. J. and Brady, S. T. The Structural Hypothesis of axonal transport: Two classes of moving elements. In D. G. Weiss (ed.), *Axoplasmic Transport.* Berlin: Springer-Verlag, 1982, pp. 397–405.
8. Van Vliet, C., Thomas, E. C., Merino-Trigo, A., Teasdale, R. D. and Gleeson, P. A. Intracellular sorting and transport of proteins. *Prog. Biophys. Mol. Biol.* 83: 1–45, 2003.
9. Hammerschlag, R., Stone, G. C., Bolen, F. A., Lindsey, J. D. and Ellisman, M. H. Evidence that all newly synthesized proteins destined for fast axonal transport pass through the Golgi apparatus. *J. Cell Biol.* 93: 568–575, 1982.

10. Maxfield, F. R. and McGraw, T. E. Endocytic recycling. *Nat. Rev. Mol. Cell Biol.* 5: 121–132, 2004.
11. Brady, S. T. Molecular motors in the nervous system. *Neuron* 7: 521–533, 1991.
12. Smith, R. S. The short term accumulation of axonally transported organelles in the region of localized lesions of single myelinated axons. *J. Neurocytol.* 9: 39–65, 1980.
13. Tsukita, S. and Ishikawa, H. The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 84: 513–530, 1980.
14. Brady, S. T., Lasek, R. J. and Allen, R. D. Video microscopy of fast axonal transport in isolated axoplasm: A new model for study of molecular mechanisms. *Cell Motil.* 5: 81–101, 1985.
15. Sahenk, Z. and Lasek, R. J. Inhibition of proteolysis blocks anterograde-retrograde conversion of axonally transported vesicles. *Brain Res.* 460: 199–203, 1988.
16. Smith, R. S. and Snyder, R. E. Reversal of rapid axonal transport at a lesion: leupeptin inhibits reversed protein transport, but does not inhibit reversed organelle transport. *Brain Res.* 552: 215–227, 1991.
17. Schroer, T. A., Brady, S. T. and Kelly, R. Fast axonal transport of foreign vesicles in squid axoplasm. *J. Cell Biol.* 101: 568–572, 1985.
18. Kristensson, K. Retrograde transport of macromolecules in axons. *Annu. Rev. Pharmacol. Toxicol.* 18: 97–110, 1987.
19. Kelly, R. B. and Grote, E. Protein targeting in the neuron. *Ann. Rev. Neurosci.* 16: 95–127, 1993.
20. Greengard, P., Benfenati, F. and Valtorta, F. Synapsin I, an actin-binding protein regulating synaptic vesicle traffic in the nerve terminal. *Adv. Second Messenger Phosphoprotein Res.* 29: 31–45, 1994.
21. McGuinness, T. L., Brady, S. T., Gruner, J. *et al.* Phosphorylation-dependent inhibition by synapsin I of organelle movement in squid axoplasm. *J. Neurosci.* 9: 4138–4149, 1989.
22. Morfini, G., Szebenyi, G., Richards, B. and Brady, S. T. Regulation of kinesin: implications for neuronal development. *Dev. Neurosci.* 23: 364–376, 2001.
23. Morfini, G., Szebenyi, G., Elluru, R., Ratner, N. and Brady, S. T. Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. *EMBO J.* 23: 281–293, 2002.
24. Morfini, G., Szebenyi, G., Brown, H. *et al.* A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons. *EMBO J.* 23: 2235–2245, 2004.
25. Horton, A. C. and Ehlers, M. D. Neuronal polarity and trafficking. *Neuron* 40: 277–295, 2003.
26. Steward, O. Targeting of mRNAs to subsynaptic microdomains in dendrites. *Curr. Opin. Neurobiol.* 5: 55–61, 1995.
27. Glanzer, J. G. and Eberwine, J. H. Mechanisms of translational control in dendrites. *Neurobiol. Aging* 24: 1105–1111, 2003.
28. Steward, O. and Schuman, E. M. Compartmentalized synthesis and degradation of proteins in neurons. *Neuron* 40: 347–359, 2003.
29. Zalfa, F. and Bagni, C. Molecular insights into mental retardation: multiple functions for the Fragile X mental retardation protein? *Curr. Issues Mol. Biol.* 6: 73–88, 2004.
30. Kalwy, S. A. and Smith, R. Mechanisms of myelin basic protein and proteolipid protein targeting in oligodendrocytes. *Mol. Membr. Biol.* 11: 67–78, 1994.
31. Baas, P. W. and Brown, A. Slow axonal transport: the polymer transport model. *Trends Cell Biol.* 7: 380–384, 1997.
32. Oblinger, M. M., Brady, S. T., McQuarrie, I. G. and Lasek, R. Differences in the protein composition of the axonally transported cytoskeleton in peripheral and central mammalian neurons. *J. Neurosci.* 7: 453–462, 1987.
33. Griffin, J. W., George, E. B., Hsieh, S. and Glass, J. D. Axonal degeneration and disorders of the axonal cytoskeleton. In S. G. Waxman, J. D. Kocsis and P. K. Stys (eds), *The Axon: Structure, Function and Pathophysiology.* New York: Oxford University Press, 1995, pp. 375–390.
34. Brown, A. Live-cell imaging of slow axonal transport in cultured neurons. *Methods Cell Biol.* 71: 305–323, 2003.
35. Roots, B. Neurofilament accumulation induced in synapses by leupeptin. *Science* 221: 971–972, 1983.
36. Lasek, R. J. and Brady, S. T. Adenylyl imidodiphosphate (AMPPNP), a nonhydrolyzable analogue of ATP, produces a stable intermediate in the motility cycle of fast axonal transport. *Biol. Bull.* 167: 503, 1984.
37. Brady, S. T. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* 317: 73–75, 1985.
38. Vale, R. D., Reese, T. S. and Sheetz, M. P. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* 42: 39–50, 1985.
39. Hirokawa, N. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* 279: 519–526, 1998.
40. Brown, M. E. and Bridgman, P. C. Myosin function in nervous and sensory systems. *J. Neurobiol.* 58: 118–130, 2004.
41. Kalhammer, G. and Bahler, M. Unconventional myosins. *Essays Biochem.* 35: 33–42, 2000.
42. Asai, D. J. and Wilkes, D. E. The dynein heavy chain family. *J. Eukaryot. Microbiol.* 51: 23–29, 2004.
43. Vale, R. D. The molecular motor toolbox for intracellular transport. *Cell* 112: 467–480, 2003.
44. Brady, S. T. A kinesin medley: biochemical and functional heterogeneity. *Trends Cell Biol.* 5: 159–164, 1995.
45. Hirokawa, N. and Takemura, R. Biochemical and molecular characterization of diseases linked to motor proteins. *Trends Biochem. Sci.* 28: 558–565, 2003.
46. Stenoien, D. S. and Brady, S. T. Immunochemical analysis of kinesin light chain function. *Molec. Biol. Cell* 8: 675–689, 1997.
47. Hirokawa, N., Pfister, K. K., Yorifuji, H., Wagner, M. C., Brady, S. T. and Bloom, G. S. Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration. *Cell* 56: 867–878, 1989.
48. Pfister, K. K., Wagner, M. C., Stenoien, D., Bloom, G. S. and Brady, S. T9. Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell Biol.* 108: 1453–1463, 1989.
49. Miki, H., Setou, M. and Hirokawa, N. Kinesin superfamily proteins (KIFs) in the mouse transcriptome. *Genome Res.* 13: 1455–1465, 2003.

50. Cyr, J. L., Pfister, K. K., Bloom, G. S., Slaughter, C. A. and Brady, S. T. Molecular genetics of kinesin light chains: Generation of isoforms by alternative splicing. *Proc. Natl Acad. Sci. U.S.A.* 88: 10114–10118, 1991.
51. Elluru, R., Bloom, G. S. and Brady, S. T. Fast axonal transport of kinesin in the rat visual system: functionality of the kinesin heavy chain isoforms. *Mol. Biol. Cell* 6: 21–40, 1995.
52. Miki, H., Setou, M., Kaneshiro, K. and Hirokawa, N. All kinesin superfamily protein, KIF, genes in mouse and human. *Proc. Natl Acad. Sci. U.S.A* 98: 7004–7011, 2001.
53. Lawrence, C. J., Dawe, R. K., Christie, K. R. *et al.* A standardized kinesin nomenclature. *J. Cell Biol.* 167: 19–22, 2004.
54. Scholey, J. M. Intraflagellar transport. *Annu. Rev. Cell Dev. Biol.* 19: 423–443, 2003.
55. Hirokawa, N. and Takemura, R. Kinesin superfamily proteins and their various functions and dynamics. *Exp. Cell Res.* 301: 50–59, 2004.
56. Baas, P. W. Microtubule transport in the axon. *Int. Rev. Cytol.* 212: 41–62, 2002.
57. Paschal, B. M., Shpetner, H. S. and Vallee, R. B. MAP1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J. Cell Biol.* 105: 1273–1282, 1987.
58. King, S. M. The dynein microtubule motor. *Biochim Biophys Acta* 1496: 60–75, 2000.
59. Schroer, T. A. Dynactin. *Annu. Rev. Cell Dev. Biol.* 20: 759–779, 2004.
60. Dillman, J. F., Dabney, L. P., Karki, S., Paschal, B. M., Holzbaur, E. L. and Pfister, K. K. Functional analysis of dynactin and cytoplasmic dynein in slow axonal transport. *J. Neurosci.* 16: 6742–6752, 1996.
61. Ahmad, F. J., Echeverri, C. J., Vallee, R. B. and Baas, P. W. Cytoplasmic dynein and dynactin are required for the transport of microtubules into the axon. *J. Cell Biol.* 140: 391–401, 1998.
62. Libby, R. T. and Steel, K. P. The roles of unconventional myosins in hearing and deafness. *Essays Biochem.* 35: 159–174, 2000.
63. Mandelkow, E. and Mandelkow, E. M. Kinesin motors and disease. *Trends Cell Biol.* 12: 585–591, 2002.
64. Morfini, G., Pigino, G. and Brady, S. T. Polyglutamine expansion diseases: failing to deliver. *Trends Mol. Med.* 11: 64–70, 2005.
65. Hafezparast, M., Klocke, R., Ruhrberg, C. *et al.* Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science* 300: 808–812, 2003.
66. LaMonte, B. H., Wallace, K. E., Holloway, B. A. *et al.* Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron* 34: 715–727, 2002.
67. Collard, J.-F., Côté, F. and Julien, J.-P. Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* 375: 61–64, 1995.
68. Williamson, T. L. and Cleveland, D. W. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat. Neurosci.* 2: 50–56, 1999.
69. Morfini, G., Pigino, G., Beffert, U., Busciglio, J. and Brady, S. T. Fast axonal transport misregulation and Alzheimer's disease. *Neuromolecular Med.* 2: 89–99, 2002.

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CHAPTER

29

Stem Cells in the Nervous System

Alison K. Hall

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How the complex interconnected neurons and glia in the adult nervous system are generated from neuroectodermal precursors in the embryo remains a fundamental problem for developmental biologists. The mature vertebrate CNS is characterized by distinct stereotyped cytoarchitecture in different anatomical regions. This regional specificity appears to be established by patterning cues during early development that direct precursor cell differentiation. That this broad array of different cells is largely derived from multipotent stem cells is remarkable but perhaps even more remarkable is that cells with characteristics of stem cells are retained during the subsequent development of the CNS and even into the adult. The presence of multipotent stem cells as well as more fate-restricted but highly proliferative 'progenitor cells' in the developing nervous system leads us to ask how environmental signals such as growth factors regulate proliferation, survival and cell fate determination in normal development. It is the enormous potential of stem cells to repair damage or disease in the adult nervous system, however, that has focused intense interest on these cells as potential resources for regenerative medicine.

STEM CELLS ARE MULTIPOTENT AND SELF-RENEWING

During development many cells undergo multiple cell divisions and generate numerous progeny. The majority of these cells are not stem cells but rather are progenitor or precursor cells. A stem cell differs from other precursor or progenitor cells in two essential ways; stem cells are *multipotent* with the ability to give rise to multiple cell types, and *self-renewing* with the virtually unlimited ability to make more of themselves. Within this broad functional definition, there are various types of stem cell that differ in terms of their temporal or tissue-specific

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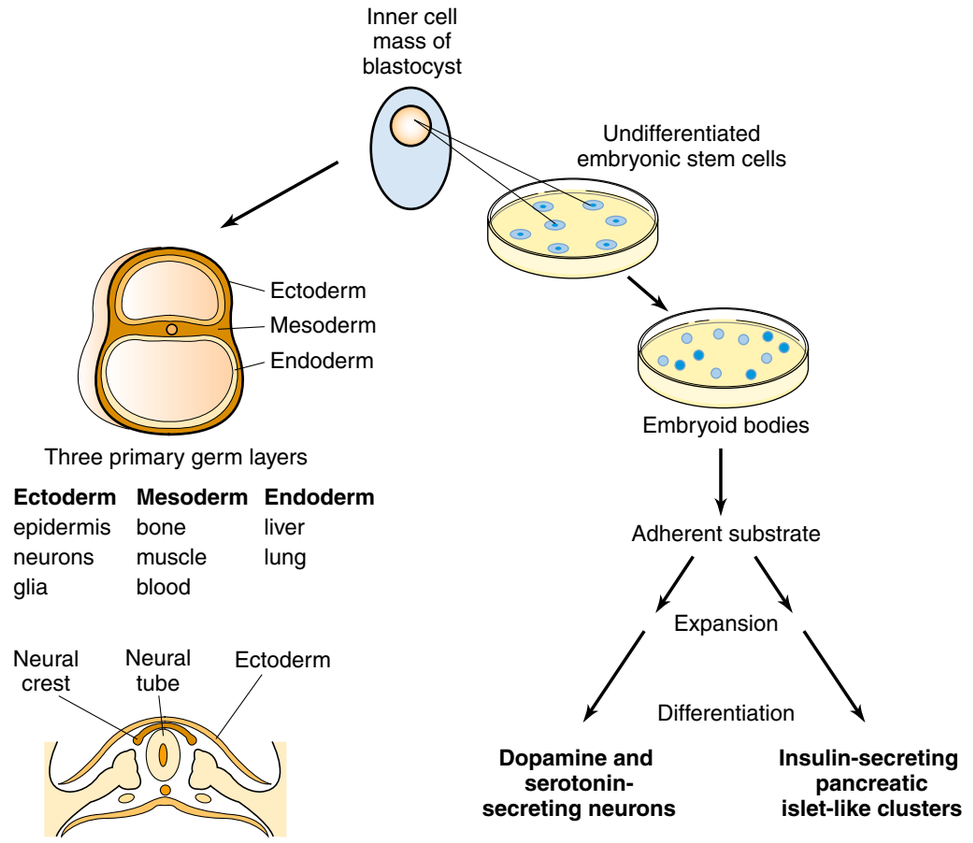

FIGURE 29-1 Embryonic stem cells self-renew and give rise to multipotent progeny. (**Left side**) In normal development, three primary germ layers are established at gastrulation, and these layers give rise to specific derivatives. For example, the outermost ectoderm gives rise to skin epidermis and the neurons and glial cells of the nervous system. Later, after neurulation, the initially sheet-like ectoderm layer gives rise to the neural tube that is the primordium of the central nervous system, and to the neural crest that gives rise to the peripheral nervous system. (**Right side**) Embryonic stem cells derived from the inner cell mass can be grown in culture as adherent, undifferentiated cells. When dissociated and grown as floating embryoid bodies, differentiated cell progeny including neurons and glia can be enriched with specific factors.

locations. For example, embryonic stem (ES) cells are derived from the inner cell mass of embryos generally at 32 cells or fewer and have the broadest range of cellular derivatives. In fact all embryonic tissues originate from ES cells *in vivo* through characteristic primary germ cell lineages (Fig. 29-1). During development, many embryonic tissues are generated from tissue-restricted fetal stem cells and progenitor cells. Once assembled, however, only a few tissues undergo cell turnover in adulthood and rely on a continued source of precursors. The best understood example of an adult tissue that continues to harbor stem cells is the blood (or hematopoietic) system. Here stem cells in bone marrow continuously produce a variety of blood and immune system cells. In other tissues, epithelial stem cells produce new skin cells throughout life, crypt cells produce intestinal lining, and mesenchymal stem cells give rise to bone, cartilage and connective tissue cells. These stem cells are found in different tissue locations, and have distinct properties. One unresolved issue is whether the different types of stem cells have similar potential to generate cells that can be utilized for neural repair.

Stem cells give rise to committed progenitors. In a tissue that depends on the continuous replacement of stem cell-derived progeny, the self renewal of stem cells and the generation of committed progenitor cells must be balanced in order not to deplete the stem cell pool and thus, the entire cell lineage over time. Retention of a stem cell pool might be accomplished in several ways. Stem cells may undergo symmetric divisions to produce two stem cell daughters, or asymmetric divisions to produce a stem cell and one of a variety of committed progenitor cells (Fig. 29-2). Committed progenitor cells typically produce particular lineages of cells and thus are considered to have a restricted cell fate, although this has recently been called into question. Likewise, committed progenitor cells may differentiate immediately into mature cells, or more likely become expanded by selective mitogens to produce many mature cells.

An important controversy remains about which factors, intrinsic or extrinsic, control the stem cell's decision to self-renew or develop into one of several committed progenitor cells. Two distinct models have been proposed to

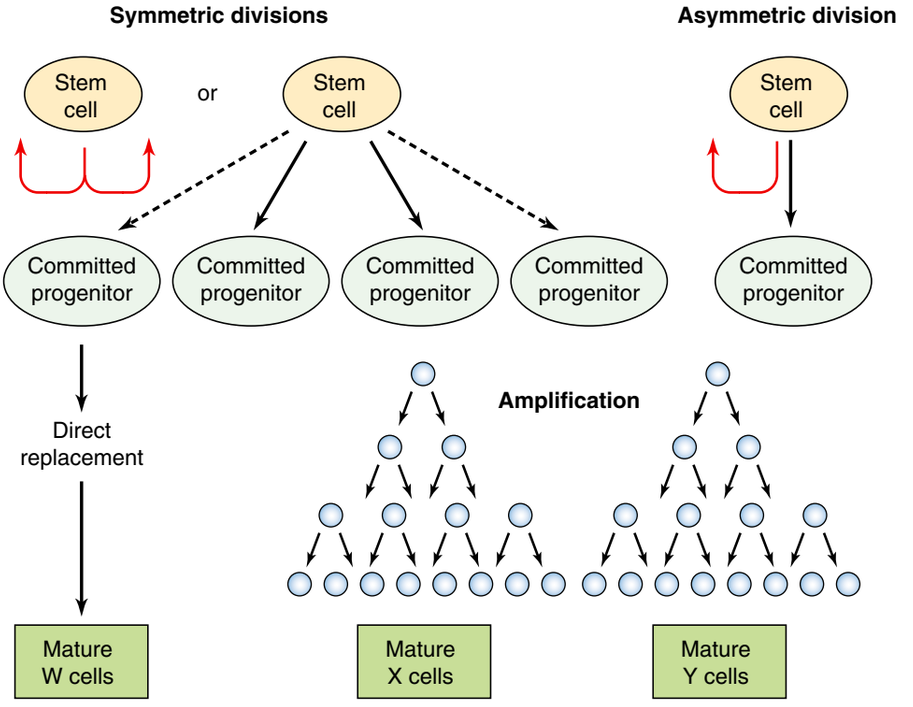

FIGURE 29-2 Stem cells undergo symmetric divisions to increase the pool size of stem cells, or asymmetric divisions to give rise to a committed progenitor cell and another stem cell. Committed progenitor cells may go on to differentiate immediately to directly replace mature cells lost in response to injury or disease. Alternatively, expansion factors may stimulate extensive division of a specific progenitor cell, and produce large numbers of a mature cell type.

describe cell commitment from a stem cell (1) in response to intrinsic molecular events, stem cells *stochastically* produce various committed progenitor cells, and growth factors act upon a particular committed cell to affect its survival or proliferation or (2) growth factors act on stem cells to *induce* the production of particular committed progenitor cells. The ability to control the generation of distinct precursor cells from stem cells is of critical importance if these cells are to be useful for various therapies and it is possible that cell commitment in the nervous system involves features of both mechanisms.

Lessons from the hematopoietic stem cell. In healthy individuals, circulating red and white cells in the blood are replaced every few weeks and new cells are generated from stem cells in the bone marrow. This turnover process suggests there are sufficient stem cells and stem cell proliferation to continue to make differentiated cells for some 90 years, and that the specific growth and differentiation signals required to produce appropriate mature cells are present throughout life. It is now clear that bone marrow contains both the hematopoietic stem cell that gives rise to blood cells and bone marrow stromal cells that can give rise to bone, cartilage, fat and connective tissue cells.

The hemopoietic stem cell is one of the best-characterized stem cells in the body and the major stem cell that is clinically applied in the treatment of diseases, including blood cancers and congenital immunodeficiency. Since the first successful allogeneic (from a genetically different person) hematopoietic stem cell transplant in 1968, thousands of patients have received transplants to treat diseases. Hematopoietic stem cells are rare in bone marrow (perhaps one in 2,000 cells) and give rise to eight quite different types of blood and immune cell. The study of hematopoietic stem cells was significantly enhanced by the now-classical observation that implanting just a few purified stem cells can completely restore an immune system that has been experimentally destroyed (Fig. 29-3A). The ability to utilize a bioassay in which one grafts selected populations of cells and assays their cellular potential *in vivo* was essential to further understanding in this field. Furthermore, hematopoietic stem cells can be prospectively isolated from the mixed bone marrow by virtue of cell surface antigens including CD34 or AC133. In the vertebrate CNS similar bioassays and prospective antibody-based isolation procedures for neural stem cells are not yet well developed.

The bone marrow is not the only source of hematopoietic stem cells. Such cells are present in low numbers in peripheral blood, and these can be expanded in number with specific factors. Peripheral blood stem cells have the major therapeutic advantage that their harvest is relatively painless and less invasive. Likewise, umbilical cord stem cells can also be obtained in a non-invasive manner from

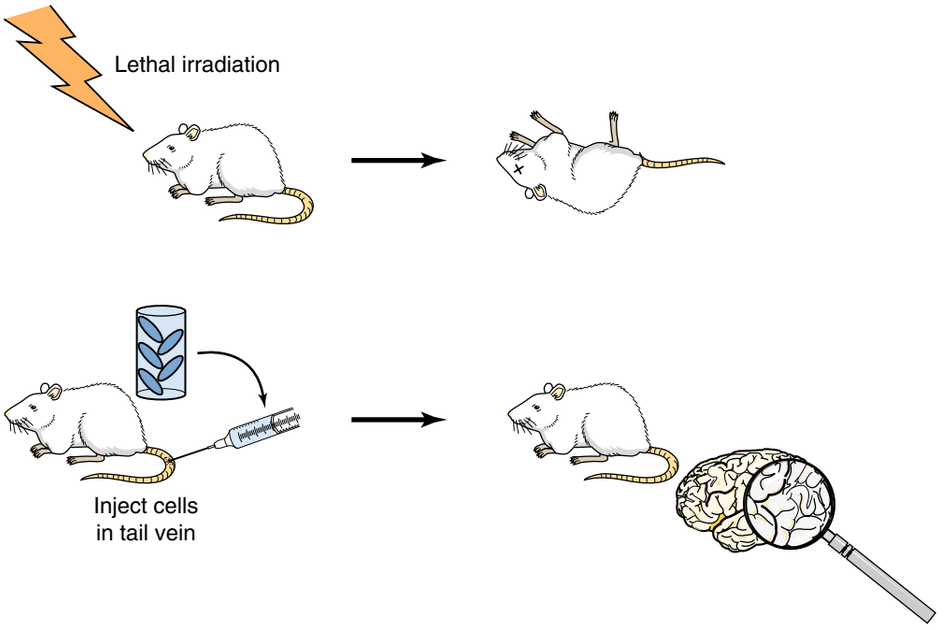

FIGURE 29-3 Intravenous administration of stem cells can reconstitute the blood forming system, and may provide cells to other tissues. In classical studies, Till and McCulloch administered dissociated bone marrow cells in the tail vein of lethally irradiated mice and found that the grafted cells repopulated the hematopoietic system and allowed the animals to survive. More recent studies suggest that blood cells also travel to sites of injury, where they may give rise to non-blood-tissue progeny.

an abundant source of tissue that is typically discarded. These cells have been used in some transplants to brain regions (see below).

The generation of distinct cell populations from hematopoietic stem cells is regulated by multiple factors. Many of the cytokines that regulate progenitor amplification and survival have been identified [1]. One well characterized signal is stem cell factor (SCF), which acts early in the lineage to stimulate proliferation of myeloid, erythroid and lymphoid progenitors, presumably by expanding a multipotent precursor cell. Regulation of each progenitor lineage appears to be mediated by the concentration or combination of specific proliferative factors. For example, a mechanism sensing the oxygen level regulates the level of the red blood cell growth stimulator erythropoietin made largely in the kidney. Erythropoietin binds to high-affinity receptors on the surface of erythroid progenitor cells to maintain their survival. Under the influence of erythropoietin, committed erythroid progenitor cells divide and differentiate. These cells then differentiate into proerythroblasts, in which hemoglobin synthesis can first be detected, and then subsequently mature and lose their nucleus. After mature cells are produced, they must be localized to body sites for function. In the hematopoietic system, long-distance cell migrations throughout the body must be employed, from marrow to lymph or spleen or to peripheral tissues. The targeting of cells to distinct tissue sites is in part mediated by the localized expression of chemotactic cytokines known as chemokines. For example, the final steps in B cell maturation are dependent on marrow-derived differentiation signals and the cells are retained in the appropriate microenvironment as a result of expression of the chemokine CXCL12 and its receptor CXCR4.

An important question is whether stem cells can be directed to produce progenitors in one lineage or another by application of specific factors. In one sense, this experiment is done *in vivo* in anemic individuals or those who have lost a lot of blood. If stem cells were *all* redirected to respond to this stress, one might anticipate a depletion of other blood cell types. This is not observed, but instead stem cells appear to routinely give rise to various progenitor cells, and it is the progenitor cells that respond to the presence or absence of mitogens to expand selected lineages. In this way, the balance of stem and progenitor cells appears maintained.

Bone marrow stem cells may not be restricted to the blood cell lineage. Stem cells that emerge during embryogenesis and in the adult are generally thought to be restricted in their developmental potential and give rise only to cells of the tissue in which they reside. Whether such a restriction is cell intrinsic or environmentally regulated is unclear. Recent studies suggest, however, that cell fate restriction may not be irreversible, as a number of stem cells appear able to produce alternative cell types to those normally produced *in vivo*. For example, adult bone marrow cells retain the ability to give rise to cell derivatives of all three germ layers, including neural cells. After lethal irradiation, bone-marrow-derived cells not only

repopulated the hematopoietic system as expected but also gave rise to cells that expressed neuron specific genes in the CNS. Transgenic mice that expressed green fluorescent protein (GFP) in all cells were used for grafting, and several months after tail vein administration of bone marrow cells, thousands of GFP-labeled cells were found throughout the CNS (Fig. 29-3B). Many transplanted cells in the brain had characteristics of macrophage/microglia. This result is perhaps not surprising given that microglial cells in the CNS are thought to have their origin in the bone marrow although trafficking between the blood and brain in the adult has been controversial. More unexpected, however was the discovery of labeled cells with neural morphologies and that expressed molecular characteristics of neural-tube-derived cells (neuronal or astrocytic markers) integrated into the host brain [2]. Tissue injury may promote the recruitment of bone marrow stem cells to additional cell types. For example, in the *mdx* mouse model of muscular dystrophy, transplanted bone marrow cells gave rise to skeletal myocytes [3]. Because of the potential ability of bone marrow cells to home to site of injury, clinical trials have begun to assess bone marrow transplant for repair of diabetic skin ulceration and myocardial infarctions.

Initially, many assumed that the nervous system might utilize similar stem-cell- and progenitor-cell-based regulatory mechanisms to produce the many types of neuron and glia, but several aspects of the nervous system may require specific mechanisms. Unlike the blood-forming system, the nervous system is largely formed during fetal and neonatal development. In the adult during normal life, neurons do not undergo significant turnover, nor do many neurons or even glial cells appear to be generated once the CNS is formed, and thus there is no great 'need' for large numbers of neural stem cells. Unlike the epithelial system, neurons form intricate connections over long distances with specific synaptic partners in functional networks. These aspects of spatial and cellular restriction suggest that neural stem cells may differ from those in other systems and that specific therapies based on stem cells face unusual challenges in the nervous system. Clearly in many cases injury to the adult nervous system is not functionally repaired by stem cells effectively, and thus neurodegenerative diseases or neural trauma have devastating consequences. If neural stem cells could be isolated, propagated and directed toward particular lineages effectively and then functionally incorporated into existing circuits, their promise as therapeutic agents in neural repair is enormous. Several ongoing studies suggest that these issues may be tractable.

STEM CELLS CONTRIBUTE TO THE DEVELOPING NERVOUS SYSTEM

A central role for neural stem cells in the development of the nervous system is becoming clear. During embryological development, the nervous system originates from ectodermal cells otherwise fated to become skin, and the molecular regulation of neural induction involves the generation of genetic competence as well as cell–cell communication events [4]. Cells that remain in the neural tube give rise to CNS neurons and glia, while cells that migrate from the dorsal portion of the neural tube disperse throughout the body in stereotypic pathways to give rise to the peripheral nervous system (PNS). Both the CNS and the PNS appear to utilize stem cells and neural progenitors in subsequent development. As with tissue-specific stem cells discussed above, it is still not clear how restricted some of these neural stem cells are, or if they are capable of contributing to neurons and glia in more than one region of the nervous system.

Central nervous system stem cells maintain an undifferentiated state and the capacity to self-renew in response to epidermal growth factor and fibroblast growth factor. An important breakthrough was made in the last decade when it was recognized that the embryonic CNS contains multipotent stem cells capable of extensive cell renewal and the generation of neurons, astrocytes and oligodendrocytes [5–7]. A critical assay used in these studies was to culture dissociated CNS cells in 20 ng/ml fibroblast growth factor (FGF)2 and epidermal growth factor (EGF)2 on a nonadherent substrate. In this medium, aggregated, floating 'neurospheres' of cells form (Fig. 29-4). When such neurospheres are plated on to an adhesive

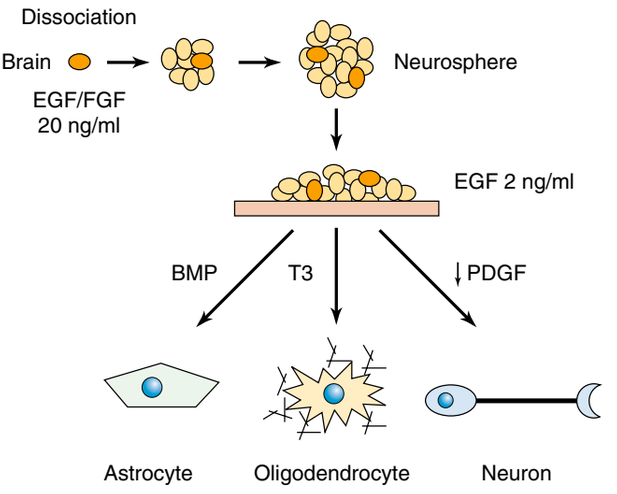

FIGURE 29-4 Neural stem cells can be isolated from the brain and propagated as neurospheres. Dissociated brain cells maintained in epidermal growth factor (*EGF*) and/or fibroblast growth factor (*FGF*) contain a small population of stem cells that continue to propagate in growth factor as floating 'neurospheres'. Neurospheres grown on an adhesive substrate give rise to the major cells of the brain, neurons, astrocytes and oligodendrocytes, *in vitro*, indicating that they contain multipotent cells. These neurospheres can be re-dissociated and give rise to more neurospheres, suggesting that they contain stem cells. *BMP*, bone morphogenetic proteins; *PDGF*, platelet-derived growth factor; *T3*, triiodothyronine.

substrate and growth factors are withdrawn they differentiate into clumps of cells containing neurons and glial cells, suggesting that neurospheres contain multipotent cells. If neurospheres are dissociated into single cells and cultured in FGF and EGF many individual cells again give rise to neurospheres *in vitro*, suggesting that some cells can self-renew. Remarkably, neurospheres could be generated not only from the embryonic CNS but also from adult brain and spinal cord. In general, neurosphere-generating cells were more easily isolated from the ventricular lining of the brain or the central canal of the spinal cord, suggesting that these regions were the preferred location of such stem cells. More recent studies have succeeded in isolating small numbers of neurosphere-forming cells from adult brain parenchyma, which may indicate a wider distribution of stem cells in the adult CNS. It is important to keep in mind, however, that the actual relationship between neurosphere generating cells *in vitro* and true stem cells *in vivo* is not well understood.

Cell lineage studies reveal close associations between distinct neural cell types. One characteristic of the vertebrate CNS is the exquisite cytoarchitectural organization of the tissue, which is retained through the life of the animal. Such a reproducible pattern of cellular development suggest that the fate of multipotential stem cells is modulated by local environmental patterning influences. Consistent with such regional specification, cell lineage studies using replication incompetent retroviruses to label clones of cells derived from one precursor *in vivo* revealed several types of precursor cells that were spatially segregated. For example, following injection into the embryonic spinal cord, clones of cells containing motor neurons and oligodendrocytes were frequently observed, suggesting that these cells share a local common precursor. Subsequent studies demonstrated that both cells originate in the same region of the ventral neural tube and share inductive cues. In more rostral regions of the CNS, precursors give rise to radially arrayed clones of neurons and glial in vertically oriented columns that span multiple cortical layers. Not all clones contain both neurons and glia. Many clones, particularly later in development, are comprised of only glial cells. Such clones contain only astrocytes or only oligodendrocytes or both. In mixed clones astrocytes were largely located in gray matter while oligodendrocytes were largely located in white matter. Several specific progenitors, including a neuron-restricted precursor, glial-restricted precursor and oligodendrocytes–astrocyte (O–2A) precursor have been described. Together these observations lead to the general concept that the multipotent and self-renewing stem cell (detected several years later) gives rise to restricted progenitors with more limited cellular potential and their fate depends on environmental cues.

The development of motor neurons and oligodendrocytes is dependent on sonic hedgehog. One of the most intensely studied cell types in the CNS is the spinal cord motor neuron. These cells arise in a distinct domain in the ventral ventricular zone of the spinal cord during a specific time in development. The induction of motor neuron precursors from 'stem cells' in the ventricular domain appears regulated by the local expression of the patterning molecule sonic hedgehog (Shh) secreted by the notochord and floorplate [8, 9]. Likewise, oligodendrocyte precursors are generated from the same domain later in development, probably from the same cells, and their induction is also dependent on Shh. Other more dorsal domains of the ventral ventricular zone generate precursors to distinct populations of interneurons and this induction is regulated by differing concentrations of Shh. Dorsal ventricular regions of the spinal cord do not produce motor neuron or oligodendrocyte precursors but rather circumferential and other distinct interneuron populations. Dorsal spinal cord has the potential to generate ventral cell types however, if stimulated by Shh. These studies suggest that multipotent stem cells are localized throughout the dorsoventral axis of the developing spinal cord, and that the fate they assume is regulated by local signals [10]. Given the diversity of cell types in the CNS, many other cues must exist to regulate cell fate. It is likely that in most cases a single cue is not sufficient to induce a distinct cell type. Rather, a series of temporal cues instruct cells in increasingly restricted pathways.

Bone morphogenetic proteins stimulate astrogenesis. Dorsal spinal cord generates distinct populations of neurons and glia and specification of their precursor fate also seems to depend on localized cues. During embryonic development members of the transforming growth factor (TGF)β superfamily, including bone morphogenetic proteins (BMPs), are expressed in dorsal regions where they contribute to the induction of dorsal neural cell populations and suppression of ventral cell fates. In rostral regions of the CNS the role of BMPs in cell fate is more complex and depends on the maturity of the responding cells. Early in development BMP2 and BMP4 stimulate neuronal differentiation in cortical cultures while later in development BMP stimulation enhances the appearance of astrocytes in cortical cultures [11, 12]. The induction of astrocytes from cultured mouse neuroepithelial cells by BMPs appears to reflect an inhibition of neuronal development, since exposure of forebrain neural progenitors to BMP2 reduces the number of neurons and increases the number of glia. Additional factors such as platelet-derived growth factor and thyroid hormone are critical in the generation of the full spectrum of neural cells. One clear cellular target for BMP actions *in vitro* is the bipotential O-2A progenitor cell. These cells usually generate oligodendrocytes but can be induced to generate a subset of astrocytes called type 2 astrocytes by a variety of signals. BMP treatment of enriched cultures of bipotential glial precursors results in an increase in astrocyte differentiation and concomitant decrease oligodendrocyte differentiation. Further, altering the spatial and temporal expression of BMPs during CNS development has significant effects

on the subsequent development of astrocytes and oligodendrocytes. In the cerebral cortex of animals that overexpress BMP4 driven by the neuron-specific enolase promoter, increased numbers of astrocytes and reduced numbers of oligodendrocytes are seen [13]. Since gliogenesis persists well into the postnatal period, the expression of regulatory signals such as BMPs is also likely to be persistent. Together these data suggest that BMPs act as a major regulator of neural cell fate determination in the vertebrate CNS, both during early patterning of CNS stem cells and later during glial precursor differentiation. Recent studies suggest that under pathological conditions cells in the adult vertebrate CNS retain the ability to respond to these early fate cues.

Neural crest stem cells give rise to neuronal or glial derivatives in the peripheral nervous system. A wide range of cells in the peripheral nervous system, including autonomic neurons, sensory neurons, neuroendocrine cells and glial cells, are derived from neural crest cell precursors that emerge from the dorsal neural tube during the period of neural tube closure and subsequently migrate throughout the body. Transplantation studies *in vivo* and cell cloning experiments *in vitro* provide strong evidence that the neural crest contains multipotent progenitor cells. The prospective isolation of mammalian neural crest cells by virtue of their p75 expression and absence of markers of differentiation has allowed the analysis of PNS stem cells *in vitro* [14, 15]. Single cell cloning studies indicate that some cells are multipotent and give rise to neurons and glia. Further, multipotent neural crest cells also give rise to multipotent progeny, suggesting the presence of a true stem cell in the neural crest.

Several growth factors have been identified that act to increase particular derivatives from PNS stem cells, but their mode of action as proliferative agents, survival factors or inductive cues is not completely understood. For example, BMPs act on neural crest stem cells to induce adrenergic neurons [16], and glial growth factor (GGF), a member of the neuregulin family, inhibits neurogenesis and enhances gliogenesis [14]. The observation that isolated stem cells can be induced to produce cultures containing numerous cells of a single type, but that such cultures still contain cells of other types, may reflect the fact that PNS stem cells stochastically produce a variety of progenitors, and specific lineages are then amplified by growth factors. This issue has proved difficult to resolve, although it is clear that neural crest populations give rise to mature derivatives largely in response to 'instructive' cues from the microenvironment.

NEUROGENESIS OCCURS IN THE ADULT BRAIN

For many years, it was thought that the adult nervous system was unable to generate new neurons; after all, neural trauma or disease is devastating and essentially irreversible. Immediate exceptions were made to the notion that neurogenesis largely ceased in the adult, and focused initially on the songbird brain. Male songbirds add large numbers of new neurons each year to vocal centers in their brains [17, 18], and their generation is related to seasonal hormone surges and growth factor production. These neurons clearly integrate into the neural circuitry of the brain, providing a strong indication that, under the appropriate conditions, neurons can decipher migratory cues and synaptic partners even within the complex network of adult functional synapses.

The ability to generate new neurons is not restricted to the adult avian CNS. Two areas of the mammalian brain, the dentate gyrus of the hippocampus and the anterior region of the subventricular zone (SVZa), which generates cells in the rostral migratory stream to the olfactory bulb, include dividing cells that gave rise to neurons even in adults (Fig. 29-5). Early thymidine birthdating studies revealed that many neurons were generated after birth in rodents and, using similar birthdating approaches, neurogenesis has been detected in the adult olfactory precursors and hippocampus. More recent studies tracing the fate of the newly generated cells suggests that at least some of the dividing precursors are multipotent and may be stem cells [19–22]. These observations suggest that many regions of the mature brain have the capacity to give rise to new neurons.

If stem cells are retained into adulthood in the CNS it becomes critical to identify, localize and characterize those cells. Neurosphere assays suggest that the majority of stem cells in the adult CNS are localized close to the ventricles, although the precise nature of the cells remains obscure. Several populations have been suggested to include adult neural stem cells, including GFAP-expressing astrocytes, Notch1-expressing ciliated ependymal cells lining the ventricles, and GFAP radial glial cells. The presence of these stem cells has largely been inferred retrospectively and in order to effectively characterize and manipulate these cells it is important to purify them prospectively. Several assays have been suggested to enrich or isolate

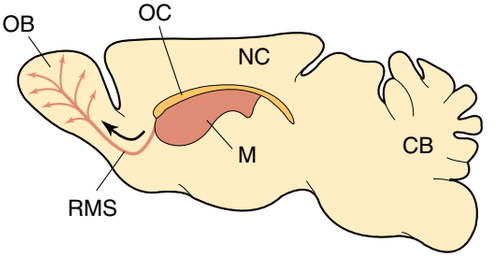

FIGURE 29-5 In the adult rodent brain, dividing cells in the subventricular zone adjacent to the lateral ventricle migrate in the rostral migratory stream (*RMS*) and differentiate into olfactory neurons in the olfactory bulb (*OB*). This is one site of neuronal turnover in the adult that appears to result from persistent generation of neurons from adult CNS stem cells. *CB*, cerebellum; *NC*, neocortex.

stem cells, although none are widely accepted. For example, stem cells may be enriched by removing other cells with features of differentiated neural cells from a mixed population. The problem with this approach is that stem cells are found in vanishingly small numbers so the yield is minimal. Until effective approaches for prospective isolation of reasonable numbers of adult stem cells are identified, it will be difficult to test many ideas about the regulation of adult CNS stem cells.

The findings that the adult CNS contain stem cells and that in some regions neurogenesis occurs in the normal brain raises the question as to why, in neurodegenerative diseases or after CNS trauma, neural repair is ineffective. At least three different phenomena may underlie the inability of the adult CNS to undergo extensive repair: (1) Although stem cells are present in the tissue there are too few to promote effective repair; (2) Although stem cells are present, they are unable to 'home' to the site of pathology; (3) Although stem cells are present in the tissue the molecular environment does not promote appropriate precursors or facilitate precursor expansion. These impediments to neural repair might be overcome if sufficient neural stem cells are delivered to areas of injury in conjunction with modulation of the growth factor environment. Recent studies on specific neural insults suggest that this type of therapy may have some application (see below).

STEM CELLS OFFER THE POTENTIAL FOR REPAIR IN THE ADULT NERVOUS SYSTEM

Mature nerve cells cannot divide to replace those that are lost. Thus, many neural disorders or injuries are characterized by the cells and functions that are lost. For example, in Parkinson's disease, neurons that make the neurotransmitter dopamine in the substantia nigra die. In amyotrophic lateral sclerosis, motor neurons of the spinal cord that activate muscles die. In multiple sclerosis, the oligodendrocytes that protect nerve fibers are lost and, following traumatic injury or stroke, multiple types of neural cell are lost. The ability to treat neurological ailments by transplanting stem cells to replace sick or missing cells in the brain has enormous promise. Much of the interest in stem cells comes from the possibility that these cells offer a source of 'new' neural cells to create functioning nerve tissue, offering new therapeutic possibilities. New findings in animals suggest that transplanted cells survive and may become incorporated in working neural circuits [23]. If stem cell therapeutic approaches are to be effective a number of critical issues have to be addressed. For example: (1) What is a good source of stem cells for neural repair? Is there a single type of stem cell, perhaps in bone marrow, that can generate the cells of any tissue? Are such cells more effective and more easily obtainable than stem cells from the adult CNS? (2) What cues are needed for the stem cells to differentiate efficiently into the desired cell type? Can *in vitro* expanded cells be effectively incorporated into the CNS? (3) What factors stimulate stem cells to home to sites of injury?

Brain cells can be derived from embryonic stem cells. One promising candidate for neural repair is the embryonic stem cell. Recently, techniques have been developed for the *in vitro* culture of ES cells, providing unprecedented opportunities for studying and understanding the regulation of human tissue differentiation. Very early human ES cells can be isolated and spread out on a feeder layer of mouse cells to produce an immortalized pluripotent human stem cell culture. These ES cells can then be stimulated to divide symmetrically to produce large numbers of undifferentiated stem cells [24, 25]. Alternatively, ES cells can be coaxed into forming aggregated clumps of cells called embryoid bodies that can then be stimulated to differentiate into a variety of cell types. One distinct advantage in the use of ES cells is that they are available *in vitro* for genetic manipulation and appear initially homogeneous, thus allowing selected transcription factors or growth factors to be genetically applied to stimulate differentiation. An early success in directed differentiation was obtained with human ES cells that were stimulated to become pancreatic islet cells that produce insulin [26].

Embryonic stem cells can also be manipulated to generate both CNS precursor cells and the terminal neuronal and glial fates of the CNS. For example, ES cells can be stimulated to give rise to dopaminergic neurons *in vitro* and have been transplanted into animal models of neurodegeneration and appear to offer functional benefit. In this case, a transcription factor essential for dopaminergic differentiation in development was transfected into ES cells *in vitro* and cell lines were established. Further neuronal differentiation was promoted with the soluble growth factors FGF8 and sonic hedgehog, with the result that some 80% of the cells contained the rate-limiting enzyme for catecholamine synthesis, tyrosine hydroxylase and dopamine itself were released *in vitro* [27]. When these cells were transplanted into the striatum of mice in which dopaminergic cells had been chemically ablated with 6-OHDA, many stimulated ES cells grafted into the site and extended processes. One and two months after grafting, animals who received these cells continued to show significant synaptic function and behavioral improvement. In another example, mouse embryonic stem cells were stimulated to differentiate into motor neurons and, when introduced into the spinal cord of embryonic chicks, motor axons formed contacts with skeletal muscle [28]. Thus, an understanding of inductive signals involved in normal pathways of neurogenesis has been very useful in directing ES cells to form specific classes of CNS neuron.

ES cells aggregated into embryoid bodies and propagated in growth factors, including platelet-derived growth factor, differentiate rapidly to include CNS glial cells.

About 40% of this population express markers of oligodendrocytes, and a similar proportion have astrocytic markers. To test the potential of these cells *in vitro*, ES cells propagated in this fashion were transplanted into the spinal cord or cerebral ventricles of myelin-deficient rats. Two weeks after transplantation, ES derived cells were present in the dorsal columns of the spinal cord both at the implant and several millimeters in both directions from that site. The mouse origin of the ES cells transplanted into rat was confirmed by analysis of mouse satellite DNA in the grafts. Similarly, intraventricular transplanted cells formed myelin in multiple brain regions [29]. These and other results support the further study of stimulated ES cells for potential therapies in the nervous system.

Even in these studies, the differentiation of stem cells is not complete and, after stimulation, the cultures contain cells at different stages and directions of differentiation. These observations may suggest that stem cells always give rise to particular progenitors and the primary role of exogenously added growth factors is to expand certain progenitor pools. One strong concern in these and other stem cell experiments is to avoid transplanting undifferentiated ES cells that cause teratoma-type cancers. Current studies are directed toward the efficient production of single cell types for use in therapies. The isolation of ES cells from early human embryos or propagation of fetal stem cells from aborted fetal tissues, however, raises many ethical, legal, religious and policy questions and the potential uses of stem cells for generating human tissues is a subject of ongoing public debate.

Brain cells may also be derived from non-neural stem cells. The isolation of human embryonic or nervous tissue for transplant therapy will always be fraught with significant ethical concerns, and thus scientists have explored other cellular sources of stem cells for neural therapies. Surprising recent data suggest that, contrary to traditional expectations, it may be possible to 'transdifferentiate' cells from one classical primary germ lineage to another. Developmental biologists have long known that three embryonic germ layers give rise to selected body cell lineages (endoderm to lining of the gut, mesoderm to blood and muscle, ectoderm to skin and nervous tissues, for example). The notion that segregation of cell fate is irreversible is being challenged by a series of recent discoveries suggesting that neural cells might be obtained from mesodermal lineages.

While it is perhaps not surprising that ES cells can generate neurons, as human embryonic stem cells develop into every tissue of the body, it is surprising that adult stem cells in particular tissues can give rise to cells of another lineage. For example, reports indicate that hemopoietic stem cells can give rise to myocytes [3], bone marrow stromal cells can give rise to astrocytes [30] and umbilical cord cells can be induced to form neurons and glia that can integrate in the brain after transplantation [31]. Further, there are reports that cells derived from the adult forebrain can reconstitute the hematopoietic system of lethally irradiated mice [32], and stem cells isolated from young or adult rodent skin proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells and adipocytes [33]. This type of work suggests that cells from completely different primary germ layers, such as bone marrow and blood, might produce nerve cells. The ability to generate neurons from a non-nervous-system cell tells us that cell potential may be broader than we imagined, and may provide a more convenient source for therapeutic cell approaches. Despite controversies concerning the plasticity of bone-marrow-derived stem cells, early clinical trials are being conducted in patients suffering from myocardial infarct, arthritic and neurological diseases using autologous bone marrow stem cells (see review in [34]). While these studies are in very early stages, one does wonder if there are multipotent stem cells in every tissue or, even more tantalizing, if there is a single type of stem cell, for example in bone marrow, that might generate the cells of any tissue.

One of the complications in this field is that relatively few differentiated cells are present in the graft, despite large numbers of transplanted cells. It is not clear why so few cells are present and whether this reflects the lack of survival factors in the adult host environment or the presence of few plastic cells in the graft. Unfortunately, the small number of differentiated neurons may also indicate that few cells have differentiated, and most cells are derived from cell fusion or other unusual cellular events. Enthusiasm for the utilization of non-neural stem cells for repair of the nervous system must also be tempered, as two recent studies have failed to repeat some of the original transdifferentiation results [35, 36]. In these studies, genetically labeled enriched hematopoietic stem cells were transplanted into lethally irradiated hosts, and non-hematopoietic derivatives were searched for. Extremely rare cells were occasionally seen in other tissues, even after damage, and, more critically, no evidence was seen for hematopoietic cells becoming neurons. The variability in studies of this type is an indication of our lack of fundamental understanding of the biology of stem cells in general.

An important remaining challenge in experimentally assaying the range of cellular derivatives made by stem cells has been to develop a bioassay in which precursors can repopulate the nervous and other systems effectively. One approach has been to inject adult neural stem cell neurospheres into the amniotic cavity of young chick embryos. In some animals, these cells integrate and give rise to cells of multiple germ layers and can be detected by virtue of introduced markers. Adult neural stem cells can also be used to form mouse chimeras with early embryos or when injected into blastocysts [37] and contribute to CNS and other organs, suggesting a very broad range of potential cell fates. These bioassay approaches are important for our understanding of stem cell biology, but are unlikely to be employed therapeutically.

STEM CELL TRANSPLANTS FOR NEURAL REPAIR

Stem cell transplantation to replenish neurons lost in Parkinson's disease. Parkinson's disease is a particularly intriguing clinical target for a cell transplant strategy, in part because it is based upon replacing a clearly defined and discrete population of neurons. In Parkinson's disease, a gradual loss of dopamineric neurons of the substantia nigra cells in the ventral midbrain leads to reduction of dopaminergic input to the striatum, and resulting in motor abnormalities. Management of individuals with Parkinson's disease has focused on symptomatic treatment, largely with drug therapy based on levodopa. Recently, however, there has been renewed interest in restorative or regenerative treatment based on transplantation of cells into the brain. The underlying premise of this approach is that injected cells survive and function as normal dopaminergic neurons and in doing so restore motor function.

Attempts have been made to replace dopaminergic neurons in diseased brain by grafting different types of dopamine secreting cell, including fetal midbrain precursors [38]. As discussed above, ES cells can be stimulated to give rise to dopaminergic neurons *in vitro* and, when transplanted into animal models of neurodegeneration, lead to long-lasting functional benefit [27, 38, 39]. Clinical studies suggest that grafted dopaminergic neurons remain for long periods despite a slow loss of the patient's own neurons. Fetal grafts have also been used as a source of dopaminergic transplants. One concern with this approach is that the results obtained are highly variable, for reasons that are poorly understood. Furthermore, involuntary movements occur in about 10% of patients. The results of these types of cell replacement therapy are encouraging but the heterogeneity of transplanted cells and risks of immunological rejection continue to make this approach challenging.

A number of studies have utilized injection of multiple types of stem cell into the damaged CNS in an attempt to enhance repair. For example, injection of mesenchymal stem cells into the adult rat brain after contusion injury or stroke models has been reported to enhance functional recovery. Likewise, transplantation of mesenchymal stem cells into the cavity created by spinal cord injury in adult rats appears to promote regeneration. In such cases, however, it seems likely that the transplanted cells are not differentiating into neural derivatives but rather acting to supply growth or survival factors that facilitate the endogenous repair process. Thus, it is important to recognize that enhanced functional recovery effected by stem cells may reflect the secretion of growth or survival factors rather than their integration into the injured CNS.

Stem cell transplantation to replenish glial cells lost in multiple sclerosis. The potential augmentation of neurons and glial cells in the pathological adult brain faces very different spatial challenges. In multiple sclerosis, CNS inflammation and widespread oligodendrocyte and axonal losses are hallmarks of the advanced disease. One therapeutic approach has focused on replacing the myelinating oligodendrocyte of the CNS. While neurons may be difficult to produce in large numbers, replacement of the more ubiquitous glia may alternatively be challenging because it is difficult to deliver cells simultaneously to many sites in the adult nervous system.

One model that has been extensively used to determine the repair capacity of glial precursors is the *shiverer* mutant mouse. This animal lacks myelin basic protein and as a consequence is unable to generate compact myelin. These animals develop an extensive tremor postnatally and die early. Classical studies demonstrated that transplantation of normal tissue into the developing shiverer CNS resulted in patchy myelin formation as a result of the integration of the transplanted cells. This type of analysis has subsequently been repeated using a variety of cell sources and delivery approaches. For example, intraventricular injection of a neural stem cell line resulted in widespread myelination and a reduction in the behavioral deficit [40]. The widespread dispersal of the injected cells suggests that either these cells are capable of long-distance migration through the developing CNS or they integrate into different sites as a result of transependymal migration. Similar studies have utilized rodent neurospheres or gliosphere-derived cells with similar results, and more recently the capacity of transplanted human stem cells to generate myelin in the *shiverer* background has been demonstrated.

While such studies are illuminating in terms of the myelinogenic capacity of the transplanted cells, the lack of endogenous compact myelin in the *shiverer* host does not make them a particularly useful model for demyelinating diseases such as multiple sclerosis. Furthermore, while extensive dispersal of glial cells may be feasible in the developing CNS, cellular migration through the adult myelinated CNS appears to be highly restricted and direct injection of stem cells into multiple sites of the adult human CNS is not practical. Remarkably, some studies have raised the possibility that delivery of regenerative stem cells via the blood stream may facilitate repair of demyelinated lesions.

Some aspects of multiple sclerosis are reflected in the animal model experimental autoimmune encephalomyelitis, which is induced by immunization of susceptible animals with appropriate encephalogenic proteins or peptides. In these animals, if cultured adult stem cell neurospheres are injected into the bloodstream, injected cells can find their way to damaged portions of the nervous system and improve function in mice. How the injected cells augmented the recovery process is unclear. One possibility is that cells recruited to the lesions differentiated into oligodendrocytes and generated new myelin sheaths, but this seems unlikely in the face of ongoing cellular destruction.

Repair in the CNS also requires transplanted cells to traverse the blood–brain barrier. To circumvent the blood–brain barrier, in other approaches cells were delivered intracerebroventricularly, and neurophysiological recovery was also noted [41]. As encouraging as these initial studies are, the animal experiment is very different from treating multiple sclerosis, which is a chronic and inflammatory disorder that involves both glial and axonal changes. Furthermore, the scale of repair required in the human CNS is substantially larger than that in murine or rodent models. We still face questions with multifocal diseases as to the best way to introduce cells throughout the nervous system and, in the case of a chronic inflammatory disease, how to keep transplanted cells from succumbing to the same underlying disease.

Hematopoietic stem cell transplants as treatment for glial disease. Hematopoietic stem cell transplants have been used successfully to treat genetic disorders that affect the nervous system, such leukodystrophies. Leukodystrophies are a group of genetic disorders characterized by the imperfect development or maintenance of the myelin sheath and include diseases like adrenoleukodystrophy, Alexander's disease, Canavan's disease, Krabbe's disease, metachromatic leukodystrophy, Pelizaeus–Merzbacher disease, Refsum's disease and phenylketonuria. Globoid-cell leukodystrophy is caused by a deficiency in galactocerebroside, a glycolipid essential for normal myelin. Depending on the particular leukodystrophy, patients either die prematurely, suffer from severe cognitive dysfunction or suffer from seizures. Children with globoid cell leukodystrophy treated with hematopoietic stem cell transplantation develop normal leukocyte galactocerebroside levels, and CNS deterioration can be reversed or prevented [42]. It remains unclear exactly how hematopoietic stem cell transplants result in this neural improvement.

EXPANDING ENDOGENOUS NEURAL STEM CELLS FOR REPAIR

The realization that many regions of the adult CNS contain neural stem cells as well as glial precursors capable of generating oligodendrocytes suggests that approaches designed to expand these cells and stimulate their appropriate differentiation may be useful therapeutically. The ability to locally expand populations of endogenous neural stem cells and have the progeny integrate successfully into the mature CNS would circumvent many of the problems associated with cellular transplantation.

In most regions of the CNS there is little or no turnover of neurons during the life of the animal and following trauma to the adult CNS recruitment of new neurons is extremely limited. This may reflect the low proliferation rate of neural stem cells or the inability of stem cell progeny to survive or target the appropriate location. It may be that recruitment is severely impaired by the neuroinflammatory response that accompanies a wide range of neural pathologies such as Alzheimer's disease, trauma and multiple sclerosis. Consistent with this notion, when a distinct population of cortical neurons is eliminated by retrograde incorporation of a cellular toxin, little activation of the inflammatory pathway occurs and some endogenous cellular repair has been observed [43]. Incorporation of BrdU demonstrated neural precursor proliferation and the newly generated cells migrated to the appropriate laminar location, assumed the correct identity and projected appropriately to the opposite brain hemisphere. In similar studies, when mature dopaminergic neurons are destroyed by the toxin MPTP, the rate of replacement of the lost neurons increases [44]. These observations suggest that under the appropriate conditions endogenous stem cells of the adult CNS might have the capacity to replace lost neurons, although the results have to be interpreted with caution since similar parallel studies have found that only glial cells were generated in the adult [45]. How the adult brain detects the loss of selected neurons and whether this loss serves to 'activate' dormant stem cells or recruit their progeny to particular fates is unknown.

The capacity of the adult CNS to generate new glial cells is well established. Following penetrating lesions or contusion injuries to the brain or spinal cord, a rapid glial response occurs in which astrocytes surrounding the lesion undergo hypertrophy and proliferate. Such a response generates a 'glial scar' that is thought to contribute to the lack of axonal regeneration. The astrocytes of the scar, however, do not appear to functionally integrate into the surrounding neuropil. Similarly, CNS injuries promote the proliferation of a population of cells that have been termed 'adult oligodendrocyte precursors', identified by expression of the NG2 epitope. In adult human white matter these presumptive glial progenitors are very common and may comprise perhaps 3% of all cells. It seems likely that the progeny of these cells contribute to the remyelination that is seen as shadow plaques in some multiple sclerosis patients. Adult oligodendrocyte precursors can also be passaged as neurospheres with the mitogenic and differentiative characteristics of stem or multipotent cells. These cells generate functional neurons and glia *in vitro*, suggesting that a broader range of cell fates are possible under some conditions. Animals implanted with expanded white-matter-derived progenitor cells contained β-tubulin-labeled neurons in the rostral migratory stream to the olfactory bulb as well as GFAP labeled astrocytes and CNP oligodendrocytes in white matter [46], suggesting that white matter $NG2^+$ cells are capable of stem-cell-like properties.

Infusion of growth factors following an injury has been suggested to greatly enhance the replacement of lost cells. For example, infusion of EGF, the major mitogen for neural precursors, was reported to enhance the

recruitment of neural stem cells or their progeny to lesioned areas. In spinal cord injury, direct injection of Shh into the lesion site resulted in dramatic increases in cell proliferation and the proliferating cells had the capacity to give rise to neurons, astrocytes and oligodendrocytes. Further, Shh injection appeared to enhance functional recovery, although it was unclear whether the improved function resulted from enhanced neural cell generation or a reduction in secondary cell death.

Cell fusion may underlie some observations of grafted cells. Better methods are needed to document the birth, proliferation and migration of new neurons. Early encouraging results that transplanted cells differentiated into primary neurons has been tempered by proof that, rather than a single grafted cell differentiating into a neuron, cell fusion events may explain some apparent transdifferentiation. For example, when female patients with various blood diseases who had received human male adult bone marrow transplants were examined postmortem for persistent transplanted cells, Y chromosome markers were present in a few Purkinje cells in the cerebellum [47]. The frequency of such Y-labeled neurons was small but appeared to reflect an immutable marker that could only have been obtained from the transplanted bone marrow. This led to questions about whether blood cells transdifferentiate from blood lineage to neuron lineage cells or if another mechanism transfers the label to adult neurons. In adult mice that received GFP-labeled bone-marrow-derived cells, again labeled Purkinje cells were observed but GFP was present only in heterokaryon products of cell fusion [47]. These heterokaryons increased in a linear fashion over several years. Cell fusion has also been demonstrated to underlie bone marrow contributions to differentiated liver cells [48, 49]. These cell fusion events are essentially equivalent to nuclear reprogramming. The fact that grafted bone marrow cells could participate in the formation or maintenance of neurons is remarkable, but these studies do suggest that some initial euphoria in response to the apparent plasticity of stem cells might need re-examination. While cell fusion may cast doubt on some cell transformation interpretations, this mechanism does suggest that a novel form of therapy might be possible.

COMMON STEM CELL THERAPY CHALLENGES

While promising, stem or progenitor cell research for neural therapeutics still needs to overcome many obstacles before it transfers effectively to the clinic. There are significant gaps in our understanding of how stem cells contribute to the normal development of the nervous system and the nature of the regulatory factors that stimulate appropriate differentiation. Developmental biologists understand that a closely regulated interplay of genetic regulation and cell–cell communication via growth factors will be required. Efficient stem cell therapies are likely to be based upon the ability to prospectively isolate and greatly expand the stem cells, and subsequently efficiently direct their differentiation, and these issues presently pose significant technical hurdles.

The source of cells used for transplantation forms a continuing discussion point. Early reports suggest that ES cells are most efficient for production of large numbers of neurons, but their use involves significant ethical and legal debates. If adult stem cells, or even stem cells from an easily harvested location such as bone marrow, were capable of significant expansion and could be directed to differentiate into a wide range of neural cells, these would be very attractive. Further, the possibility of expanding the patient's own endogenous stem cells would alleviate concerns about immune rejection and the use of immunosuppression drugs. At this point, it is unclear if neurons can be efficiently derived from any source other than ES cells.

No matter how many cells might be generated in the laboratory, this approach is useless if the adult brain does not accept and incorporate the graft correctly. The functional integration of immature cells into the host neural circuitry is essential for neuronal replacement. Animal studies suggest that transplanted cells can incorporate and function in circuits, but the extent of the damaged brain's ability to allow regenerative responses is not entirely clear. Further, little is known about the regulation of injury factors that might direct stem cells to localize in injured regions, or how to deliver cells effectively to large regions of the damaged nervous system. Indeed, very little is known about the possibility that transplanted cells might succumb to the same disease process that affected original cells. By contrast, a remaining major concern is to prevent transplantation of cells that continue to proliferate and create tumors. The long term side effects of transplanting new neural cells is largely unknown.

CONCLUSIONS

In the last decade, the roles of stem and progenitor cells in the differentiation of the embryonic nervous system have become illuminated, leading to fundamental advances in our knowledge of the regulation of cell fates in the brain. Much is still needed for us to understand the interplay between genetic and cell communication events that leads to normal generation of the complex nervous system. These issues are important to resolve if we are to learn how disease or trauma affect the growing brain.

We have also caught a glimpse of how various stem cells might repair damage or disease in the adult nervous system. Embryonic stem cells form an important source of new neurons, but accompanying this success are many ethical issues about their use. Some reports have suggested other non neural tissues might serve as a reservoir of stem cells that might transdifferentiate into neurons and glial

cells, but questions about the interpretation of these experiments leaves this approach uncertain.

Finally, for the field to move forward in an effective way it is critical that new bioassays that efficiently isolate and monitor stem and progenitor cells in the intact nervous system and directly assess their restorative potential are developed. Such advances will allow us to effectively compare what cell fates are possible *in vitro* with their differentiation in a normal or pathologic cellular environment.

REFERENCES

1. Kondo, M., Wagers, A. J., Manz, M. G. *et al.* Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu. Rev. Immunol.* 21: 759–806, 2002.
2. Brazelton, T. R., Rossi, F. M., Keshet, G. I. and Blau, H. M. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 290: 1775–1779, 2000.
3. Gussoni, E., Soneoka, Y., Strickland, C. D. *et al.* Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401: 390–394, 1999.
4. Hemmati-Brivanlou, A. and Melton, D. Vertebrate neural induction. *Annu. Rev. Neurosci.* 20: 43–60, 1997.
5. Reynolds, B. A. and Weiss, S. Generation of neurons and astrocytes from isolated cells from the adult mammalian central nervous system. *Science* 255: 1707–1710, 1992.
6. Reynolds, B. A. and Weiss, S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* 175: 1–13, 1996.
7. Gage, F. H. Mammalian neural stem cells. *Science* 287: 1433–1438, 2000.
8. Liem, K. J., Tremmel, G., Roelink, H. and Jessel, T. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* 82: 969–979, 1995.
9. Liem, K. J., Tremml, G., Roelink, H. and Jessel, T. A role for the roof plate and its resident TGFβ-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* 91: 127–138, 1997.
10. Zhu, G., Mehler, M. F., Zhao, J., Yu Yung, S. and Kessler, J. A. Sonic hedgehog and BMP2 exert opposing actions on proliferation and differentiation of embryonic neural progenitor cells. *Dev. Biol.* 215: 118–129, 1999.
11. Mehler, M., Mabie, P., Zhu, G., Gokhan, S. and Kessler, J. Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineages. *Dev. Neurosci* 22: 74–85, 2000.
12. Gross, R. E., Mehler, M. F., Mabie, P. C., Santschi, L., Ngo, P. and Kessler, J. A. Promotion of astroglial lineage commitment from mammalian subventricular zone progenitor cells by bone morphogenetic proteins. *Neuron* 17: 1–20, 1996.
13. Gomes, W. A., Mehler, M. F. and Kessler, J. A. Transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment. *Dev. Biol.* 255: 164–177, 2003.
14. Shah, N. M., Groves, A. K. and Anderson, D. J. Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell* 85: 331–343, 1996.
15. Bixby, S., Kruger, G. M., Mosher, J. T., Joseph, N. M. and Morrison, S. J. Cell intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neuronal diversity. *Neuron* 35: 643–656, 2002.
16. Schneider, C., Wicht, H., Enderich, J., Wegner, M. and Rohrer. H. Bone morphogenetic proteins are required *in vivo* for the generation of sympathetic neurons. *Neuron.* 24: 861–870, 1999.
17. Nottebaum, F. A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. *Science* 214: 1368–1370, 1981.
18. Tramontin, A. D. and Brenowitz, E. A. Seasonal plasticity in the adult brain. *Trends Neurosci.* 23: 251–258, 2000.
19. Lois, C. and Alvarez Buylla, A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl Acad. Sci. U.S.A.* 90: 2074–2077, 1993.
20. Alvarez Buylla, A. and Garcia Verdugo, J. M. Neurogenesis in adult subventricular zone. *J. Neurosci.* 22: 629–634, 2002.
21. Luskin, M. B. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11: 173–189, 1993.
22. Gage, F. H., Ray, J. and Fisher, L. J. Isolation, characterization and use of stem cells from the CNS. *Annu. Rev. Neurosci* 18: 159–192, 1995.
23. Englund, U., Bjorklund, A., Wictorin, K., Lindvall, O. and Kokaia, M. Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. *Proc. Natl Acad. Sci. U.S.A.* 99: 17089–17094, 2002.
24. Smith, A. G., Heath, J. K., Donaldson, D. D. *et al.* Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336: 688–690, 1988.
25. Stavridis, M. P. and Smith, A. G. Neural differentiation of mouse embryonic stem cells. *Biochem. Soc. Trans.* 31: 45–49, 2003.
26. Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. and McKay, R. D. G. Differentiation of embroyonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292: 1389–1394, 2001.
27. Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418: 50–56, 2002
28. Wichterle, H., Lieberam, I., Porter, J. A. and Jessell, T. M. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110: 385–397, 2002.
29. Brustle, O., Jones, K. N., Learish, R. D. *et al.* Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285: 754–756, 1999.
30. Kopen, G. C., Prockop, D. J. and Phinney, D. G. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl Acad. Sci. U.S.A.* 96: 10711–10716, 1999.
31. Buzanska, L., Stachowiak, E., Stachowiak, M. and Domanska-Janik, K. Neural stem cell line derived from human umbilical cord blood – morphological and functional properties. *J. Neurochem.* 85(Suppl. 2): 33, 2003.
32. Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. and Vescovi, A. L. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo. Science* 283: 534–537, 1999.

33. Toma, J. G., Akhavan, M., Fernandes, K. J. *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat. Cell Biol.* 3: 778–784, 2001.
34. Tao, H. and Ma, D. D. Evidence for transdifferentiation of human bone marrow-derived stem cells: recent progress and controversies. *Pathology* 35: 6–13, 2003.
35. Castro, R. F., Jackson, K. A., Goodell, M. A., Robertson, C. S., Liu, H. and Shine, H. D. Failure of bone marrow cells to transdifferentiate into neural cells *in vivo*. *Science* 297: 1299, 2002.
36. Wagers, A. J., Sherwood, R. I., Christensen, J. L. and Weissman, I. L. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* 297: 2256–2259, 2002.
37. Clarke, D. L., Johansson, C. B., Wilbertz, J. *et al.* Generalized potential of adult neural cells. *Science* 288: 1660–1663, 2000.
38. Studer, L., Tabar, V. and McKay, R. D. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nat. Neurosci.* 1: 290–295, 1998.
39. Wu, P., Tarasenko, Y. I., Gu, Y., Huang, L. Y., Coggeshall, R. E. and Yu, Y. Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. *Nat. Neurosci.* 5: 1271–1278, 2002.
40. Yandava, B. D., Billinghurst, L. L. and Snyder, E. Y. 'Global' cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. *Proc. Natl Acad. Sci. U.S.A.* 96: 7029–7034, 1999.
41. Pluchin, S., Quattrini, A., Brambilla, E. *et al.* Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422, 688–694, 2003.
42. Krivit, W., Shapiro, E. G., Peters, C. *et al.* Hematopoietic stem cell transplantation in globoid cell leukodystrophy. *N. Engl. J. Med.* 338: 1119–1127, 1998.
43. Magavi, S. S., Leavitt, B. R. and Macklis, J. D. Induction of neurogenesis in the neocortex of adult mice. *Nature* 405: 951–955, 2000.
44. Zhao, M., Momma, S., Delfani, K. *et al.* Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc. Natl Acad. Sci. U.S.A.* 100: 7925–7930, 2003.
45. Lie, D. C., Dziewczapolski, G., Willhoite, A. R., Kaspar, B. K., Shults, C. W. and Gage, F. H. The adult substantia nigra contains progenitor cells with neurogenic potential. *J. Neurosci.* 22: 6639–6649, 2002.
46. Nunes, M. C., Roy, N. S., Keyoung, H. M. *et al.* Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat. Med.* 9: 439–447, 2003.
47. Weimann, J. M., Johansson, C. B., Trejo, A. and Blau, H. M. Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. *Nat. Cell Biol.* 5: 959–966, 2003.
48. Vassilopoulos, G., Wang, P. R. and Russell, D. W. Transplanted bone marrow forms liver by cell fusion. *Nature* 422: 901–904, 2003.
49. Wang, X., Willenbring, H., Akkari, Y. *et al.* Cell fusion is the princple source of bone marrow derived hepatocytes. *Nature* 422: 897–901, 2003.

CHAPTER

30

Axonal Growth in the Adult Mammalian Nervous System: Regeneration and Compensatory Plasticity

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SUMMARY AND CONCLUSIONS 526

The adult mammalian brain and spinal cord have limited capacity for spontaneous functional and structural recovery after injury. However, over the past 20 years a virtual explosion of scientific evidence has shown us that damaged adult neurons are indeed capable of regrowth given the proper conditions, and a number of strategies in which adult CNS neurons can be influenced to regrow following injury have been found. These strategies include suppression of action of growth-inhibitory molecules, which are primarily found in myelin, reduction of scar barriers, enhancement of the neuronal response to axotomy through supplementation with growth factors and enhancement of the growth potential of the injured neuron. These investigations, which will be discussed in this chapter, lay the foundation for understanding CNS repair mechanisms and give hope to a situation previously thought to be hopeless.

There are two important structural processes in which the brain and spinal cord regain function after damage, i.e. regeneration and compensatory plasticity (Fig. 30-1). In regeneration, the cut axon begins to regrow from the

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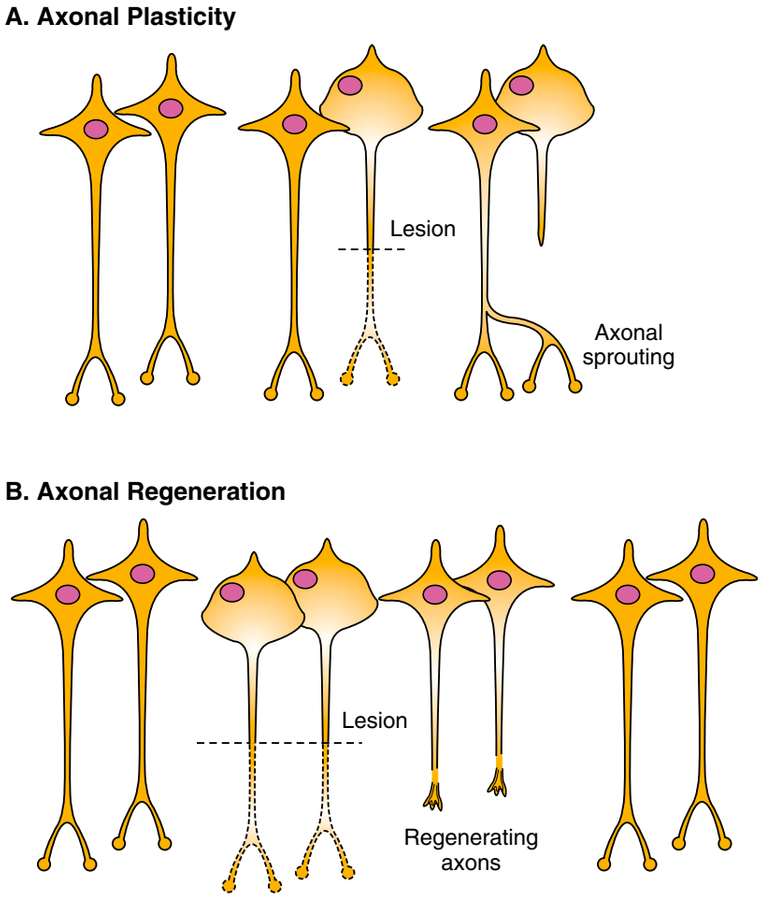

FIGURE 30-1 Simplified drawing depicting the difference between axonal plasticity (**A**), where following axonal injury the cut axon does not regrow, but other undamaged neurons grow new axons to reinnervate denervated targets. In axonal regeneration (**B**), cut axons regrow from the damaged sites and reconnect with denervated targets.

damaged end and elongates either through or around damaged tissue to eventually reconnect with deafferented targets. On the other hand, compensatory plasticity is conceptually quite different, i.e. it is the *undamaged* spared neurons that grow new axonal connections to deafferented targets, and this source of new axonal growth can be quite distant from the original injury site. Interestingly, these two types of recovery mechanisms most probably share a similar molecular basis for promoting axonal regrowth.

REGENERATION IN THE PERIPHERAL NERVOUS SYSTEM

Peripheral nerve injuries are caused by a variety of factors including acute trauma, chronic repetitive insults, and inheritable or acquired metabolic disorders (Ch. 36). As opposed to the CNS, which has traditionally been thought to be fixed, axonal injury in the PNS often results in some degree of spontaneous regeneration, although it is not always completely successful in terms of targeting or full functional recovery.

Wallerian degeneration is the secondary disruption of the myelin sheath and axon distal to the injury. This process was first described in 1850 in frog peripheral nerves by the British physiologist Augustus Waller and was subsequently termed Wallerian degeneration. When either central or peripheral axons are damaged, Wallerian degeneration occurs, but with important differences between these two systems. These differences may underlie at least in part their different regenerative potentials (Table 30-1).

The molecular and cellular events during Wallerian degeneration in the peripheral nervous system transform the damaged nerve into an environment that supports regeneration. First, the cut axon degenerates distal to the injury site and, within 18–48 hours, cytoskeletal disintegration of axonal neurofilaments and microtubules occurs, which is due to proteases such as calpains

TABLE 30-1 Major differences in Wallerian degeneration

Peripheral nervous system	Central nervous system
Schwann cell survival and differentiation	Apoptotic death of oligodendrocytes
Rapid phagocytosis of injured axons and myelin sheaths by invading macrophages	Slow removal of injured axons, myelin sheaths and persistence of some myelin inhibitory factors
Formation of new myelin sheaths	No new myelin reforming

that are stimulated by an increase in intra-axonal concentration of calcium ion [1] (Fig. 30-2). The Schwann cell processes surrounding the axon retract their myelin but do not die, and are supported by autocrine trophic factors. Axotomy induces the production of inflammatory cytokines such as tumor necrosis factor (TNF)α and interleukin (IL)-1α in Schwann cells almost immediately. These cytokines play a critical role in macrophage recruitment to the injury site through endothelial cell activation and chemokine production [2]. After 1–2 weeks, fairly rapidly as compared to the CNS, degenerating axonal and myelin debris is removed primarily by macrophages migrating in from the bloodstream and partly by endogenous endoneurial macrophages and Schwann cells themselves. Invading macrophages also serve a key role by secreting a variety of cytokines, such as IL-1 and platelet-derived growth factor (PDGF), that stimulate Schwann cells to divide, dedifferentiate and proliferate distal to the injury. These stimulated Schwann cells are induced to secrete growth factors such as nerve growth factor (NGF), insulin-like growth factor (IGF)-1 and ciliary neurotrophic factor (CNTF) which increase the chance of neurons to survive the trauma of axotomy and stimulate axons to regenerate.

Both Schwann cells and basal lamina are required for axonal regeneration to proceed. Each axon–Schwann cell unit in a peripheral nerve is surrounded by a basal lamina sheath of collagen, laminin and fibronectin. Following injury, this entire basal lamina/Schwann cell cylinder is known as the band of Büngner, and this unit forms pathways to guide regenerating nerves back to their appropriate target structures. In a localized crush injury, the basal lamina sheaths surrounding the nerves are usually left intact, and therefore axonal regrowth and functional recovery are much better than in a transection injury, where the lamina sheath is cut and guidance mechanisms are therefore lost.

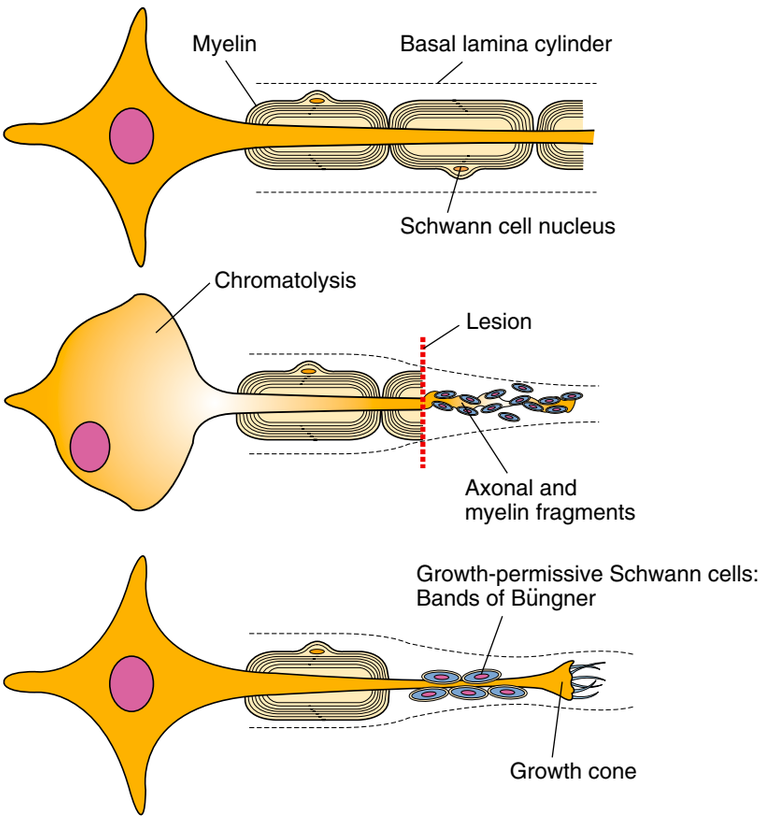

FIGURE 30-2 Wallerian degeneration in the PNS. After an axon is injured, resulting chromatolysis, i.e. stress reaction and increased protein synthesis, occurs in the neuronal cell body, with axonal and myelin degeneration distal to the injury. Growth-permissive Schwann cells secrete growth factors that stimulate axons to regenerate.

Cell surface adhesion molecules, which promote regeneration, are expressed on plasmalemma of both Schwann cells and regenerating peripheral axons. Adhesion molecules such as L1, neural cell adhesion molecule (N-CAM) and *N*-cadherin promote axonal regeneration by homophilic interactions between axons and Schwann cell surfaces (see Ch. 7). The expression of p75 (low affinity NGF receptor, Ch. 27) is also increased at the Schwann cell surface after injury. Extracellular matrix molecules, such as tenascin and proteoglycans, increase the regenerative potential of damaged peripheral nerves by binding to integrins on the axonal surface.

Structural and biochemical changes occur after axotomy. At the site of axonal damage, and within approximately one hour, the cut end of the axon seals off and formation of a motile growth cone-like structure is formed. Within 1–2 days, depending upon how far from the cell body the cut is, major changes in gene expression and protein production occur within the cell body and new proteins are transported to the growing axon tip. These include structural proteins such as tubulin alpha 1, pro-regenerative proteins such as Reg-2 [3] and the growth associated protein GAP-43. This phosphoprotein (also known as B-50, F1, pp46, 48K 4.5) is present on the inside of the growth cone membrane where it interacts with various kinases and plays an important role in communication between the growth cone and its micro-environment [4, 5]. Following axotomy, massive upregulation of GAP-43 occurs in both sensory and motor neurons, and levels remain high until axons reconnect with their targets at which time expression returns to baseline levels.

REGENERATION IN THE CENTRAL NERVOUS SYSTEM

For regeneration in the mammalian CNS to occur, the injured neuron must first survive, and its damaged axon must regrow sometimes long distances through or around the injury site, eventually connecting with appropriate targets. As in the PNS, the axon is driven forward by the growth cone, a dynamic motile structure at the distal tip of regenerating axons containing the molecular machinery for growth and guidance to navigate through the intact and injured CNS. Once contact is made, the axon needs to be remyelinated and functional synapses formed on target cells.

After a CNS axon is injured, Wallerian degeneration distal to the cut end does occur, as in the PNS, but with important differences (Table 30-1). The degenerating myelin and axonal debris persist much longer, with less recruitment of macrophages from the peripheral blood to eliminate the debris; thus myelin inhibitory factors are present much longer than in the injured PNS. Microglia are the first glial cells to react, within a few hours after injury. Astrocytes and meningeal cells are activated and collaborate in forming the glial scar, with inhibitory molecules such as chondroitin sulfate proteoglycans remaining for several weeks to months. Oligodendrocytes persist or die slowly.

As opposed to the PNS, where growth-associated molecules such as GAP-43 are uniformly upregulated by the lesioned neurons after axotomy, CNS neurons vary greatly in their responses to injury. The pattern of expression of GAP-43 in the CNS during development is ubiquitous but, after synaptogenesis is completed and critical periods for activity dependent plasticity are over, GAP-43 is downregulated in most adult CNS neurons. However, some neurons continue to express high levels of GAP-43, including the dopaminergic neurons of the substantia nigra, and neurons associated with adult plasticity, such as hippocampal cells and cells within the olfactory bulb, where axons continue to grow into adulthood. After injury, dopaminergic neurons do not increase their expression of GAP-43, although most central neurons do have some transitory increased response, with greater upregulation of proteins the closer the injury is to the neuronal cell body.

Despite extensive study, the role of Wallerian degeneration in nerve degeneration and regeneration is not completely understood. However, the discovery of a mutant mouse strain has the potential to add some insight into this important reparative process. The Wld^s (Wallerian degenerate, slow) mouse is a spontaneously occurring mutant strain with an interesting phenotype of slow Wallerian degeneration and prolonged axonal survival after both CNS and PNS nerve injury [6]. The Wld^s mutation is made by the splicing of fragments of two genes within an 85 kb triplication on chromosome 4. This splice results in a new open reading frame and codes for a 42 kDa chimeric protein unique to the Wld^s mouse, with an as of yet unknown function. However, introduction of the Wld^s gene into cultured rat DRG neurons made these previously susceptible neurons resistant to axonal degeneration in a model of toxic neuropathy. Further studies are needed to fully elucidate the potential therapeutic role of the Wld^s gene in axonal survival and repair [7].

That the regenerative ability of adult mammalian neurons can be altered by their environment was first suggested by Ramon y Cajal and later demonstrated in the now classic experiment by David and Aguayo [8] (Fig. 30-3). When sciatic nerve segments taken from rats were used as 'bridges' between the medulla oblongata and spinal cord, damaged CNS axons at both of these levels grew into the peripheral nerve grafts for long distances, i.e. for several centimeters in length. Importantly, the suggestion that the peripheral nerve glial environment was permissive to central axonal regrowth led the way for experiments investigating the differences between central oligodendrocytes and peripheral Schwann cells and their products, i.e. myelin.

Central nervous system myelin contains molecules that inhibit neurite growth. One of the major obstacles to new neurite outgrowth in the adult CNS is the presence

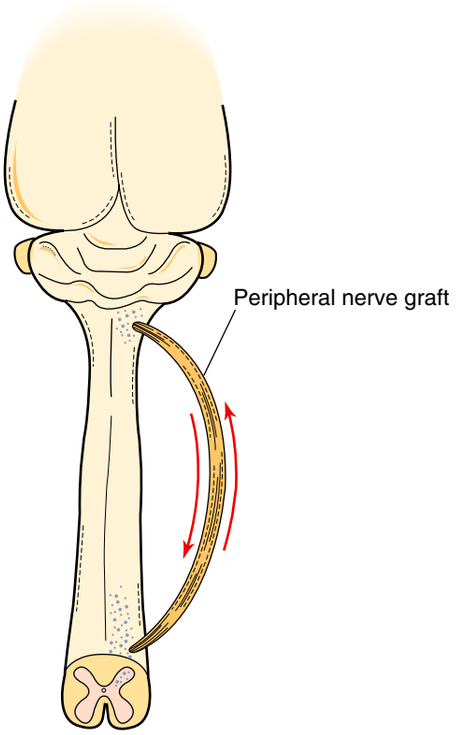

FIGURE 30-3 Diagram of the dorsal surface of the rat CNS, showing a peripheral nerve 'bridge' linking the medulla oblongata and the thoracic spinal cord. As indicated by the arrows, damaged CNS axons from both sites of the nerve implantation grew into the graft and continued for long distances, demonstrating the growth potential of CNS neurons when given the microenvironment of the PNS. (Modified with permission from reference [8].)

of inhibitory molecules in myelin. Studies have elucidated three inhibitors associated with myelin, namely Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) [9].

Nogo-A is a potent inhibitor of neurite growth and blocks axonal regeneration in the central nervous system. Early *in vitro* experiments showed that neurite outgrowth was impeded across a culture dish coated with CNS myelin whereas neurites would actively grow on a dish coated with PNS myelin. Biochemical fractionation of rat brain myelin led to the identification of two membrane proteins of about 250 kDa and 35 kDa with strong inhibitory properties when added to growing neurites. Further purification of the corresponding bovine 220 kDa protein enabled sequencing of six peptides, which ultimately led to the cloning of the cDNA of a gene that was aptly named *Nogo* [10]. So far, three distinct isoforms of Nogo have been identified, Nogo-A, B and C, with Nogo-A being the isoform most studied (Fig. 30-4).

Nogo-A is a membrane protein expressed in the adult mammalian CNS primarily by oligodendrocytes [11]. Neuronal expression is also seen in the dorsal root ganglia, sympathetic ganglia, motor neurons, hippocampal pyramidal cells and cerebellar Purkinje cells of the developing nervous system. Nogo-B is found in many tissues and cell types including adult neurons and oligodendrocytes, and Nogo-C is most highly expressed outside of the CNS, in skeletal muscle. While the functions of Nogo-B and Nogo-C are not entirely known, Nogo-B is found highly expressed in cultured endothelial and smooth muscle cells, as well as in intact blood vessels, and it may be a regulator of vascular homeostasis and remodeling [12].

Peptide fragment analysis of Nogo-A has shown that inhibition of neurite growth, collapse of growth cones and inhibition of fibroblast spreading are associated with two or three distinct regions of the molecule [13]. A principal inhibitory region is found in the middle of the Nogo-A- specific sequence, termed 'Amino-Nogo'. A 66-residue loop between the two hydrophobic regions (Nogo-66) is also able to inhibit neurite growth and to induce growth cone collapse [14] (Fig. 30-5).

The Nogo gene is a member of the reticulon superfamily. Nogo-A comprises approximately 1200 amino acids with two long hydrophobic stretches (transmembrane segments or loops) close to the C-terminus. Only about the last 180 amino acids show homologies to a previously known family of three genes, the reticulons (RTN). The RTN proteins are named after the main subcellular localization of RTN-1, which is in the endoplasmic reticulum. Nogo, i.e. RTN-4, not only colocalizes with the endoplasmic reticulum but is also found on oligodendrocyte

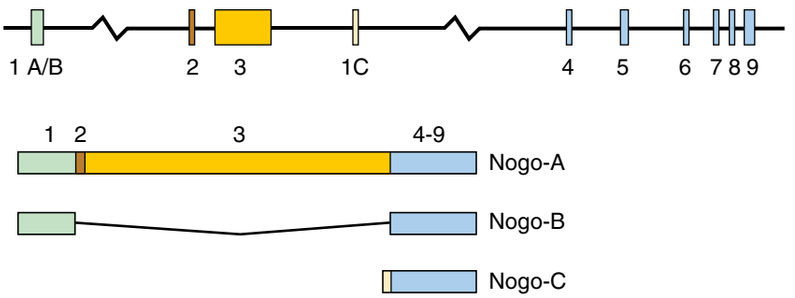

FIGURE 30-4 Isoforms of Nogo. Nogo-A, -B and -C have a common carboxy terminus of about 180 amino acids (the common domain – *blue*– contains Nogo-66). Nogo -A and -B share an amino terminus of about 172 amino acids (*green*).

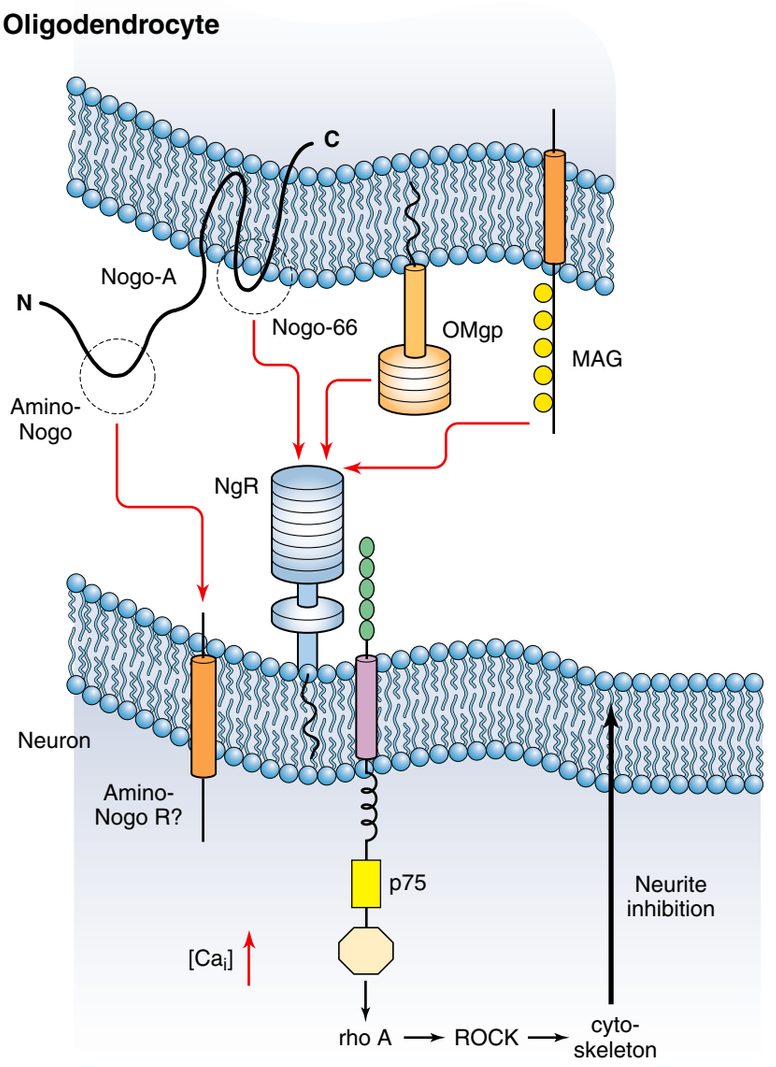

FIGURE 30-5 Nogo-A, MAG and OMgp are the principal inhibitors of neurite growth in CNS myelin. A portion of Nogo-A, i.e. Nogo-66, interacts with the same receptor, NgR, as do MAG and OMgp. NgR in the neuronal membrane complexes with p75 and other possible co-receptors to activate Rho A and its downstream target, ROCK, and may result in neurite inhibition. Another major inhibitory area of Nogo-A, known as Amino-Nogo, interacts with a receptor that is not yet known. [Ca_i], intracellular calcium. (Modified with permission from reference [9].)

plasma membranes. The RTN family is evolutionarily very old and occurs in all eukaryotes including plants and fungi. The RTN family is distributed widely throughout the CNS and other tissues in animals [15].

Most of the known RTN proteins have a relatively short amino (N)-terminal sequence. The very long N-terminal sequence of Nogo-A with its potent inhibitory regions appears late in vertebrate evolution, which suggests that Nogo-A is the result of a fusion between an ancient RTN homology domain at the C-terminus and two to three open reading frame sequences at the N-terminus, allowing the evolved protein to adopt a new function, i.e. that of a neurite-growth inhibitor. The lack of Nogo-A in fish and salamanders would explain the high regeneration potential of the spinal cord after lesions in the latter animals. Whereas Nogo/RTN-4 has a known neurite-growth inhibitory function, more generalized cellular functions relating to the endoplasmic reticulum are suggested for the other proteins in the RTN family as well as for Nogo-A, -B and -C, for example roles in intracellular trafficking or apoptosis.

Nogo-A neutralizing antibodies lead to axonal growth and functional recovery *in vivo*. After cell culture experiments, the next step was to show the ability to block myelin inhibitors and achieve axonal regrowth after spinal cord injury. The monoclonal antibody IN-1 was initially raised against myelin fractions enriched for

Nogo-A. Treatment with IN-1 promoted axonal regeneration and behavioral recovery in rats after thoracic spinal cord injury. These exciting results have since been confirmed using local intrathecal pump infusions of recombinant IN-1 Fab fragments or novel anti-Nogo-A antibodies [9].

Lines of knockout mice null for the Nogo genes have been developed. Different lines of knockout mice for Nogo-A, Nogo-A/-B and Nogo-A/-B/-C have been studied (see [9] for review). It should be noted that, as is often done, hybrids of two different mouse strains that differed greatly in various relevant respects, such as neuroinflammatory response, cell death at lesion sites, scarring response and overall behavior, were used. After spinal cord lesion, the Nogo-A-specific knockout mice showed a moderate but clearly detectable increase in regenerative sprouting and elongation. The same phenotype was quantitatively enhanced in one of the Nogo-A/-B knockout lines. Because Nogo-B was greatly upregulated in the Nogo-A knockout, the Nogo-66 site seemed to compensate partially for the absent Nogo-A-specific active site. At odds with these observations were the results from a different laboratory, in which neither the Nogo-A/-B knockout lines nor the survivors from a single Nogo-A/-B/-C knockout mouse that escaped lethality showed major enhancement of sprouting or regeneration of the lesioned corticospinal tract [9]. The analysis of backcrossed strains on a pure genetic background and conditional knockout mice will hopefully help to resolve these issues in the future.

Myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein are myelin components that have growth-inhibitory activity. MAG is a member of the immunoglobulin (Ig) superfamily and a sialic acid-binding transmembrane glycoprotein. In the CNS, MAG is found in the periaxonal myelin membrane and in the PNS, in the inner and outermost membranes of the myelin sheath [16]. MAG has long been known to have potent neurite inhibitory activity *in vitro*. There is evidence that immunization with myelin or recombinant Nogo-66/MAG promotes axonal regeneration after corticospinal tract lesions in adult mice [17]. However, MAG knockout mice did not show long-distance regeneration in the lesioned spinal cord.

A third myelin inhibitory protein, OMgp, is a GPI-linked protein expressed by oligodendrocytes [18]. OMgp is a relatively minor component of myelin, believed to be localized to the paranodal loops, next to the node of Ranvier. OMgp contains a leucine-rich repeat (LRR) domain and a C-terminal domain with serine/threonine repeats. Like MAG, OMgp is also found in the PNS. Like Nogo, OMgp is also expressed in adult neurons.

To date, the possible roles played by these inhibitors that are found within neurons is not known. In one recent study a high degree of Nogo-A expression was found in rodent regenerating axons in the central and especially the peripheral nervous system, suggesting that neuronal, as distinct from oligodendroglial-derived Nogo also may be involved in axonal growth and guidance [19].

Inhibition of neurite growth is mediated through surface receptors and intracellular signaling molecules. Interestingly, one receptor that binds to Nogo-A, MAG and OMgp has been characterized; it is an 85 kDa GPI-linked, leucine-rich repeat glycoprotein named Nogo receptor (NgR) [14]. This receptor is expressed on the surface of various neurons and was named NgR since it binds to the 66-amino-acid region in the C-terminal domain that is common to Nogo-A, -B and -C, i.e. Nogo-66. Surprisingly, NgR was found to be complexed with the low-affinity p75 nerve growth factor (NGF) receptor (discussed in Ch. 27), which acts as a signal transducing subunit [16] (Fig. 30-5). Recently a third component, LINGO-1, a transmembrane protein that links to NgR and p75, has been identified as an important functional component of this signaling complex [20]. Amino-Nogo, the additional inhibitory domain for Nogo-A, does not require this NgR complex for activity, and the receptor for Amino-Nogo has not yet been found. In studies using NgR-blocking peptides, when infused locally over rodent spinal cord lesions or applied systemically, corticospinal tract regeneration and functional recovery was seen even when given one week after lesions [21]. Interestingly, another *in vivo* study examining the regeneration in $p75^{NGR}$ knockout mice after spinal cord lesion showed no enhancement of regeneration, suggesting that the $p75^{NGR}$ component of the NgR is not the sole mediator of inhibitory function [22].

Two intracellular signaling components of neurite-growth inhibition have been identified so far, namely, calcium ion and the Rho-A/Rho kinase (ROCK) pathway (Fig. 30-5). The inhibitory effects on neurons as mediated by RhoA, a small GTPase, are thought to be via intracellular regulation of cytoskeletal assembly (see Ch. 19). Importantly, inhibition of these intracellular pathways by appropriate blockers can prevent myelin- or Nogo-A-induced collapse of growth cones and inhibition of neurite growth *in vitro*; and these blockers can also induce corticospinal tract sprouting after spinal cord lesions *in vivo* [23]. Finally, several studies have shown that elevated levels of cAMP can cancel the repulsive and inhibitory effects of several inhibitors, including MAG and Nogo [16]. cAMP may act locally on growth cones, as well as on neuronal cell bodies to mediate growth-enhancing effects.

Axon growth is inhibited by the glial scar. Another barrier to CNS regeneration is the glial scar, which consists mainly of reactive astrocytes and proteoglycans. As early as Ramon y Cajal [24], glial scar tissue was implicated as an impediment to adult axonal regeneration after CNS injury. More recent studies since the early 1990s have

demonstrated that failure of axonal regeneration may be due to the nonpermissiveness to neurite growth of extracellular matrix molecules (ECM), including the chondroitin sulfate proteoglycans (CSPGs) and keratin sulfate proteoglycans in the scar [25]. *In vitro*, the inhibitory nature of CSPGs has been attributed both to the core protein and to associated chondroitin sulfate side chains [26]. *In vivo* studies have shown that, after CNS injuries, various CSGPs that are normally expressed on immature glial cells are re-expressed in the glial scar. These include NG2, neurocan, brevican, versican, aggrecan and phosphacan. Degradation of associated chondroitin sulfates after spinal cord injury partially enhanced axonal growth and functional recovery, indicating that reduction in selective scar components can increase regenerative growth in the adult CNS [27]. Furthermore, CSGPs appear to have a role in regulating visual cortical synaptic plasticity. After CSPG degradation in adult rats, monocular deprivation caused an ocular dominance shift toward the nondeprived eye, indicating that the mature ECM is inhibitory for experience-dependent plasticity [28].

Astrocytes play a key role in scar formation in experiments with double knockout mice deficient in vimentin and GFAP and subjected to lateral hemisection of the thoracic spinal cord, axonal sprouting of the corticospinal tract and raphe-spinal pathways was observed and correlated with hindlimb functional recovery on a grid walk test [29]. However, the role of reactive astrocytes in CNS injury is not completely understood. Another study involving adult transgenic mice with selective and conditionally targeted gene deletions has shown that reactive astrocytes actually protected tissue and preserved function after spinal cord injury [30]. This protection was attributed to the action of reactive astrocytes in blood brain barrier repair and restriction of the inflammatory response. Presently, one is left with the conclusion that inflammation, including the role of astrocytes in CNS injury, is a complex and inadequately understood area warranting further study. In particular, a main problem is to overcome the inhibitory environment of the glial scar, without compromising the positive effects of glial cells.

Neurotrophic factors promote both cell survival and axon growth after adult central nervous system injury *in vivo*. Neurotrophic factors comprise several complex families of molecules that support axonal regrowth and neuronal survival in the adult CNS (see detailed discussion in Ch. 27). These molecules include the nerve growth factor (NGF) superfamily (NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, neurotrophin (NT)-4/5), the neurokine or ciliary neurotrophic factor (CNTF) family, the glial-derived neurotrophic factor (GDNF) family and factors such as the bone morphogenetic proteins (BMPs) the IGFs and FGFs, with multiple roles including neurotrophic functions. The trk family of receptor tryrosine kinases is the high-affinity, signal-transducing receptor for the NGF family, with NGF the primary ligand for TrkA, BDNF and NT-4/5 for TrkB, and NT-3 for TrkC. In experiments using neurotrophins to study regenerative properties of adult rubrospinal neurons, Tetzlaff and colleagues found that the application of BDNF or NT-4/5 (but not NGF or NT-3) prevented the atrophy of axotomized rubrospinal neurons, stimulated the expression of GAP-43 and Tα1-tubulin in these neurons, and promoted the regeneration of axotomized neurons into grafts of peripheral nerves implanted into spinal cord lesions [31]. Interestingly, the treatment was most effective when neurotrophic factors were applied in the vicinity of the cell bodies themselves and not at the spinal cord injury site, except for NT-3, which stimulated corticospinal tract regenerative sprouting if applied to the injury site [32]. Surprisingly, it was found that even when animals were treated up to 1 year after axotomy, local infusion of BDNF was still capable of rescuing these axotomized neurons and of stimulating them to regenerate into peripheral nerve implants [33]. These exciting results indicate that the regenerative capabilities of adult CNS neurons are not necessarily lost even after long-standing chronic injury.

Other ways to use neurotrophins in CNS repair paradigms are to combine them with growth-promoting cells or bridging materials or to transplant into lesion sites cells that are genetically engineered to produce specific neurotrophins. In one example, engineered Schwann cells that expressed BDNF were used to stimulate growth of supraspinal axons across the transected adult rat spinal cord. A polymer channel was placed in between the two stumps, and engineered Schwann cells expressing BDNF were seeded into the channel. Although BDNF did provide increased selection for TrkB-expressing axons, neurites did not leave the substrate of the guidance channel, once again demonstrating the importance of a permissive environment in order to achieve long-distance functional regeneration [34].

Despite the extensive use of neurotrophins to induce adult axonal regrowth and neuronal plasticity, results have been mixed, and many challenges remain. Neurotrophins have roles in the CNS that go beyond cell survival and neurite outgrowth and include such diverse and basic activities as modulation of membrane excitability, regulation of cell differentiation and intracellular molecular trafficking. A better understanding of the role of neurotrophins in cellular regulation and control would augment their use as important therapeutic agents in the repair of CNS injury.

CENTRAL NERVOUS SYSTEM INJURY AND COMPENSATORY PLASTICITY

Neonatal brain damage results in compensatory plasticity. Although, without intervention, axonal growth in the injured adult CNS is limited, the immature CNS responds to injury with a remarkable rerouting of neuronal pathways from undamaged areas to re-innervate

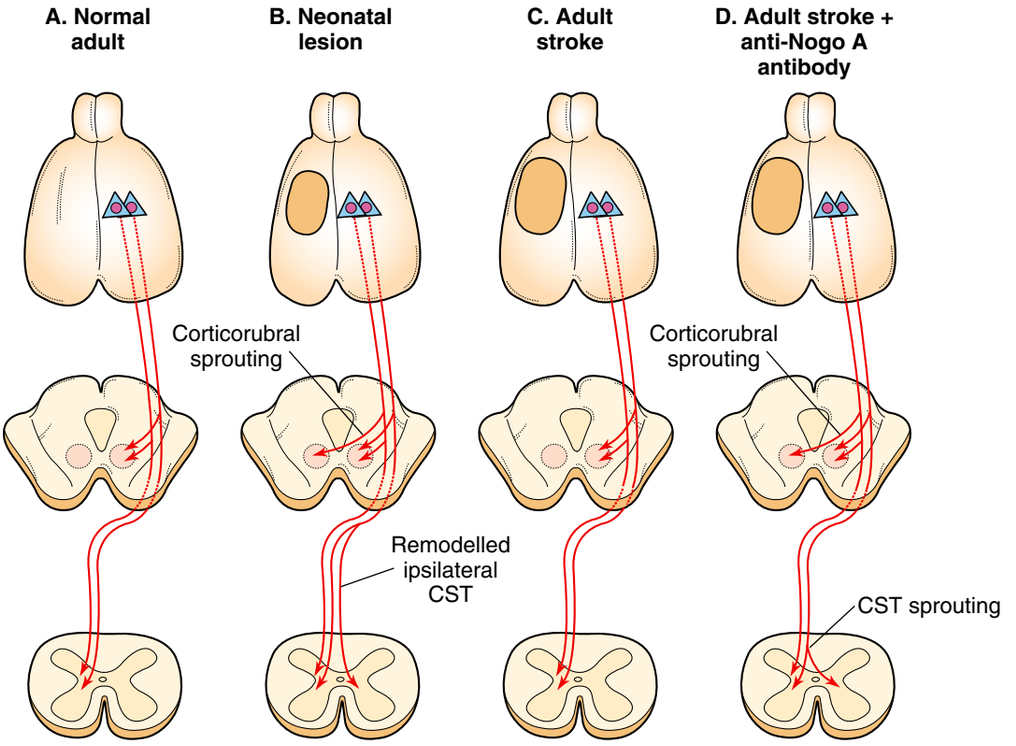

FIGURE 30-6 Diagram depicting cortico-efferent projections from the rodent motor cortex to subcortical areas including the red nucleus and the spinal cord. In the normal adult (**A**), cortical neurons project to the ipsilateral red nucleus and the contralateral spinal cord gray column. After a neonatal cortical lesion (**B**), cortico-efferent fibers spontaneously sprout to the contralateral, denervated red nucleus and the ipsilateral, denervated spinal cord (gray). After an adult stroke lesion (**C**), there is very little spontaneous sprouting seen. However, after adult stroke and treatment with anti-Nogo-A antibody (**D**), plasticity to the denervated red nucleus and spinal cord is again seen, correlating with functional recovery. CST, corticospinal tract

denervated areas. This is called *structural compensatory plasticity*. This lesion-induced plasticity following perinatal brain damage occurs in all systems studied, including the visual, auditory, sensorimotor and limbic systems. For example, following lesion to the motor cortex of 2-day old rat pups, the motor cortex from the opposite, unlesioned hemisphere reroutes corticoefferent connections to bilaterally innervate subcortical areas including the striatum, thalamus, red nucleus, basilar pontine nuclei and spinal cord (see [35] for review, and Fig. 30-6). This new pathway formation is thought to underlie the better functional outcome often seen after neonatal brain injury as compared to the same injuries sustained in adulthood [36]. Therefore, ways to enhance structural plasticity after adult CNS lesions would greatly improve functional recovery and thereby quality of life.

Compensatory plasticity and functional recovery can be enhanced in the injured adult central nervous system through blockade of Nogo-A. Interestingly, some of the same molecules that restrict regeneration in the adult CNS, e.g. Nogo-A, are also responsible for shaping adult lesion-induced neuronal plasticity. In a series of experiments, adult rats were subjected to unilateral ischemic brain damage through middle cerebral artery occlusion and given antibodies to block Nogo-A. A dramatic recovery in skilled forelimb use was seen that correlated with new corticoefferent plasticity from the unlesioned sensorimotor cortex to subcortical motor areas such as the red nucleus and the spinal cord [37, 38] (Fig. 30-6). Furthermore, electrophysiological mapping studies of the opposite, unlesioned hemisphere showed a functional reorganization of the motor map, with new areas of the motor cortex now innervating the previously impaired forelimb [39]. The specificity of these new pathways indicates that developmental mechanisms of neuronal target recognition and synapse specification probably persist throughout life, thus assuring that under conditions of enhanced axonal plasticity and regeneration, functional networks can be formed [40, 41].

These findings of enhanced axonal plasticity and functional recovery after stroke and anti-Nogo-A therapy have now been extended to the aged rat. 24-month-old rats were trained on a reaching task and given anti-Nogo-A antibody treatment via intraventricular pump 1 week after experimental stroke. These old animals showed dramatic returns in skilled forelimb function with this therapy, indicating that even the aged CNS retains the proper molecular cues to allow for neuronal plasticity and functional recovery given the proper conditions.

Another important aspect of this study was that the time window for starting the treatment was extended to 1 week after stroke, demonstrating that anti-Nogo therapy is effective even beyond the time of acute brain damage. These findings open the possibility of treatment options for those suffering from chronic brain or spinal cord injuries.

Even in the case of spinal cord injury where application of anti-Nogo antibodies results in regeneration of the cut axons, an additional important element for functional recovery is enhanced fiber growth from the unlesioned fibers, i.e. compensatory plasticity, as discussed above. After high corticospinal tract injury in the rat at the level of the medullary pyramid and treatment with anti-Nogo antibodies, rubrospinal pathways were shown to sprout into deafferented areas of the spinal cord, resulting in high levels of functional recovery, i.e. a 'functional switch' in the remodeled pathway [42].

Furthermore, various studies suggest that neutralizing Nogo antibodies can induce a transient growth response in the intact adult CNS [40]. Interestingly, while Purkinje cells of the cerebellum do not upregulate GAP-43 at all after axotomy, increased expression of GAP-43 as well as regeneration is seen after application of anti-Nogo antibodies to the intact cerebellum [43].

SUMMARY AND CONCLUSIONS

Research over the past 20 years has shown that the adult mammalian CNS is not a static structure and, in fact, under certain conditions retains a high capacity for at least short-distance axonal growth. Such growth results in structural remodeling of new neural pathways from intact neurons or, under appropriate experimental or therapeutic conditions, regeneration from the cut ends of injured axons. These processes are correlated with a high degree of functional recovery in animal models of CNS injury. A better understanding of the complex molecular and cellular mechanisms underlying regeneration and plasticity is currently unfolding, and future studies are aimed at turning these basic science concepts into therapeutic tools to improve functional outcome and quality of life in patients with brain, spinal cord or peripheral nerve injury.

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REFERENCES

1. Fawcett, J. W., Rosser, A. E. and Dunnett, S. B. Peripheral nerve regeneration. In *Brain Damage, Brain Repair*. New York: Oxford University Press, 2001.
2. Shamash S., Reichert, F. and Rotshenker, S. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1-alpha, and interleukin-1-beta. *J. Neurosci.* 22: 3052–3060, 2002.
3. Livesey, F. J., O'Brien, J. A., Li, M., Smith, A. G., Murphy L. J. and Hunt, S. P. A Schwann cell mitogen accompanying regeneration of motor neurons. *Nature* 390: 614–618, 1997.
4. Benowitz, L. I. and Routtenberg, A. GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* 20: 84–91, 1997.
5. Meiri, K. F., Saffell, J. L., Walsh, F. S. and Doherty, P. Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. *J. Neurosci.* 18: 10429–10437, 1998.
6. Perry, V. H., Brown, M. C., Lunn, E. R., Tree, P., and Gordon, S. Evidence that very slow Wallerian degeneration in C57BL/Ola mice is an intrinsic property of the peripheral nerve. *Eur. J. Neurosci.* 2: 802–808, 1990.
7. Wang, M. S., Fang, G., Culver, D. G., Davis, A. A., Rich, M. M., and Glass, J. D. The Wld^s protein protects against axonal degeneration: a model of gene therapy for peripheral neuropathy. *Ann. Neurol.* 50: 773–779, 2001.
8. David, S. and Aguayo, A. J. Axonal elongation into peripheral nervous system 'bridges' after central nervous system injury in adult rats. *Science* 214: 931–933, 1981.
9. Schwab, M. E. Nogo and axon regeneration. *Curr. Opin. Neurobiol.* 14: 118–124, 2004.
10. Chen, M. S., Huber, A. B., Van Der Haar, M. E. *et al.* Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403: 434–439, 2000.
11. Huber, A. B., Weinmann, O., Brösamle, C., Oertle, T. and Schwab, M. E. Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesion. *J. Neurosci.* 22: 3553–3567, 2002.
12. Acevedo, L., Yu, J., Erdjument-Bromage, H. *et al.* A new role for Nogo as a regulator of vascular remodeling. *Nat. Med.* 17: 382–388, 2004.
13. Oertle, T., Van Der Haar, M. E., Bandtlow, C. E. *et al.* Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. *J. Neurosci.* 23: 5393–5406, 2003.
14. Fournier, A. E., Grandpre, T., Strittmatter, S. M. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* 409: 341–346, 2001.
15. Oertle, T. and Schwab, M. E. Nogo and its partners. *Trends Cell Biol.* 13: 187–194, 2003.
16. Filbin, M. T. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat. Rev. Neurosci.* 4: 703–713, 2003.
17. Sicotte, M., Tsatas, O., Jeong, S. Y., Cai, C-Q., He, Z. and David, S. Immunization with myelin or recombinant Nogo-66/MAG promotes axon regeneration and sprouting after corticospinal tract lesions in the spinal cord. *Mol. Cell. Neurosci.* 23: 251–263, 2003.
18. Wang, K. C., Koprivica, V., Kim, J. A. *et al.* Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* 417: 941–944, 2002.
19. Hunt, D., Coffin, R. S., Prinjha, R. K., Campbell, G., and Anderson, P. N. Nogo-A expression in the intact and injured nervous system. *Mol. Cell. Neurosci.* 24: 1083–1102, 2003.
20. Mi, S., Lee, X., Shao, Z. *et al.* LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat. Neurosci.* 7: 221–228, 2004.

21. Li, S. and Strittmatter, S. M. Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury. *J. Neurosci.* 23: 4219–4227, 2003.
22. Song, X-Y., Zhong, J., Wang, X. and Zhou, X-F. Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. *J. Neurosci.* 24: 542–546, 2004.
23. Fournier, A. E., Takizawa, B. T. and Strittmatter, S. M. Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J. Neurosci.* 23: 1416–1423, 2003.
24. Cajal, Ramon Y. *Degeneration and Regeneration of the Nervous System.* New York: Hafner Publishing Co., 1928.
25. Silver, J. and Miller, J. H. Regeneration beyond the glial scar. *Nat. Neurosci. Rev.* 5: 146–156, 2004.
26. Ughrin, Y. M., Chen, Z. J. and Levine, J. M. Multiple regions of the NG2 proteoglycan inhibit neurite growth and induce growth cone collapse. *J. Neurosci* 23: 175–186, 2003.
27. Bradbury, E. J., Moon, L. D., Popat, R. J. *et al.* Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416: 636–640, 2002.
28. Pizzorusso, T., Medini, P., Berardi, N., Chierzi, S., Fawcett, J. W. and Maffei, L. Reactivation of ocular dominance plasticity in the adult visual cortex. *Science* 298: 1248–1251, 2002.
29. Gimenez y Ribotta, M., Gaviria, M., Menet, V. and Privat, A. Strategies for regeneration and repair in spinal cord traumatic injury. *Prog. Brain Res.* 137: 191–212, 2002.
30. Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, M. B. and Sofroniew, M. V. Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J. Neurosci.* 24: 2143–2155, 2004.
31. Kobayashi, N. R., Fan, D-P., Giehl, K. M., Bedard, A. M., Wiegand, S. J. and Tetzlaff, W. BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and Tα1-tubulin mRNA expression, and promote axonal regeneration. *J. Neurosci.* 17: 9583–9595, 1997.
32. Schnell, L., Schneider, R., Kolbeck, R., Barde, Y. A. and Schwab M. E. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* 367: 170–173, 1994.
33. Kwon, B. K., Liu, J., Messerer, C. *et al.* Survival and regeneration of rubrospinal neurons one year after spinal cord injury. *Proc. Natl Acad. Sci. U.S.A.* 99:3246–3251, 2002.
34. Menei, P., Montero-Menei, C., Whittemore, S. R., Bunge, R. P. and Bunge, M. B. Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. *Eur J. Neurosci.* 10: 607–621, 1998.
35. Castro, A. J. Plasticity in the motor system. In B. Kolb and R. C. Tees (eds), *The Cerebral Cortex of the Rat.* Cambridge, MA: MIT Press, 1990, pp. 563–588.
36. Z'Graggen, W. J., Fouad, K., Raineteau, O., Metz, G. A. S., Schwab, M. E. and Kartje, G. L. Compensatory sprouting and impulse rerouting after unilateral pyramidal tract lesion in neonatal rats. *J. Neurosci.* 20: 6561–6569, 2000.
37. Papadopoulos, C. M., Tsai, S-Y., Alsbiei, T., O'Brien, T. E., Schwab, M. E. and Kartje, G. L. Functional recovery and neuroanatomical plasticity following middle cerebral artery occlusion and IN-1 antibody treatment in the adult rat. *Ann. Neurol.* 51: 433–441, 2002.
38. Wiessner, C., Bareyre, F. M., Allegrini, P. R. *et al.* Anti-Nogo-A antibody infusion 24 hours after experimental stroke improved behavioral outcome and corticospinal plasticity in normotensive and spontaneously hypertensive rats. *J. Cereb. Blood Flow Metab.* 23: 154–165, 2003.
39. Emerick, A. J., Neafsey, E. J., Schwab, M. E. and Kartje, G. L. Functional reorganization of the motor cortex in adult rats after cortical lesion and treatment with monoclonal antibody IN-1. *J. Neurosci.* 23: 4826–4830, 2003.
40. Bareyre, F. M., Haudenschild, B. and Schwab, M. E. Long-lasting sprouting and gene expression changes induced by the monoclonal antibody IN-1 in the adult spinal cord. *J. Neurosci.* 22: 7097–7110, 2002.
41. Bareyre, F. M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T. C., Weinmann, O. and Schwab, M. E. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. *Nat. Neurosci.* 3: 269–277, 2004.
42. Raineteau, O. and Schwab, M. E. Plasticity of motor systems after incomplete spinal cord injury. *Nat. Rev. Neurosci.* 2: 263–274, 2001.
43. Buffo, A., Zagrebelsky, M., Huber, A. B. *et al.* Application of neutralizing antibodies against NI-35/250 myelin-associated neurite growth inhibitory proteins to the adult rat cerebellum induces sprouting of uninjured Purkinje cell axons. *J. Neurosci.* 20: 2275–2286, 2000.

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CHAPTER

31

Energy Metabolism of the Brain

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INTRODUCTION

The human brain is an 'expensive' organ in terms of energy usage since it accounts for only ≈2% of the body weight, yet the energy required to maintain brain function amounts to ≈20% of an individual's resting metabolic rate [1 and references therein]. In tissues such as heart and skeletal muscle, energy use increases greatly during mechanical work. Metabolic activity in brain varies much less than in tissues that do mechanical work. This is explained by the fact that the resting state typically requires considerable energy for maintaining membrane potentials. As a comparison, the ATP consumption per gram per minute used for signaling in the brain is equal to the energy used by a human leg muscle running a marathon or the osmotic work in the kidney [1 and references therein, 2].

Processes related to signaling require the largest proportion of brain energy utilization but considerable energy is also used to maintain basic cellular functions. Approximately 75% of the energy consumed by brain is related to signaling, while the remaining 25% of energy utilization serves to maintain essential nonsignaling cellular activity including protein synthesis and degradation, nucleotide turnover, phospholipid turnover, axoplasmic transport and mitochondrial proton leak [1] (Table 31-1). The overall energy use by human brain is estimated to be 21 μmol ATP/g/min. Metabolic rates are considerably higher in gray matter (rate in rat neocortex is ≈33–55 μmol ATP/g/min) than in white matter as measured in either rat or human brain (Table 31-2).

Metabolism in brain is compartmentalized. The brain contains two major classes of cells: neurons, which include a large number of phenotypes reflecting the many different properties of structure and function found among neurons, and glia, which include astrocytes, oligodendroglia and microglia (see Ch. 1). The proportion of energy used by glial cells is unclear with estimates ranging from about 17.5% (recalculated from [1]) to as high as 40% of oxidative metabolism [9]. *In vivo* studies suggest that glia utilize from ≈14% of brain metabolism [10] to as much as 30% of total tricarboxylic acid (TCA) cycle activity [11]. Some processes with high energy cost, such as

TABLE 31-1 Energy use by brain

Use	Energy expense (% of total energy)
Signaling	**75**
Action potentials	35.3
Postsynaptic receptors	25.5
Resting potentials	9.8
Glutamate recycling	2.3
Postsynaptic Ca^{2+}	2.3
Basic cellular activities	**25**
Phospholipids turnover & membrane distribution	≈5
Turnover of proteins & oligonucleotides	≈2
Axoplasmic transport	*
Mitochondrial proton leak	†

Source: adapted from Attwell and Laughlin [1].
*Energy cost unknown.
†Value not available for brain but is 20% in a variety of other tissues.

TABLE 31-2 Cerebral metabolic rates (CMR) are regionally and activity-dependent

	Rat	Human
Resting CMR (whole brain)		
Oxygen consumption (CMR_{O2}, μmol(g min)$^{-1}$)	3.4 [3]*	1.5
Glucose utilization (CMR_{glc}, μmol(g min)$^{-1}$)	0.7 [4]	0.23 [5]–0.3 [2]
CMR_{O2}/CMR_{glc}	4.9	5
Respiratory quotient†		0.97 [2]
Regional differences in CMR_{glc} (μmol(g min)$^{-1}$)		
Cortex	1–1.5 [6, 7]	0.40 [4]–0.45 [2]
White matter	0.3	0.2
Whole brain	0.7–0.9 [6, 7, 8]	0.23–0.37 [8]
Focal stimulation		0.63 [8]
Anesthesia‡	0.35–0.65 [5, 6]	
Coma§		0.13–0.15 [2]

References in square brackets.
*Calculated from ml/100 g/min by using the normal volume of gas at normal pressure (22.4 l/mol)
†Ratio between CO_2 produced and oxygen consumed.
‡Anesthesia with light barbiturate, halothane or α-chloralose.
§Coma induced by barbiturate, diabetes or, hypoglycemia; values for CMR_{glc} are calculated from $CMR_{O2}/6$.

phospholipid turnover and maintenance of phospholipid polarization in membranes, are likely to be higher in glia than in neurons. Most of the biochemical pathways of energy metabolism in the brain are similar to those in other tissues. However, there is compartmentation of metabolism due to the highly specialized cellular and subcellular localization of transporters, enzymes and metabolic pathways that makes brain cells specialized to perform specific functions and also uniquely interdependent. Metabolic compartmentation is defined as the presence in a tissue of more than one distinct pool of a given metabolite. These separate pools of a metabolite are not in rapid equilibrium with each other but maintain their own integrity and turnover rates [12]. Compartmentation makes metabolism in the brain complex and the continuous interactions of neurons and glial cells essential for brain function [13].

SUBSTRATES FOR CEREBRAL ENERGY METABOLISM

Energy-yielding substrates enter the brain from the blood through the blood–brain barrier. The brain requires a continuous supply of oxygen and energy-yielding substrate(s). Glucose is essential for developing brain and is the most important energy-yielding substrate used by adult brain. The rates of glucose transport and utilization are lower in developing brain and increase with maturity [14–16]. Monocarboxylic acids, including lactate and the ketone bodies acetoacetate and β-hydroxybutyrate, are also important energy-yielding substrates. These compounds are also incorporated into lipids, amino acids and proteins in developing brain [17, 18]. Lactate appears to have a special role as a readily available source of energy during the immediate postnatal period [19]. The ketone bodies are essential substrates for brain metabolism throughout the suckling period, which is consistent with the high concentration of fat in the milk of humans and other mammals [16]. In conjunction with the importance of monocarboxylic acids for developing brain, the rates of transport of these substrates from the circulating blood through the blood–brain barrier and into brain are high in animals and humans during the suckling period, when the activity of enzymes for oxidative use of glucose are low, and decline with maturity as glucose metabolism increases [16].

Endothelial cells of the blood–brain barrier and brain cells have specific transporters for the uptake of glucose and monocarboxylic acids. Substrates used by the brain are taken up from the circulation via transporters in the endothelial cells that form the blood–brain barrier (see also discussion of facilitated transport in Ch. 5). The main energy nutrient transporters in the blood–brain barrier are the highly glycosylated 55 kDa form of the glucose transporter GLUT1 and the monocarboxylic acid transporter MCT1 (Fig. 31-1). Other isoforms of these transporters are discussed below. High amounts of MCT1 and GLUT1 and low levels of MCT2 are present in the blood–brain barrier of developing brain [16, 20]. The intense labeling of MCT1 protein in brain microvasculature during the suckling period declines significantly after 14 days of age in rat brain, coincidentally with a dramatic increase in GLUT1 content and with the switch to glucose as the predominant fuel entering brain [16]. The change in substrate use during development is related to the availability of circulating substrates as well as to the developmental changes in the transport properties of the blood–brain barrier (see detailed review by Vannucci and Simpson [16]).

The uptake of energy substrates by brain cells is influenced by the type and distribution of transporters unique to each cell type. The selective distribution of the individual isoforms of transporter molecules contributes to the regulation of overall substrate use in individual types of brain cells and in brain (Fig. 31-1). Although astrocyte end feet cover ≈96% of the blood–brain barrier, experimental results suggest that approximately equal proportions of glucose are taken up by neurons and glia in brain [21]. The 45 kDa form of glucose transporter GLUT1 is found on astrocytes and the choroid plexus [15]. The high-capacity glucose transporter GLUT3 is found only on neurons [14, 15], and GLUT5 is localized primarily on microglia [22]. The levels of both GLUT1 and GLUT3 increase dramatically with cerebral maturation and synaptogenesis. GLUT3 is relatively low in nonvascular portions of rat brain at 7 days of age, then increases almost linearly about 10-fold by 28 days of age [15, 16]. The increase in GLUT1 occurs at

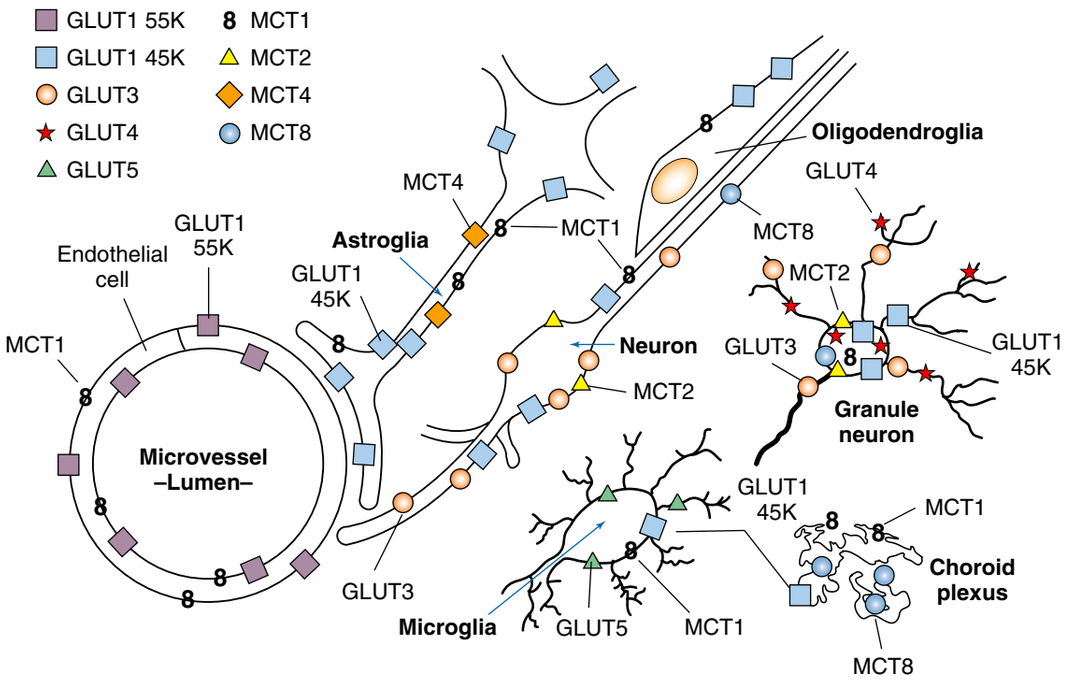

FIGURE 31-1 Glucose and monocarboxylic acid transporters in brain. Specific glucose and monocarboxylic acid transporters in brain are localized on different types of brain cells. (Courtesy of Ian Simpson and Susan Vannucci.)

a slightly later age; levels remain low in nonvascular portions of rat brain until 14 days of age, then increase severalfold until 28–35 days of age [16]. Although the distribution and properties of different transporters give insight into the potential capabilities of brain cells to take up specific substrates, there is still uncertainty about the proportions of glucose actually taken up and metabolized by astrocytes and neurons. Label from infused glucose rapidly appears in glutamate in brain [23] and later in glutamine (see below). These findings illustrate the importance of the high capacity transporter GLUT3 in providing glucose to neurons.

Reports on the distribution of MCT transporters in brain cells are somewhat conflicting, in part as a result of differences between data from *in vivo* studies and data obtained from studies with cultured brain cells, differences in mRNA expression and the contents of transporter proteins, and differences among species [16, 24, 25]. There is evidence that both MCT1 and MCT2 are found in all types of brain cells in developing brain, which is consistent with the importance of monocarboxylic acids as substrates at early ages [16, 20, 26]. MCT1 is localized on the blood–brain barrier and on astrocytes of mature brain, but is not found on all astrocytes *in vivo* [16, 26]. Kinetic studies suggest the possibility of a lower-affinity astrocytic MCT transporter [27], which would be consistent with the identification of MCT4 on some glial cells [28, 29]. The higher-affinity monocarboxylate transporter MCT2 is localized primarily on neurons in mature brain [24, 29–32]. Kinetic properties for lactate transport by neuronal preparations isolated from brain are not totally consistent with the properties of MCT2, suggesting the possible presence of another neuronal MCT transporter [32, 33]. While eight different members of the proton-coupled MCT transporter family have been identified, only the MCT1–4 transporters have been characterized in detail thus far. Although the staining for MCT in brain slices appears more diffuse with maturation, the level of MCT1 in the nonvascular tissue remains fairly constant, and the level of MCT2 transporter protein in neurons increases with development [16].

The transporters of the blood–brain barrier can be altered under pathological conditions. The uptake of glucose and amount of GLUT1 on the blood–brain barrier can both be increased by hypoglycemia [34–36]. There are controversial reports about the effects of chronic hyperglycemia and diabetes mellitus, with some studies showing decreased glucose uptake and others reporting no difference (for review see [37]). A rat model of diabetes mellitus showed no alterations in glucose transport or blood–brain barrier GLUT1 protein content compared to controls, yet there was significantly increased GLUT1 gene expression in the blood–brain barrier of the diabetic rats [34]. MCT1 on the blood–brain barrier may be upregulated by a ketogenic diet and in other conditions with high levels of circulating monocarboxylic acids, such as in diabetes mellitus and cancer [38].

AGE AND DEVELOPMENT INFLUENCE CEREBRAL ENERGY METABOLISM

The rate and pathways of metabolism change during development. The energy metabolism of the brain and the blood flow that sustains the metabolic rate vary considerably from birth to old age. Data on the cerebral metabolic rate in human infants during the early postnatal period have been published [39], and are consistent with results of *in vitro* measurements in animal brain preparations and inferences drawn from cerebral blood flow measurements in intact animals [2]. Such studies suggest that cerebral oxygen consumption is low at birth, rises rapidly during the period of cerebral growth and development and reaches a maximal level at about the time maturation is completed. Significant utilization of ketone bodies by the brain is normal in the neonatal period [16–18, 40]. The newborn infant tends to be hypoglycemic but becomes ketotic when it begins to nurse because of the high fat content of the mother's milk. During the suckling period the brain is highly dependent on the use of ketone bodies for energy since the activities of the enzymes for utilization of these substrates are high, whereas activities of pyruvate dehydrogenase and of some oxidative enzymes are lower [41].

The first two enzymes in the pathway of ketone utilization are D-β-hydroxybutyrate dehydrogenase and acetoacetyl-succinyl-CoA transferase. These exhibit a postnatal pattern of development that is well adapted to the nutritional demands of the brain [41]. At birth, the activities of these enzymes in the brain are low; activities rise rapidly with the ketosis that develops at the onset of suckling. The activities reach their peaks just before weaning and then gradually decline after weaning to normal adult rates of approximately one-third to one-fourth the maximal rates attained at the earlier stages [18, 41]. Ketone bodies are used for energy and are incorporated into the lipids and proteins formed in the rapidly developing brain [17, 41]. When weaned onto the normal, relatively high-carbohydrate diet, the ketosis disappears and cerebral ketone utilization decreases considerably. Studies have been carried out mainly in the newborn rat, but there is evidence that the situation is similar in the human infant [42]. The reports of ketoacidosis and developmental delay in infants with impaired ability to utilize ketone bodies underscore the importance of these substrates during the suckling period [43].

Cerebral metabolic rate increases during early development. Glucose is an essential substrate for developing brain [16, 40]. When ketone bodies and lactate are utilized primarily for energy, the requirement for glucose may reflect metabolism via the pentose phosphate shunt for formation of ribose-5-phosphate as well as production of the NADPH required for lipid biosynthesis. In general, the whole brain local glucose utilization is very significantly correlated with postconceptional age [39, 44]. In the brain of suckling rat, even during intense use of ketone bodies, the postnatal increases in local glucose use appear to be critically linked to the acquisition of new functions and neurological competence [40]. This may also be true for neonatal human brain since local increases in the glucose metabolic rate reflect the functional maturation of these regions [44]. The overall rise in oxidative glucose use is associated with the developmental switch in transporters on the blood–brain barrier. These changes are also consistent with the progressive increases in the levels of a number of enzymes of oxidative metabolism in the brain, and with the increase in the activity of enzymes associated with the malate–aspartate shuttle [2, 16]. The increased use of glucose for energy as the brain matures is accompanied by increased oxidation of glycolytically derived pyruvate via the TCA cycle.

The rate of blood flow in different structures of the brain reaches peak levels at different developmental stages, depending on the maturation rate of the particular structure. In structures that consist predominantly of white matter, the peaks coincide roughly with maximal rates of myelination. From these peaks, blood flow and, probably, cerebral metabolic rate decline to the levels characteristic of adulthood [2, 39, 44].

Cerebral metabolic rate declines from developmental levels and plateaus after maturation. Reliable quantitative data on the changes in cerebral circulation and metabolism in humans from the middle of the first decade of life to old age have been reported [2, 39, 44]. By 6 years of age, cerebral blood flow and oxygen consumption already have attained high rates, and they decline thereafter to the rates of normal young adulthood [45]. Oxygen is utilized in the brain almost entirely for the oxidation of carbohydrates [46]. The equation for the complete oxidation of glucose is:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$$

A respiratory quotient (CO_2 production/O_2 consumption) of 0.97 for brain indicates that it depends almost exclusively on carbohydrate oxidation. The consumption of O_2 provides the energy required for intense activity. Cerebral oxygen consumption of 5.2 ml/100 g brain tissue/min in a 5–6-year-old child corresponds to total oxygen consumption by the brain of ≈60 ml/min, or more than 50% of the total body basal oxygen consumption, a proportion markedly greater than that occurring in adulthood. The reasons for the extraordinarily high cerebral metabolic rates in children are unknown, but presumably they reflect the extra energy requirements for the biosynthetic processes associated with growth and development. By early adulthood the rate of cerebral oxygen consumption has decreased to 3.5 ml/100 g brain tissue/min, a rate that is subject to minimal decline in normal elderly brain [2]. Calculated in other terms, the total cerebral metabolic rate in adults is approximately 20 W, or 0.25 kcal/min,

equal to 20% of the whole body metabolism under most conditions (Table 31-2). Even during sleep there is only a relatively small decrease in cerebral metabolic rate; indeed, it may even be increased in rapid eye movement (REM) sleep [2].

REGULATION OF THE CEREBRAL METABOLIC RATE

Both excitatory and inhibitory neuronal signals utilize energy derived from metabolism. As mentioned above, the greatest proportion of energy utilization by brain serves signaling-related processes (Table 31-1). Brain function represents balances between excitatory and inhibitory neuronal signaling integrated with respect to time and location within the brain. Both excitatory and inhibitory signaling depends on transmembrane electrical potentials, which in turn are maintained or restored by energy-dependent ion transport (see Chs 5 and 6). The continual signaling is reflected in the persistent electrochemical activity of the brain. Thus, brain energy metabolism and the flow of energy-yielding substrates via the cerebral blood flow are linked to or regulated by brain function and, in fact, are used as measures of regional brain function (see Ch. 58). The process or processes by which this regulation is achieved is a subject of current research. The regional regulation of cerebral blood flow may be via chemical signals that affect the cerebral vasculature.

Continuous cerebral circulation is required to sustain brain function. Not only does the brain utilize O_2 at a very rapid rate, but the brain is absolutely dependent on uninterrupted oxidative metabolism for maintenance of its functional and structural integrity. Even at its maximal rate, anaerobic glycolysis can provide only a small portion of the required energy. Since the amount of O_2 stored in brain is extremely small compared with its rate of utilization, the brain requires a continuous supply of O_2 from the circulation, and brief disruptions in this supply have an immediate effect on brain function and consciousness.

The oxygen concentration in cerebral venous blood is substantially lower than in the cerebral arterial blood because of the high oxygen extraction. Whereas the glucose extraction is 10%, that for oxygen is 50–70% [47]. Thus, glucose delivery to the neural tissue can be elevated by solely increasing the extraction ratio without change in the blood flow, but for oxygen there is less extraction reserve and blood flow increase is needed [47]. During brain activation, venous oxygen content falls due to a mismatch between increases in cerebral blood flow and oxygen metabolism [48], resulting in decreased tissue deoxyhemoglobin content [49]. Because oxygen reaches the brain cells purely by diffusion and because there are no intracellular oxygen stores in the brain (in contrast to the myoglobin that stores oxygen in muscle), the net consumption of oxygen, defined as the cerebral metabolic rate of oxygen ($CMRO_2$), is reflected in the oxygen concentration gradient across the blood–brain barrier. The precise mechanism by which increased electrical activity, and thus increased metabolism, triggers these massive increases in cerebral blood flow is not totally understood.

In contrast to oxygen, which can freely diffuse across cell membranes, glucose is transported across the blood–brain barrier by facilitated diffusion (see above and Ch. 5). Net uptake of glucose by brain, defined as the cerebral metabolic rate of glucose (CMR_{glc}), is largely independent of the plasma glucose concentration [50]. The brain, like the liver but in contrast to muscle, has a significant tissue glucose concentration (≈1–2 mmol/l) that is about three-fold lower than in blood, and the brain has a high physical distribution space for glucose [51, 52]. Glucose transport is not likely to play a major role in regulating cerebral glucose utilization under physiologic conditions, since at normoglycemia the average intracellular concentration of glucose is well above 0.05 mM, the K_m of hexokinase (see below).

Glucose is the main obligatory substrate for energy metabolism. Brain glucose metabolism exhibits large regional variations at rest (consider, for example, the difference between gray and white matter) and is profoundly affected by local brain activation [8], suggesting a strong link between energy metabolism and brain function (Table 31-2). (See Brain Imaging in Ch. 58 for detailed discussion.) It is of interest that brain glucose concentration appears to be very constant across brain regions, despite strong metabolic differences between white and gray matter and among different brain nuclei. Cerebral blood volume and blood flow, however, differ with respect to the brain region, consistent with differences in regional resting metabolic activity [53]. However, brain glucose concentrations can change acutely, consistent with the facilitative nature of glucose transport, and are higher when metabolism is reduced (e.g. under barbiturate anesthesia). Brain glucose can become rate-limiting to metabolism, e.g. during profound activation, seizures and hypoglycemia. When the intracellular concentration of glucose approaches the K_m of hexokinase, significant facilitated transport of glucose into brain still occurs *in vivo* but cerebral blood flow (CBF) is acutely increased [54], possibly regulated by adenosine and K^+-ATP channels [55]. Glycogen, which is present at only low levels in brain, is the only fuel store endogenous to the brain that can provide significant amounts of energy (in the form of glucosyl equivalents) in periods during which the supply of glucose does not meet the metabolic demand (see below).

In addition, the brain can make limited use of other substrates for metabolism, e.g. during fasting (see above). Under normal circumstances, the venous lactate concentration is slightly higher than the arterial concentration,

suggesting a small net lactate output from the brain. Increased plasma lactate concentrations have recently been reported to lead to a reduced brain glucose metabolic rate, suggesting that the adult human brain can use lactate as a fuel (see below). Despite the ability of the brain to use other fuels, glucose remains the main obligatory substrate for energy metabolism.

METABOLISM IN THE BRAIN IS HIGHLY COMPARTMENTALIZED

Over the past few decades it has become quite clear that the metabolism of neurons and glial cells is interrelated and that these cells function in an integrated fashion. In this context it must be noted that several important enzymes, e.g. pyruvate carboxylase and glutamine synthetase, are found only in astrocytes [56, 57] (Table 31-3). Moreover, transporters for glucose, monocarboxylic acids, glutamate and glutamine are differentially distributed on brain neural and glial cells (see above and Chs 5 and 15). The compartmentation of transporters and enzymes leads to metabolic specialization of brain cells that leads in turn to the requirement for intercellular trafficking of metabolites.

One result of the selective localization of brain enzymes is that astrocytes must provide certain substrates (e.g. glutamine) to neurons for replenishment of the neuronal TCA cycle and for neurotransmitter synthesis [58]. Thus, astrocytes and neurons are essential partners in brain function. See also discussion in Chapter 15.

TABLE 31-3 Selective distribution of brain enzymes

Neurons	Astrocytes
Cytosolic	
Choline acetyltransferase	Glutamine synthetase‡
Neuronal enolase‡	Malic enzyme *
LDH1*	LDH5*
Glutamic acid decarboxylase†	
Mitochondrial	
Phosphate-activated glutaminase†	Pyruvate carboxylase‡
Mitochondrial malic enzyme†	Neurosteroid synthesis†
Ubiquitous mitochondrial creatine kinase	Phosphate-activated glutaminase (low)

* Enriched.
† Highly enriched.
‡ Very highly enriched.
LDH, lactate dehydrogenase.

Glucose has numerous metabolic fates in brain. It is crucial to remember that brain glucose does not function solely as an energy substrate but fulfills many roles, including that of a substrate for inositol biosynthesis and glycogen formation (Fig. 31-2). Moreover, its carbon skeleton may be incorporated into acetylcholine, lactate, glutamate, glutamine, aspartate, GABA and alanine. In developing brain, metabolism via the pentose phosphate shunt (PPS) is particularly important to provide ribose-5-phosphate for nucleotide synthesis and the NADPH required for lipid biosynthesis. The carbon skeleton of glucose is also incorporated into lipids [59].

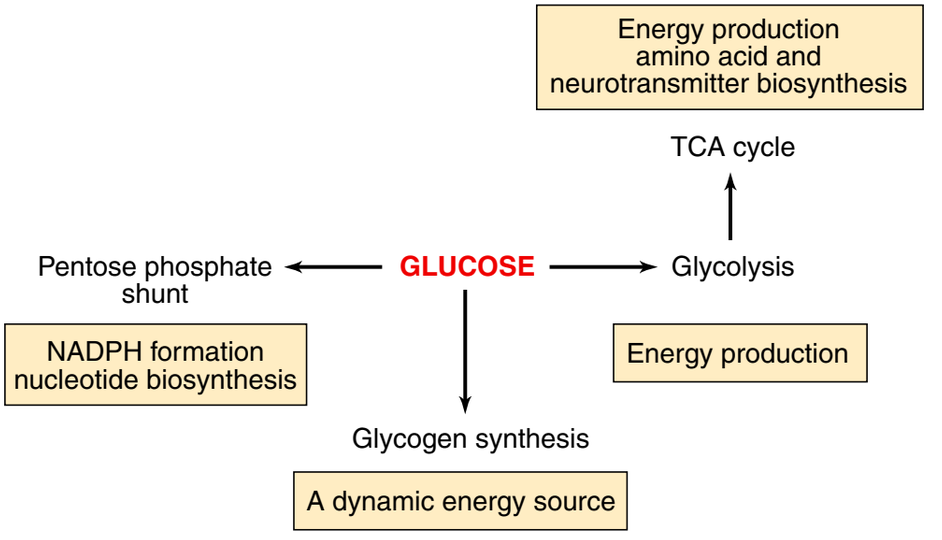

FIGURE 31-2 Glucose has multiple metabolic fates. Glucose is the main substrate for energy production via glycolysis and TCA cycle metabolism. Furthermore, glucose metabolism is closely connected to amino acid and neurotransmitter biosynthesis via TCA cycle intermediates. Glucose is the main precursor for glycogen synthesis. Metabolism of glucose via the pentose phosphate shunt provides ribose-5-phosphate for synthesis of nucleotides and NADPH for lipid biosynthesis and maintenance of reduced glutathione.

GLYCOGEN IS ACTIVELY SYNTHESIZED AND DEGRADED IN BRAIN, PROVIDING A DYNAMIC SOURCE OF CARBOHYDRATE

Compared to liver and muscle, glycogen is normally present in a relatively low concentration in brain, 2–6 μmol glucosyl units per gram wet weight for brain [60], compared to 100–500 μmol/g for liver [61]. However, concentrations as high as 8–12 μmol/g have been reported for rat brain [62]. Yet the brain glycogen concentration normally exceeds that of free brain glucose and glycogen is the only significant energy reserve in brain. The role of glycogen as a significant, endogenous store of fuel has been discounted in brain because of its low concentration compared to the total brain glucose utilization rate (Table 31-2). Indeed, if glycogen were the only source of fuel, it would be utilized in a few minutes. However, glycogen content is not rapidly depleted during hypoglycemia, as it needs to supply only a fraction of total glucose requirement, since glucose continues to be transported across the blood–brain barrier even in hypoglycemia [63].

Glycogen is a unique energy reserve that requires no energy (ATP) for mobilization (with the exception of the ATP required to convert phosphorylase b to phosphorylase a). The evidence that rapid, continual breakdown and synthesis of glycogen occurs at a rate of about 2% of the normal cerebral metabolic rate of glucose in brain [63] and is subject to elaborate control mechanisms suggests that local carbohydrate reserves in the form of glycogen are important for normal brain function. An accepted role of glycogen is that of a carbohydrate reserve utilized when glucose falls below need, yet its precise role remains to be determined.

The steady-state concentration of glycogen is regulated precisely by the coordination of separate synthetic and degradative enzymatic processes. The enzymatic regulation exists at several metabolic steps [2]. The amount of glucose-6-phosphate (Glc-6-P), the initial substrate for glycogen synthesis, usually varies inversely with the rate of brain glycolysis. Thus, a decline in Glc-6-P during energy need slows glycogen formation. The enzymes involved in brain glycogen synthesis and metabolism have different kinetic and regulatory properties from those involved in other tissues.

Glycogen and its enzymes are compartmentalized. Glycogen granules are only present in astrocytes of adult animals but are found in both astrocytes and neurons of immature animals. Of the enzymes involved in glycogen metabolism, glycogen phosphorylase is found in astrocytes only. Under steady-state conditions, it is probable that less than 10% of phosphorylase in brain is in the unphosphorylated b form (requiring AMP). This form is probably not very active at the low AMP concentrations present when intracellular glucose is sufficient to maintain ATP synthesis. Brain phosphorylase b kinase is activated indirectly by cAMP and by the molar concentrations of Ca^{2+} released during neuronal excitation, providing a molecular mechanism through which glycogen metabolism can be related to brain activity.

Synthesis of glycogen from UDP-glucose is catalyzed by glycogen synthase, which is rate-controlling. As in other tissues, glycogen synthase occurs in both a phosphorylated form, synthase-b, which depends on Glc-6-P as a positive modulator, and a dephosphorylated, independent synthase-a form, which is much more active.

As discussed above, glycogen can provide fuel to support metabolism of neurons. However, this is achieved in the form of glucose equivalents, not glucose *per se*, because most glycogen is broken down to Glc-6-P and the brain lacks substantial glucose-6-phosphatase activity. The glycogen-derived substrate transported from astrocytes to neurons is most likely lactate, consistent with recent proposals that glial lactate may be a fuel for neurons (see below, Fig. 31-4). This concept is supported by the observation that astroglial glycogen can support neuronal function [61, 63–65].

Hormones and neuronal activity affect brain glycogen metabolism. Glycogen is affected by hormones endogenous to the brain including vasoactive intestinal peptide and noradrenaline, as well as circulating hormones, such as insulin [61, 63, 64]. The mechanism whereby insulin exerts an effect on glycogen metabolism in brain has not been determined [63]. Glycogen metabolism in brain, unlike in other tissues, is controlled locally, due to differential local metabolic rates.

Brain glycogen concentrations are increased in conditions of decreased electrical activity (such as deep pentobarbital anesthesia) and its metabolism is likely to be affected by focal activity [61]. In addition, glycogen concentrations and enzyme expression are modulated by sleep deprivation. Moreover, glycogen is required to maintain intense firing in the isolated axon even in the presence of adequate extracellular glucose [61]. Altogether, experimental evidence suggests that even under normal conditions, glycogen has an important role in brain function.

GLYCOLYSIS: CONVERSION OF GLUCOSE TO PYRUVATE OR LACTATE

The terms aerobic and anaerobic glycolysis have historically been related to the extent of conversion of glucose to pyruvate or lactate under conditions of 'adequate' oxygen and no oxygen, respectively. Thus, anaerobic glycolysis leads to a complete conversion of glucose to lactate, which allows the maintenance of an optimal cytoplasmic NAD^+/NADH ratio (Fig. 31-3). Glycolytic flux is the rate at which glucose is converted to pyruvate in the cytosol [2]. The first step of glucose metabolism and thus of glycolysis involves phosphorylation of glucose by hexokinase. This essentially irreversible reaction is one key point in the

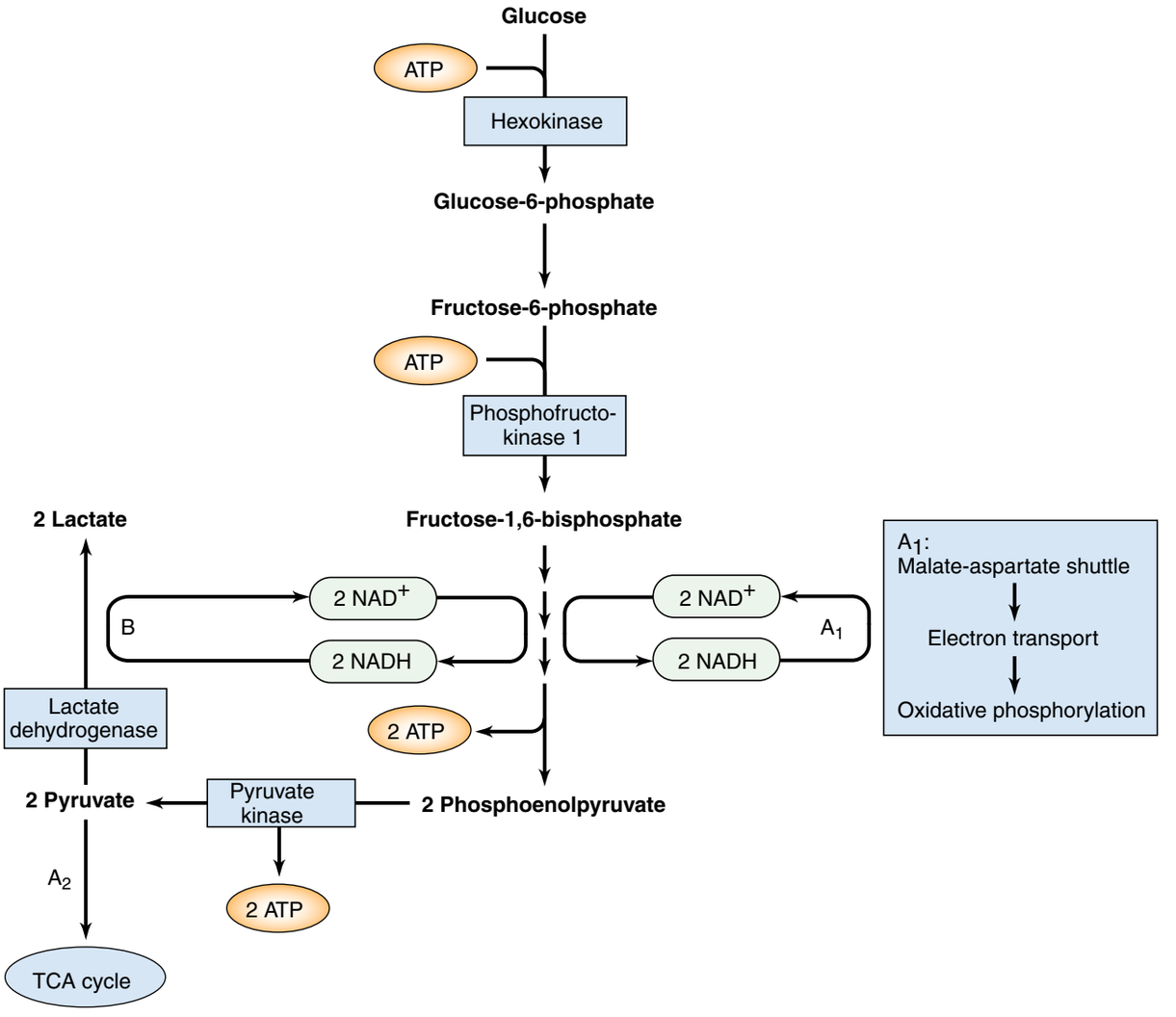

FIGURE 31-3 Glycolysis. A schematic representation of aerobic (A_1 and A_2) and anaerobic (B) glycolysis. Glucose is phosphorylated to glucose-6-phosphate and subsequently to fructose-1,6-bisphosphate via fructose-6-phosphate and phosphofructokinase 1, the main regulating enzyme in brain glycolysis. In one of the following steps, NADH is produced in the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. The NADH produced is either oxidized in the lactate dehydrogenase reaction reducing pyruvate to lactate (B), or the reducing equivalent from NADH is transferred to the mitochondria via the malate aspartate shuttle to be oxidized in the electron transport chain for oxidative phosphorylation (A_1). Pyruvate from aerobic glycolysis is subsequently metabolized via the tricarboxylic acid (*TCA*) cycle (A_2).

regulation of carbohydrate metabolic rate in brain. The second major regulatory enzyme is phosphofructokinase, discussed later. Figure 31-3 outlines the flow of glycolytic substrates in brain.

Brain hexokinase is inhibited by its product glucose-6-phosphate and to a lesser extent by adenosine diphosphate. The isoenzyme of hexokinase found in brain may be soluble in the cytosol or be attached firmly to mitochondria [2 and references therein]. An equilibrium exists between the soluble and the bound enzyme. The binding changes the kinetic properties of hexokinase and its inhibition by Glc-6-P resulting in a more active enzyme. The extent of binding is inversely related to the ATP:ADP ratio, i.e. conditions in which energy utilization exceeds supply will shift the solubilization equilibrium towards the bound form and produce a greater potential capacity for initiating glycolysis to meet the energy demand. This mechanism allows ATP to function both as the substrate of the enzyme and as a regulator to decrease ATP production through its influence on enzyme binding. It also confers preference on glucose in the competition for the ATP or more precisely its magnesium complex, MgATP ($MgATP^{2-}$) generated by mitochondrial oxidative phosphorylation. Thus, a process that will sustain ATP production continues at the expense of other energy requiring processes.

In addition to acting on enzyme kinetics, Glc-6-P solubilizes hexokinase, thus reducing the efficiency of the enzyme when the reaction product accumulates. The total

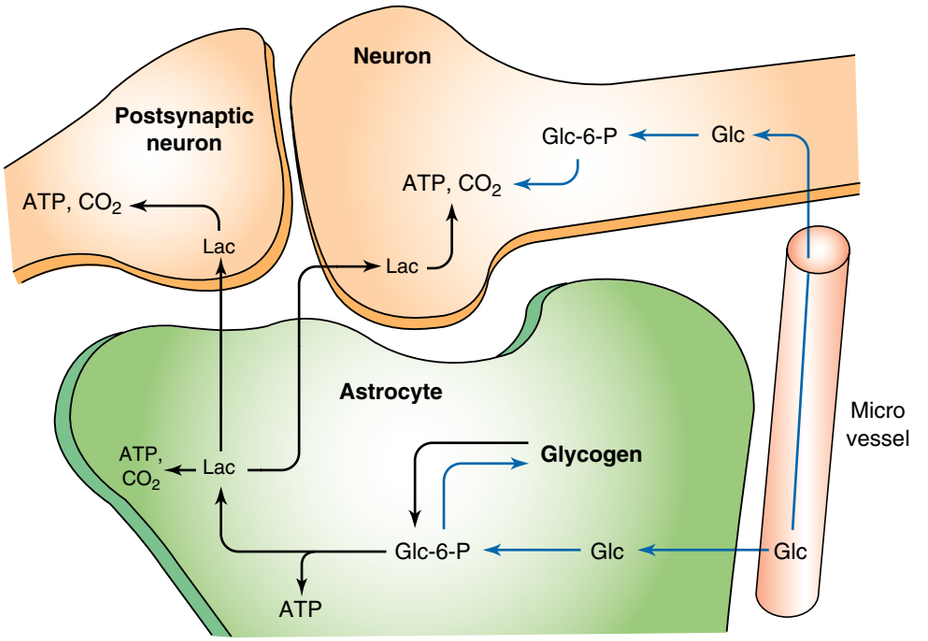

FIGURE 31-4 Scheme of compartmentalized glial glycogen metabolism. When glucose phosphorylation is limited by the low brain glucose concentration, astrocytic glycogenolysis can provide the necessary glucosyl units to maintain ATP synthesis in the glial compartment (*black lines*). Glycogen can provide fuel to neurons, presumably in the form of lactate, during hypoglycemia and thus reduce the energy deficit in the neuronal compartment. When the glucose supply is sufficient, glucose is stored in glial glycogen (*blue lines*). *Glc,* glucose; *Glc-6-P,* glucose-6-phosphate; *Lac,* lactate.

sum of these mechanisms is a fine-tuning of the activity of the initial enzyme in glycolysis in response to changes in the cellular environment. Glc-6-P represents a branch point in glucose metabolism because it is a common substrate for enzymes involved in glycolysis, pentose phosphate shunt (PPS) and glycogen-forming pathways (Fig. 31-2). There is also slight but detectable Glc-6-Pase activity in brain, the significance of which is not clear.

Brain glycolysis is regulated mainly by phosphofructokinase. Fructose-6-phosphate is the substrate of phosphofructokinase-1, a key regulatory enzyme controlling glycolysis [6]. The other substrate is MgATP. Like other regulatory reactions, it is essentially irreversible. It is modulated by a large number of metabolites and cofactors, whose concentrations under different metabolic conditions have a great effect on glycolytic flux. Prominent among these are the availability of ~P and citrate. Brain phosphofructokinase 1 is inhibited by ATP, Mg^{2+} and citrate and stimulated by NH_4^+, K^+, PO_4^{3-}, 5'-AMP, 3',5'-cAMP, ADP and fructose-2,6-bisphosphate. In the steady state, the concentrations of ATP and citrate in brain are apparently sufficient to keep phosphofructokinase-1 relatively inhibited as long as the concentration of positive modulators, or disinhibitors, is low. When the steady state is disturbed, activation of this enzyme produces an increase in glycolytic flux, which takes place almost as fast as events changing the internal milieu.

The glycolytic pathway in brain involves a number of enzymatic reactions that are similar in neurons and glia and similar to those in other tissues. One difference is that neurons contain a specific isoform of enolase called neuronal enolase (NSE). Immunocytochemical detection of the enolases makes them useful in determining neuron:glia ratios in tissue samples. Brain phosphoenolpyruvate kinase controls an essentially irreversible reaction that requires not only Mg^{2+}, as do several other glycolytic enzymes, but also K^+ or Na^+. It should be noted that two reactions are responsible for ATP production in glycolysis, namely phosphoglycerate kinase and pyruvate kinase. The latter step also may be regulatory, since the enzyme can be phosphorylated and dephosphorylated, and is inactive in its phosphorylated form. Overall, the glycolytic pathway consumes two and produces four molecules of ATP, thus providing a net of two ATP molecules per glucose molecule.

THE PENTOSE PHOSPHATE SHUNT IS ACTIVE IN BRAIN

Under basal conditions 5% of brain glucose is metabolized via the pentose phosphate shunt (PPS), also termed the hexose monophosphate pathway [66], a pathway active in both neurons and astrocytes. The PPS has

relatively high activity in developing brain, reaching a peak during myelination. Its main contribution is probably to produce the NADPH needed for reductive reactions in lipid synthesis (see Ch. 3). NADPH is also necessary for the maintenance of reduced glutathione, which is required for the inactivation of reactive oxygen species (see Ch. 32). Thus, the activity of the PPS responds to oxidative stress. The shunt also provides pentose for nucleotide synthesis; however, only a small fraction of the activity of this pathway would be required. Pentose phosphate flux apparently is regulated by the concentrations of Glc-6-P, $NADP^+$, glyceraldehyde-3-phosphate and fructose-6-phosphate. Since transketolase, an enzyme in this shunt, requires thiamine pyrophosphate as a cofactor, poor myelin maintenance in thiamine deficiency may reflect failure of this pathway to provide sufficient NADPH for lipid synthesis [2, 67] (see Peripheral Neuropathy in Ch. 36).

GLYCEROL PHOSPHATE DEHYDROGENASE IS AN NADH OXIDIZING ENZYME RELATED TO GLYCOLYSIS

Glycerol phosphate dehydrogenase (GPDH) is indirectly associated with glycolysis and reduces dihydroxyacetone phosphate to glycerol-3-phosphate, oxidizing NADH in the process. Under hypoxic conditions, glycerol-3-phosphate and lactate increase initially at comparable rates, although the amount of lactate produced greatly exceeds that of glycerol-3-phosphate. The relative concentrations of the oxidized and reduced substrates of the GPDH and lactate dehydrogenase (LDH) catalyzed reactions indicate much higher local concentrations of NADH in brain than are found by gross measurements. In fact, the relative proportions of oxidized and reduced substrates of the reactions that are linked to the pyridine nucleotides may be a better indicator of local redox states (NAD^+/NADH) in brain than the direct measurement of pyridine nucleotides themselves [2, 68].

THE MALATE–ASPARTATE SHUTTLE HAS A KEY ROLE IN BRAIN METABOLISM

The malate–aspartate shuttle is the most important pathway for transferring reducing equivalents from the cytosol to the mitochondria in brain. This shuttle involves both the cytosolic and mitochondrial forms of aspartate aminotransferase and malate dehydrogenase, the mitochondrial aspartate–glutamate carrier and the dicarboxylic acid carrier in brain (Fig. 31-5) [69]. The electrogenic exchange of aspartate for glutamate and a

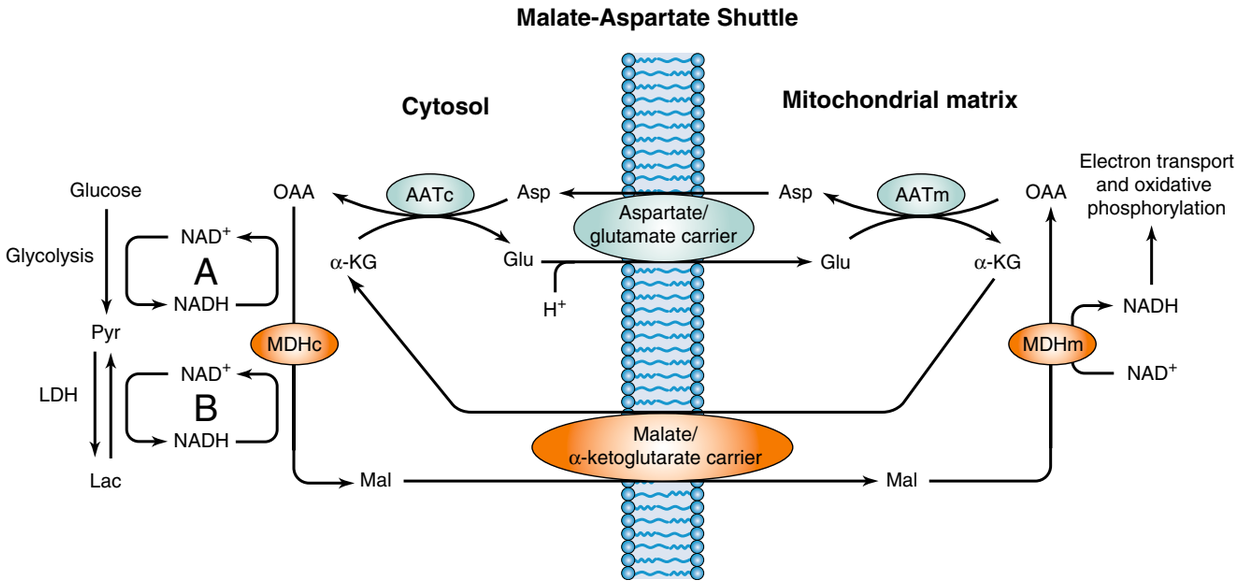

FIGURE 31-5 The malate–aspartate shuttle. NADH is produced in glycolysis (*A*) and in the conversion of lactate (*Lac*) to pyruvate (*Pyr*) via lactate dehydrogenase (*LDH, B*). The reducing equivalents from NADH are transferred via the malate aspartate shuttle to be oxidized via electron transport and oxidative phosphorylation. Oxaloacetate (*OAA*) is converted to malate (*Mal*) by cytosolic malate dehydrogenase (*MDHc*) and NADH is oxidized to NAD^+. Malate is transported into the mitochondrial matrix by a carrier and converted into OAA, and NAD^+ is reduced to NADH via mitochondrial malate dehydrogenase (MDH_m). Within the mitochondria oxaloacetate is converted into aspartate, and α-ketoglutarate (*α-kg*) is formed from glutamate (*Glu*) via mitochondrial aspartate aminotransferase (AAT_m). Aspartate leaves the mitochondrial matrix in exchange for a molecule of glutamate and a proton. In the cytosol aspartate is converted back to oxaloacetate via cytosolic aspartate aminotransferase (AAT_c).

proton via the aspartate–glutamate carrier is irreversible; the exchange favors efflux of aspartate from and entry of glutamate into mitochondria. Exchange via this carrier, which appears to be the overall rate-limiting step of the shuttle [71], is stimulated by Ca^{2+} binding to a domain on the outer side of the inner mitochondrial membrane. Two Ca^{2+}-sensitive aspartate–glutamate carriers have been identified; citrin (AGC2) is found primarily in liver and kidney, and aralar1 (AGC1) is found in skeletal muscle and brain [71]. Aralar1 is highly enriched on neuronal mitochondria and is often found in areas with high levels of cytochrome oxidase [72]. A considerably lower level of aralar1 and malate–aspartate shuttle activity is present in astrocytes [72].

The activity of the malate–aspartate shuttle increases during development in parallel with synaptogenesis, which is consistent with the high activity and importance of this shuttle in neurons and synaptic terminals. Evidence of highly regulated malate–aspartate shuttle in adult human brain has been documented [73 and references therein].

The malate–aspartate shuttle has a role in linking metabolic pathways in brain. It is essential for coordinating the activity of the glycolytic pathway and the TCA cycle by maintaining a low redox state ($NADH/NAD^+$) essential for continuation of glycolysis [74]. Activity of the shuttle is much higher in neurons than astrocytes, which is consistent with the involvement of the shuttle in the synthesis of neurotransmitter glutamate [75] and the enrichment of aralar1 on neuronal mitochondria [72]. The activity of the malate–aspartate shuttle is impaired in pathological conditions including hypoxic/ischemic brain damage [76]. Impaired shuttle activity limits the ability of neurons to oxidize lactate for energy [32].

THERE IS DYNAMIC METABOLISM OF LACTATE IN BRAIN

Lactate is formed under both aerobic and anaerobic conditions in brain. Under anaerobic conditions or upregulation of glycolysis, considerable lactate is produced, presumably in astrocytes, via glycolysis to provide ATP [77, 78]. Conversion of lactate to pyruvate by lactate dehydrogenase (LDH) is necessary for further metabolism of lactate through the tricarboxylic acid (TCA) cycle. Lactate dehydrogenase exists in different isoforms named LDH1–5, consisting of tetramers formed from two distinct subunits. The A (m-type) and B (h-type) subunits of LDH are the products of separate genes and are differentially regulated [79]. These isoforms have slightly different kinetic properties regarding rates of catalysis and affinities for pyruvate. Although the A subunit, hence LDH4 and LDH5 (corresponding to A4 and A3B) are the predominant isoforms in astrocytes [80], and the B subunit, hence LDH1 and LDH2 (corresponding to B4 and B3A) are enriched in synaptic terminals, the enrichment of these isoforms *per se* does not control the direction of metabolism. However, upregulation of LDH may contribute to the cerebral lactate accumulation seen in thiamine deficiency [81]. The expression of LDH isozymes is developmentally regulated. Lactate dehydrogenase functions in the cytoplasm to oxidize NADH, which accumulates as a result of the activity of glyceraldehyde-3-phosphate dehydrogenase in glycolysis. This permits glycolytic ATP production to continue under anaerobic conditions. Lactate dehydrogenase also functions under aerobic conditions because NADH cannot penetrate mitochondrial membranes. Therefore, oxidation of NADH in the cytoplasm depends on this reaction and on the activity of the malate–aspartate shuttle (see above), and to a much lesser extent the glyceraldehyde-3-phosphate shuttle that transfers reducing equivalents to mitochondria. Under normal circumstances there is dynamic turnover of a significant portion of brain lactate in the presence of sufficient O_2 [82 and references therein]. However, the functional significance of this dynamic lactate metabolism is unclear.

Compartmentation of the pyruvate–lactate pool is unexpectedly complex. Studies of cultured brain cells and of *in vivo* brain glucose metabolism have provided evidence for unexpected complexity of the pyruvate–lactate relationship. Using carbon-13 labeling and nuclear magnetic resonance (NMR) spectroscopy, it has been shown that alanine produced from pyruvate exhibits different labeling depending upon whether the pyruvate is derived from glucose or from exogenously supplied lactate [83]. There is much convincing evidence that distinct pools of a given metabolite can exist within the cytosol of a cell [84–86]. This means that the cytosol must not be considered as a free solution but rather as a superbly organized gel containing many proteins and surfaces on which directed transfer of metabolites and cofactors effectively restricts diffusion. Thus, the high protein content and affinity of enzymes for their substrates and co-factors can restrict the diffusion of small molecules through the cytosol [87].

The astrocyte–neuron lactate shuttle hypothesis is controversial. In recent years the possibility that lactate, formed within the brain and released by astrocytes, is an important neuronal substrate both for energy and incorporation into neurotransmitters has been the subject of many studies and considerable controversy. There is evidence that suggests transient release of lactate in human brain on stimulation [48, 8, 88]. Little is known about the highly active metabolism that takes place in the many elaborate, lamellar distal processes of astrocytes dispersed through the neuropil and interacting with an estimated >100,000 synapses [82, and references therein]. However, it is well established that astrocytes do respond to neuronal activity [89]. For example, in the isolated mouse optic nerve preparation, upon stimulation, astrocytic glycogen

is mobilized and transferred as lactate to sustain nerve function [61].

In vitro data demonstrate formation of lactate from metabolism of glucose and from the degradation of glycogen to lactate that is released from cultured astrocytes [77, 90]. The carbon skeleton from lactate is readily incorporated into the neurotransmitters glutamate and GABA in primary cultures of neurons [91, 92]. The high rate of transport and oxidation of lactate in isolated synaptosomes from mature brain, even in the presence of added glucose, is consistent with a role for lactate metabolism in neurons [32]. Lactate is an advantageous substrate for neurons since energy is obtained as NADH without the expense of ATP. The highly localized distribution of MCT2 in neurons (see above and Fig. 31-1) [16, 28, 29] and the findings that in nonvascular tissue the level of MCT1 remains fairly constant with development, whereas the level of MCT2 in neurons increases with maturation, are consistent with a role for these transporters and lactate metabolism in mature brain [16].

There is considerable evidence to support the overall concept of release of lactate, derived from glucose metabolism in astrocytes, and subsequent use by neurons in brain. However, the extent and specific circumstances in which neurons *in vivo* utilize lactate formed within astrocytes for energy and incorporation into neurotransmitters is not known. The likelihood that some trafficking of lactate between astrocytes and neurons has a role in brain metabolism is high, but there is no evidence [1] to support any stoichiometric conversion of brain glucose to lactate or any stochiometric coupling of neuronal/glial lactate transfer to the neuronal/glial glutamate/glutamine cycle as published [93].

Although astrocytes release lactate, they can oxidize lactate for energy and can produce it from TCA cycle intermediates [86, 94]. Astrocytes control the microenvironment of brain and respond to neurotransmitters as well as to changes in the concentrations of substrates in the extracellular milieu [95]. Certain substrates, such as lactate, glutamine [96] and ketone bodies [97], can be produced as well as consumed by astrocytes, depending on the composition of substrates in the extracellular milieu. It is important to recognize the complexity of lactate balance since changes in the lactate level can be the net result of many processes, including: plasma lactate level, altered transport into and release from brain cells, changes in the glycolytic rate, alterations in the rates of the malate–aspartate shuttle activity, activity of the pyruvate dehydrogenase complex, TCA cycle activity, the cytoplasmic pH and redox state, the rate of glycogenolysis in astrocytes and the rate of lactate efflux from brain [82]. Subcellular compartmentation in astrocytes is well documented [12, 13, 58, 65, 83, 84] and it is likely that the astrocyte processes interacting closely with nerve terminals represent a highly metabolically active compartment that may supply energy metabolites *in vivo* [82]. However, the information about *in vivo* metabolism in such discrete areas and short time frames is not yet available [82]. Additionally, astrocytic processes surround capillaries of the blood–brain barrier or interact with other astrocytes forming a syncytium capable of transmitting signals and buffering metabolism [82, 98].

THE COMPLETE OXIDATION OF GLUCOSE REQUIRES TRICARBOXYLIC ACID CYCLE ACTIVITY

The pyruvate dehydrogenase complex plays a key role in regulating oxidation of glucose. In order for glucose to be oxidized to CO_2, pyruvate formed in glycolysis needs to enter the TCA cycle. This is accomplished via the pyruvate dehydrogenase (PDH) complex localized in the mitochondria, which controls the rate of pyruvate entry into the TCA cycle as acetyl coenzyme A (acetyl-CoA)(see also mitochondrial metabolism in Ch. 42). Pyruvate dehydrogenase is a mitochondrial multienzyme complex consisting of pyruvate decarboxylase and two other enzymes, lipoate acetyltransferase and lipoamide dehydrogenase, as well as the coenzymes thiamine pyrophosphate, lipoic acid, CoA, FAD and NAD^+. PDH is inactivated by being phosphorylated at the decarboxylase moiety by a tightly bound Mg^{2+}/ATP^{4-}-dependent protein kinase. PDH is activated by being dephosphorylated by a loosely bound Mg^{2+}- and Ca^{2+}-dependent phosphatase. About half the brain enzyme is usually active. Pyruvate protects the complex against inactivation by inhibiting the kinase. ADP is a competitive inhibitor of Mg^{2+} for the inactivating kinase. Under conditions of greater metabolic demand, increases in pyruvate and ADP and decreases in acetyl-CoA and ATP make the complex more active. Pyruvate dehydrogenase is inhibited by NADH, thereby decreasing formation of acetyl-CoA during hypoxia and allowing more pyruvate to be reduced by lactate dehydrogenase, thus forming the NAD^+ necessary to sustain glycolysis. Pyruvate dehydrogenase defects do occur in several mitochondrial enzyme-deficiency states [99] (see Ch. 42).

Acetyl coenzyme A formed from glucose is the precursor for acetylcholine. Although acetylcholine synthesis normally is controlled by the rate of choline uptake and choline acetyltransferase activity (see Ch. 11), the supply of acetyl coenzyme A (acetyl-CoA) can be limiting under adverse conditions. Choline uptake is, however, independent of the acetyl-CoA concentration. The mitochondrial membrane is not permeable to the acetyl-CoA produced within it, but there is efflux of its condensation product, citrate. Acetyl-CoA can subsequently be formed from citrate in the cytosol by ATP citrate lyase. The acetyl moiety of acetylcholine is formed in a compartment, presumably representing the synaptic terminal, having a rapid glucose turnover. The cytosol of cholinergic nerve endings is rich in citrate lyase, and it is likely that citrate shuttles the acetyl-CoA from the mitochondrial

compartment to the cytosol. During hypoxia or hypoglycemia, acetylcholine synthesis can be inhibited by failure of the acetyl-CoA supply.

Pyruvate carboxylation in astrocytes is the major anaplerotic pathway in brain. Anabolic pathways lead to an ongoing drain on brain TCA cycle intermediates, which would be depleted if there were no mechanism to replenish these compounds. Pyruvate carboxylase, which has been shown to be primarily, if not exclusively, an astrocytic enzyme [56], fulfills this 'anaplerotic' or refilling function in brain by adding a CO_2 to pyruvate forming oxaloacetate which is then combined with acetyl-CoA to form citrate. Although malic enzyme or the combined action of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase can fix CO_2, they do not perform this function to a significant extent in brain [100, 101]. To export net amounts of citrate, α-ketoglutarate or oxaloacetate from the TCA cycle, the supply of dicarboxylic acids must be replenished. The CO_2 fixation rate sets the upper limit at which biosynthetic reactions can occur. The pyruvate carboxylase reaction in astrocytes is essential for neurons because they are continuously releasing amino acid neurotransmitters and thus require metabolites from astrocytes for the replenishment of the neuronal TCA cycle and neurotransmitter biosynthesis [13].

ENERGY OUTPUT AND OXYGEN CONSUMPTION ARE ASSOCIATED WITH TCA CYCLE ACTIVITY

The actual flux through the TCA cycle depends on glycolysis and acetyl coenzyme A production. However, activity of the TCA cycle is subject to control at several enzymatic steps of the cycle [2, 67] and by the local ADP concentration, which is a prime activator of the mitochondrial respiration to which the TCA cycle is linked (Fig. 31-6). As in other tissues, there are two isocitrate dehydrogenases in brain. One is active primarily in the cytoplasm and requires nicotinamide adenine dinucleotide phosphate ($NADP^+$) as cofactor; the second is the mitochondrial isocitrate dehydrogenase that requires NAD^+ and participates in the TCA cycle. The NAD^+-linked enzyme catalyzes an essentially irreversible reaction and has allosteric properties, i.e. it is inhibited by ATP and NADH and may be stimulated by ADP. The function of cytoplasmic $NADP^+$ isocitrate dehydrogenase is uncertain but it has been postulated that it supplies the NADPH necessary for many reductive synthetic reactions. The relatively high activity of this enzyme in immature brain and white matter is consistent with such a role. α-Ketoglutarate dehydrogenase, which oxidatively decarboxylates α-ketoglutarate, requires the same cofactors as pyruvate dehydrogenase.

Succinate dehydrogenase is the enzyme that catalyzes the oxidation of succinate to fumarate and is also part of the respiratory chain. It is bound tightly to the inner mitochondrial membrane and is an integral part of the respiratory chain, as it constitutes complex II (Fig. 31-6). Isocitrate and succinate concentrations in brain are affected little by changes in the flux of the TCA cycle as long as an adequate glucose supply is available. The highly unfavorable free energy change of the malate dehydrogenase reaction is overcome by the rapid removal of oxaloacetate. The latter is maintained at a very low concentration under steady-state conditions by the condensation reaction with acetyl-CoA catalyzed by citrate synthase [67].

Malate dehydrogenase is one of several enzymes in the TCA cycle present in both the cytoplasm and mitochondria. Both the mitochondrial and cytoplasmic enzymes are components of the malate–aspartate shuttle. This represents a branch point in metabolism since mitochondrial malate dehydrogenase (MDH_m) can lead to metabolism via the TCA cycle or via the malate–aspartate shuttle. The direction of metabolism depends on the redox state and on the association of MDH_m into *metabolons* with either citrate synthase plus α-ketoglutarate dehydrogenase, or with aspartate aminotransferase plus glutamate dehydrogenase [102 and references therein]. The association of MDH_m into transient multienzyme complexes called metabo-lons, and thus directing the pathway of metabolism, is influenced by the concentration of key metabolites in the mitochondria (e.g. oxaloacetate, α-ketoglutarate and citrate) [102].

Citrate is a multifunctional compound predominantly synthesized and released by astrocytes. The steady-state concentration of citrate in brain is relatively high compared to other TCA cycle and glycolytic intermediates, reaching 0.4 mmol/l in the cerebrospinal fluid [103]. This is compatible with the ability of astrocytes to synthesize and release a considerable amount of citrate *in vitro* at a rate reflecting the activity of pyruvate carboxylase; however, the extent to which this occurs *in vivo* is not known. Citrate synthesis is compartmentalized in astrocytes; pyruvate carboxylation has a specific role in the formation of a releasable pool of citrate, but has less of a role in synthesis of the smaller intracellular pool [104, 105]. Bicarbonate stimulates the release of citrate, which is threefold greater from cerebellar astrocytes than from cortical astrocytes [104]. This difference reflects the functional specialization of astrocytes in different brain regions, possibly in response to the neuronal specialization. It has been shown that citrate can attenuate the inhibitory action of Zn^{2+} on NMDA-receptor-mediated glutamate release (see NMDA receptors in Ch. 15). The ability of astrocytes to release large amounts of citrate may be related to the ability of these cells to significantly upregulate glycolysis under certain conditions [105]. This may be so since the release of citrate would decrease its intracellular concentration, thereby relieving the inhibitory action of citrate on PFK1 in the glycolytic pathway (Fig. 31-3).

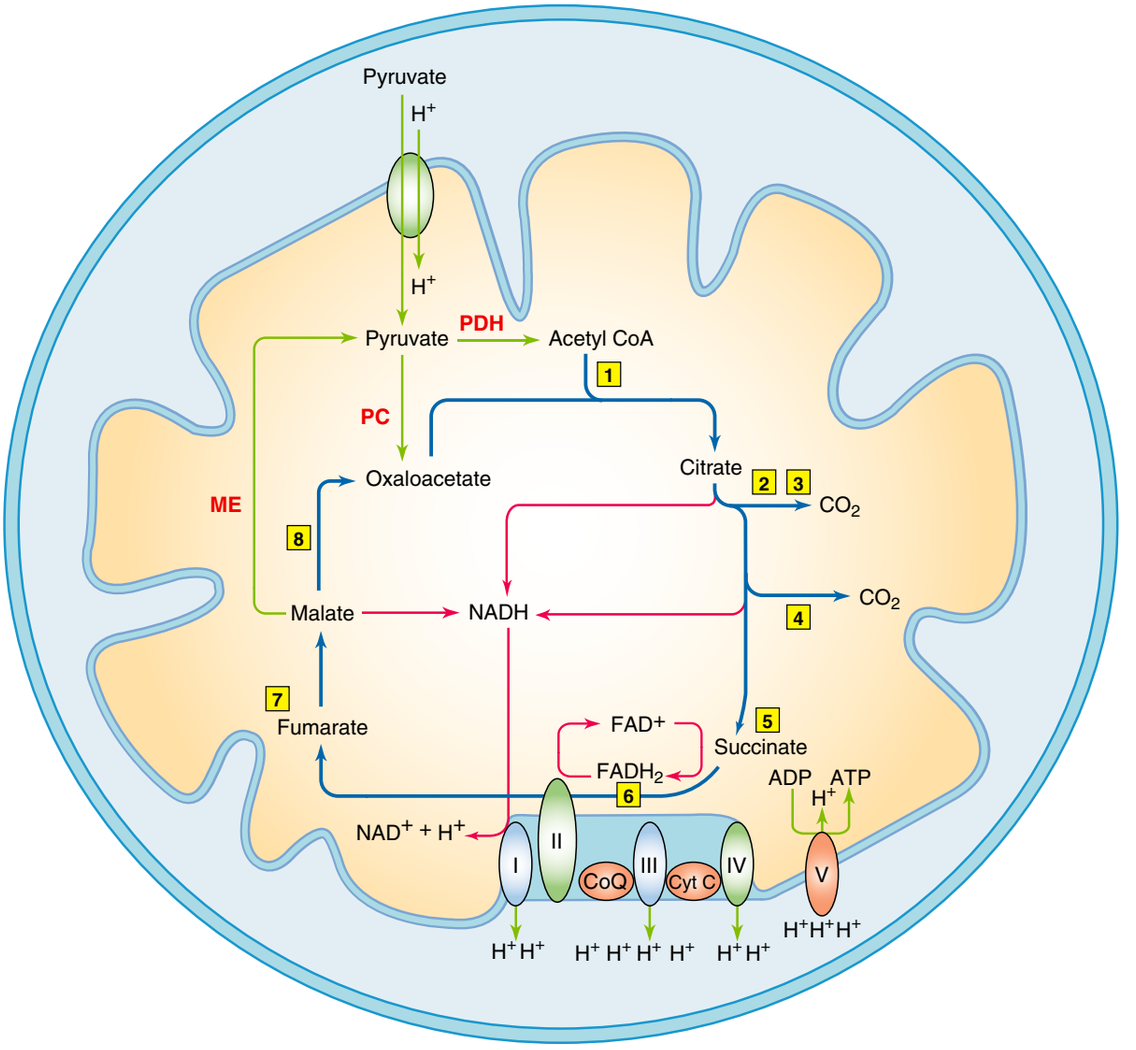

FIGURE 31-6 Tricarboxylic acid cycle and oxidative phosphorylation. Pyruvate is carried into the mitochondrial matrix for oxidative decarboxylation to acetyl-CoA via the pyruvate dehydrogenase complex (*PDH*) or for carboxylation to oxaloacetate via pyruvate carboxylation (*PC*). Acetyl-CoA is condensed via citrate synthase (*1*) to citrate, which is converted to α-ketoglutarate via aconitase (*2*) and isocitrate dehydrogenase (*3*). α-ketoglutarate is subsequently decarboxylated via the α-ketoglutarate dehydrogenase complex (*4*) to succinyl-CoA. Succinate is formed from succinyl-CoA via succinyl CoA synthase (*5*). Complex II of the respiratory chain/succinate dehydrogenase (*6*) oxidizes succinate to fumarate, which is converted into malate via fumarase (*7*). Malate is oxidized to oxaloacetate via malate dehydrogenase (*8*) or it can be converted to pyruvate via malic enzyme (ME). NADH is re-oxidized in complex I, and FADH in complex II of the electron transport chain. The electrons are carried by the complexes, coenzyme Q (*CoQ*) and cytochrome C (*Cyt c*) to O_2, which is reduced to H_2O in complex IV. The electron transport generates a proton gradient over the inner membrane driving the ATP synthesis in complex V.

MANY TCA CYCLE ENZYMES AND COMPONENTS OF OXIDATIVE PHOSPHORYLATION ARE DIFFERENTIALLY DISTRIBUTED IN NEURONAL AND GLIAL MITOCHONDRIA

Mitochondria are distributed with varying densities throughout the central nervous system with the more vascular parts containing most of the mitochondria. Mitochondria have their greatest concentration in neurons and are present throughout the perikaryon, cytoplasm, dendrites, axons and nerve endings (see Ch. 1 and [106, 107]). Regions of particular abundance are nerve endings, the axon hillock and nodes of Ranvier. Mitochondrial heterogeneity has been demonstrated in the brain by separation of mitochondria of synaptic and nonsynaptic (neuronal cell bodies and glial) origin, which showed differences in distribution of enzymes catalyzing the TCA cycle reactions [107]. Another example of mitochondrial heterogeneity is the dramatically different distribution of respiratory chain components in neurons and astrocytes [108]. This may be due in part to the fact that most of the TCA cycle enzymes are encoded by the nuclear DNA in

the host cell and imported into the mitochondrial matrix, in contrast to enzymes of the respiratory chain complexes, which are encoded by the mitochondrial DNA (see Ch. 42 for genetic abnormalities of mitochondria in brain and muscle).

Mitochondrial heterogeneity leads to multiple simultaneous TCA cycles in astrocytes and neurons. While it is clear that mitochondrial heterogeneity exists in the brain as a whole it is less clear if mitochondria in individual cells may exhibit such heterogeneity. However, circumstantial evidence from detailed metabolic studies of cultured brain cells points toward such a possibility [105]. The presence of multiple simultaneous TCA cycles in astrocytes, neurons and freshly isolated synaptic terminals provides additional evidence for mitochondrial heterogeneity [74, 84, 104, 109]. Furthermore, discrete subfractions of synaptic mitochondria have considerable heterogeneity of mitochondrial enzymes that are thought to reflect subcellular compartmentalization and functional metabolic specialization [74]. Moreover, studies of the distribution of α-ketoglutarate dehydrogenase in mitochondria of individual astrocytes provide evidence of distinct subpopulations of mitochondria within individual cells [110]. The question of subcellular compartmentation is of fundamental importance with regard to the full understanding of the extent and intricacies of metabolic compartmentation in brain. The existence of distinct microdomains within astrocytes [111] and neurons [28] are other examples of functionally important subcellular compartmentalization.

Partial TCA cycles can provide energy in brain. Under certain metabolic conditions with low levels of acetyl-CoA, such as in hypoglycemia, only a part of the TCA cycle may operate, particularly in neurons and synaptic terminals [112, 113]. This truncated cycle, which enables utilization of glutamine/glutamate as energy substrates, consists of the steps from α-ketoglutarate to oxaloacetate and leads to aspartate production and sometimes accumulation [112]. The majority of the reducing equivalents produced in the complete TCA cycle are derived from these reactions [67]. The energy production from this truncated TCA cycle corresponds to 75% of that produced from the entire cycle (see below).

Adenosine triphosphate production in brain is highly regulated. Oxidative steps of carbohydrate metabolism normally contribute 36 of the 38 high-energy phosphate bonds (~P) (assuming a P:O ratio of 3:1) generated during aerobic metabolism of a single glucose molecule. However, it is not likely that there is a net production of 38 ATP equivalents per mole of glucose (Table 31-1) since a fraction of the glucose taken up is converted to lactate, and there is a proton leak as well as specific non-ATP synthase uses of protons (e.g. malate–aspartate shuttle, phosphate transport and ATP transport) across the mitochondrial membrane. The steady-state concentration of ATP is high and represents the net sum of very rapid synthesis and utilization. On average, half of the terminal phosphate groups turn over in about 3 seconds; the turnover is probably much faster in certain regions [2]. The level of ~P is kept constant by regulation of ADP phosphorylation in relation to ATP hydrolysis. The active adenylyl kinase reaction, which forms equivalent amounts of ATP and AMP from ADP, prevents any great accumulation of ADP. Only a small amount of AMP is present under steady-state conditions; thus, a relatively small decrease in ATP may lead to a relatively large increase in AMP, which is a positive modulator of AMP kinase, which in turn regulates many reactions that lead to increased ATP synthesis. Such an amplification factor provides a sensitive control for maintenance of ATP levels [68], which are surprisingly constant *in vivo*.

Phosphocreatine has a role in maintaining adenosine triphosphate levels in brain. The concentration of phosphocreatine (PCr) in brain is even higher than that of ATP, and creatine phosphokinase (CPK) is extremely active. The PCr level is exquisitely sensitive to changes in oxygenation, providing ~P for ADP phosphorylation and, thus, maintaining ATP levels. The CPK system also may function in regulating mitochondrial activity. In neurons with a very heterogeneous mitochondrial distribution, the creatine/phosphocreatine shuttle may play a critical role in energy transport [114]. The precise role of CPK in brain (whether as an energy buffer or as transport of ATP equivalents) is uncertain. However, it should be noted that knocking out the CPK enzymes in mouse brain had an effect on spatial learning but not on motor function [115].

Substrates other than glucose provide energy for brain cells (e.g. glutamate, glutamine, lactate, fatty acids, ketone bodies). It is important to note that all of the machinery for using monocarboxylic acids for energy is present in adult brain [116, 117] (see also Ch. 58). Normally, there are no significant cerebral arteriovenous differences for the ketone bodies D-β-hydroxybutyrate and acetoacetate, which are formed in the course of the catabolism of fatty acids by liver. In prolonged starvation, the carbohydrate stores of the body are exhausted and the rate of gluconeogenesis is insufficient to provide glucose fast enough to meet the requirements of the brain; blood ketone concentrations rise as a result of the rapid fat catabolism. The brain then apparently turns to the ketone bodies as the source of its energy supply. Cerebral utilization of ketone bodies appears to follow passively their concentrations in arterial blood [116]. In normal adults, ketone concentrations are very low in blood and cerebral utilization of ketones is negligible. In ketotic states resulting from starvation; fat-feeding or ketogenic diets; diabetes; or any other condition that accelerates the mobilization and catabolism of fat, cerebral utilization of ketones is increased more or less in direct proportion to the degree of ketosis [116].

The enzymes responsible for their metabolism, D-β-hydroxybutyrate dehydrogenase, acetoacetate-succinyl-CoA transferase and acetoacetyl-CoA-thiolase, are present in

brain tissue in sufficient amounts to convert them into acetyl-CoA and to feed them into the TCA cycle at a sufficient rate to satisfy the metabolic demands of the brain [116]. Considerable uptake of ketone bodies by the brain during prolonged fasting for several weeks has been reported [117]. If one assumed that the substances were oxidized completely, their rates of utilization would have accounted for more than 50% of the total cerebral oxygen consumption, more than that accounted for by the glucose uptake. D-β-hydroxybutyrate uptake was several times greater than that of acetoacetate, a reflection of its higher concentration in the blood. It should be noted that D-β-hydroxybutyrate is incapable of maintaining or restoring normal cerebral function in the absence of glucose in the blood [2]. This suggests that, although it can partially replace glucose, it cannot fully satisfy the cerebral energy needs in the absence of some glucose consumption. One explanation may be that the first product of D-β-hydroxybutyrate oxidation, acetoacetate, is metabolized further by its displacement of the succinyl moiety of succinyl-CoA to form acetoacetyl-CoA. A certain rate of glucose utilization may be essential to drive the TCA cycle, to provide enough succinyl-CoA to permit the further oxidation of acetoacetate and, hence, to pull along the oxidation of D-β-hydroxybutyrate.

Several studies document the use of lactate [77, 118] and ketone bodies [119] by mature brain in the presence of glucose. Octanoate and other short chain fatty acids can be used by brain astrocytes *in vitro* for energy [120]. It is important that such backup systems are present to protect the brain when glucose is low [9, 32, 94]. Glucose derived carbohydrates and amino acids together form a large pool of carbon in brain, i.e. ≈15 µmol carbohydrate/g plus another 25 µmol/g in the aggregate amino acid pool that are in a dynamic state of use and replenishment [82]. There is evidence that amino acids can be oxidized *in vivo* by the brain for energy [121]. Since glutamate is formed within brain from glucose and monocarboxylic acids it is not likely to be a significant source of energy. However, under specific circumstances such as hypoglycemia the oxidation of glutamate is an important mechanism for providing energy via a partial TCA cycle from α-ketoglutarate to oxaloacetate (see above). Malic enzyme is required for the complete oxidation of glutamate. Leucine enters the brain at a high rate and can be converted to 3-hydroxybutyrate and subsequently oxidized for energy by brain cells. The use of amino acids for energy requires the disposal of the NH_3 removed.

GLUTAMATE/GLUTAMINE METABOLISM IS LINKED TO ENERGY METABOLISM

Glutamate enters the mitochondria via the aspartate–glutamate carrier or the glutamate carrier. These carriers are illustrated in Figure 31-7. Glutamate participates in a number of metabolic reactions catalyzed by the enzymes glutamate dehydrogenase, a number of aminotransferases, glutamine synthetase, phosphate-activated glutaminase and glutamate decarboxylase (Fig. 31-8). Additionally, glutamate participates in metabolic pathways such as biosynthesis of ornithine and peptides such as glutathione and proteins. While all of these reactions occur in the brain as a whole, no single type of cell is able to perform all of these reactions [122]. This has to do with the fact that many enzymes exhibit selective cellular distribution in the brain (Table 31-3). Hence, glutamate decarboxylase is mainly expressed in GABAergic neurons and glutamine synthetase is present in glial cells. Phosphate activated glutaminase has a much higher activity in neurons than in astrocytes [123], but astrocytes are capable of oxidizing glutamine for energy [13, 94].

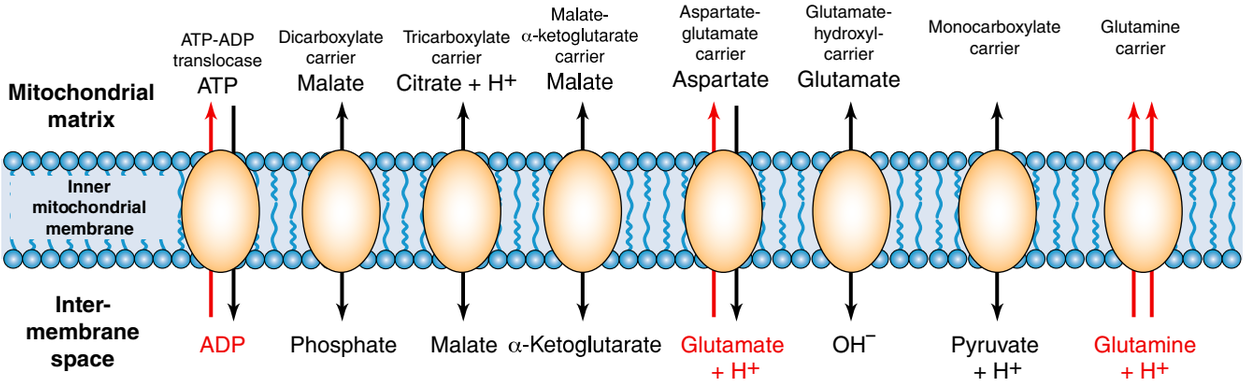

FIGURE 31-7 Mitochondrial carriers. Ions and small molecules enter the intermembrane space, since the outer mitochondrial membrane is not a significant permeability barrier. However, the inner mitochondrial membrane is impermeable to ions except those for which there are specific carriers. Most of the carriers are reversible, as indicated by *two-headed arrows*. Compounds transported in one direction are indicated in red. The ATP/ADP translocase and the aspartate–glutamate carrier are both electrophoretic; their transport is driven in the direction of the mitochondrial membrane potential, as indicated by red arrows. Glutamine is carried into the matrix by an electroneutral carrier. The unimpaired functioning of mitochondrial carriers is essential for normal metabolism. (Adapted with permission from reference [70].)

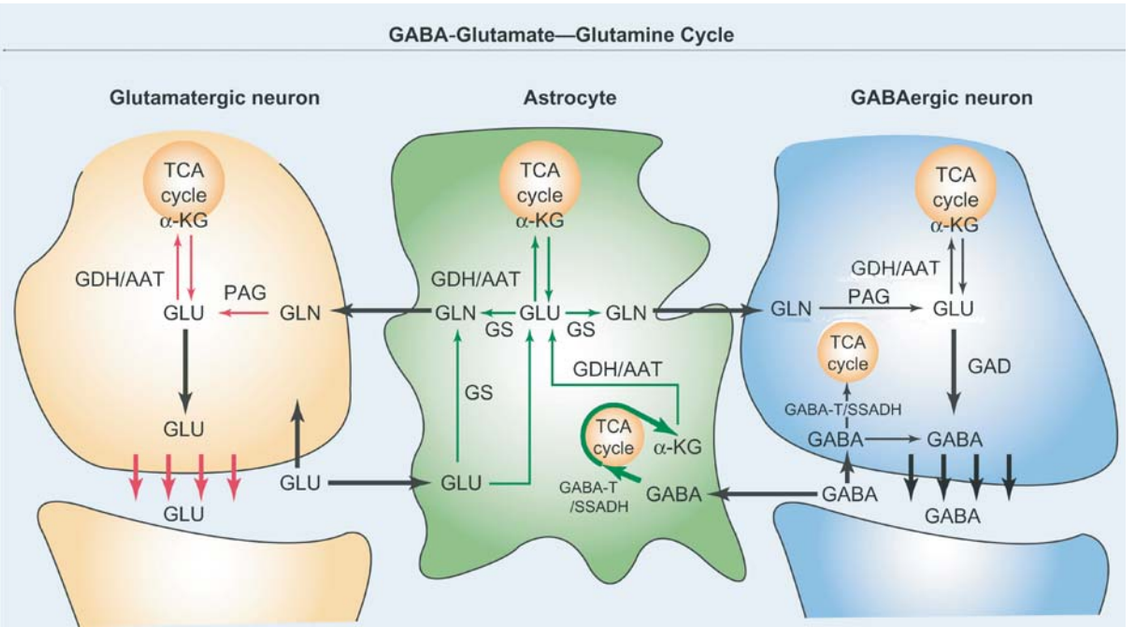

FIGURE 31-8 GABA–glutamate–glutamine cycle. Simplified schematic representation of key metabolic processes and release and uptake of neurotransmitters in glutamatergic and GABAergic synapses interacting with a surrounding astrocyte. The glutamate–glutamine cycle including the glutamine synthetase (*GS*) reaction is indicated in the glutamatergic neuron – astrocyte interaction. Analogously, the GABA–glutamate–glutamine cycle, including the GABA transaminase (*GABA*-T), succinate semialdehyde dehydrogenase (*SSADH*) and glutamate decarboxylase (*GAD*) reactions, is indicated in the GABAergic neuron–astrocyte interaction. The close association of the neurotransmitters, GABA and glutamate, to TCA cycle metabolism is indicated in all three cells. Glutamate is converted to α-ketoglutarate via either glutamate dehydrogenase (*GDH*) or aspartate aminotransferase (*AAT*). *GLN*, glutamine; *GLU*, glutamate; *α-KG*, α-ketoglutarate; *PAG*, phosphate activated glutaminase; *TCA*, tricarboxylic acid.

It should be noted that the reactions catalyzed by glutamate dehydrogenase and several aminotransferases convert glutamate into α-ketoglutarate and thus associate glutamate with the TCA cycle. The aspartate aminotransferase activity in brain is high in comparison with flux through the TCA cycle. Thus amino acids made by transamination of TCA cycle intermediates (e.g. aspartate and glutamate) in the mitochondrial matrix are in equilibrium with the TCA cycle intermediates. However, transport across the inner mitochondrial membrane may influence this process (see above). In spite of the fact that the thermodynamic equilibrium of the glutamate dehydrogenase reaction favors glutamate production, reductive amination of α-ketoglutarate normally does not take place in the brain [122]. This is likely to be related to the high K_m of the enzyme for ammonia and to the fact that brain NAD^+/NADH ratio is high.

Glutamate and glutamine metabolism is compartmentalized in brain. Since the enzymes of glutamate and glutamine metabolism are differentially distributed in astrocytes and neurons, glutamate metabolism in brain is compartmentalized (see above). A small glutamate pool that is precursor for glutamine and a large glutamate pool with a slow turnover characterized the two glutamate compartments originally described in the brain [124]. These pools reflect the glial and neuronal glutamate pools respectively. This is compatible with the cycle of GABA, glutamate and glutamine metabolism shown in Figure 31-8. Synaptically released glutamate is predominantly taken up into astrocytes and converted into glutamine, which subsequently is transferred back to the glutamatergic neuron. This is in keeping with the preferential localization of high-affinity glutamate transporters and glutamine synthetase in astrocytes and the enrichment of phosphate activated glutaminase in neurons [125, 126].

It is important to emphasize, that the glutamate/glutamine cycle does not operate in a stoichiometric fashion, because glutamate in astrocytes is oxidatively metabolized in addition to being converted into glutamine [13, 86, 112, 122]. Furthermore, glutamine can also be oxidized in neurons after conversion to glutamate and subsequently enter the TCA cycle as α-ketoglutarate. In this context it should be noted that the carbon skeleton of glutamate has to exit the TCA cycle in the step of malate or oxaloacetate and subsequently reenter as acetyl-CoA from pyruvate to be completely oxidized. This is part of the 'pyruvate recycling' pathway that has been reported in rat brain [127]. Pyruvate recycling in cultured cells takes place in astrocytes but only to a very small extent [128]. It is conceivable that this process becomes more pronounced during hypoglycemia. However, the opposite is the case with *in vitro* studies; recycling was abolished in astrocytes and did not appear in neurons in culture [129]. To the extent that the carbon skeletons of glutamate and glutamine are quantitatively oxidized to CO_2 obviously there is a need for replenishment of the glutamate pool, which occurs via the anaplerotic enzyme pyruvate carboxylase in astrocytes

(see above). This consideration may also apply to GABA (see below). Because the cycle is not stoichiometric, glutamatergic and GABAergic neurons are incapable of replenishing their neurotransmitter pools unless precursors are received from surrounding astrocytes.

A specialized glutamate–glutamine cycle operates in GABAergic neurons and surrounding astrocytes. The cycle also operates between GABAergic neurons and astrocytes and is of quantitative importance for the maintenance of the neurotransmitter GABA pool [130] (see GABA neurotransmission in Ch. 16). It should be noted that it is a prerequisite for the GABA-glutamate–glutamine cycle to operate that GABA can be converted to glutamate in the astrocytic compartment. This is possible since the carbon skeleton of GABA has access to the TCA cycle by the concerted action of GABA transaminase and succinate semialdehyde dehydrogenase. After conversion to oxaloacetate and condensation with acetyl-CoA, α-ketoglutarate and thus glutamate can be formed. In GABAergic neurons the simultaneous presence of GABA transaminase, succinate semialdehyde dehydrogenase and glutamate decarboxylase provides a means by which the α-ketoglutarate dehydrogenase and succinyl CoA synthetase reactions can be circumvented. This is referred to as the *GABA shunt* of the TCA cycle, the activity of which constitutes approximately 10% of the normal flux in the TCA cycle (see also Fig. 16.1).

Several shuttles act to transfer nitrogen in brain. While the glutamate–glutamine cycle accounts for the transfer of the carbon skeleton of glutamate (between neurons and astrocytes) it does not account for the fate of the ammonia released in the glutaminase reaction and utilized in the glutamine synthetase reaction. In the metabolic communication between muscle and liver, the nitrogen transfer is well known to occur as an alanine–glucose exchange. An analogous mechanism involving alanine and lactate has been proposed for brain, according to which alanine is responsible for the transfer of the ammonia nitrogen between the neuronal (glutamatergic) and the astrocytic compartments. There is evidence that leucine and α-ketoisocaproic acid may also have a role in the transfer of amino groups between neurons and glia [122, 131].

HOW COMPARTMENTALIZED BRAIN METABOLISM IS STUDIED

Since the brain utilizes energy almost exclusively from oxidative metabolism of glucose, brain metabolism has been studied by focusing on net oxygen and glucose uptake. Oxygen consumption was classically measured as the arteriovenous difference of O_2 content. When a substance is exchanged between brain and blood, the difference between its steady state of delivery to brain in the arterial blood and removal in the venous blood must be equal to the net rate of its utilization or production by brain. This relation can be expressed as follows:

$$CMR = CBF(\mathrm{A}-\mathrm{V})$$

See reference [2] for a discussion of these classical methods. Additionally, oxygen consumption can be measured directly by using positron emission tomography (PET) or polarography, or indirectly, by measuring the changing oxygenation of deoxyhemoglobin. The latter method has been exploited by the blood-oxygen-level-dependent (BOLD) functional magnetic resonance imaging (fMRI) or infrared spectroscopy methods (see detailed discussions in Ch. 58). Inherent in all methods designed to measure cerebral oxygen metabolism directly is the intrinsic problem that oxygen can rapidly diffuse across the blood–brain barrier. PET measurements of cerebral metabolic rate $(CMR)O_2$ contain a number of correction factors and are complicated to perform; yet some intriguing results under certain conditions have been reported such as the lack of increase in cerebral oxygen consumption during focal stimulation [8]. Assessment of cerebral oxygen metabolism by the BOLD mechanism, on the other hand [49], is heavily model-dependent, as the apparent increase in venous deoxyhemoglobin during focal stimulation is a transient process critically dependent on the blood flow and volume changes [132]. Nonetheless, imaging brain function using BOLD fMRI has led to renewed interest and insight into cognitive neuroscience, mainly due to its simplicity and ease of use. Other indirect methods to measure cerebral oxygen metabolism have been developed since the late 1980s by measuring the turnover of carbon-13 label from glucose into glutamate C4, which through label exchange with the TCA cycle intermediate α-ketoglutarate, can reflect the rate of the TCA cycle [10]. This approach offers the elegance of trapping the label in an intracellular metabolite and thus does not suffer from the drawback of the direct oxygen methods, such as diffusion of the metabolic product and interference with non-metabolism-related label flow.

In addition to $CMRO_2$, the cerebral metabolic rate of glucose (CMR_{glc}) can be studied using radiotracers. However, the study of metabolism using radiotracers in general is cumbersome due to the need to separate the products of metabolism and thus is not applicable *in vivo*. To alleviate this problem, Sokoloff pioneered an elegant method that consists of applying the radiotracer in a non-metabolizable form, e.g. 2-deoxyglucose, and measuring the amount of radioactivity in the phosphorylation product [133]. This approach has led to the widely used autoradiography approach for experimental animal studies and to the ^{18}F-2-deoxyglucose (FDG) method (FDG-PET) for human use, which is now routinely used for various purposes including cancer detection and developmental studies [44]. See Chapter 58 for detailed discussion.

A drawback to all methods discussed above, however, is that they fail to directly address the metabolic compartmentation inherent in the brain. For example, direct or indirect measurement of oxygen metabolism using labeled

O_2 will measure overall oxygen consumption irrespective of the cellular localization. The same holds true for the deoxyglucose method, although some evidence suggests that astrocytes may be the preferential site of increased glucose uptake during stimulation [134]. Lastly, the measurement of glutamate turnover directly (see above) does not take into account the fact that glutamate, and its metabolism, is known to be compartmentalized in the CNS. For example, as pointed out earlier, studies in the 1960s using carbon-14 labeling reported that glutamate and glutamine have a different product-precursor relationship depending on whether carbon-14-labeled glucose or carbon-14-labeled acetate was administered [135]. From these experiments the existence of at least two TCA cycles associated with a large glutamate and a small glutamine pool was deduced. Indeed, it was demonstrated that acetate selectively enters astrocytes *in vivo* [136]. Since the use of radiotracers fails to separate the tracer not only in different molecules but also in different positions within the molecule, studying compartmentation with radiotracers, although very sensitive, is tedious and complicated.

Carbon-13 nuclear magnetic resonance spectroscopy is a powerful method for studying brain metabolism. On the other hand, ^{13}C-NMR spectroscopy not only allows the simultaneous and separate detection of signals from different compounds, but also within different atoms in a given molecule. For example, carbon-13 from glucose labeled at the C1 (or C6) position is incorporated via the PDH reaction into the C2 of acetyl-CoA, and subsequently into the C4 of α-ketoglutarate (Fig. 31-9), which labels the

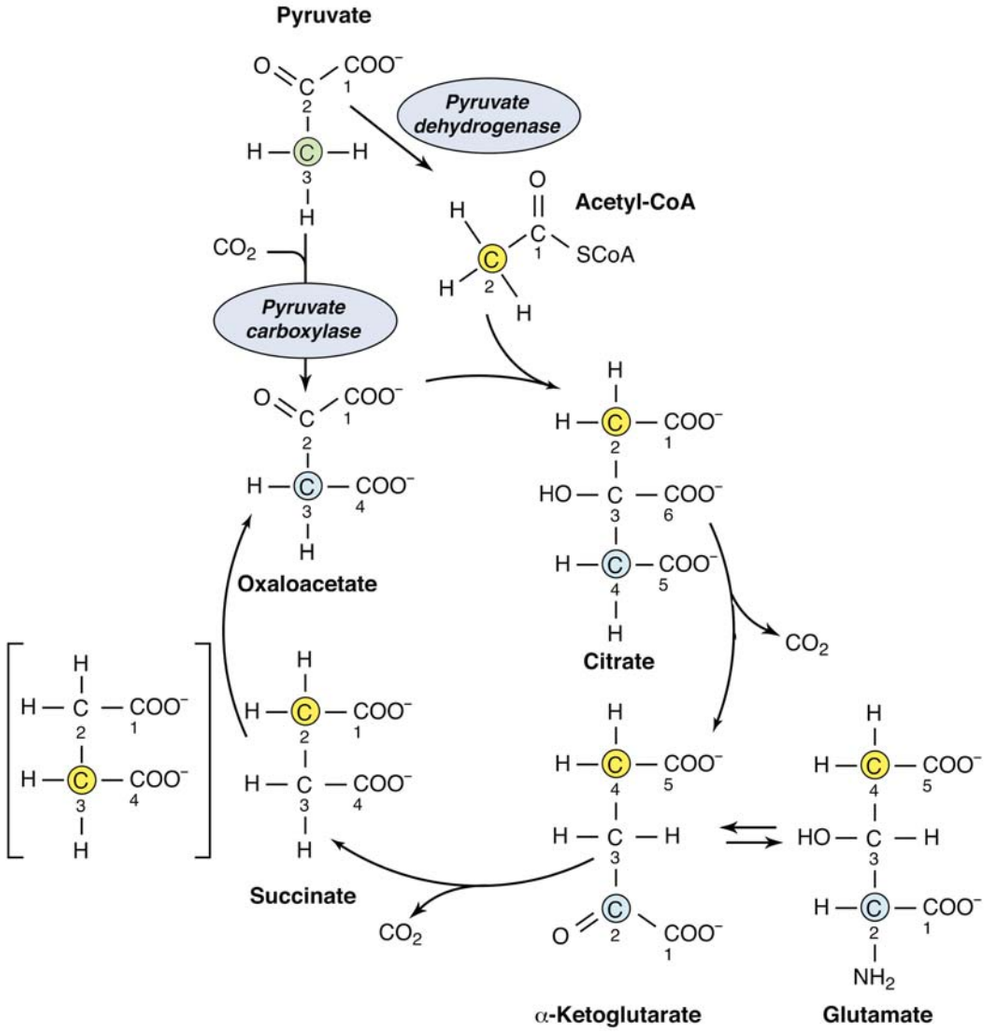

FIGURE 31-9 Labeling pattern for metabolism of [1-^{13}C] or [6-^{13}C] glucose by NMR spectroscopy. Glucose labeled in the C1 or C6 position will give rise to pyruvate labeled in the C3 position (*green*). Metabolism via the pyruvate dehydrogenase pathway (*yellow*) gives rise to acetyl COA labelled in the C2 position, and subsequently to glutamate and glutamine labeled in the C4 position. Metabolism in the TCA: the label is scrambled at the level of succinate, leading to equal labeling of the C2 and C3 of aspartate. Acetate or β-hydroxybutyrate labeled at the C2 position leads to a similar labeling pattern as metabolism of C1- or C6-labeled glucose via pyruvate dehydrogenase. Metabolism via pyruvate carboxylase (*blue*), which occurs only in astrocytes, gives rise to glutamate and glutamine labeled in the C2 position. See text for further details.

C4 of glutamate, which can be measured by NMR spectroscopy. In the TCA cycle, the label is scrambled at the level of succinate, leading to equal labeling of the C2 and C3 of aspartate (Fig 31-9), and in subsequent turns glutamate and glutamine. On the other hand, action of pyruvate carboxylase on C3 labeled pyruvate leads to labeling of the C3 position of oxaloacetate, which labels the C2 of glutamate and glutamine. In addition to determining the amount of label in a given position in a molecule such as glutamate, the amount of different molecules with multiple labels (isotope isomers or isotopomers) such as singly or doubly labeled glutamate C4 can be determined due to the magnetic interaction between adjacent carbon-13 nuclei [127]. A labeling pattern similar to that obtained with glucose as a precursor is obtained when using acetate labeled at the C2 or β-hydroxybutyrate labeled at the C2 (or C4) positions. Because of selective uptake by astrocytes, acetate provides specific information about metabolism in these cells, and about the trafficking of compounds from astrocytes to neurons. β-hydroxybutyrate is taken up by both astrocytes and neurons, and can provide information about TCA cycle activity even when metabolism via pyruvate dehydrogenase is impaired [127].

Cultured neurons and astrocytes are useful for studying subcellular compartmentation and identifying pathways of metabolism. An important approach to study the brain is based on brain cell cultures [128, 129], cell lines [137] and tissue slices [138]. Detailed biochemical information is obtained without interference from metabolism of peripheral organs such as liver or obstructions such as the blood–brain barrier. Cells taken directly from the organism and subsequently grown for at least 24 hours *in vitro* are considered to be primary cultures. Different procedures have been used, often in combination, to enable the establishment of monotypic cultures from mixed cell suspensions. Brain cells are obtained after an initial mechanical and/or enzymatic dissociation of the nervous tissue of newborn mice, rats or other animals [139]. Primary cultures of cortical and cerebellar granule neurons are important models since 90% of the synapses in the brain utilize either glutamate or GABA as the neurotransmitter. Because of the relative abundance of neurotransmitter glutamate, cerebellar granule cells are the preferred model system for excitatory neurotransmission. GABAergic cerebral cortex neurons are the model for inhibitory neurotransmission. Primary cultures of astrocytes are an important model since these cells are responsible for the removal and metabolism of neurotransmitter glutamate and for control of the pH and ion concentrations of the extracellular milieu in brain [13, 95]. Since astrocytes from different brain regions have different properties, astrocytes as counterparts for studies using specific neurons are taken from the same brain region as the neurons [140]. Co-cultures of astrocytes and neurons are a valuable tool for studying neuronal-glial interactions [104]. Cultured cell lines are sometimes used in the study of particular aspects of metabolism, e.g. compartmentalized pyruvate metabolism in C-6 glioma cells using carbon-13-labeled glucose and lactate [137].

Preparations such as brain slices, axons, synaptosomes and isolated mitochondria are tools for studying specific aspects of metabolism. Brain slices are a step closer to the *in vivo* situation without the confounding presence of the blood–brain barrier. Valuable information has been obtained about metabolic pathways and pathological situations from brain slices [138]. Furthermore, subcellular preparations have also been used to analyze particular aspects of metabolism. Studies with synaptosomes can reveal information about the highly specialized metabolism in nerve terminals and neuron-neuron interaction [94, 109, 112]. Isolated optic nerve preparation is a valuable model for studying axonal metabolism [61]. In addition, studies with isolated mitochondria can help to dissect the regulation of mitochondrial enzymes and TCA cycle metabolism [74, 107]; whereas the activity and regulation of cytosolic enzymes can be studied in the soluble fraction from brain [102]. Uptake studies, *in situ* hybridization and Western blots have been invaluable tools for studying the transport of substrates by brain cells [16].

***In vivo* nuclear magnetic resonance spectroscopy provides information about the regulation of metabolism in the intact brain.** Given the compartmentalization of compounds such as glutamate, glutamine and glycogen and related enzymes (see above), important insights into the regulation of brain energy metabolism can be obtained by *in vivo* NMR spectroscopy studies (Fig. 31-10 and Ch. 58). Particularly exciting in this regard is the ability of ^{13}C-NMR to measure glutamine labeling completely noninvasively [50]. Since glutamate measured in tissue *in vivo* mainly reflects the neuronal glutamate pool, the flow of label from glutamate to glutamine thus may reflect glutamate neurotransmission. ^{13}C-NMR spectroscopy thus has opened the avenue of quantifying glutamate neurotransmission *in vivo*, which provides a translational bridge between the information obtained from cellular studies (see above) and studies in patients. In addition to observing the flux from glucose labeled at the C1 (or C6) position into the C4 of glutamate and glutamine, label can be observed directly in other positions of the molecule, such as the C2 position (Fig. 31-10), which is important in assessing pyruvate carboxylase activity which appears to be significant *in vivo* [141, 144]. In addition, by analyzing the flow of label into the other positions in glutamate and glutamine, insight into the regulation of subcellular fluxes can be gained [141]. Lastly, *in vivo* ^{13}C-NMR spectroscopy is the only method capable of assessing *in vivo* brain glycogen metabolism in the human brain [142] (Fig. 31-10).

In vivo H- and ^{31}P-NMR spectroscopy can be used to assess the concentration of clinically relevant compounds

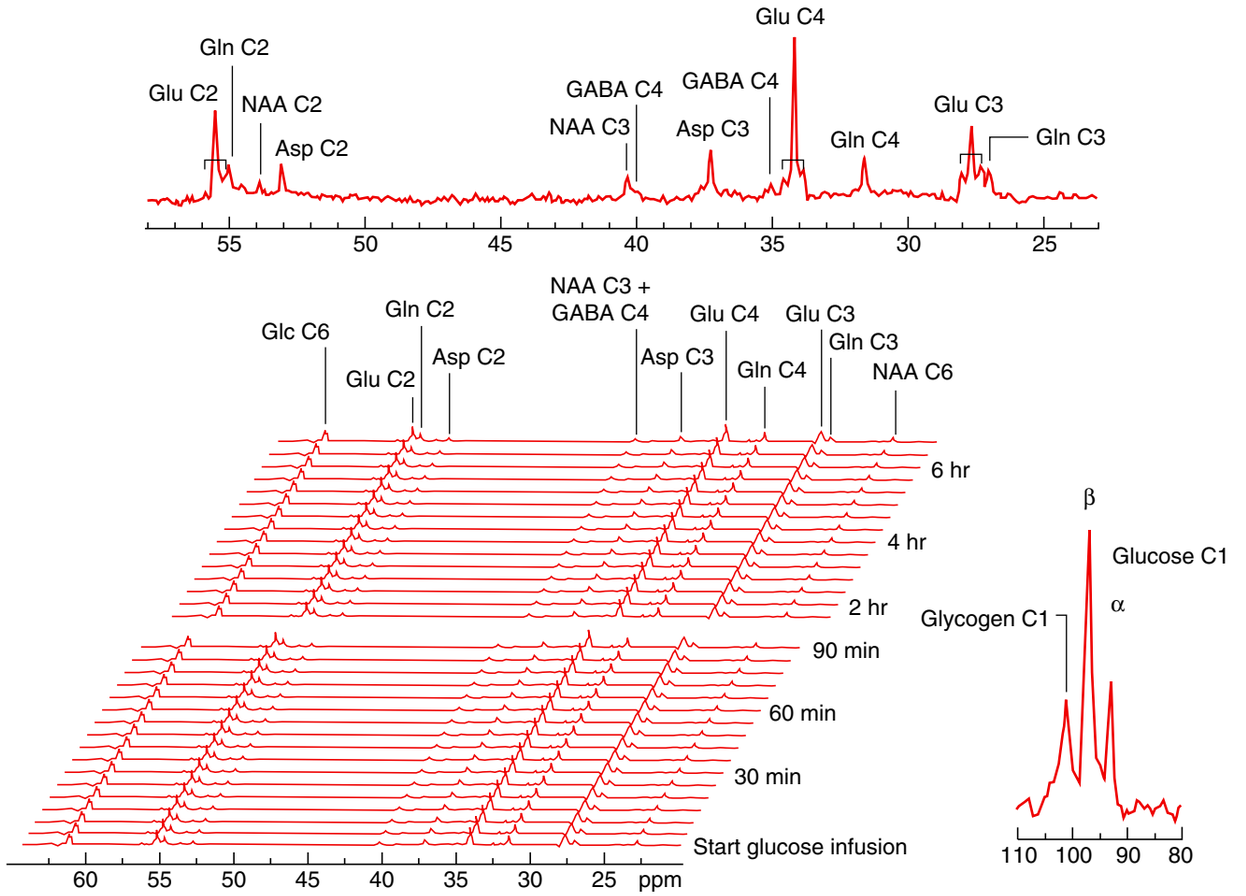

FIGURE 31-10 Cerebral amino acids and carbohydrates incorporate ^{13}C label from infused glucose. The **top panel** shows a ^{13}C NMR spectrum obtained from a gray-matter-rich volume in the human head. (From reference [141].) The **right panel** shows label incorporation into brain glycogen and glucose in humans. (From reference [142].) The **stack plot** illustrates the rate of label incorporation into many compounds and carbons in the rat brain. (From reference [143].) In all studies, glucose labeled at the 1 or 6 position was administered intravenously.

and high-energy phosphates [145]. Determinations of the *N*-acetyl aspartate (NAA), creatine, choline and lactate, and ratios of these compounds are frequently used as indices of the integrity of metabolism and tissue in developing brain [146, 147]. Measurement of high-energy phosphates provides information about the energy status of the brain [145]. These methods, as well as ^{13}C-NMR spectroscopy are used for research and clinically as NMR becomes more widely available.

RELATION OF ENERGY METABOLISM TO PATHOLOGICAL CONDITIONS IN THE BRAIN

Using newer instrumentation, such as PET and NMR, it has been shown that energy metabolism is perturbed in defined brain areas in patients with neurological or psychiatric diseases [145, 146]. Detailed information about pathological conditions that adversely affect brain metabolism can be found in many other chapters of this book. For detailed discussions, see respective chapters: oxidative stress, Chapter 32, hepatic encephalopathy, Chapter 34, psychiatric diseases, Chapters 54 and 55, and imaging techniques, Chapter 58.

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REFERENCES

1. Attwell, D. and Laughlin, S. B. An energy budget for signaling in the grey matter of the brain. *J. Cereb. Blood Flow Metab.* 21: 1133–1145, 2001.
2. Clarke, D. D. and Sokoloff, L. Circulation and energy metabolism. In G. J. Siegel, B. W. Agranoff, R. W. Albers, S. K. Fisher, and M.D. Uhler (eds), *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, 6th edn. Philadelphia: Lippincott Williams & Wilkins, 1999, pp. 638–669.

3. Nilsson, B. and Siesjo, B. K. A method for determining blood flow and oxygen consumption in the rat brain. *Acta Physiol. Scand.* 96: 72–82, 1976.
4. Linde, R., Schmalbruch, I. K., Paulson, O. B. *et al.* The Kety–Schmidt technique for repeated measurements of global cerebral blood flow and metabolism in the conscious rat. *Acta Physiol. Scand.* 165: 395–401, 1999.
5. Hasselbalch, S. G., Madsen, P. L., Knudsen, G. M. *et al.* Calculation of the FDG lumped constant by simultaneous measurements of global glucose and FDG metabolism in humans. *J. Cereb. Blood Flow Metab.* 18: 154–160, 1998.
6. Nakao, Y., Itoh, Y., Kuang, T. Y. *et al.* Effects of anesthesia on functional activation of cerebral blood flow and metabolism. *Proc. Natl Acad. Sci. U.S.A.* 98: 7593–7598, 2001.
7. Ueki, M., Mies, G. and Hossmann, K. A. Effect of alpha-chloralose, halothane, pentobarbital and nitrous oxide anesthesia on metabolic coupling in somatosensory cortex of rat. *Acta Anaesthesiol. Scand.* 36: 318–322, 1992.
8. Fox, P. T., Raichle, M. E., Mintun, M. A. *et al.* Nonoxidative glucose consumption during focal physiologic neural activity. *Science* 241: 462–464, 1988.
9. Hertz, L. and Hertz, E. Cataplerotic TCA cycle flux determined as glutamate-sustained oxygen consumption in primary cultures of astrocytes. *Neurochem. Int.* 43: 355–361, 2003.
10. Rothman, D. L., Novotny, E. J., Shulman, G. I. *et al.* ^{1}H-[^{13}C] NMR measurement of [4-^{13}C] glutamate, *Proc. Natl Acad. Sci. U.S.A.* 89: 9603–9606, 1992.
11. Oz, G., Berkich, D. A., Henry, P.-G. *et al.* Neuroglial metabolism in the awake rat brain: fixation increases with brain activity *J. Neurosci.* 24: 11273–11279, 2004.
12. Berl, S. and Clarke, D. D. Introduction. In S. Berl, D. D. Clarke and D. Schneider (eds), *Metabolic Compartmentation and Neurotransmission: Relation to Brain Structure and Function.* New York: Plenum Press, 1975, pp. xiii–xvii.
13. Hertz, L., Dringen, R., Schousboe, A. *et al.* Astrocytes: glutamate producers for neurons. *J. Neurosci. Res.* 57: 417–428, 1999.
14. Dwyer, D. S., Vannucci, S. J. and Simpson, I. A. Expression, regulation and functional role of glucose transporters (GLUTs) in brain. *Int. Rev. Neurobiol.* 51: 159–188, 2002.
15. Vannucci, S. J., Maher, F. and Simpson, I. A. Glucose transporter proteins in brain: delivery of glucose to neurons and glia. *Glia* 21: 2–21, 1997.
16. Vannucci, S. J. and Simpson, I. A. Developmental switch in brain nutrient transporter expression in the rat. *Am. J. Physiol. Endocrinol. Metab.* 285: E1127–E1134, 2003.
17. Cremer, J. E. Substrate utilization and brain development. *J. Cereb. Blood Flow Metab.* 2: 394–407, 1982.
18. Nehlig, A. Brain uptake and metabolism of ketone bodies in animal models. *Prostaglandins Leukot. Essent. Fatty Acids* 70: 265–275, 2004.
19. Medina, J. M. The role of lactate as an energy substrate for the brain during the early neonatal period. *Biol. Neonate* 48: 237–244, 1985.
20. Baud, O., Fayol, L., Gressens, P. *et al.* Perinatal and early postnatal changes in the expression of monocarboxylate transporters MCT1 and MCT2 in the rat forebrain. *J. Comp. Neurol.* 465: 445–454, 2003.
21. Wittendorp-Rechenmann, E., Lam, C. D., Steibel, J. *et al.* High resolution tracer targeting combining microautoradiographic imaging by cellular ^{14}C-trajectography with immunohistochemistry: a novel protocol to demonstrate metabolism of [^{14}C]2-deoxyglucose by neurons and astrocytes. *J. Trace Microprobe Tech.* 20: 505–515, 2002.
22. Vannucci, S. J., Clark, R. R., Koehler-Stec, E. *et al.* Glucose transporter expression in brain: relationship to cerebral glucose utilization. *Dev. Neurosci.* 20: 369–379, 1998.
23. Fitzpatrick, S. M., Hetherington, H. P., Behar, K. L. *et al.* The flux from glucose to glutamate in the rat brain *in vivo* as determined by ^{1}H-observed, ^{13}C-edited NMR spectroscopy. *J. Cereb. Blood Flow Metab.* 2: 170–179, 1990.
24. Pierre, K., Magistretti, P. J., Pellerin, L. MCT2 is a major neuronal monocarboxylate transporter in the adult mouse brain. *J. Cereb. Blood Flow Metab.* 22: 586–595, 2002.
25. Hanu, R., McKenna, M., O'Neill, A. *et al.* Monocarboxylic acid transporters, MCT1 and MCT2, in cortical astrocytes *in vitro* and *in vivo*. *Am. J. Physiol. Cell. Physiol.* 278, C921–C930, 2000.
26. Leino, R. L., Gerhart, D. Z. and Drewes, L. R. Monocarboxylate transporter (MCT1) abundance in brains of suckling and adult rats: a quantitative electron microscopic immunogold study. *Brain Res. Dev. Brain Res.* 113: 47–54, 1999.
27. Tildon, J. T., McKenna, M. C., Stevenson, J. H. *et al.* Transport of L-lactate by cultured rat brain astrocytes. *Neurochem. Res.* 18: 177–184, 1993.
28. Bergersen, L., Rafiki, A. and Ottersen, O. P. Immunogold cytochemistry identifies specialized membrane domains for monocarboxylate transport in the central nervous system. *Neurochem. Res.* 27: 89–96, 2002.
29. Rafiki, A., Boulland, J. L., Halestrap, A. P. *et al.* Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. *Neuroscience* 122: 677–688, 2003.
30. Bergersen, L., Waerhaug, O., Helm, J. *et al.* A novel postsynaptic density protein: the monocarboxylate transporter MCT2 is co-localized with delta-glutamate receptors in postsynaptic densities of parallel fiber-Purkinje cell synapses. *Exp. Brain Res.* 136: 523–34, 2001.
31. Broer, S., Broer, A., Schneider, H. P. *et al.* Characterization of the high-affinity monocarboxylate transporter MCT2 in *Xenopus laevis* oocytes. *Biochem. J.* 341: 529–535, 1999.
32. McKenna, M. C., Tildon, J. T., Stevenson, J. H. *et al.* Lactate transport by cortical synaptosomes from adult rat brain: Characterization of kinetics and inhibitor specificity. *Dev. Neurosci.* 20: 300–309, 1998.
33. Mac, M. and Nalecz, K. A. Expression of monocarboxylic acid transporters (MCT) in brain cells. Implication for branched chain alpha-ketoacids transport in neurons. *Neurochem. Int.* 43: 305–309, 2003.
34. Simpson, I. A., Appel, N. M., Hokari, M. *et al.* Blood–brain barrier glucose transporter: effects of hypo- and hyperglycemia revisited. *J. Neurochem.* 72: 238–247, 1999.
35. McCall, A. L. Cerebral glucose metabolism in diabetes mellitus. *Eur. J. Pharmacol.* 490: 147–158, 2004.
36. Kumagai, A. K., Kang, Y. S., Boado, R. J. and Pardridge, W. M. Upregulation of blood–brain barrier GLUT1 glucose transporter protein and mRNA in experimental chronic hypoglycemia. *Diabetes* 44: 1399–1404, 1995.
37. Pelligrino, D. A., LaManna, J. C., Duckrow, R. B. *et al.* I. Hyperglycemia and blood–brain barrier glucose transport. *J. Cereb. Blood Flow Metab.* 12: 887–899, 1992.

38. Leino, R. L., Gerhart, D. Z., Duelli, R. *et al.* Diet-induced ketosis increases monocarboxylate transporter (MCT1) levels in rat brain. *Neurochem. Int.* 38: 519–527, 2001.
39. Kinnala, A., Suhonen-Polvi, H., Aarimaa, T. *et al.* Cerebral metabolic rate for glucose during the first six months of life: an FDG positron emission tomography study. *Arch. Dis. Child. Fetal Neonat. Ed.* 74: F153–F157, 1996.
40. Nehlig, A. Age-dependent pathways of brain energy metabolism: the suckling rat, a natural model of the ketogenic diet. *Epilepsy Res.* 37: 211–221, 1999.
41. Patel, M. S., Johnson, C. A., Rajan, R. *et al.* The metabolism of ketone bodies in developing human brain: development of ketone-body-utilizing enzymes and ketone bodies as precursors for lipid synthesis. *J. Neurochem.* 25: 905–908, 1975.
42. Settergren, G., Lindblad, B. S. and Persson, B. Cerebral blood flow and exchange of oxygen, glucose, ketone bodies, lactate, pyruvate and amino acids in infants. *Acta Paediatr. Scand.* 65: 343–353, 1976.
43. Tildon, J. T. and Cornblath, M. Succinyl-CoA: 3-ketoacid CoA-transferase deficiency. A cause for ketoacidosis in infancy. *J. Clin. Invest.* 51: 493–498, 1972.
44. Takahashi, T., Shirane, R., Sato, S. *et al.* Developmental changes of cerebral blood flow and oxygen metabolism in children. *Am. J. Neuroradiol.* 20: 917–922, 1999.
45. Kennedy, C. and Sokoloff, L. An adaptation of the nitrous oxide method to the study of the cerebral circulation in children; normal values for cerebral blood flow and cerebral metabolic rate in childhood. *J. Clin. Invest.* 36: 1130–1137, 1957.
46. Sokoloff, L. The metabolism of the central nervous system *in vivo*. In J. Field, H. W. Magoun and V. E. Hall (eds), *Handbook of Physiology – Neurophysiology*, vol. 3. Washington, DC: American Physiological Society, 1960, pp. 1843–1864.
47. Paulson, O. B. Blood–brain barrier, brain metabolism and cerebral blood flow. *Eur. Neuropharmacol.* 12: 495–501, 2002.
48. Fox, P. T. and Raichle, M. E. Focal physiological uncoupling of cerebral blood flow and oxidative metabolism during somatosensory stimulation in human subjects. *Proc. Natl Acad. Sci. U.S.A.* 83: 1140–1144, 1986.
49. Ogawa, S., Lee, T. M., Kay, A. R. *et al.* Brain magnetic resonance imaging with contrast dependent on blood oxygenation. *Proc. Natl Acad. Sci. U.S.A.* 87: 9868–9872, 1990.
50. Gruetter, R., Novotny, E. J., Boulware, S. D. *et al.* Non-invasive measurements of the cerebral steady-state glucose concentration and transport in humans by ^{13}C magnetic resonance. In L. Drewes and A. Betz (eds), *Frontiers in Cerebral Vascular Biology: Transport and its Regulation*, vol. 331. New York: Plenum Press, 1993, pp. 35–40.
51. Pfeuffer, J., Tkac, I., Provencher, S. W. *et al.* Toward an *in vivo* neurochemical profile: quantification of 18 metabolites in short-echo-time ^{1}H NMR spectra of the rat brain. *J. Magn. Reson.* 141: 104–120, 1999.
52. Hasselbalch, S. G., Knudsen, G. M., Capaldo, B. *et al.* Blood–brain barrier transport and brain metabolism of glucose during acute hyperglycemia in humans. *J. Clin. Endocrinol. Metab.* 86: 1986–1990, 2001.
53. Heiss, W. D., Pawlik, G., Herholz, K. *et al.* Regional kinetic constants and cerebral metabolic rate for glucose in normal human volunteers determined by dynamic positron emission tomography of [^{18}F]-2-fluoro-deoxy-D-glucose. *J. Cereb. Blood Flow Metab.* 4: 212–223, 1984.
54. Gruetter, R., Novotny, E. J., Boulware, S. D. *et al.* Localized ^{13}C NMR spectroscopy of amino acid labeling from [1–^{13}C] D-glucose in the human brain. *J. Neurochem.* 63: 1377–1385, 1994.
55. Horinaka, N., Kuang, T. Y., Pak, H. *et al.* Blockade of cerebral blood flow response to insulin-induced hypoglycemia by caffeine and glibenclamide in conscious rats. *J. Cereb. Blood Flow Metab.* 17: 1309–1318, 1997.
56. Yu, A., Drejer, J., Hertz, L. *et al.* Pyruvate carboxylase activity in primary cultures of astrocytes and neurons. *J. Neurochem.* 41: 1484–1487, 1983.
57. Martinez-Hernandez, A., Bell, K. P. and Norenberg, M. D. Glutamine synthetase: glial localization in rat brain. *Science* 195: 1356–1358, 1977.
58. Schousboe, A., Westergaard, N., Sonnewald, U. *et al.* Glutamate and glutamine metabolism and compartmentation in astrocytes. *Dev. Neurosci.* 15: 359–366, 1993.
59. Tabernero, A., Vicario, C. and Medina, J. M. Lactate spares glucose as a metabolic fuel in neurons and astrocytes from primary culture. *Neurosci. Res.* 26: 369–376, 1996.
60. Choi, I. Y. and Gruetter, R. *In vivo* ^{13}C NMR assessment of brain glycogen concentration and turnover in the awake rat. *Neurochem. Int.* 43: 317–322, 2003.
61. Brown, A. M. Brain glycogen re-awakened. *J. Neurochem.* 89: 537–552, 2004.
62. Cruz, N. F. and Dienel, G. A. High glycogen levels in brains of rats with minimal environmental stimuli: implications for metabolic contributions of working astrocytes. *J. Cereb. Blood Flow Metab.* 22: 1476–1489, 2002.
63. Gruetter, R. Glycogen: the forgotten cerebral energy store. *J. Neurosci. Res.* 74:179–183, 2003.
64. Swanson, R. A. Physiologic coupling of glial glycogen metabolism to neuronal activity in brain. *Can. J. Physiol. Pharmacol.* 70: S138–S144, 1992.
65. Wiesinger, H., Hamprecht, B. and Dringen, R. Metabolic pathways for glucose in astrocytes. *Glia* 21: 22–34, 1997.
66. Ben Yoseph, O., Camp, D. M., Robinson, T. E. *et al.* Dynamic measurements of cerebral pentose phosphate pathway activity *in vivo* using [1,6–$^{13}C_2$,6,6–$^{2}H_2$]glucose and microdialysis. *J. Neurochem.* 64: 1336–1342, 1995.
67. Matthews, C. K., and van Holde, K. E. *Biochemistry*, 2nd edn. Redwood City, CA: Benjamin Cummings, 1996, pp. 474–477.
68. Lowry, O. H. and Passoneau, J. V. The relationships between substrates and enzymes of glycolysis in brain. *J. Biol. Chem.* 239: 31–32. 1964.
69. Palmieri, L., Pardo, B., del Arco, A. *et al.* Citrin and aralar1 are Ca^{2+}-stimulated aspartate/glutamate transporters in mitochondria. *EMBO J.* 20: 5060–5069, 2001.
70. Berg, J. M., Tymoczko, J. L. and Stryer, L. *Biochemistry*, 5th edn. New York: W. H. Freeman, 2002.
71. Del Arco, A., Morcillo, J., Martinez-Morales, J. R. *et al.* Expression of the aspartate/glutamate mitochondrial carriers aralar1 and citrin during development and in adult rat tissues. *Eur. J. Biochem.* 269: 3313–3320, 2002.
72. Ramos, M., del Arco, A., Pardo, B. *et al.* Developmental changes in the Ca^{2+}-regulated mitochondrial aspartate–glutamate carrier aralar1 in brain and prominent expression in the spinal cord. *Brain Res. Dev. Brain Res.* 143: 33–46, 2003.

73. Choi, I. Y., Lei, H. and Gruetter, R. Effect of deep pentobarbital anesthesia on neurotransmitter metabolism *in vivo*: on the correlation of total glucose consumption with glutamatergic action. *J. Cereb. Blood Flow Metab.* 22: 1343–1351, 2002.
74. McKenna, M. C., Stevenson, J. H., Huang, X. *et al.* Differential distribution of the enzymes glutamate dehydrogenase and aspartate aminotransferase in cortical synaptic mitochondria contributes to metabolic compartmentation in cortical synaptic terminals. *Neurochem. Int.* 37: 229–241, 2000.
75. Palaiologos, G., Hertz, L. and Schousboe, A. Evidence that aspartate aminotransferase activity and ketodicarboxylate carrier function are essential for biosynthesis of transmitter glutamate. *J. Neurochem.* 51: 317–320, 1988.
76. Cheeseman, A. J. and Clark, J. B. Influence of the malate–aspartate shuttle on oxidative metabolism in synaptosomes. *J. Neurochem.* 50: 1559–1565, 1988.
77. Itoh, Y., Esaki, T., Shimoji, K. *et al.* Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia *in vitro* and on glucose utilization by brain *in vivo*. *Proc. Natl Acad. Sci. U.S.A.* 100: 4879–84, 2003.
78. Almeida, A., Almeida, J., Bolanos, J. P. *et al.* Different responses of astrocytes and neurons to nitric oxide: The role of glycolytically generated ATP in astrocyte protection. *Proc. Natl Acad. Sci. U.S.A.* 98: 15294–15299, 2001.
79. Buono, R. J. and Lang, R. K. Hypoxic repression of lactate dehydrogenase-B in retina. *Exp. Eye Res.* 69: 685–693, 1999.
80. Bittar, P. G., Charnay, Y., Pellerin, L. *et al.* Selective distribution of lactate dehydrogenase isoenzymes in neurons and astrocytes of human brain. *J. Cereb. Blood Flow Metab.* 16: 1079–1089, 1996.
81. Navaro, D., Zwingmann, C., Hazell, A. S. and Butterworth, R. F. Brain lactate synthesis in thiamine deficiency: a re-evaluation using ^{1}H-^{13}C nuclear magnetic resonance spectroscopy. *J. Neurosci. Res.* 79: 33–41, 2005.
82. Dienel, G. A. and Cruz, N. F. Neighborly interactions of metabolically-active astrocytes *in vivo*. *Neurochem. Int.* 43: 339–354, 2003.
83. Schousboe, A., Sonnewald. U. and Waagepetersen, H. S. Differential roles of alanine in GABAergic and glutamatergic neurons. *Neurochem. Int.* 43: 311–315, 2003.
84. McKenna, M. C., Tildon, J. T., Couto, R. *et al.* The metabolism of malate by cultured rat brain astrocytes. *Neurochem. Res.* 15: 1211–1220, 1990.
85. Bakken, I. J., White, L. R., Aasly, J. *et al.* Lactate formation from [U-^{13}C]aspartate in cultured astrocytes: compartmentation of pyruvate metabolism. *Neurosci. Lett.* 237: 117–120, 1997.
86. McKenna, M. C., Sonnewald, U., Huang, X. *et al.* Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes. *J. Neurochem.* 66: 386–393, 1996.
87. Wheatley, D. N. Diffusion theory, the cell and the synapse. *Biosystems* 45: 151–163, 1998.
88. Prichard, J., Rothman, D., Novotny, E. *et al.* Lactate rise detected by ^{1}H NMR in human visual cortex during physiologic stimulation. *Proc. Natl Acad. Sci. U.S.A.* 88: 5829–5831, 1991.
89. Magistretti, P. J., Olivier, S., Naichen, Y. *et al.* Neurotransmitters regulate energy metabolism in astrocytes: Implications for the metabolic trafficking between neural cells. *Dev. Neurosci.* 15: 306–312, 1994.
90. Pellerin, L. Lactate as a pivotal element in neuron-glia metabolic cooperation. *Neurochem. Int.* 43: 331–338, 2003.
91. Waagepetersen, H., Bakken, I. J., Larsson, O. M. *et al.* Metabolism of lactate in cultured GABAergic neurons studied by ^{13}C NMR spectroscopy. *J. Cereb. Blood Flow Metab.* 18: 109–117, 1998.
92. Waagepetersen, H., Bakken, I. J., Larsson, O. M. *et al.* Comparison of lactate and glucose metabolism in cultured neocortical neurons using ^{13}C NMR spectroscopy. *Dev. Neurosci.* 20: 310–321, 1998.
93. Magistretti, P. J., Pellerin, L., Rothman, D. L. *et al.* Energy on demand. *Science* 22: 283, 496–497, 1999.
94. McKenna, M. C., Tildon, J. T., Stevenson, J. H. Jr *et al.* Regulation of energy metabolism in synaptic terminals and cultured rat brain astrocytes: differences revealed using aminooxyacetate. *Dev. Neurosci.* 15: 320–329, 1993.
95. Hansson, E., Muyderman, H., Leonova, J. *et al.* Astroglia and glutamate in physiology and pathology: aspects on glutamate transport, glutamate-induced cell swelling and gap-junction communication. *Neurochem. Int.* 37: 317–29, 2000.
96. Yudkoff, M., Nissim, I. and Pleasure, D. Astrocyte metabolism of [^{15}N]glutamine: implications for the glutamine–glutamate cycle. *J. Neurochem.* 51: 843–850, 1988.
97. Auestad, N., Korsak, R. A., Morrow, J. W. *et al.* Fatty acid oxidation and ketogenesis by astrocytes in primary culture. *J. Neurochem.* 56: 1376–1386, 1991.
98. Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S. *et al.* Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* 247: 470–473, 1990.
99. Morgan-Hughes, J. A. Mitochondrial disease. *Trends Neurosci.* 9: 15–19. 1986.
100. Patel, M. S. The effect of ketone bodies on pyruvate carboxylation by rat brain mitochondria. *J. Neurochem.* 23, 865–867, 1974.
101. McKenna, M. C., Tildon, J. T., Stevenson, J. H., Huang, X. and Couto, R. Regulation of mitochondrial and cytosolic malic enzymes from cultured rat brain astrocytes. *Neurochem. Res.* 20: 1491–1501 1995.
102. Malik, P., McKenna, M. C. and Tildon, J. T. Regulation of malate dehydrogenases from neonatal, adolescent and mature rat brain. *Neurochem. Res.* 18: 247–257, 1992.
103. Bell, J. D., Brown, J. C. C., Sadler, P. J. *et al.* High resolution proton nuclear magnetic resonance studies of human cerebrospinal fluid. *Clin. Sci.* 72: 563–570, 1987.
104. Westergaard, N., Sonnewald, U., Unsgård, G. *et al.* Uptake, release and metabolism of citrate in neurons and astrocytes in primary cultures. *J. Neurochem.* 62: 1727–1733, 1994.
105. Waagepetersen, H. S., Sonnewald, U., Larsson, O. M. *et al.* Multiple compartments with different metabolic characteristics are involved in biosynthesis of intracellular and released glutamine and citrate in astrocytes. *Glia* 35: 246–252, 2001.
106. Friede, L. and Pax, R. A. Mitochondria and mitochondrial enzymes. A comparative study of localization in the cat's brain stem. *Z. Zellforsch. Microsk. Anat. Histochem.* 2: 186–191, 1961.
107. Lai, J. C., Walsh, J. M., Dennis, S. C. *et al.* Synaptic and non-synaptic mitochondria from rat brain: isolation and characterization. *J. Neurochem.* 28: 625–631, 1977.

108. Bolanos, J. P., Heales, S. J., Land, J. M. *et al.* Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. *J. Neurochem.* 64: 1965–1972, 1995.
109. McKenna, M. C., Tildon, J. T., Stevenson, J. H. *et al.* Energy metabolism in cortical synaptic terminals from weanling and mature rat brain: evidence for multiple compartments of tricarboxylic acid (TCA) cycle activity. *Dev. Neurosci.* 16: 291–300, 1994.
110. Waagepetersen, H. S., Hansen, G. H., Fenger, K. *et al.* Cellular mitochondrial heterogeneity in cultured astrocytes as demonstrated by immunogold labelling of α-ketoglutarate dehydrogenase. *Glia*, in press, 2005.
111. Grosche, J., Matyash, V., Moller, T. *et al.* Microdomains for neuron–glia interaction: parallel fiber signaling to Bergmann glial cells. *Nat. Neurosci.* 2: 139–143, 1999.
112. Yudkoff, M., Nelson, D., Daikhin, Y. *et al.* Tricarboxylic acid cycle in rat brain synaptosomes. Fluxes and interactions with aspartate aminotransferase and malate/aspartate shuttle. *J. Biol. Chem.* 269: 27414–27420, 1994.
113. Sonnewald, U. and McKenna, M. C. Metabolic compartmentation in cortical synaptosomes: Influence of glucose and preferential incorporation of endogenous glutamate into GABA. *Neurochem. Res.* 27: 43–50, 2002.
114. Meyer, R. A. and Sweeney, H. L. A simple analysis of the 'phosphocreatine shuttle'. *Am. J. Physiol.* 246: 365–377, 1984.
115. Jost, C. R., Van Der Zee, C. E., In 't Zandt, H. J. *et al.* Creatine kinase B-driven energy transfer in the brain is important for habituation and spatial learning behaviour, mossy fibre field size and determination of seizure susceptibility. *Eur. J. Neurosci.* 15: 692–706, 2002.
116. Krebs, H. A., Williamson, D. H., Bates, M. W. *et al.* The role of ketone bodies in caloric homeostatis *Adv. Enzyme Regul.* 9: 387–409. 1971.
117. Klee, C. B. and Sokoloff, L. Changes in D(-)-β-hydroxybutyric dehydrogenase activity during brain maturation in the rat. *J. Biol. Chem.* 242: 3880–3883. 1967.
118. Smith, D., Pernet, A., Hallett, W. A. *et al.* Lactate: a preferred fuel for human brain metabolism *in vivo*. *J. Cereb. Blood Flow Metab.* 23: 658–664, 2003.
119. Pan, J. W., de Graaf, R. A., Petersen, K. F. *et al.* [2, 4–$^{13}C_2$]-β-Hydroxybutyrate metabolism in human brain. *J. Cereb. Blood Flow Metab.* 22: 890–898, 2002.
120. Edmond, J., Robbins, R. A., Bergstrom, J. D. *et al.* Capacity for substrate utilization in oxidative metabolism by neurons, astrocytes, and oligodendrocytes from developing brain in primary culture. *J. Neurosci. Res.* 18: 551–561, 1987.
121. Zielke, H. R., Collins, R. M., Baab, P. J. *et al* Compartmentation of [^{14}C]glutamate and [^{14}C]glutamine oxidative metabolism in the rat hippocampus as determined by microdialysis. *J. Neurochem.* 71: 1315–1320, 1998.
122. Yudkoff, M., Nissim, I., Daikhin, Y. *et al.* Brain glutamate metabolism: neuronal-astroglial relationships. *Dev. Neurosci.* 15: 343–50, 1993.
123. Kvamme, E., Torgner, I. A. and Roberg, B. Kinetics and localization of brain phosphate activated glutaminase. *J. Neurosci. Res.* 66: 951–958, 2001.
124. Van den Berg, C. J. and Garfinkel, D. A simulation study of brain compartments: Metabolism of glutamate and related substances in mouse brain. *Biochem. J.* 23: 211–218, 1971.
125. Danbolt, N. C. Glutamate uptake. *Prog. Neurobiol.* 65: 1–105, 2001.
126. Hogstad, S., Svenneby, G., Torgner, I. A. *et al.* Glutaminase in neurons and astrocytes cultured from mouse brain: kinetic properties and effects of phosphate, glutamate, and ammonia. *Neurochem. Res.* 13: 383–388, 1988.
127. Cerdan, S., Kunnecke, B. and Seelig, J. Cerebral metabolism of [1, 2–$^{13}C_2$]acetate as detected by *in vivo* and *in vitro* ^{13}C NMR, *J. Biol. Chem.* 265: 12916–12926, 1990.
128. Waagepetersen, H. S., Qu, H., Hertz, L. *et al.* Demonstration of pyruvate recycling in primary cultures of neocortical astrocytes but not in neurons, *Neurochem. Res.* 27: 1431–1437, 2002.
129. Bakken, I. J., White, L. R., Aasly, J. *et al.* [U-^{13}C] aspartate metabolism in cultured cortical astrocytes and cerebellar granule neurons studied by NMR spectroscopy. *Glia* 23: 271–277, 1998.
130. Sonnewald, U., Westergaard, N., Schousboe, A. *et al.* Direct demonstration by [^{13}C]NMR spectroscopy that glutamine from astrocytes is a precursor for GABA synthesis in neurons. *Neurochem. Int.* 22: 19–29, 1993.
131. Yudkoff, M. Brain metabolism of branched-chain amino acids. *Glia* 21: 92–98, 1997.
132. Tsekos, N. V., Zhang, F., Merkle, H. *et al.* Quantitative measurements of cerebral blood flow in rats using the FAIR technique: correlation with previous iodoantipyrine autoradiographic studies, *Magn. Reson. Med.* 39: 564–573, 1998.
133. Sokoloff, L., Reivich, M., Kennedy, C. *et al.* The [^{14}C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.* 28: 897–916, 1977.
134. Tsacopoulos, M. and Magistretti, P. Metabolic coupling between glia and neurons. *J. Neurosci.* 16: 877–885, 1996.
135. Berl, S., Nicklas, W. J. and Clarke, D. D. Compartmentation of citric acid cycle metabolism in brain: labelling of glutamate, glutamine, aspartate and gaba by several radioactive tracer metabolites. *J. Neurochem.* 17: 1009–1015, 1970.
136. Waniewski, R. A. and Martin, D. L. Preferential utilization of acetate by astrocytes is attributable to transport. *J. Neurosci.* 18: 5225–5233, 1998.
137. Bouzier, A. K., Voisin, P., Goodwin, R. *et al.* Glucose and lactate metabolism in C6 glioma cells: evidence for the preferential utilization of lactate for cell oxidative metabolism, *Dev. Neurosci.* 20: 331–338, 1998.
138. Bachelard, H. and Badar-Goffer, R. NMR spectroscopy in neurochemistry. *J. Neurochem.* 61: 412–429, 1993.
139. Hertz, L., Peng, L. and Lai, J. C. Functional studies in cultured astrocytes. *Methods* 16: 293–310, 1998.
140. Qu, H., Konradsen, J. R., van Hengel, M. *et al.* Effect of glutamine and GABA on [U-(^{13})C]glutamate metabolism in cerebellar astrocytes and granule neurons. *J. Neurosci. Res.* 66: 885–890, 2001.
141. Gruetter, R., Seaquist, E. R. and Ugurbil, K. A mathematical model of compartmentalized neurotransmitter metabolism in the human brain. *Am. J. Physiol.* 281, E100–112, 2001.
142. Oz, G., Henry, P. G., Seaquist, E. R. *et al.* Direct, noninvasive measurement of brain glycogen metabolism in humans. *Neurochem. Int.* 43: 323–329, 2003.

143. Henry, P. G., Tkac, I. and Gruetter, R. ^{1}H-localized broadband ^{13}C NMR spectroscopy of the rat brain *in vivo* at 9. 4 Tesla. *Magn. Reson. Med.* 50: 684–692, 2003.
144. Sibson, N. R., Mason, G. F., Shen, J. *et al. In vivo* ^{13}C NMR measurement of neurotransmitter glutamate cycling, anaplerosis and TCA cycle flux in rat brain during [2–^{13}C]glucose infusion. *J. Neurochem.* 76: 975–978, 2001.
145. Kreis, R. Quantitative localized ^{1}H MR spectroscopy for clinical use. *Prog. NMR Spectrosocpy* 31: 155–195, 1997.
146. Blumberg, R. M., Cady, E. B., Wigglesworth, J. S. *et al.* Relation between delayed impairment of cerebral metabolism and infarction following transient focal hypoxia-ischemia in the developing brain. *Exp. Brain Res.* 113: 130–137, 1997.
147. Tkac, I., Rao, R., Georgieff, M. K. *et al.* Developmental and regional changes in the neurochemical profile of the rat brain determined by *in vivo* ^{1}H NMR spectroscopy. *Magn. Reson. Med.* 50: 24–32, 2003.

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CHAPTER

32

Hypoxic–Ischemic Brain Injury and Oxidative Stress

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HYPOXIA–ISCHEMIA AND BRAIN INFARCTION

Hypoxic–ischemic brain injury continues to be the third leading cause of death in the United States, affecting over half a million new victims each year. Of these, nearly one third will die and another third will be left with severe and permanent disability. Unlike ischemic injury to many other tissues, the severity of disability is not predicted well by the amount of brain tissue lost. For example, damage to a small area in the medial temporal lobe may lead to severe disability, such as loss of speech, while damage to a greater volume elsewhere has little effect on function. The degree of disability does not simply reflect the severity or distribution of impaired blood supply. Populations of cells lying side by side in the brain can display dramatically different vulnerabilities to equivalent degrees of ischemia. Although a great deal has been learned about how nervous system anatomy, physiology and biochemistry interact to modify hypoxic–ischemic brain injury, much remains to be learned about what features contribute to the special vulnerability of the brain to stroke and of specific cell populations to hypoxic–ischemic injury during stroke.

Energy failure, an early consequence of hypoxia–ischemia, causes disruption of ionic homeostasis and accumulation of extracellular neurotransmitters. As discussed in Chapter 31, normal energy metabolism in

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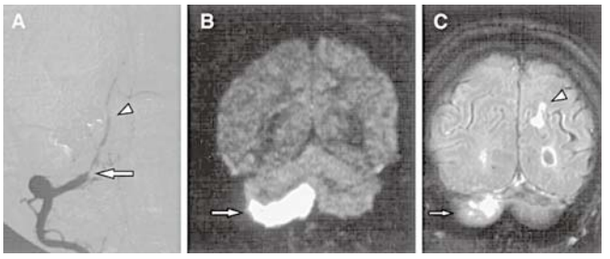


FIGURE 32-1 Focal ischemia produces a core of infarction caused by occlusion of the vessel supplying the affected brain tissue. A 53-year-old man presented with imbalance, various cranial neuropathies and hiccups. (**A**) Angiography revealed high-grade stenosis and clot in his right vertebral artery (*arrow*) and nonfilling (*arrowhead*) of his posterior inferior cerebellar artery (*PICA*). (**B**) Diffusion-weighted magnetic resonance (*MR*) study demonstrates infarction and edema in the right inferior cerebellum (*arrow*), in the distribution of the occluded PICA. (**C**) Fluid-attenuated inversion recovery (*FLAIR*) MR image: in addition to the cerebellar infarct (*arrow*), a small infarct in the left occipital lobe (*arrowhead*) was found, likely resulting from an embolus into the left posterior cerebral artery from plaques in the vertebral artery. (Courtesy of Dr DeWitte Cross, Mallinckrodt Institute of Radiology, Washington University School of Medicine.)

the brain has several unusual features, including a high metabolic rate, limited intrinsic energy stores and critical dependence on aerobic metabolism of glucose. Reflecting this special metabolic status, as well as the existence of several unique injury mechanisms discussed below, the brain exhibits higher vulnerability to ischemic injury than most other tissues. Ischemic brain injury occurs in several clinical settings. The most common is *stroke*, focal disruption of blood supply to a part of the brain (Fig. 32-1). Other settings include transient impairment of blood flow to the entire brain, *global ischemia*, as occurs during cardiac arrest. When brain hypoxia or ischemia occurs, tissue energy demands cannot be met, so ATP levels fall. Loss of ATP results in decreased function of active ion pumps, such as the Na,K-ATPase, the most important transporter for maintaining high intracellular concentrations of K^+ (≈155 mmol/l) and low intracellular concentrations of Na^+ (≈12 mmol/l) (see Ch. 5). Loss of ion pump function allows rundown of transmembrane ion gradients (Fig. 32-2), leading to membrane depolarization, the opening of voltage-sensitive ion channels and a cascade of subsequent events, which, if sustained, lead ultimately to cell death. Depending on the circumstances, this death may be restricted to selectively vulnerable neuronal populations or may involve all cells in a region of brain, termed *tissue infarction*.

Within seconds of an ischemic insult, normal brain electrical activity ceases, as a result of the activation of membrane K^+ channels and widespread neuronal hyperpolarization [1]. The hyperpolarization may be due to opening of K^+ channels responding to acute changes in local concentrations of ATP, H^+ or Ca^{2+}, or it may reflect altered nonheme metalloprotein association with and regulation of specific K^+ channels [2]. This response, presumably protective, however fails to preserve high-energy phosphate levels in tissue as concentrations of phosphocreatine (PCr) and ATP fall within minutes after ischemia onset [3]. The fall in Po_2 during ischemia leads to enhanced lactic acid production as cells undergo a Pasteur shift from a dependence on aerobic metabolism to a dependence on glycolysis. The resulting lactic acidosis decreases the pH of the ischemic tissue from the normal 7.3 to intra-ischemic values ranging from 6.8 to 6.2, depending, in part, on the pre-ischemic quantities of glucose available for conversion to lactic acid. In addition, efflux of K^+ from depolarizing neurons results in prolonged elevations in extracellular $[K^+]$ (Fig. 32-2) and massive cellular depolarization, a state known as *spreading depression*, which can propagate in brain tissue. Rapid inactivation of O_2-sensitive K^+ channels by decreased Po_2 may represent one mechanism whereby neurons put a brake on this ongoing K^+ efflux [2]. Other cellular ion gradients are also lost; thus intracellular Na^+ and Ca^{2+} rise (Fig. 32-3) and intracellular Mg^{2+} falls. Extracellular concentrations of many neurotransmitters are increased during hypoxia–ischemia. Depolarization-induced entry of Ca^{2+} via voltage-sensitive Ca^{2+} channels stimulates release of vesicular neurotransmitter pools, including the excitatory amino acid neurotransmitter glutamate. At the same time, Na^+-dependent uptake of certain neurotransmitters, including glutamate, is impaired (Ch. 5). High capacity uptake of glutamate by the glutamate transporter couples the uptake of one glutamate and two Na^+ with the export of one K^+ and one HCO_3^- (or OH^-) (see Fig. 15–7). When the cellular ion gradients are discharged, the driving force for glutamate uptake is lost. In addition, glutamate uptake by the widely expressed astrocyte high-affinity glutamate transporter GLT-1, or excitatory amino acid transporter-2 (EAAT2), and the neuronal transporter, or EAAT3, can be downregulated by free-radical-mediated oxidation of a redox site on the transporter [4]. Furthermore, since the transporter is electrogenic, i.e. normally transferring a positive charge inward, membrane depolarization can lead to reversal of the transporter, producing glutamate efflux [5]. Thus, both impaired glutamate uptake and enhanced glutamate release contribute to sustained elevations of extracellular glutamate in the ischemic brain. Microdialysis of ischemic rat brain has detected an increase from the resting extracellular glutamate concentrations of 1–2 μmol/l up to concentrations in the high micromolar or even low millimolar range.

Focal and global ischemia produce different distributions of injury. Ischemic injury to the brain can result from several different processes. Focal ischemia, which accounts for a majority of strokes, occurs when an artery supplying a region of the brain is occluded, either by an embolus, which is generally material broken off from a plaque in a large artery or a thrombus in the heart, or by a thrombus or platelet plug that forms directly in the affected artery (Fig. 32-1). While focal ischemic insults generally reflect the distribution of vascular supply to a region, the area of infarction is typically less than the entire distribution of the occluded artery because of the presence of collateral circulation at the borders of the

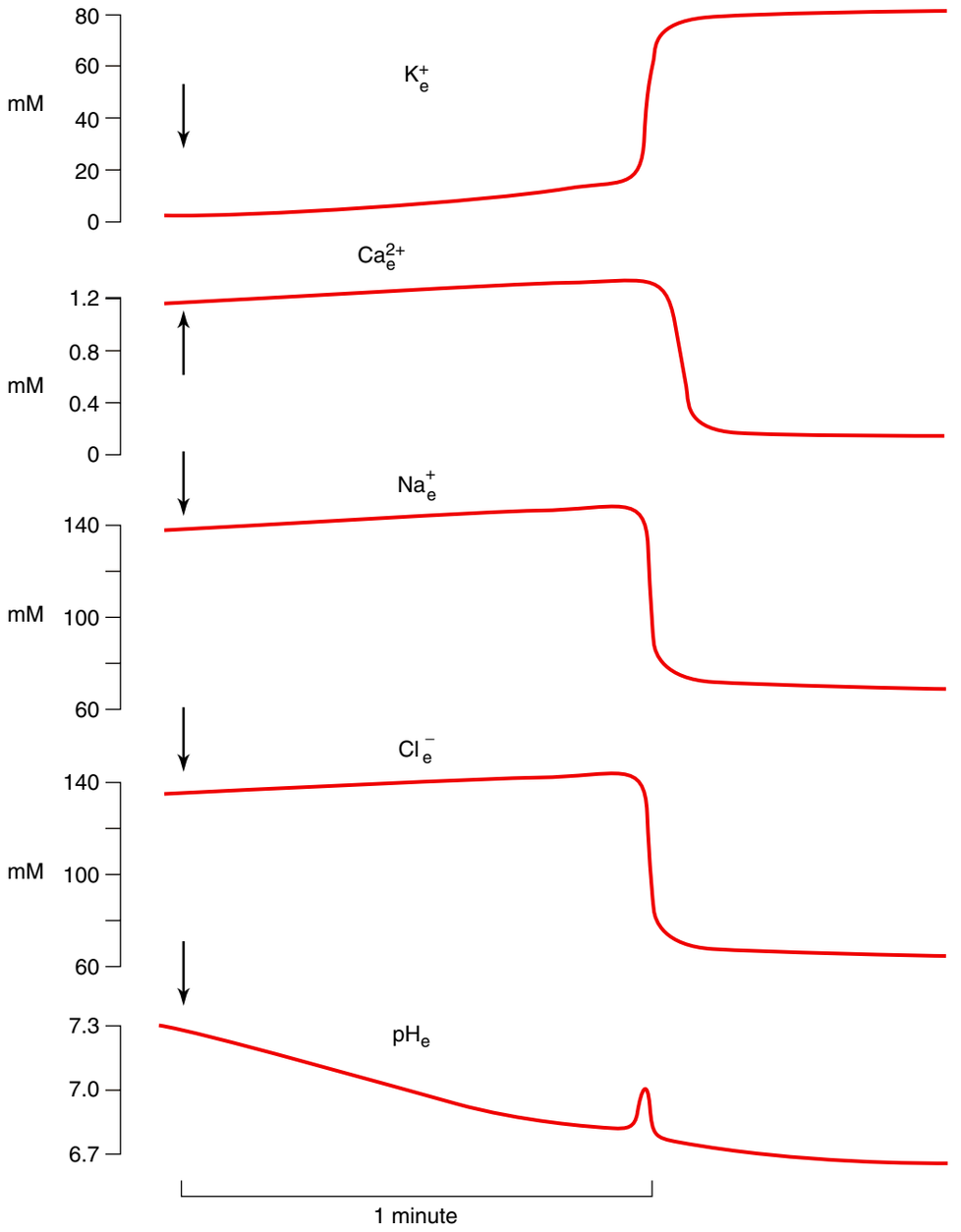


FIGURE 32-2 Changes in extracellular ion concentrations following ischemia. Extracellular pH starts to decrease immediately after the onset of ischemia. This change is accompanied by slight increases in the extracellular concentrations of K^+, Cl^- and Na^+. After about 1 min of ischemia, a dramatic shift of ions occurs, with K^+ leaving cells and Ca^{2+}, Cl^- and Na^+ leaving the extracellular space (from [1] with permission).

region supplied by the occluded vessel. The ultimate area of infarction will depend on the duration and degree of vascular occlusion and the availability of collateral blood supply [6]. The region of the brain supplied uniquely by the occluded artery develops the most severe injury, termed the *ischemic core*, while the rim of tissue surrounding the core, termed the *penumbra*, which has the benefit of some maintained blood flow supplied by collateral circulation, sustains less severe injury. Focal ischemia may also accompany other acute brain insults, such as intracerebral hemorrhage or trauma.

Reversible global ischemia, such as occurs during cardiac arrest and resuscitation, reflects a transient loss of blood flow to the entire brain and generally results in the death of certain selectively vulnerable neuronal populations. Hypoxia accompanies ischemic insults but may also occur without loss of blood flow, for example during near-drowning or carbon monoxide poisoning. Hypoglycemia produces brain injury that has several features in common with ischemic injury. Neurons are more susceptible than glial cells to ischemia, hypoxia or hypoglycemia; and the phylogenetically newer regions of the brain, including the cortex

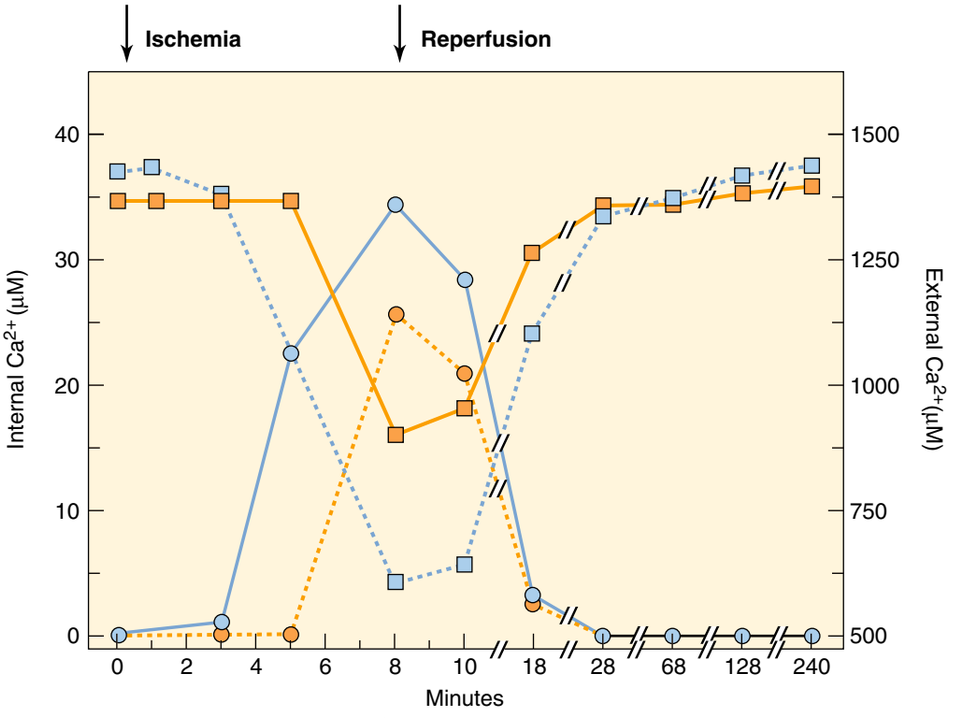


FIGURE 32-3 Changes in intracellular and extracellular Ca^{2+} during ischemia–reperfusion and the effects of the *N*-methyl-D-aspartate receptor antagonist MK-801. Intracellular Ca^{2+}, *circles*; extracellular Ca^{2+}, *squares*; with MK-801, *orange*; without MK-801, *blue*. (From Silver and Erecinska, in [3].)

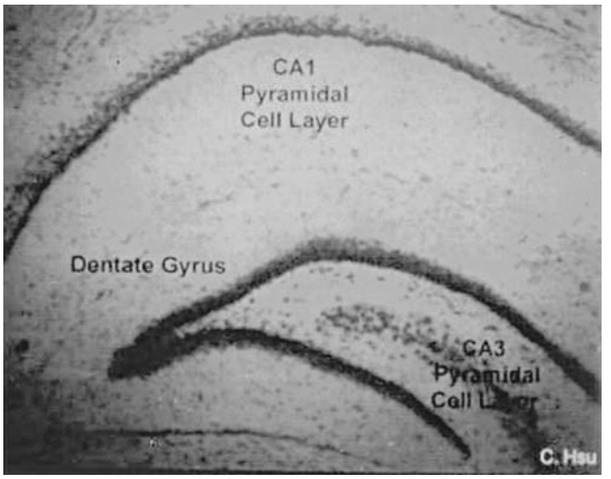
and cerebellum, are affected to a greater extent than the brainstem [6].

The 'Selective vulnerability' of specific neurons or glial cells is not explained by vascular distribution. As first recognized by Vogt and Vogt (see [6]), the juxtaposition of relatively vulnerable and relatively resistant neuronal populations within a single vascular distribution suggests that intrinsic tissue factors contribute heavily to ischemic neuronal vulnerability. For example, pyramidal neurons in the CA1 subfield of the hippocampus die after 5–10 min of global ischemia, while neurons in the nearby CA3 region are preserved. Populations of neurons that are selectively vulnerable to ischemia include cortical pyramidal neurons, cerebellar Purkinje cells, hippocampal CA1 pyramidal neurons and subpopulations in the amygdala, striatum, thalamus and brainstem nuclei (Fig. 32-4). Some of the mechanisms that may contribute to selective vulnerability of certain cell populations to ischemic injury are discussed further below.

MICROVASCULAR INJURY IN HYPOXIA–ISCHEMIA

Hypoxia–ischemia disrupts the blood–brain barrier and damages endothelial cells. Damage to the blood–brain barrier (BBB), which occurs gradually following ischemia–reperfusion, reflects the vulnerability of the cellular components of the BBB to damage, both directly by the ischemic insult itself and indirectly as a consequence of brain parenchymal responses. Damage to microvascular endothelial cells can lead to vasospasm and promote adhesion of platelets and leukocytes, leading to vessel plugging. Maneuvers that limit the postischemic recruitment of inflammatory cells to the ischemic zone and the subsequent inflammatory response can protect the integrity of the BBB and improve postischemic cerebral blood flow [3]. Transgenic mice that do not express intercellular adhesion molecule (ICAM)-1, a protein that is important in promoting adhesion of leukocytes to endothelial cells (see Ch. 7), are less vulnerable to ischemic injury than littermate controls and have improved cerebral blood flow.

Metalloproteinases may be involved in cerebrovascular injury. Matrix metalloproteinases (MMPs) also contribute to disruption of the blood–brain-barrier during ischemia. MMPs are Zn^{2+} endopeptidases that are secreted as the precursor zymogen, and are then cleaved to the active form of the protein. MMP-2 and MMP-9 are type IV collagenases that have been shown to degrade the perivascular extracellular matrix following ischemia, causing disruption of the BBB. Inhibition of MMPs limits BBB opening and decreases edema formation following ischemia [7]. More recently, MMPs have been suggested to have a role in promoting hemorrhagic transformation after stroke, especially in the context of tissue plasminogen activator administration as thrombolytic therapy to stroke patients [8].



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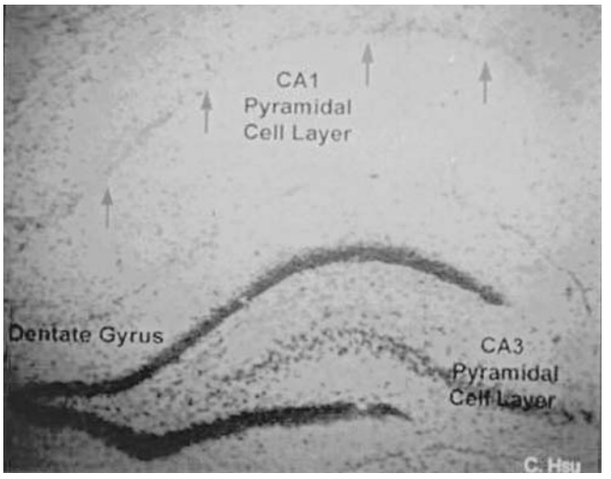

B

FIGURE 32-4 Rat hippocampus showing neuronal populations that are selectively vulnerable to ischemic damage. (**A**) control; (**B**) ischemia. A brief period of global ischemia causes nearly complete loss of neurons in the CA1 region (*arrows*) of the hippocampus, while neurons in the nearby CA3 region are almost completely spared. (Courtesy of Dr Chung Y. Hsu, Washington University School of Medicine.)

Besides increased ischemia, another consequence of damage to the endothelial cells or astrocytes that comprise the BBB may be the entry of components of the vascular compartment, such as cytokines and inflammatory cells, to the brain parenchyma. Agents that protect endothelial cells, such as antioxidants, can help preserve BBB integrity following ischemia–reperfusion [9]. The response of nonvascular or parenchymal cells to the ischemic insult also affects BBB function. For example, *N*-methyl-D-aspartate (NMDA) receptor antagonists, which reduce neuronal death and tissue infarction after focal ischemia (see below), decrease BBB disruption, possibly by blocking neuronal production of reactive oxygen species (ROS) triggered by NMDA receptor activation, thus reducing their deleterious effects on endothelial or astrocyte cell function.

Edema may lead to secondary ischemia, which can produce further brain damage. As a result of the breakdown of the BBB after ischemia, the tissue content of water in the affected area of the brain may increase, leading to swelling or edema of the ischemic region. Some of the increased tissue water is intracellular, reflecting impaired cellular ion homeostasis due to energy failure, as well as the action of glutamate, which triggers excess cation influx in neurons and glia (Ch. 15). Some of the increased tissue water is extracellular, because of breakdown of the BBB followed by leakage of solute and water from the intravascular space.

Aquaporins can contribute to brain edema. Recently, aquaporins have been implicated in edema formation after ischemia. Aquaporins are small, homotetrameric membrane channels that mediate rapid water flux through cell membranes. The main aquaporin in brain, AQP4, is assembled into multimeric square arrays (orthogonal arrays) in astrocyte foot process membranes adjacent to capillary basement membranes. Like AQP1, AQP4 is constitutively active and is therefore believed to be involved in normal regulation of brain water flux. However, AQP4 knockout mice develop less postischemic edema and smaller infarcts than littermate controls, suggesting that AQP4 may contribute to early edema formation during ischemia–reperfusion injury [10]. Finally, because tissue edema in the rigid intracranial cavity can restrict blood flow, secondary ischemia can ensue. If edema is substantial enough, uncal or central brainstem herniation may result, leading to respiratory arrest and death.

EXCITOTOXIC INJURY IN HYPOXIA–ISCHEMIA

Both NMDA and AMPA/kainate receptors contribute to excitotoxic neuronal degeneration of neurons and glia. Excitotoxicity (toxic glutamate receptor activation) is a key mediator of central neuronal loss consequent to hypoxic–ischemic insults [11]. While brain gray matter contains glutamate concentrations in the average range of 10 mmol/l, much of which is stored in synaptic vesicles for release as a neurotransmitter, the extracellular concentration of glutamate is normally maintained in the range of ≈1 μmol/l by Na^+-flux-coupled transport (see Ch. 5). During hypoxia–ischemia, as cellular energy reserves and Na^+ gradients fall, increased release and impaired uptake of glutamate mediate a toxic buildup of extracellular glutamate, leading to overstimulation of glutamate receptors and consequent neuronal cell death. Excitotoxicity can be exacerbated by decreased cellular energy stores.

In vitro studies on excitotoxicity suggest that while both NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate (KA) receptors can mediate excitotoxicity (see Ch. 15), these classes of glutamate receptors do not do so equally. Experiments with cortical or hippocampal cell cultures suggest that much of the neuronal death associated with brief, intense glutamate exposure is mediated by NMDA receptor activation, probably because this can induce lethal amounts of Ca^{2+} influx more rapidly than can AMPA/KA receptor stimulation.

However, overactivation of AMPA or KA receptors can also lead to intracellular Ca^{2+} overload and neurodegeneration. This may be especially true under conditions where NMDA-receptor activity is reduced by extracellular acidity or a buildup of extracellular Zn^{2+} [12]. It is also true with respect to specific neuronal subpopulations that express AMPA-sensitive Ca^{2+} channels (see Ch. 15). G-protein-linked metabotropic glutamate receptors (mGluRs) appear not to mediate excitotoxicity directly but, rather, to modify the degree of excitotoxic injury.

Excitotoxicity leads to increased Ca^{2+} and Zn^{2+}, which can activate cytotoxic intracellular pathways. The prolonged availability of extracellular glutamate during hypoxia–ischemia is transduced by neuronal membrane receptors into potentially lethal intracellular ionic derangements, in particular, intracellular Na^+ and Ca^{2+} overload. Excitotoxic neuronal cell death, which tends to occur by necrosis, correlates well with measures of total Ca^{2+} influx, and removal of extracellular Ca^{2+} attenuates glutamate-induced neuronal death. Sustained elevations in intracellular Ca^{2+} can initiate 'toxic cascades', which are capable of ultimately killing the cell (Fig. 32-5). These cascades include activation of catabolic enzymes, such as proteases, phospholipases and endonucleases [13]. Elevated concentrations of intracellular Ca^{2+} can further lead to initiation of protein-kinase and lipid-kinase cascades (Ch. 22), impairment of metabolism and generation of ROS. While many of these events occur early and can result in rapid cell death, others, such as energy compromise and ROS formation, may initiate more delayed death processes, discussed in more detail in the next section. In addition, most of these Ca^{2+}-dependent events may be common to both NMDA- and AMPA/KA-receptor-mediated excitotoxicity.

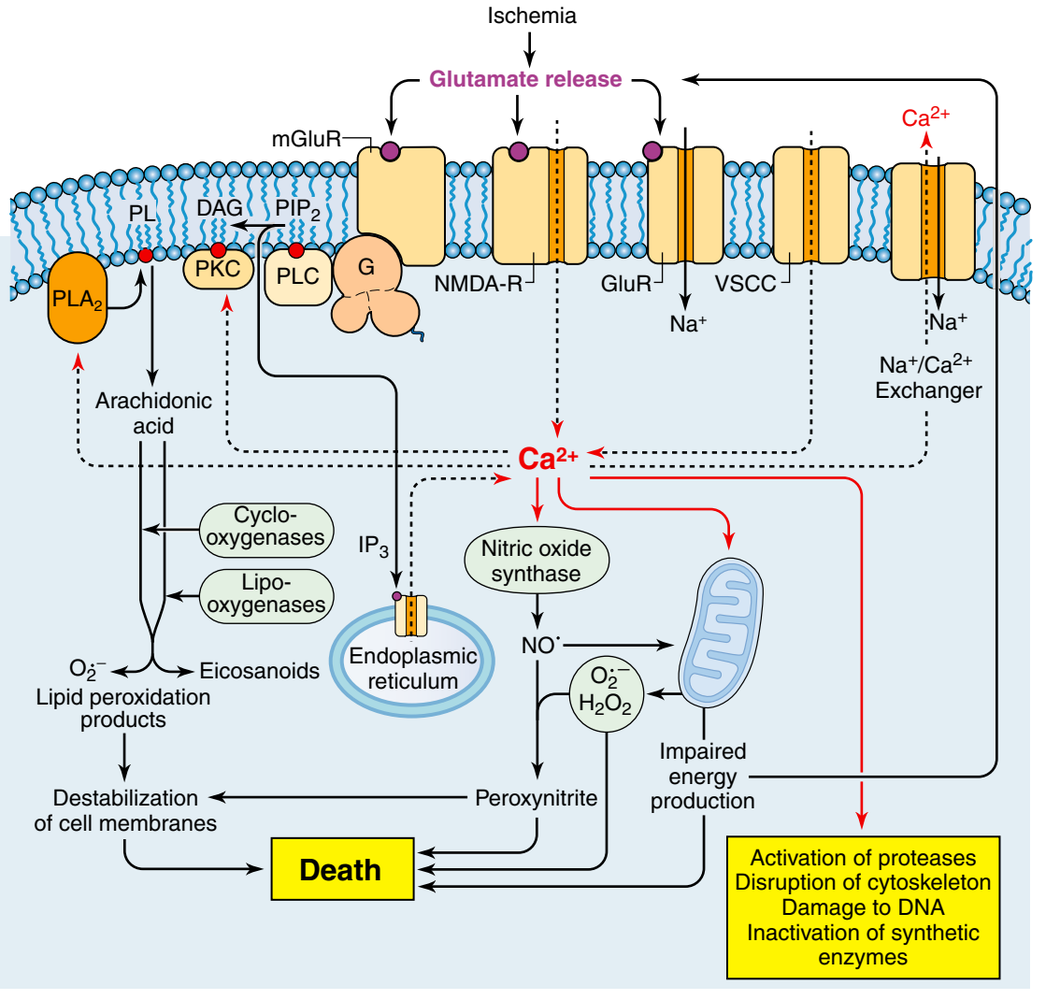

FIGURE 32-5 Mechanisms contributing to neuronal injury during ischemia–reperfusion. Simplified diagram showing several pathways believed to contribute to excitotoxic neuronal injury in ischemia. *DAG*, diacylglycerol; *G*, G protein; *GluR*, AMPA/Kainate type of glutamate receptors; H_2O_2, hydrogen peroxide; IP_3, inositol 1,4,5-trisphosphate; *mGluR*, metabotropic glutamate receptor; *NMDA-R*, *N*-methyl-D-aspartate receptor; $NO^{\cdot}$, nitric oxide; $O^{\cdot-}_2$, superoxide radical; PIP_2, phosphatidylinositol 4,5-bisphosphate; *PKC*, protein kinase C; *PL*, phospholipids; PLA_2, phospholipase A_2; *PLC*, phospholipase C; *VSCC*, voltage-sensitive Ca^{2+} channel.

Ca^{2+}-dependent activation of the cysteine protease calpain occurs early in ischemia and may lead to disruption of the cytoskeleton [3]. Endonuclease activation can be triggered by influx of large amounts of Ca^{2+}, which may lead to DNA breakdown during hypoxia–ischemia [14]. Increased intracellular Ca^{2+} also activates a variety of protein kinases. In particular, Ca^{2+}/calmodulin kinase (CaMK) and the classical Ca^{2+}-dependent isoforms of protein kinase C (PKC) can modify the function of many ion channels, including NMDA- and AMPA/KA- receptor-gated channels and voltage-gated Ca^{2+} channels [15]. An especially important deleterious consequence of Ca^{2+} overload following excitotoxic glutamate receptor activation is formation of ROS. Free radical production is linked to elevated $[Ca^{2+}]i$ in several ways: (1) Ca^{2+}-dependent activation of phospholipase A2, with liberation of arachidonic acid and further metabolism, leading to 'by-product' free radical production and lipid peroxidation (see Chs 3 and 33); (2) stimulation of NMDA receptors, leading to activation of nitric oxide synthase (NOS) and the release of nitric oxide (NO), which can then interact with ROS from other sources to generate highly reactive peroxynitrite [16]; and (3) dysfunction of the mitochondrial electron transport chain, enhancing mitochondrial production of free radicals [17]. Activation of NMDA receptors triggers Ca^{2+}-dependent production of ROS from the mitochondrial electron-transport chain, although the exact mechanism behind this process is still not well understood. In cell cultures, ROS production is stimulated by concentrations of NMDA that are not neurotoxic and, in awake rats, NMDA receptors are responsible for a low baseline production of ROS, suggesting that even 'physiological' NMDA receptor activity may trigger production of ROS. However, cell culture studies suggest that substantially greater amounts of mitochondrial ROS are generated when NMDA receptors are overstimulated sufficiently to produce excitotoxicity. Since AMPA/KA receptor activation, possibly in conjunction with the group 1 metabotropic glutamate receptors (see Ch. 15), may also elicit enhanced mitochondrial ROS production, it is likely that excitotoxicity may be an important trigger for mitochondrial free radical production in ischemia–reperfusion injury. Supporting the idea that free radicals are important downstream mediators of excitotoxicity, treatment with free radical scavengers can attenuate NMDA- or AMPA-receptor-mediated neuronal death.

Excitatory amino acid-receptor antagonists can provide neuroprotection in experimental models of hypoxia–ischemia. Supporting the glutamate hypothesis of hypoxic–ischemic brain damage, substantial evidence indicates that antagonists of ion channel-linked glutamate receptors can reduce infarct volume in animal models of focal brain ischemia. This is true both for NMDA antagonists and for AMPA/KA antagonists [11]. The magnitude of infarct reduction is model-dependent but is typically on the order of 25–30%, and the reduction can exceed 50%.

An additional injury mechanism, ischemic apoptosis (discussed below), may evolve in parallel with excitotoxic necrosis, but apoptosis has little chance to be manifested under circumstances in which fulminant excitotoxicity predominates. Evidence favoring the concurrence of apoptosis and necrosis can be found in the observation that the application of an NMDA antagonist drug together with a drug designed to inhibit apoptosis leads to greater neuroprotection than does either drug treatment alone. Each agent alone, administered at optimal doses systemically just after the ischemic insult, reduces infarction by about half, but combination therapy can reduce infarct volume by as much as 80%.

Growing evidence suggests that, in addition to Ca^{2+}, the ubiquitous metal ion Zn^{2+} may contribute to neuronal injury during cerebral ischemia-hypoxia. Zn^{2+} is necessary for the proper function of many metalloenzymes and transcription factors. In the nervous system, Zn^{2+} appears to serve an additional function as a mediator of central neural signaling [12]. Low concentrations of Zn^{2+} can modify the function of Na^{+}, K^{+} and Ca^{2+} channels as well as certain subtypes of $GABA_A$ receptor, and Zn^{2+} strongly attenuates activation of the NMDA subclass of glutamate receptors. Free Zn^{2+} is present in synaptic vesicles within excitatory boutons throughout the brain, and the neuropil of the hippocampus and neocortex is especially rich in releasable pools of Zn^{2+}. Neuronal firing causes a Ca^{2+}-dependent release of Zn^{2+}; with intense neuronal activity, it has been estimated that Zn^{2+} concentrations of several hundred micromoles per liter could be achieved in synaptic clefts. A pathogenic role for Zn^{2+} in ischemic brain damage has been suggested, based on the following observations: presynaptic Zn^{2+} translocates into selectively vulnerable hippocampal hilar neurons, which degenerate after transient forebrain ischemia, and into limbic or cortical neurons, which degenerate after seizures induced by KA or perforant path stimulation; and chelation of extracellular Zn^{2+} by Ca^{2+}-EDTA reduces selective neuronal death in multiple regions of rat brain in an experimental model of transient global ischemia [18].

ISCHEMIC APOPTOSIS

Hypoxia–ischemia may initiate apoptosis in parallel with excitotoxicity. In 1972, Wyllie and colleagues (see [19]) described two distinct patterns of cell death. One form, apoptosis, progressed through a set of morphological changes characterized by condensation and margination of large nuclear chromatin aggregates and extrusion of membrane-bound cytoplasmic and nuclear material, termed apoptotic bodies, with progressive loss of cell volume. Since these features were prominent in cells dying by programmed cell death during normal development, the concept emerged that apoptosis reflects the execution of a death 'program', in many cases requiring new protein synthesis. The mechanisms underlying apoptosis are

outlined in more detail in Chapter 35. As discussed above, growing evidence has implicated not only excitotoxicity but also apoptosis as important processes leading to brain cell death after hypoxic–ischemic insults. Inhibitors of macromolecular synthesis and of caspase activity can limit apoptotic death of neurons exposed to hypoxia–ischemia. In addition, transgenic mice overexpressing the antiapoptotic gene *bcl*-2 have smaller infarcts than their littermates after hypoxia–ischemia. Intrinsic mechanisms of neural cell apoptosis are discussed in more detail in Chapters 27 and 35.

Triggers of ischemic apoptosis may include decreased supply or sensitivity to neurotrophins, oxidative stress, exposure to inflammatory cytokines or damage to mitochondria. One factor that may promote apoptosis after ischemic insults is deprivation of growth factor support. Deprivation may result from damage to neuronal or glial targets responsible for providing growth factor support. However, tissue concentrations of several growth factors increase in the brain following hypoxic–ischemic insults, suggesting that either there may be decreased sensitivity of neurons to neurotrophins after ischemia, or increased concentrations of neurotrophins are required to counter pro-apoptotic stimuli, such as free-radical exposure. Addition of exogenous growth factors, such as nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) (see Ch. 27) [20], can reduce hypoxic–ischemic damage. However, while neurotrophins may attenuate ischemic apoptosis, they may, in contrast, have deleterious effects by enhancing the excitotoxic necrosis induced by ischemia.

In addition, oxidative stress may trigger apoptosis following hypoxia–ischemia. Exposure of neurons to a free radical stress, either by application of H_2O_2, exposure to ultraviolet irradiation or depletion of antioxidant defenses, such as glutathione or superoxide dismutase (SOD; see below), may trigger apoptosis. Free radicals not only may serve as inducers of apoptotic cell death, they may also be a signal in the apoptotic cascade: neurons deprived of growth factors demonstrate an early increase in oxygen free radical formation and can be rescued from growth factor deprivation-induced apoptosis by application of antioxidants [21]. Thus, free radicals may serve as triggers, signals or effectors of neuronal apoptosis.

Persistent impairment of cellular energy metabolism after an ischemic insult may also play a role in triggering apoptotic neuronal degeneration. Studies in cell culture and animal models of stroke suggest that inhibition of mitochondrial function by mitochondrial toxins such as 3-nitropropionic acid or rotenone not only worsens excitotoxic injury but also can trigger apoptotic neuronal death. Prolonged deficits in mitochondrial function and energy metabolism have been observed after ischemia–reperfusion and may represent a trigger for apoptotic neurodegeneration after ischemia.

Increased expression and enhanced concentrations of inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)α and transforming growth factor (TGF)β, are observed in brain following ischemia and may derive from inflammatory cells, such as macrophages or microglia, as well as neurons and glia. Because cytokines are capable of triggering apoptosis in many cell types, including neurons, increased concentrations of these molecules might be expected to trigger apoptotic cell death in vulnerable cells. Thus, ischemic apoptosis may be induced by free radicals, cytokines, metabolic insults and changes in growth factor sensitivity, which may result from excitotoxic damage to intracellular systems such as the cytoskeleton, axonal transport, and mitochondria.

FREE RADICALS IN HYPOXIA–ISCHEMIA

The brain has a number of characteristics that make it especially susceptible to free- radical-mediated injury. Brain lipids are highly enriched in polyunsaturated fatty acids and many regions of the brain, for example, the substantia nigra and the striatum, have high concentrations of iron. Both these factors increase the susceptibility of brain cell membranes to lipid peroxidation. Because the brain is critically dependent on aerobic metabolism, mitochondrial respiratory activity is higher than in many other tissues, increasing the risk of free radical 'leak' from mitochondria; conversely, free radical damage to mitochondria in brain may be tolerated relatively poorly because of this dependence on aerobic metabolism.

Oxygen free radicals are required intermediates in many biological reactions but may damage macromolecules during oxidative stress. Free radicals are molecules that possess an outer electron orbital with a solitary unpaired electron; these include the hydrogen atom ($H^{\cdot}$); the diatomic oxygen molecule O_2, which possesses two unpaired electrons with the same spin in two separate orbitals; $NO^{\cdot}$; superoxide ($O_2^{\cdot -}$); hydroxyl radical ($^{\cdot}OH$); and transition metals, such as copper and iron. While O_2 qualifies as a radical by having two unpaired electrons, its reactivity with nonradical compounds is limited because the unpaired electrons in O_2 have the same spin state. The two electrons in a covalent bond have opposite spins so, in order for O_2 to react with a nonradical, one of the electrons must undergo 'spin inversion' so that both are antispin to the electrons on O_2, an extremely slow process. O_2 does react readily with radicals, accepting one electron at a time to form the very reactive superoxide radical $O_2^{\cdot -}$, which has one unpaired electron [22].

Although some radical species may persist for prolonged periods, most are generally unstable and will attempt to donate their unpaired electron to a nearby molecule or to remove or 'abstract' a second electron, usually in the form of a hydrogen atom, from a neighboring molecule to pair with their free electron. Free-radical reactions are intrinsic to a majority of the metabolic and

synthetic reactions carried out by eukaryotic cells and, as such, are required for life. ATP production by the mitochondrial electron chain, for example, uses a controlled set of oxygen radical reactions to couple the reduction of free-radical electrons with the movement of protons across the mitochondrial membrane. Cytochrome oxidase, complex IV of the mitochondrial electron transport chain (see Ch. 31), catalyzes transfer of these free electrons to molecular oxygen as the final acceptor with water as the end product.

The addition of O_2 to macromolecules, such as in the metabolism of arachidonic acid to eicosanoids or oxidation of small molecules by P450 enzymes, requires 'activation' of molecular oxygen to permit transfer of atomic oxygen ($O^{\cdot}$) from O_2 to the biological compound. Most enzymes that catalyze biological radical reactions bind a metal ion (Fe, Cu, Co or the group VI element Se), which destabilizes the O_2 molecule. These reactions also involve cofactors such as flavin adenine dinucleotide or flavin mononucleotide to help stabilize the resulting oxygen atoms until the reaction is complete.

Although such reactions are generally very efficient, there is often some small amount of leak of ROS, including radicals such as $O_2^{\cdot-}$, its acid HO_2, hydroxyl radical ($^{\cdot}OH$) and $NO^{\cdot}$, as well as nonradicals such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O\Delta g$) and hypochlorous acid (HOCl).

While H_2O_2 is not a free radical, it can be rapidly decomposed via the *Fenton reaction*:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

In addition, superoxide, hydrogen peroxide and hydroxyl radicals can be interconverted via the so-called *Haber–Weiss reaction*:

$$Fe^{3+} + O_2^{\cdot-} \rightleftharpoons Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$
$$O_2^{\cdot-} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2$$

Cuprous and cupric ions may substitute for ferrous and ferric ions in the Haber–Weiss reaction [22]. Peroxynitrite can be formed from the reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$:

$$NO^{\cdot} + O_2^{\cdot-} \rightarrow ONOO^- \rightarrow NO_2^{\cdot-} + {}^{\cdot}OH$$

Oxidative stress generally describes a condition in which cellular antioxidant defenses are inadequate to completely detoxify the free radicals being generated, because of excessive production of ROS, loss of antioxidant defenses or, typically, both [23]. This condition may occur locally, as antioxidant defenses may become overwhelmed at certain subcellular locations while remaining intact overall, and selectively with regard to radical species, as antioxidant defenses are radical-specific – for example SOD for superoxide and catalase or glutathione peroxidase for H_2O_2.

A major consequence of oxidative stress is damage to cellular macromolecules. Addition of a free radical electron to a fatty acid causes fragmentation of the lipid or alteration of its chemical structure (Fig. 32-6). For example, the unconjugated *cis* double bonds in unsaturated fatty acids may be shifted to produce a conjugated *trans* double bond system (see Ch. 3). Peroxyl or hydroxyl groups may be added to the unsaturated fatty acid, or the fatty acid carbon chain may be cleaved during reaction with the free electron to generate a fatty aldehyde, both processes termed *lipid peroxidation*. Fatty aldehydes such as 4-hydroxynonenal can then react with free thiol groups such as cysteines on proteins to produce thioesters, which may affect protein function and stability [22]. Free-radical damage to proteins may cause cross-linking, carbonyl formation and protein denaturation. DNA bases may be modified by oxidation, resulting in single- and double-strand breaks or mispairing of purine and pyrimidine during DNA replication.

Reactive oxygen species generated during ischemia–reperfusion contribute to the injury. Evidence for free radical production and oxidative stress during ischemia–reperfusion comes from a variety of studies. While free radicals may be generated to a small extent during ischemia, far greater production of reactive oxygen intermediates occurs after reintroduction of oxygen during reperfusion. Early studies with animal models of ischemia–reperfusion injury showed decreased brain concentrations of antioxidants such as ascorbic acid, vitamin E and glutathione, as well as production of aldehydic lipid peroxidation products, detected as thiobarbituric acid-reactive substances. Additional evidence for ROS generation has been obtained with intra-ischemic microdialysis with solutions containing salicylic acid or spin-trapping agents, which react with ROS to form relatively stable products that can be detected in the microdialysis solution, or with oxidation-sensitive fluorescent probes, such as dihydroethidine, which detects superoxide radical. Oxidative damage to macromolecules has provided another index of ROS-mediated injury in ischemia–reperfusion. Lipid- and protein-oxidation products, as well as DNA-oxidation products, have been detected in brain tissue after ischemia–reperfusion.

Treatment with antioxidants such as vitamin E, 21-aminosteroids, and spin-trapping agents such as phenyl tert-butyl *N*-nitrone (PBN), can reduce ischemic brain infarction. In addition, transgenic mice that overexpress the cytosolic antioxidant enzyme CuZn-SOD (SOD1; see below) [24] or the extracellular SOD3 [25] are resistant to ischemic brain injury compared to wild-type controls. Conversely, reduction of cellular antioxidant defenses can potentiate ischemic injury. Transgenic mice that are *SOD1*$^{-/-}$ exhibit enhanced susceptibility to ischemic brain injury, even though they have a grossly normal CNS phenotype under unstressed conditions, for example in the absence of ischemia or nerve transection. This suggests that other antioxidant defenses are adequate to handle physiological concentrations of cytosolic superoxide anion.

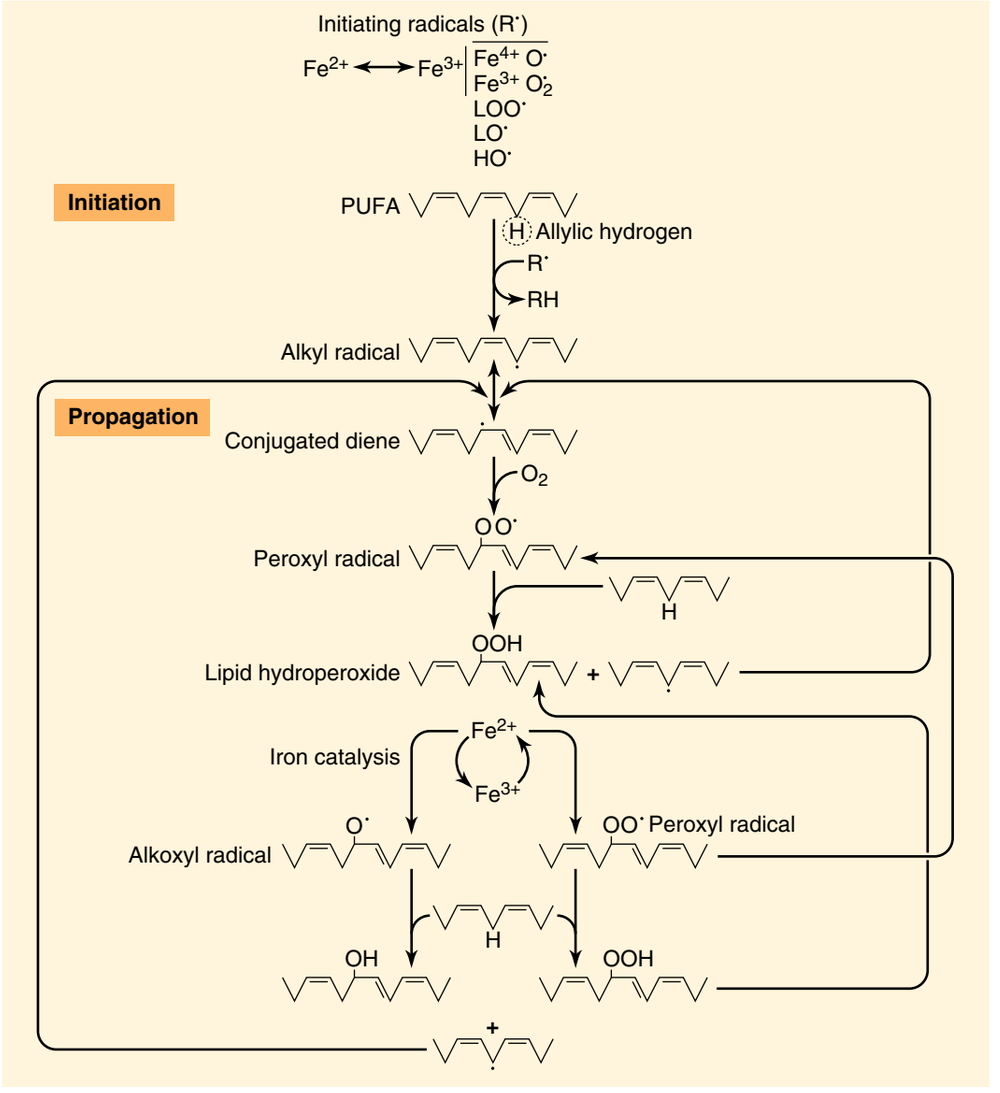

FIGURE 32-6 Lipid peroxidation leads to fragmentation or oxidation of polyunsaturated fatty acids (PUFA). *HO·*, hydroxyl radical; *LO·*, lipid alkoxylradical; *LOO·*, lipid peroxyl radical; $O^{\cdot-}_2$, superoxide radical; *O·*, atomic oxygen radical. (From Hall in [3].)

In contrast, mice lacking the mitochondrial antioxidant enzyme Mn^{2+}-SOD (SOD2) die soon after birth, and mice that are heterozygous for the *sod2* gene, which exhibit half the SOD2 activity of wild-type mice, have larger areas of infarction after focal ischemia. A large body of literature now links oxidative stress, produced by both increased production of ROS and impaired defenses against ROS, to ischemic injury (reviewed in [24]).

Mitochondria, nitric oxide synthase and arachidonic acid metabolism are sources of reactive oxygen species during ischemia–reperfusion injury. ROS generation during ischemia–reperfusion may come from several sources, including NOS activity, mitochondrial electron transport, multiple steps in the metabolism of arachidonic acid and, in some species, xanthine oxidase, which is produced by hydrolysis of xanthine dehydrogenase. In addition, the decreased intracellular pH accompanying ischemia may alter the binding of transition metals such as iron, increasing their participation in the Haber–Weiss reaction [22]. P450 enzymes and NAD(P)H oxidase are two additional potential sources of oxygen radicals whose contribution to enhanced radical production during CNS ischemia has not been systematically explored. Mitochondria were among the earliest sites of cellular ROS production to be identified. Studies on isolated mitochondria have suggested that as much as 3–4% of O_2 utilized by resting mitochondria may be leaked as superoxide and H_2O_2. Mitochondrial production of ROS can be enhanced by increased electron-transport activity, as well

as by disturbances in electron transfer down the transport chain. The lipid electron-transport molecule ubiquinone (Q9, Q10) is the major site of free radical leak from mitochondria via the ubiquinone cycle. Ubiquinone undergoes a two-electron reduction, first to a semiquinone and then to a diol, at mitochondrial complex I (NADH dehydrogenase, NADH:ubiquinone oxidoreductase) or complex II (succinate dehydrogenase) and subsequently delivers the two electrons to the iron–sulfur center of complex III (cytochrome bc1 in mammalian mitochondria). Inhibition of either complex I or complex III will impair the efficiency of electron transfer and may allow a free semiquinone to be produced. The semiquinone can then interact with O_2 to generate $O_2^{\cdot-}$ (Fig. 32-7). Elevated intracellular Ca^{2+}, exposure to fatty acids or other molecules that alter the physical properties of the mitochondrial membrane and inhibition of mitochondrial respiratory components may enhance this leak of ROS from mitochondria. Mitochondria appear to be an important and perhaps dominant source of free radicals in brain tissue subjected to ischemia–reperfusion. Microdialysis studies using salicylate to detect ROS release from ischemic brain cortex show that mitochondrial inhibitors such as rotenone eliminate ROS production during ischemia–reperfusion. Elevated concentrations of intracellular Ca^{2+} and Na^+, a consequence of energy failure and excitotoxic glutamate receptor stimulation, can be expected to inhibit complex I as well as overproduction of superoxide anion. The resultant oxidative stress may lead to further inhibition of mitochondrial respiratory components, promoting further free radical production in a vicious, feed-forward cycle. Although xanthine oxidase has been implicated in animal models of ischemia, its role in human stroke is less clear.

Nitric oxide and peroxynitrite contribute to oxidative damage. As discussed above, NOS has been identified as a source of ROS with special relevance to pathological conditions. NOS is homologous to P450 cytochrome *c* reductase; cofactors in the reaction are flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin and NAD(P)H (Fig. 32-7). NOS normally converts arginine and molecular oxygen to citrulline and $NO^{\cdot}$, a free radical gas; this process is itself leaky and associated with free radical generation [16]. $NO^{\cdot}$, originally identified as endothelial-derived relaxing factor (EDRF), is a potent vasodilator. $NO^{\cdot}$ also binds to guanylyl cyclase to increase GTP production and can nitrosylate the small G protein Ras on a specific cysteine to cause constitutive activation. Three family members have been identified: Ca^{2+}/calmodulin-activated brain NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). High concentrations of nNOS are found in a subpopulation of neurons previously identified as NAD(P)H-diaphorase cells. $NO^{\cdot}$, which has limited radical reactivity, can combine readily with $O_2^{\cdot-}$ and possibly H_2O_2 to produce a highly oxidizing, nonradical compound, peroxynitrite [16]. When protonated, peroxynitrite has reactivity similar to that of $^{\cdot}OH$. Cell culture studies suggest that, while $NO^{\cdot}$ itself is relatively nontoxic, peroxynitrite is quite neurotoxic. Peroxynitrite reacts

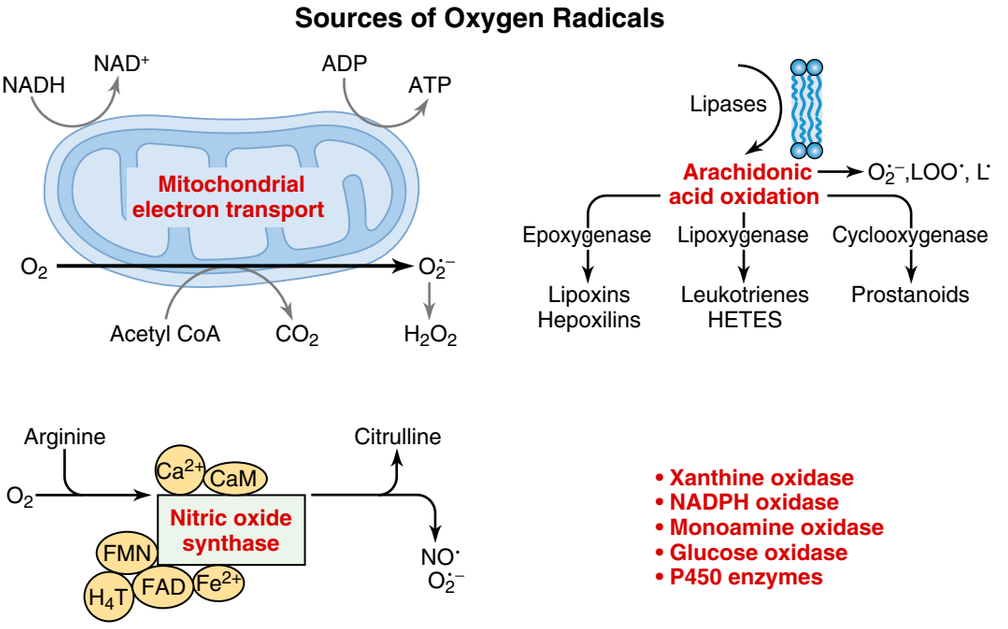

FIGURE 32-7 Sources of free radical formation which may contribute to injury during ischemia–reperfusion. Nitric oxide synthase, the mitochondrial electron-transport chain and metabolism of arachidonic acid are among the likely contributors. *CaM*, calcium/calmodulin; *FAD*, flavin adenine dinucleotide; *FMN*, flavin mononucleotide; H_4T, tetrahydrobiopterin; *HETES*, hydroxyeicosatetraenoic acids; *L·*, lipid alkoxyl radical; *LOO·*, lipid peroxyl radical; *NO·*, nitric oxide; $O^{\cdot-}_2$, superoxide radical.

with protein tyrosine residues to produce nitrotyrosine. Potential targets of tyrosine nitrosylation include many important cellular proteins, including Cu,Zn-SOD, aconitase, Mn-SOD and cytochrome oxidase (mitochondrial complex IV), all of which are inactivated by nitrosylation. Peroxynitrite may cause lipid peroxidation but may also generate nitrosolipids, which decrease lipid peroxidation by acting as peroxidation 'chain-breakers'. In addition, peroxynitrite targets DNA, leading to chain breaks and DNA base oxidation. Involvement of nNOS as a mediator of ischemic injury is suggested by the ability of nNOS inhibitors to reduce ischemic injury and by the neuroprotective effect of nNOS gene deletion in knockout mice. Peroxynitrite-mediated DNA damage can activate poly (ADP-ribose) polymerase (PARP or PARS), an enzyme involved in DNA repair. It has been suggested that activation of PARP-1 contributes to ischemic injury by causing release of apoptosis-inducing factor (AIF) from mitochondria, with subsequent entry of AIF into the nucleus to promote components of the nuclear changes associated with apoptosis [26]. Pharmacological studies *in vitro* and experiments with PARP knockout mice have suggested that inhibition of PARP provides substantial neuroprotection against ischemic neuronal injury. Another potentially neuroprotective agent may be hydrogen sulfide which, although a toxic gas, is present physiologically in brain in 10–150 μM concentrations and has been shown to inhibit peroxynitrite-mediated tyrosine nitration and inactivation of α_1 antiproteinase and peroxynitrite-induced cytotoxicity, protein nitration and oxidation in human neuroblastoma SH-SY5Y cells [26a]. H_2S also has been shown to protect neurons against glutamate-induced stress in primary cultures of embryonic rat cortical neurons via increasing levels of glutathione, which is an important anti-oxidant (see below) [26b].

Production of eicosanoids from polyunsaturated fatty acids such as arachidonic acid may generate reactive oxygen species. Arachidonic acid metabolism may also be a source of ROS production [27]. As discussed in Chapters 3 and 33, arachidonic acid undergoes an extensive array of reactions to biologically active lipids, the eicosanoids. These reactions may be accompanied by free-radical production. In particular, cyclooxygenase isoforms and 5-lipoxygenase contain heme iron and may generate a low concentration of superoxide anion constitutively. 12-lipoxygenases, which possess nonheme iron, may not release superoxide anion but may induce lipid peroxidation after translocation to membranes.

Brain antioxidant defenses modify ischemia–reperfusion injury. The high metabolic rate of brain cells implies a high baseline ROS production, and brain cells possess high concentrations of both enzymatic and small-molecule antioxidant defenses. SOD1 may represent as much as 1% of total protein in brain; it converts $O_2^{\cdot-}$ to H_2O_2, which is then further metabolized to water and oxygen by catalase and glutathione peroxidase. The SOD1 gene is located on chromosome 21 and codes for a 16–18 kDa subunit that binds one Cu^{2+} and one Zn^{2+}; the active enzyme is a homodimer. SOD2 is a homotetramer and the gene is on chromosome 6. An extracellular, glycosylated form, SOD3, has been shown in rodents to overlap in activity with SOD1. Several other enzymes unrelated to CuZn-SOD, for example, the *atx1-HAH* gene product, are also capable of acting as SODs. It remains to be determined to what extent SOD3 and these alternative dismutases complement SOD1 and SOD2 as antioxidant systems in humans.

Catalase and glutathione peroxidase provide two important cellular systems for eliminating H_2O_2. Catalase, a 56 kDa cytosolic hemoprotein homotetramer that can act without a cofactor, although it may bind NAD(P)H, functions as a peroxidase to convert H_2O_2 to water. It can be irreversibly inactivated by oxidation and demonstrates decreased activity after ischemia–reperfusion. Catalase is more abundant in astrocytes than in neurons and in white matter than in gray matter, but it can be induced in neurons by neurotrophins. There is substantially less catalase activity in brain than in other tissues, such as liver.

The human genes have been identified for four members of the glutathione peroxidase (GPx) family of selenoproteins. Classical GPx (GPx1) is a complex of four 23 kDa subunits. Plasma GPx (GPx3), GPx2 and a fourth enzyme, phospholipid hydroperoxide glutathione peroxidase (PHGPx), are monomers with extensive homology to classical GPx1. All four enzymes contain one selenium atom per subunit in the form of a selenocysteine and all use glutathione (GSH) as a cofactor to convert H_2O_2 to water. PHGPx is unique in its ability to detoxify not only H_2O_2 but fatty acid and cholesterol lipid hydroperoxides directly. In addition, it has a cytosolic isoform and an isoform with a mitochondrial targeting sequence. Both GPx1 and PHGPx proteins are present in the brain. The components of the glutathione peroxidase system, GPx, GSH, glutathione reductase and NAD(P)H, are present in the mitochondria as well as the cytoplasm. Several other proteins, including glutathione *S*-transferase, may contribute minor peroxidase activity.

Small-molecule antioxidants include glutathione, ascorbic acid (vitamin C), vitamin E and a number of dietary flavonoids. Because humans, in contrast to most other animals, are unable to synthesize vitamin C, this important antioxidant must be supplied entirely from dietary intake. Other proteins, such as thioredoxin and metallothionein, may also contribute to some extent to the cellular antioxidant pool.

Reactive oxygen species may modify both the excitotoxic and the apoptotic components of ischemic brain damage. In addition to direct effects of oxidative injury during ischemia–reperfusion, ROS may modify ischemic excitotoxicity by downregulating current through NMDA receptors. However, exposure to oxidative stress can be

expected to enhance NMDA-receptor-mediated neurotoxicity by depleting intracellular antioxidant defenses. Free radicals also contribute to apoptosis at several points in the apoptotic cascade, serving as initiators, early signals and possibly late effectors of apoptotic neuronal death. It may be this interaction of injury mechanisms, including excitotoxicity, ischemic apoptosis, oxidative injury, inflammation and impaired metabolism, that, in part, makes the brain so vulnerable to ischemic damage.

NEUROPROTECTIVE STRATEGIES FOR HYPOXIC–ISCHEMIC INJURY

Endogenous protective programs may include ischemic preconditioning and the heat shock response. Ischemic preconditioning (or ischemic tolerance) is 'the acute and/or chronic reaction to a potentially noxious stimulus, such as hypoxia, ischemia or inflammation … which protects the brain against subsequent ischemia' [28]. In a general sense, preconditioning is produced by exposure of tissue to a stimulus that can cause damage at close to (but below) the threshold for injury, and it is now clear that one stressor can induce tolerance against a different type of subsequent injury ('cross-tolerance'), indicating that changes associated with ischemic tolerance, *per se*, are not necessarily specific for ischemia. Preconditioning produces an extensive array of presumably protective responses that range from changes in excitotoxic cascades, to inhibition of inflammation and apoptosis, to enhancement of late repair mechanisms (reviewed in [28]).

One specific process that may contribute to protection in preconditioning is the *heat shock* response. This process was first described in response to mild thermal stress, which causes denaturation of proteins and induces expression of heat-shock proteins (HSPs). HSPs are now known to be induced by any stress that results in protein denaturation. Denatured proteins bind to HSP90, causing association with, and suppression of, HSFs (heat-shock factor proteins). Dissociation of HSFs from HSP90 allows them to be phosphorylated to the active form. Activated HSFs form trimers that then bind to the promoters of various HSPs to increase transcription. HSPs (which include a number of protein chaperones) bind to denatured proteins to restore their native conformation and activity. Overexpression of a number of HSPs, including HSP70 and HSP27, has been shown to protect cells from both necrotic and apoptotic death. In addition, HSP70 protects brain against ischemic injury or prolonged seizures *in vivo* [29]. Strategies that seek to induce the heat shock response have been targeted as potential therapies in ischemia in many organs, including brain.

Thrombolytics and drugs targeting several injury pathways have shown efficacy in models of hypoxia–ischemia. A number of neuroprotective strategies have been identified in animal models of ischemia, and many of these have been tested in human stroke patients. One therapeutic modality that has shown efficacy in humans is thrombolysis using either intravenous or intra-arterial infusion of tissue plasminogen activator. In addition to thrombolysis, many other drug therapies have shown protection in animal models of ischemic injury, including agonists of GABA receptors, antagonists of ionic glutamate (i.e. NMDA and AMPA), glycine, dopamine, opioid, endothelin and angiotensin receptors, ion channel blockers, phosphodiesterase inhibitors, minocycline (an antibiotic that has anti-inflammatory properties), estrogen, erythropoietin and a spectrum of antioxidants. Although most of these therapies have not consistently shown significant neuroprotection in clinical trials, a few appear to produce improvement after acute ischemic injury. Ebselen, an anti-inflammatory selenocompound that mimics glutathione peroxidase, inhibits DNA damage and lipid peroxidation after focal ischemic injury in animal studies, and has been found to reduce functional deficits after stroke in humans. Another compound, NXY-059, a nitrone spin trap with antioxidant properties, is currently undergoing clinical testing in stroke. Future studies on stroke therapy will focus on (1) engaging endogenous neuroprotective programs, such as ischemic preconditioning or the heat shock response, (2) combining thrombolytics with neuroprotective agents, such as antioxidants, and (3) maximizing the ability of the ischemic brain to recover tissue integrity and function.

REFERENCES

1. Kristian, T. and Siesjo, B. K. Changes in ionic fluxes during cerebral ischaemia. *Int. Rev. Neurobiol.* 40: 27–45, 1997.
2. Haddad, G. G. and Jiang, C. O_2-sensing mechanisms in excitable cells: role of plasma membrane K^+ channels. *Annu. Rev. Physiol.* 59: 23–42, 1997.
3. Welch, K. M. A., Caplan, L. R., Reis, D. J., Siesjo, B. K. and Weir, B. (eds.) *Primer on Cerebrovascular Diseases.* San Diego, CA: Academic Press, 1997.
4. Trotti, D., Rizzini, B. L., Rossi, D. *et al.* Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. *Eur. J. Neurosci.* 9: 1236–1243, 1997.
5. Nicholls, D. and Attwell, D. The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* 11: 462–468, 1990.
6. Graham, D. I. and Brierly, J. B. Vascular disorders of the central nervous system. In J. H. Adams, J. A. N. Corsellis and L. W. Duchen (eds), *Greenfield's Neuropathology.* New York: John Wiley & Sons, 1984, pp. 157–207.
7. Gasche, Y., Copin, J. C., Sugawara, T., Fujimura, M. and Chan, P. H. Matrix metalloproteinase inhibition prevents oxidative stress-associated blood–brain barrier disruption after transient focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 21: 1393–1400, 2001.
8. Lo, E. H., Broderick, J. P. and Moskowitz, M. A. tPA and proteolysis in the neurovascular unit. *Stroke* 35: 354–356, 2004.
9. Hall, E. D., Andrus, P. K., Smith, S. L. *et al.* Neuroprotective efficacy of microvascularly-localized versus brain-penetrating antioxidants. *Acta Neurochir. Suppl. (Wien)* 66: 107–113, 1996.

10. Agre, P., King, L. S., Yasui, M. *et al.* Aquaporin water channels–from atomic structure to clinical medicine. *J. Physiol.* 542: 3–16, 2002.
11. Choi, D. W. and Rothman, S. M. The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death. *Annu. Rev. Neurosci.* 13: 171–182, 1990.
12. Frederickson, C. J. Neurobiology of zinc and zinc-containing neurons. *Int. Rev. Neurobiol.* 31: 145–238, 1989.
13. Choi, D. W. Calcium: still center-stage in hypoxic–ischemic neuronal death. *Trends Neurosci.* 18: 58–60, 1995.
14. Chopp, M., Chan, P. H., Hsu, C. Y., Cheung, M. E. and Jacobs, T. P. DNA damage and repair in central nervous system injury: National Institute of Neurological Disorders and Stroke Workshop Summary. *Stroke* 27: 363–369, 1996.
15. Smart, T. G. Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. *Curr. Opin. Neurobiol.* 7: 358–367, 1997.
16. Beckman, J. S. and Koppenol, W. H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* 271: C1424–C1437, 1996.
17. Dykens, J. A. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+}: implications for neurodegeneration. *J. Neurochem.* 63: 584–591, 1994.
18. Koh, J. Y., Suh, S. W., Gwag, B. J., He, Y. Y., Hsu, C. Y. and Choi, D. W. The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science* 272: 1013–1016, 1996.
19. Wyllie, A. H., Kerr, J. F. and Currie, A. R. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68: 251–306, 1980.
20. Koketsu, N., Berlove, D. J., Moskowitz, M. A., Kowall, N. W., Caday, C. G. and Finklestein, S. P. Pretreatment with intraventricular basic fibroblast growth factor decreases infarct size following focal cerebral ischemia in rats. *Ann. Neurol.* 35: 451–457, 1994.
21. Greenlund, L. J., Deckwerth, T. L. and Johnson, E. M. Jr. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* 14: 303–315, 1995.
22. Halliwell, B. Reactive oxygen species and the central nervous system. *J. Neurochem.* 59: 1609–1623, 1992.
23. Davies, K. J. The paradox of aerobic life. *Biochem. Soc. Symp.* 61: 1–31, 1995.
24. Sugawara, T. and Chan, P. H. Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. *Antioxid. Redox Signal.* 5: 597–607, 2003.
25. Sheng, H., Bart, R. D., Oury, T. D., Pearlstein, R. D., Crapo, J. D. and Warner, D. S. Mice overexpressing extracellular superoxide dismutase have increased resistance to focal cerebral ischemia. *Neuroscience* 88: 185–191, 1999.
26. Hong, S. J., Dawson, T. M. and Dawson, V. L. Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. *Trends Pharmacol. Sci.* 25: 259–264, 2004.

26a. Whiteman, M., Armstrong, J. S., Chu, S. H., Jia-Ling, S. *et al.* The novel neuromodulator hydrogen sulfide: and endogenous peroxynitrite 'scavenger'? *J. Neurochem.* 90: 765–768, 2004.

26b. Kimura, Y and Kimura, H, Hydrogen sulfide protects neurons from oxidative stress, *The FASEB Journal express article* 10.1096/fj.04-1815fje. Published online May 20, 2004.

27. Schreiber, J., Eling, T. E. and Mason, R. P. The oxidation of arachidonic acid by the cyclooxygenase activity of purified prostaglandin H synthase: spin trapping of a carbon-centered free radical intermediate. *Arch. Biochem. Biophys.* 249: 126–136, 1986.
28. Dirnagl, U., Simon, R. P. and Hallenbeck, J. M. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci.* 26: 248–254, 2003.
29. Kelly, S. and Yenari, M. A. Neuroprotection: heat shock proteins. *Curr. Med. Res. Opin.* 18 Suppl 2: s55–s60, 2002.

BOX 32-1

Hydrogen Sulfide: Potential Neuroprotectant and Neuromodulator

George J. Siegel

A novel potentially neuroprotective reducing agent is hydrogen sulfide which, although a toxic gas and potent inhibitor of purified cytochrome oxidase c (Ki 0.3 μM [1]), is present physiologically in brain of humans and various species in 50 to 150 μM concentrations. It has been shown to inhibit peroxynitrite-mediated tyrosine nitration and inactivation of α_1 antiproteinase and peroxynitrite-induced cytotoxicity, protein nitration and oxidation in human neuroblastoma SH-SY5Y cells [2]. H_2S also has been shown to protect neurons against glutamate-induced stress in primary cultures of embryonic rat cortical neurons via increasing levels of glutathione, which is an important anti-oxidant (see Ch. 32) [3]. Of further interest is that H_2S induces non-glutamate calcium ion entry and calcium waves in primary rat embryonic astrocyte cultures and in hippocampal slices [4] in addition to enhancing NMDA receptor responses to glutamate and LTP in hippocampus (see Ch. 10 [5]). H_2S activates smooth muscle ATP-dependent K^+ channels (see Ch. 6) resulting in membrane hyperpolarization and muscle relaxation [6]. It has been reported that brain concentrations of H_2S are reduced in Alzheimer's disease [7].Thus, H_2S has complex biochemical effects in brain function not all understood and the conditions determining the balance between potentially toxic versus physiologically beneficial effects in intact brain are not known. Comparable metabolic together with physiologic data from intact brain under H_2S exposure are not yet available. A report that H_2S can induce a reversible state resembling hibernation with decreased core body temperature and decreased metabolic rate in mice may be of considerable significance with respect to protection of brain function under conditions of metabolic compromise if confirmed by further investigation and analysis of intact brain parameters [8].

References

1. Petersen, L.C. The effects of inhibitors on the oxygen kinetics of cytochrome c oxidase. *Biochim. Biophys. Acta*, 460: 299–307, 1977.
2. Whiteman, M., Armstrong, J. S., Chu, S. H., Jia-Ling, S. *et al.*, The novel neuromodulator hydrogen sulfide: and endogenous peroxynitrite 'scavenger'? *J. Neurochem.* 90: 765–768, 2004.
3. Kimura, Y. and Kimura, H. Hydrogen sulfide protects neurons from oxidative stress, *The FASEB Journal express article* 10.1096/fj.04-1815fje. Published online May 20, 2004.
4. Nagai, Y., Tsugane, M., Oka, J.-I. and Kimura, H. Hydrogen sulfide induces calcium waves in astrocytes, *The FASEB Journal express article* 10.1096/fj.03-1052fje. Published online Jan.20, 2004.
5. Abe, K. and Kimura, H., The possible role of hydrogen sulfide as an endogenous neuromodulator, *J. Neurosci.* 16: 1066–1071, 1996.
6. Zhao, W., Zhang, J., Lu, Y. and Wang, R. The vasorelaxant effects of H_2S as a novel endogenous gaseous K_{ATP} channel opener, *EMBO J.* 20: 6008–6016, 2001.
7. Eto, K., Asada, T., Arima, K., Makifuchi, T. and Kimura, H. Brain hydrogen sulfide is severely depressed in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 293: 1485–1488, 2002.
8. Blackstone, E., Morrison, M. and Roth, M.B. H_2S induces a suspended animation-like state in mice. *Science* 308: 518, 2005.

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CHAPTER

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Eicosanoids, Docosanoids, Platelet-Activating Factor and Inflammation

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STORAGE OF LIPID MESSENGERS IN NEURAL MEMBRANE PHOSPHOLIPIDS

Excitable membranes maintain and rapidly modulate substantial transmembrane ion gradients in response to stimuli. This function requires the presence of ion pumps, neurotransmitter receptors and other associated membrane proteins. Excitable membranes have a phospholipid composition that differs from other membranes, a property assumed to be related to their highly specialized functions. The understanding of excitable membrane organization has conceptually evolved from the lipid bilayer with embedded proteins to a highly dynamic, heterogeneous patchwork of microdomains that contain ion channels, receptors, transporters and other proteins. Cellular membranes in the nervous system were divided in the past into relatively more fluid membranes (e.g. those of cells of gray matter) and relatively more rigid membranes (e.g. oligodendrocyte plasma membrane that spirals around the axon to form the myelin), according to the higher or lower content of polyunsaturated fatty acids (PUFA) in phospholipids (see also Chs 1–4). Currently in neurons, glia and endothelial cells of the cerebrovasculature, several phospholipid pools are recognized as reservoirs of lipid messengers. In addition, *lipid rafts* have been described, isolated and their chemical composition studied in specific cellular compartments of the nervous system, including dendrites, where they are associated with specific postsynaptic proteins. Lipid rafts are microdomains enriched in cholesterol and sphingolipids. Interestingly, there are subtypes of lipid raft, which vary by their resistance to detergent extraction, density and their raft marker proteins, Thy-1 and caveolin. Several proteins have been found segregated in these microdomains, including glycophosphatidyl (GPI) anchors, signaling proteins, proteins interacting with the actin cytoskeleton and proteins involved in cell trafficking and in endocytosis. PSD-95, GRIP and glutamate AMPA receptors have been found in lipid rafts isolated from rat brain. Moreover, the normal density of synapses and dendritic spines seems to depend on lipid rafts, since changes in cholesterol availability modify rafts and in turn the properties of synapses and spines. The same study also demonstrated that AMPA receptor internalization is affected by compositional changes in the rafts (see also Ch. 15).

Specific lipid messengers are cleaved from reservoir phospholipids by phospholipases upon activation by various stimuli. These stimuli include neurotransmitters, neurotrophic factors, cytokines, membrane depolarization, ion channel activation and others. Lipid messengers regulate and interact with multiple other signaling cascades, contributing to the development, differentiation, function, protection and repair of the cells of the nervous system.

Under physiologic conditions, the balance of membrane lipid metabolism, particularly that of arachidonoyl and docosahexaenoyl chains, favors a very small and tightly controlled cellular pool of free arachidonic acid (AA, 20:4n-3) and docosahexaenoic acid (DHA, 22:6n-3), but levels increase very rapidly upon cell activation, cerebral ischemia, seizures and other types of brain trauma [1, 2]. Other free fatty acids (FFAs) in addition to AA, released during cell activation and the initial stages of focal and global cerebral ischemia, are stearic acid (18:0), palmitic acid (16:0) and oleic acid (18:1).

Phospholipids in synaptic membranes are an important target in seizures, head injury, neurodegenerative diseases and cerebral ischemia. Synaptic membranes are excitable membranes enriched in phospholipids esterified with the polyunsaturated fatty acids AA and DHA which form a significant proportion of the FFAs rapidly released during ischemia, seizure activity and other brain trauma.

Some molecular species of phospholipids in excitable membranes are reservoirs of bioactive lipids that act as messengers. Signals, such as those resulting from neurotransmitter receptor occupancy, trigger the release of phospholipid moieties via the activation of phospholipases. Some of the breakdown products are bioactive, such as inositol 1,4,5-trisphosphate (IP3), diacylglycerol (DAG; see Ch. 20) and AA. Another product, lysoplatelet-activating factor (lyso-PAF), upon further metabolism, gives rise to the bioactive lipid PAF (1-*O*-alkyl-2-acetyl-sn-3-glycerol-3-phosphocholine). Eicosanoids are derived from enzyme-mediated oxygenation of AA. AA and its metabolites function as intra- and intercellular messengers. In contrast, the significance of DHA remains an enigma. DHA-containing phospholipids may provide a specific milieu in which the membrane proteins of excitable membranes function. For example, the high concentrations of DHA phospholipids of outer segments of retinal rod photoreceptors provide a highly specialized membrane environment for rhodopsin (see also Ch. 49). In fact, a specific requirement for DHA phospholipids in rhodopsin function has been documented [3]. Recently, the isolation and structural characterization of neuroprotectin

D1 (10,17*S*-docosatriene) from DHA using tandem liquid chromatography-photodiode array-electrospray ionization-tandem mass spectrometry (LC-PDA-ESI-MS-MS)-based lipidomic analysis have been documented in ischemic brain [4] and retinal pigment epithelium [5]. This new lipid is called neuroprotectin D1 (1) because of its *neuroprotective* properties in brain ischemia–reperfusion [4] and in oxidative stress-challenged retinal pigment epithelial cells [5]; (2) because of its potent ability to *in*activate proapoptotic signaling (see apoptosis, Ch. 35) [5]; and (3) because it is the first identified neuroprotective mediator derived from DHA.

Mammalian phospholipids generally contain polyunsaturated fatty acyl chains almost exclusively esterified to the second carbon of glycerol. Phospholipase (PL)A_2 activation cleaves the acyl chains at C2 of the glycerol backbone, thus releasing free polyunsaturated fatty acids AA and DHA. There are a number of potential fates for the phospholipase products. They may be reincorporated into membrane lipids or further metabolized to biologically active derivatives. The remaining lysophospholipid can be either re-esterified and reincorporated into a membrane phospholipid or further metabolized. The discovery of neuroprotectin D1 [5] from DHA suggests that docosanoids may arise as well.

This chapter surveys the neurochemistry of lipid messengers, as well as the mechanisms by which bioactive lipids accumulate upon stimulation in response to injury, cerebral ischemia, seizures, neurotrauma or neurodegenerative diseases, and their significance in pathophysiology. Emphasis is placed on three groups of bioactive lipids: AA and its metabolites, known collectively as eicosanoids; PAF, a highly potent ether phospholipid; and the newly identified DHA-derived mediator, neuroprotectin D1.

AA metabolites and PAF have initially been studied in terms of their roles in the inflammatory response, such as increased vascular permeability and the activation of and infiltration by inflammatory cells. It is now becoming apparent, however, that these bioactive lipids have significant neurobiological actions in ion channel functions, receptors, neurotransmitter release, synaptic plasticity and neuronal gene expression.

Moreover, bioactive lipids may be considered dual messengers: they modulate cell functions as messengers and they become part of the response of the nervous tissue to injury, broadly referred to as the inflammatory response. This response occurs in ischemia–reperfusion damage associated with stroke, various forms of neurotrauma, infectious diseases and neurodegenerative diseases such as Alzheimer's disease. Inflammation in the nervous system differs from that in other tissues. If the blood-brain barrier is broken, blood-borne inflammatory cells (e.g. polymorphonuclear leukocytes, monocytes, macrophages) invade the intercellular space and glial cells are activated, particularly microglia, which play a prominent role in the inflammatory response. These responses may lead to neuronal cell injury and death [6]. In addition, ischemia, seizures and other forms of injury upregulate lipid signaling in neurons, mainly through *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (see also details in Ch. 15) (Fig. 33-1). As a consequence, PLA_2 is activated, AA is released, eicosanoids and PAF are synthesized and cyclooxygenase (COX)-2 is induced in neurons. Other neuronal correlates of the inflammatory response include signaling by cytokines, nitric oxide and various growth factors. Activation of arriving inflammatory cells also plays a role in initial defenses against injury, removal of cellular debris, and the longer-term repair/wound healing of the nervous system. Several lipid messengers are released from these cells and may participate in beneficial actions. Much remains to be learned in this area of integration of our knowledge of the inflammatory response and neuroimmune/repair signaling.

Identification of lipids with biological activity has progressed remarkably over the last few years. While the inositol phosphates have been known for some time to play fundamental roles in cell biology, others, such as lysophosphatidic acids, previously considered only as intermediates in phospholipid metabolism, have recently been found to possess important functions, and lysophosphatidic acid and sphingosine receptors have recently been cloned [16–18]. Although the specific roles of many of these bioactive lipids and lipid derivatives in neuroscience remain sparsely explored, this review should give the reader a broad appreciation of the diversity of bioactive lipids, particularly eicosanoids, docosanoids and PAF, which are produced in brain during injury (e.g. trauma or ischemia), during seizures and under inflammatory conditions.

PHOSPHOLIPASE A_2

Phospholipase A_2 catalyzes the cleavage of the fatty acyl chain from the second carbon of the glycerol backbone of phospholipids. There are a wide variety of PLA_2 types [19] that include, in addition to intracellular PLA_2, a low-molecular-weight secretory PLA_2 ($sPLA_2$) that synergizes glutamate-induced neuronal damage [13]. Whereas pathways leading to PLA_2 activation are part of normal neuronal function, ischemia–reperfusion enhances these events, overproducing PLA_2-derived lipid messengers, such as enzymatically produced AA- and DHA-oxygenation derivatives, and nonenzymatically generated lipid-peroxidation products and other reactive oxygen species (ROS), all of which may be involved in neuronal damage. Among the consequences of PLA_2 activation by ischemia are alterations in mitochondrial function by the rapid increase in the brain FFA pool size, for example the uncoupling of oxidative phosphorylation from electron transport in the mitochondrial respiratory chain and the generation of ROS. Intracellular PLA_2 types are located either in the cytosol or in noncovalent association with membranes and consist of Ca^{2+}-dependent types ($iPLA_2$).

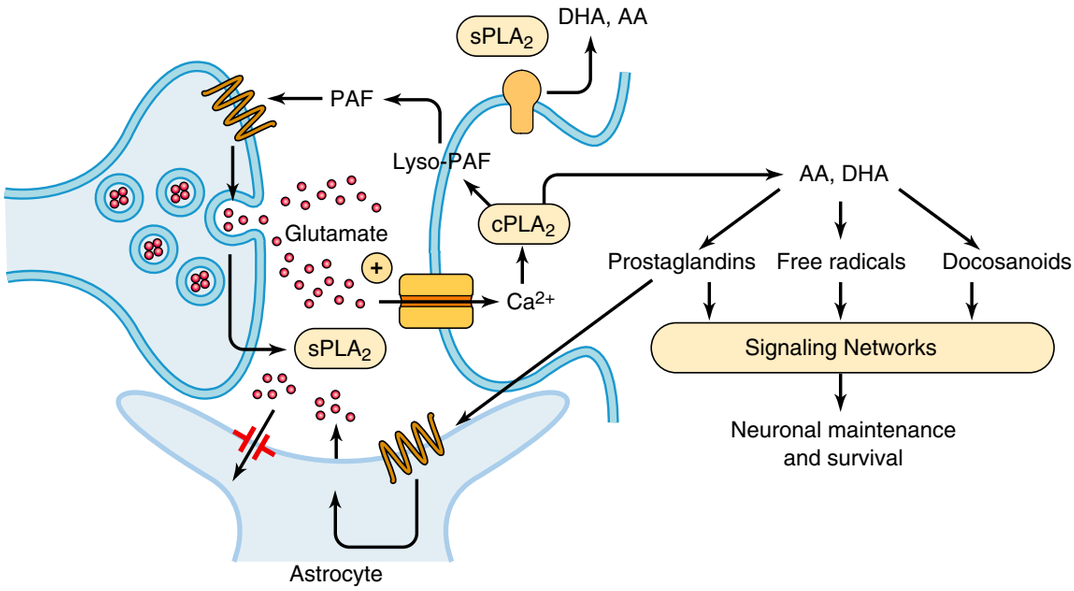

FIGURE 33-1 A depolarizing stimulus at the presynaptic terminal triggers glutamate release. Glutamate binds to the NMDA receptor and as a consequence an influx of calcium ions occurs in the postsynaptic neuron. Calcium-mediated activation of the cytoplasmic PLA_2 (*cPLA*$_2$) results in the release of arachidonic acid (*AA*), docosahexaenoic acid (*DHA*) and lyso-PAF, the PAF precursor. Although PAF has a very short biological half-life, on repeated stimulus sufficient PAF accumulates to diffuse back across the synaptic cleft. Experimental evidence for this was provided by injecting PAF into the postsynaptic neuron and monitoring neurotransmitter release [7]. PAF binds to its presynaptic receptor and enhances glutamate exocytosis by an as yet undefined mechanism. During synaptic plasticity events, PAF may also activate gene expression that in turn is probably involved in long-term alterations of synaptic function (not shown here). Cell-surface PAF-receptor antagonists confer neuroprotection during ischemia–reperfusion and inhibit PAF-induced glutamate release from hippocampal neurons and CA1 LTP formation, presumably through the same mechanism. The inhibitory effects of this antagonist on glutamate release could account in part for its neuroprotection in ischemia–reperfusion. Secretory PLA_2 (*sPLA*$_2$) may be released from the presynaptic terminal [8]: $sPLA_2$ binding sites are present in neurons [9–12], and $sPLA_2$ promotes active AA remodeling in neurons in culture [13] and may also promote DHA release. DHA may also be released by $cPLA_2$. Free DHA may subsequently follow enzyme-mediated oxygenation pathways and lead to the synthesis of docosanoids, messengers made in the retina [14] and brain [4]. Free radicals would accumulate during oxidative stress. Downstream lipid signaling modulates neuronal function and survival. Synapses are intimately surrounded by astrocytes, which express glutamate transporters that remove the excitatory neurotransmitter from the vicinity of the synaptic cleft. Astrocytes also respond to prostaglandins by releasing glutamate through a Ca^{2+}-dependent mechanism [15].

Calcium-ion-dependent phospholipase A_2 with a preference for polyunsaturated fatty acyl chains is involved in bioactive lipid formation. Calcium-ion-dependent phospholipase A_2 ($cPLA_2$) is regulated by transcription, post-translational modulation of enzyme activity and membrane translocation. Membrane translocation of $cPLA_2$ to endoplasmic reticulum and nuclear membranes is mediated via a specific Ca^{2+}-dependent domain similar to those seen in protein kinase C, phospholipase C and GTPase-activating protein [20, 21]. This site is consistent with that of other enzymes of AA metabolism, such as prostaglandin G/H synthases (PGS), also termed cyclooxygenases (COX-1 or -2) [22], as well as 5-lipoxygenase and its activator protein [23].

The catalytic activity of $cPLA_2$ is stimulated by phosphorylation catalyzed by the mitogen-activated protein kinase (MAPK) at Ser505. This modification stimulates enzyme activity only, indicating that translocation and phosphorylation are independent mechanisms of $cPLA_2$ regulation [21].

Synaptic stimulation, ischemia or seizure activates phospholipase A_2 and releases arachidonic and docosahexaenoic acids. Ischemia or seizure triggers accumulation of free AA, DHA and other FFA in the brain(see also Chs 32, 37). This reflects PLA_2 activation in excitable membranes [24]. While little is known about the mechanisms that control its activity, the importance of $cPLA_2$ in ischemic brain injury is strongly supported by the recent finding that $cPLA_2$-knockout mice have substantially reduced infarcts and neurologic deficits in a model of stroke [25].

Shifts in intracellular pH may be another mechanism by which intracellular PLA_2 activity can be regulated. Glutamate-induced AA release in mouse cortical neuronal cultures is mediated in part by a membrane-associated PLA_2 activity, which is upregulated in alkaline pH and is therefore sensitive to the shifts in pH induced by excitatory neurotransmission.

There are also mechanisms in the brain for the downregulation of intracellular PLA_2 activity by lipocortins, a

family of Ca^{2+}- and phospholipid-binding proteins that act as endogenous inhibitors of PLA_2. The steroid-inducible lipocortin-1 is present in neuronal and glial cells, especially in the hippocampus, where it may act as an endogenous neuroprotective agent [26]. Exogenous lipocortin-1 administered intracerebroventricularly to rats significantly reduces the infarct size and edema induced by cerebral ischemia and attenuates excitotoxic damage mediated by NMDA receptors. Glucocorticoid hormones inhibit lipocortin synthesis.

Secretory phospholipases A_2 are of relatively low molecular weight and have a high number of disulfide bridges, making them relatively more resistant to denaturation. The mammalian $sPLA_2$ types can be subdivided on the basis of amino acid sequence into several groups that include pancreatic or group I $sPLA_2$, the members of which function in pancreatic secretions, smooth muscle contraction, cell proliferation and fertilization; and synovial or group II PLA_2, the members of which function in inflammatory responses. Both have been found also to be expressed in the nervous system.

There are high-affinity receptors that bind secretory phospholipases A_2. The muscle (M-type) and neuronal (N-type) receptors are structurally and pharmacologically distinct. The M-type consists of a single ≈180 kDa subunit and binds both OS1 and OS2 $sPLA_2$ (purified from the venom of the Australian taipan snake *Oxyuranus scutellatus scutellatus*). The N-type is composed of three major polypeptides of 34, 48 and 82 kDa and binds OS2 and bee venom $sPLA_2$ but not OS1 [24, 27]. Expression of the M- and N-type receptors is not limited to their nominal sites. In fact, both types are widely distributed in different cells and tissues. The M-type receptor has been cloned from rabbit and human tissues and, independently, from mouse and bovine tissues; it mediates group I $sPLA_2$ cellular actions. The receptor consists of a single 180–200 kDa glycoprotein subunit that bears significant sequence homology to the macrophage mannose receptor and to other members of the C-type lectin superfamily. The recombinant receptor binds both mammalian group I and group II $sPLA_2$ with high affinity. Studies using enzymatically inactive mutants of group I $sPLA_2$ to stimulate prostaglandin E_2 (PGE_2) synthesis in rat mesangial cells suggest that at least some of the effects mediated through the receptor are independent of $sPLA_2$ enzymatic activity. Potential ligands for the N-type receptor may be the group II $sPLA_2$ induced in rat brain during ischemia.

EICOSANOIDS

Arachidonic acid is converted to biologically active derivatives by cyclooxygenases and lipoxygenases. These metabolites, referred to collectively as eicosanoids (Figs 33-2, 33-3), are potent messengers that modulate cell function and are also involved in pathophysiology. Several nervous system functions engage eicosanoids. In addition, these messengers participate in inflammatory responses and other pathologic processes in the nervous system. COXs are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin or ibuprofen. Mainly as a consequence of studies on NSAIDs, the significance of prostaglandins as critical modulators of immune responses, pain, fever, inflammation, mitogenesis and apoptosis has been established.

Prostaglandins are very rapidly released from neurons and glial cells. Synaptic activation and certain forms of injury, such as ischemia–reperfusion or seizures, trigger prostaglandin synthesis and rapid efflux into the intercellular space [28]. These lipid mediators, in turn, elicit their signaling through autocrine and paracrine routes. Several prostaglandin receptors have been cloned. Figure 33-4 depicts the PGE_2 receptor (EP receptor), which in turn generates cAMP by activating adenylyl cyclase. The PGE_2 receptor belongs to the seven-transmembrane-domain receptor family and is coupled via G proteins to cAMP signaling (see Ch. 19). This depends on the activity of protein kinase A (PKA), a heteromeric enzyme that, upon binding cAMP to its regulatory subunit, releases the catalytic subunit. The catalytic subunit, in turn, activates gene transcription by phosphorylation of a DNA-binding protein, namely, the cAMP response element binding (CREB) protein (discussed in detail in Ch. 26). Several genes contain consensus sequences for CREB. Expression of these genes in turn is modulated by this lipid signaling pathway, as well as by other signaling pathways. CREB has been implicated in plasticity changes of synaptic circuits, memory formation, and in behavior.

Arachidonic acid is also the substrate for lipoxygenase and, as in the case of cyclooxygenase, molecular oxygen is required. The exact mechanisms controlling the channeling of AA through COXs or lipoxygenases are not clearly understood. However, there is growing evidence that subcellular compartmentalization is a major factor in the channeling of AA through either pathway.

PLATELET-ACTIVATING FACTOR

Phospholipid molecules of membranes from neurons and glial cells store a wide variety of lipid messengers. Receptor-mediated events and changes in $[Ca^{2+}]i$, such as occur during excitatory neurotransmission and activity-dependent synaptic plasticity, activate phospholipases that catalyze the release of bioactive moieties from phospholipids, which then participate in intra- and/or intercellular signaling pathways.

Platelet-activating factor is a very potent and short-lived lipid messenger. It is known to have a wide range

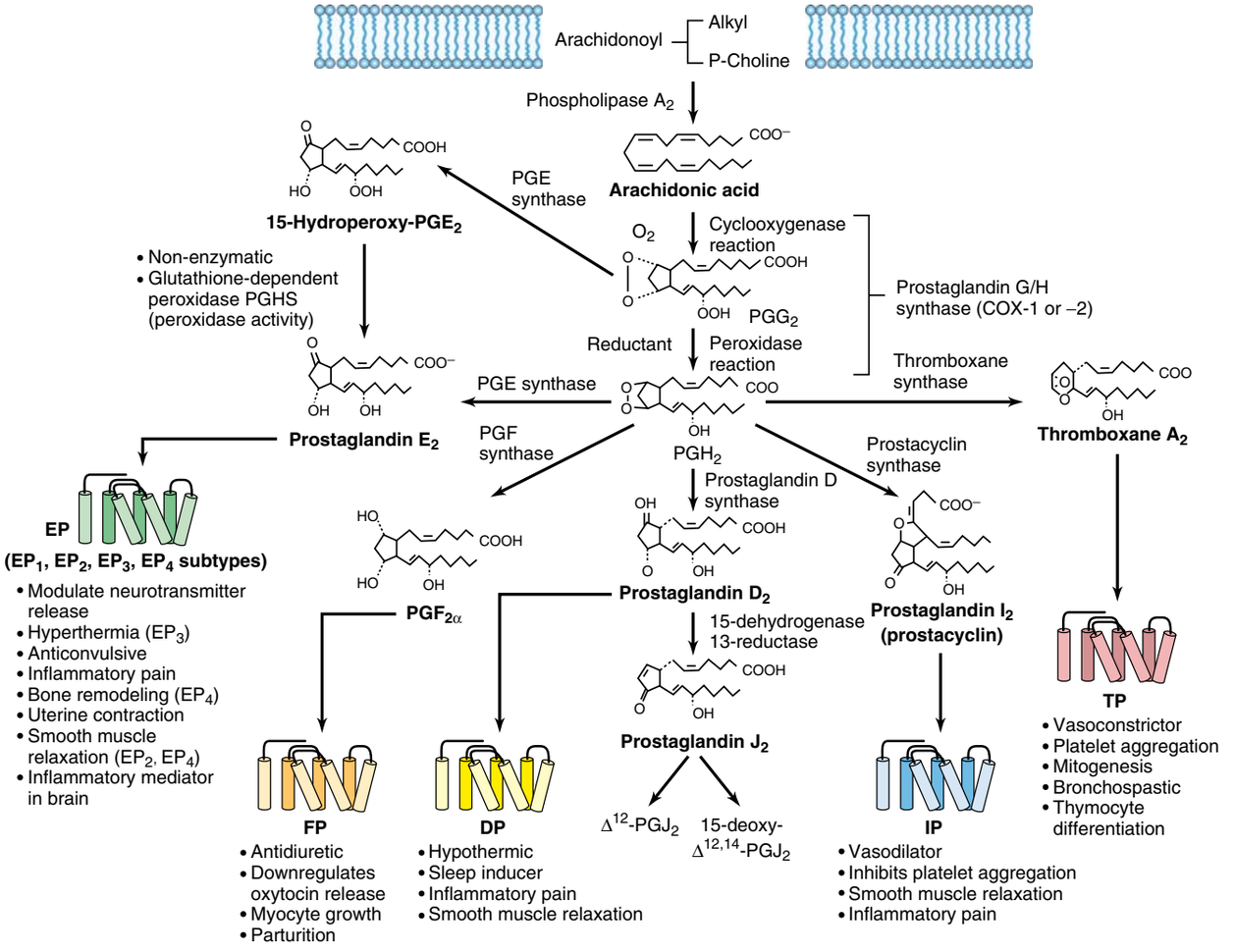

FIGURE 33-2 The arachidonic acid cascade. Free AA released by phospholipase A_2 (or PLC-diacylglycolipase-monoacylglycerollipase) undergoes cyclooxygenation and peroxidation mediated by prostaglandin G/H synthase. Specific tissue and product-specific isomerases mainly utilize PGH_2 to generate prostaglandins, prostacyclin and thromboxane. Receptors as well as some of the bioactivities of specific eicosanoids are illustrated.

of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as a potent inducer of gene expression in neural systems (Fig. 33-5). Thus, in addition to its acute roles, PAF can potentially mediate longer-term effects on cellular physiology and brain functions.

PAF enhances glutamate release in synaptically paired rat hippocampal neurons in culture [27]. The PAF analog methylcarbamoyl (mc-PAF), but not the biologically inactive lyso-PAF, increases excitatory synaptic responses. Action of the inhibitory neurotransmitter GABA is unaffected by mc-PAF under these conditions. The presynaptic PAF-receptor antagonist BN 52021 blocks the mc-PAF-enhanced glutamate release. In addition, mc-PAF increases presynaptic glutamate release since it does not augment the effects of exogenously added glutamate, and it evokes spontaneous synaptic responses characteristic of enhanced neurotransmitter release. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (LTP) [7, 30], synaptic plasticity and memory formation (see LTP and memory in Ch. 53) [31, 32].

Ischemia and seizures increase platelet-activating factor content in the brain. Furthermore, the brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive lyso-PAF [33, 34]. Presynaptic membranes display PAF binding that can be displaced by BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree. It is likely that this PAF-binding site is identical to a seven-transmembrane PAF receptor. BN 52021 inhibits both PAF-induced glutamate release [27] and long-term potentiation [7]. Moreover, this antagonist is neuroprotective in ischemia–reperfusion damage in the gerbil brain [35]. These findings together indicate that PAF, when overproduced at the synapse during ischemia, promotes enhanced glutamate release, which in turn contributes to excitotoxicity through the activation of postsynaptic receptors.

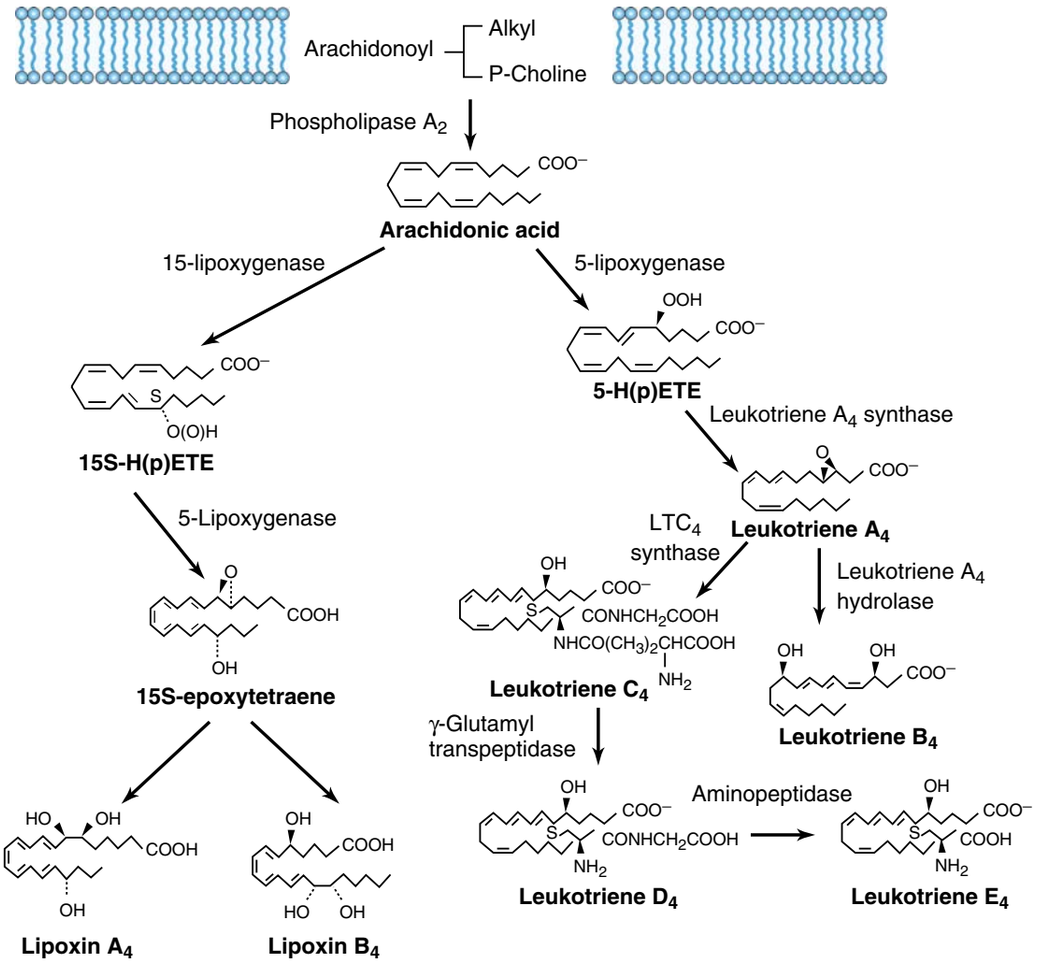

FIGURE 33-3 Arachidonic acid lipoxygenation. Lipoxygenase enzymes oxygenate arachidonic acid in different positions, thus they are named the 5-, 8-, 11-, 12- and 15-lipoxygenases and catalyze reactions that produce bioactive arachidonic acid derivatives. This figure illustrates as an example the synthesis of leukotriene B_4 and of sulfidopeptide leukotrienes (C_4, D_4 and E_4), the 5-lipoxygenase pathway. It also depicts the synthesis of lipoxins by 15-lipoxygenase.

CYCLOOXYGENASES

Cyclooxygenase-1 is a constitutive enzyme that converts arachidonic acid to prostaglandin H_2. COX-2 is inducible by cytokines, glutamate, growth factors, PAF and other mediators and is inhibited by glucocorticoids. COX-2 is encoded by an early-response gene, and its mRNA has a short half life (Table 33-1). In the human neocortex, the half-life is about 3 hours, as compared to 12 hours for COX-1 mRNA. In most tissues, stimulation, injury, inflammatory stimuli and other forms of cellular stress trigger expression of the COX-2 gene. However, brain, macula densa of kidney, testes and the female reproductive system also display constitutive levels of COX-2. In brain, the relatively high constitutive COX-2 expression appears to be almost exclusively neuronal. Dendrites and the perinuclear region are enriched in COX-2. Moreover, COX-2 expression seems to be regulated by synaptic activity [36]. Recently, COX-3 was characterized as generated from COX-1 intron-1 retention [37]. COX-3 is expressed in brain [37], brain microvasculature [38] and has been proposed to be a target of the analgesic/antipyretic acetaminophen [37, 39].

COXs thus catalyze the same first committed step of the AA cascade (Fig. 33-2). COX-2, however, is expressed in response to mitogenic and inflammatory stimuli and encoded by an early-response gene. To date we do not understand how COX-3 expression is regulated. In contrast, COX-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal COX-2 expression [36]. This expression is modulated by synaptic activity, such as long-term potentiation, and involves the NMDA glutamate receptors [36, 40].

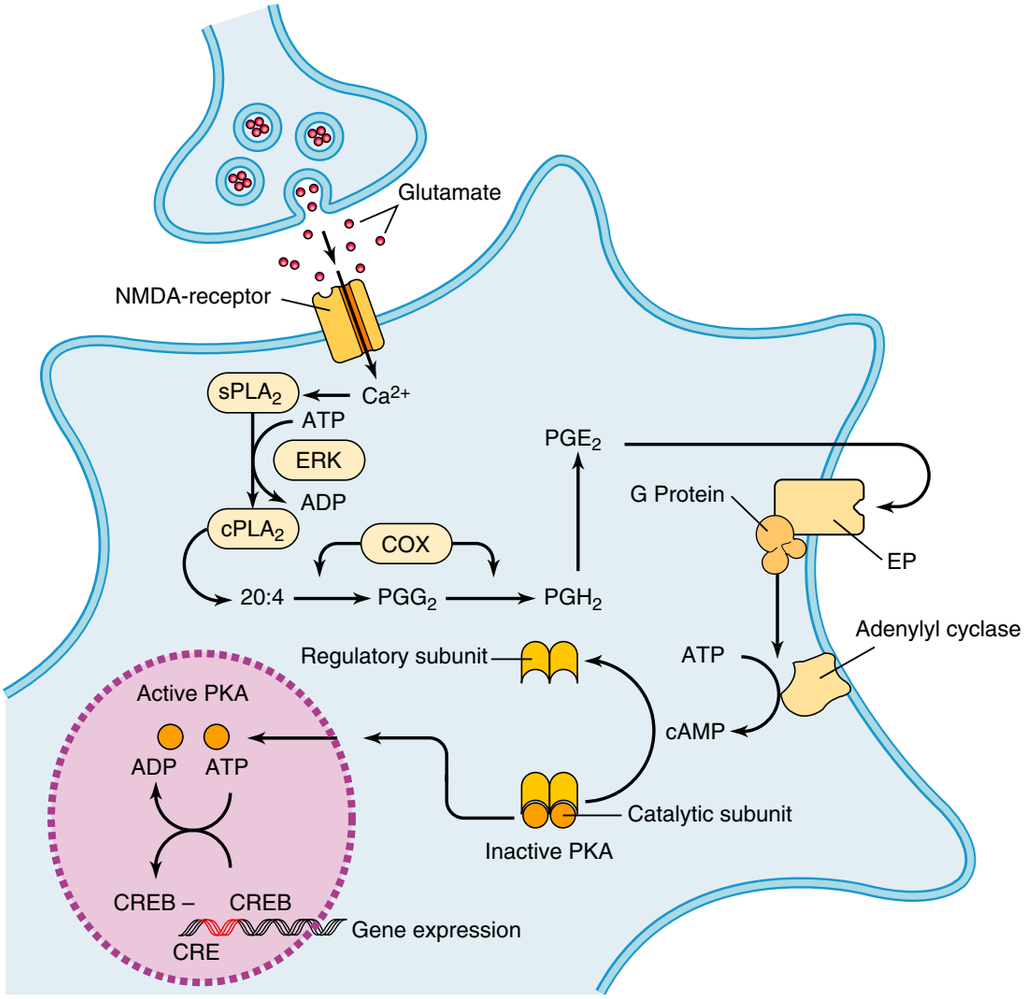

FIGURE 33-4 Prostaglandin signaling pathway triggered by the excitatory amino acid neurotransmitter glutamate. *COX*, cyclooxygenase; *cPLA$_2$*, calcium-dependent phospholipases A$_2$; *CRE*, cAMP response element; *CREB*, cAMP-response element binding protein; *EP receptor*, prostaglandin E2 receptor; *ERK*, extracellular signal-regulated kinase; *NMDA*, *N*-methyl-D-aspartate; *PGG$_2$* and *PGH$_2$* are short-lived intermediates in the synthesis of prostaglandin E$_2$ (*PGE$_2$*); *PKA*, protein kinase A; *sPLA$_2$*, secretory phospholipase A$_2$.

Platelet-activating factor is a transcriptional activator of cyclooxygenase-2. PAF induces mouse COX-2-promoter-driven luciferase activity transfected in neuroblastoma cells, such as NG108–15 or SH-SY5Y, and in NIH 3T3 cells (Fig. 33-5). The intracellular PAF antagonist BN 50730 inhibits PAF activation of this construct [41].

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures. Several early-response genes encode transcription factors, which in turn modulate the expression of other genes, whereas others encode inducible enzymes (Fig. 33-5). The glutamate analog kainic acid promotes extensive neuronal damage, particularly in the hippocampus, and induces early-response genes such as the transcription factor *zif-268*. *COX-2* is also induced under these conditions, but there are striking differences in the magnitude and duration of the induction of *COX-2* as compared with *zif-268*. *COX-2* mRNA, 2 hours after kainic acid injection, showed a 35-fold increase in hippocampus, as compared to only a 5.5-fold increase for *zif-268* [42]. Also, a peak in *COX-2* mRNA abundance was evident at 3 hours, a 71-fold increase, as compared with 1 hour for *zif-268*, a 10-fold increase. The *zif-268* mRNA time course of changes in the hippocampus corresponds to the expected profile of early-response genes, that is, a rapid decrease in abundance after the peak. *COX-2*, however, displayed sustained upregulation for several hours after kainic acid injection, a 5.2-fold increase remaining at 12 hours [42].

Cyclooxygenase-2 participates in aberrant synaptic plasticity during epileptogenesis. Neuronal COX-2 expression is also upregulated in experimental epileptogenesis, when aberrant synaptic plasticity is thought to

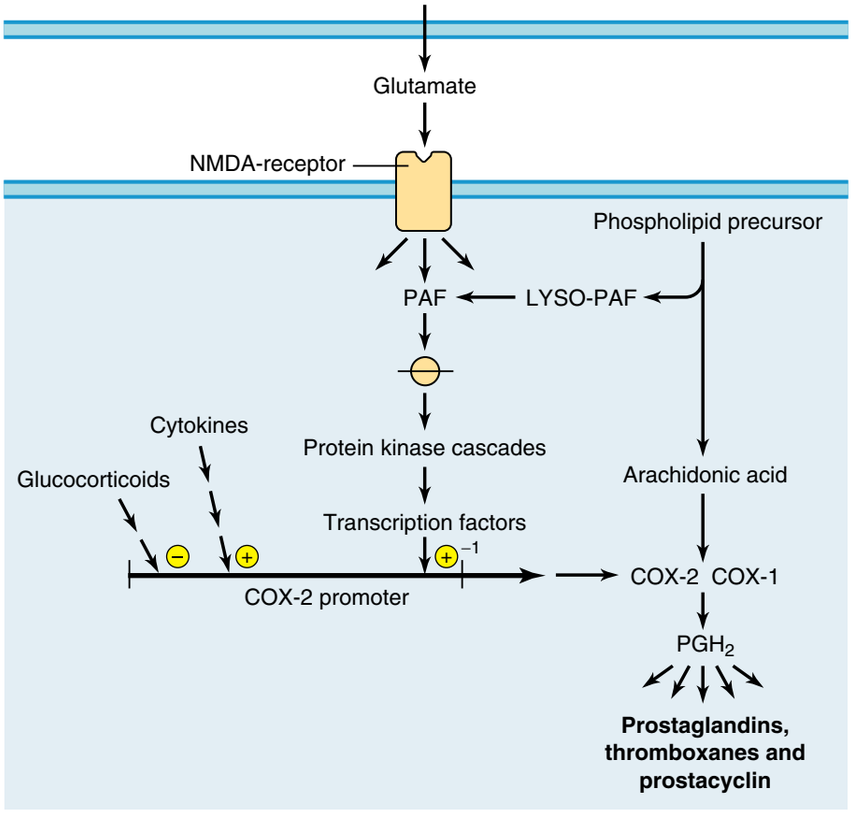

FIGURE 33-5 Seizure- or ischemia-triggered signaling events linking synapse activation and cyclooxygenase-2 (*COX-2*) gene expression in neurons. *N*-methyl-D-aspartate (NMDA) receptor activation by glutamate leads to phospholipase A_2 (*PLA_2*) activation and the generation of platelet-activating factor (*PAF*) and of arachidonic acid. PAF is synthesized through other metabolic routes as well [29], and elicits its actions through a PAF receptor. PAF activates COX-2 gene expression through protein kinase cascades and transcription factors. The COX-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). COX-2 protein then catalyzes the conversion of arachidonic acid into prostaglandin H2 (PGH2), the precursor of eicosanoids. Constitutive activity of COX-1 also catalyzes this metabolic step.

TABLE 33-1 Characteristics of cyclooxygenases or prostaglandin synthetases (IUPAC:IUB EC 1.14.99.1)

	Cyclooxygenase-1	Cyclooxygenase-2
	Constitutive	Inducible in neurons, macula densa of kidney; testes and female reproductive system also contain constitutive COX-2
Chromosome localization		1q25 10 exons, 7.5 kb
mRNA	≈5.2 kb ≈2.8 kb	≈4.3–4.6 kb ≈2.8 kb
Half-life	≥12 h	≤3 h (human neocortex) ≈1–2 h (IMR-90 cells) ≈1–3 h (IM9, HUVEC)
Function	Primarily cellular homeostasis	Inflammation and mitogenesis, neuronal Constitutive COX-2 is involved in synaptic plasticity Plays a role in neurodegenerative disease, epilepsy and stroke

be activated. The experimental kindling model resembles aspects of mesial temporal lobe epilepsy [43]. Kindling epileptogenesis is triggered by repeated subconvulsive stimulation, which gradually results in intensified seizures (Ch. 37). Both COX-2 and $cPLA_2$ expression are activated, indicating that the free AA released is converted into prostaglandins during epileptogenesis [44]. Nimesulide, a COX-2 blocker, decreases kindling epileptogenesis. The inability of nimesulide to completely inhibit kindling development suggests either a limited bioavailability of the drug to neuronal COX-2 to attain full blockade and/or a redundancy of the signaling involved. For example, COX-1, which is not inhibited by nimesulide, may catalyze the synthesis of prostaglandins, minimizing the action of nimesulide.

The exact mechanism by which COX-2 inhibition attenuates kindling epileptogenesis is not understood. Notwithstanding, COX-2 inhibition may diminish prostaglandin and/or PAF synthesis, lipid messengers that are both involved in synaptic facilitation [45]. Moreover, kindling epileptogenesis promotes selective neuronal COX-2

expression, initially in the hippocampus and subsequently in the neocortex. The stimulating electrode in this particular experiment was placed in the ventral hippocampus. Taken together, these studies suggest that the spreading of kindling-induced COX-2 expression from hippocampal neurons to neocortical neurons is a major event in the permanent facilitation of aberrant functional connectivity, and that COX-2 is a mediator [44].

Several tissue-specific, eicosanoid-selective isomerases catalyze the conversion of PGH2 into PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxanes (Fig. 33-2). An alternative pathway is the direct conversion of PGG_2 into PGE_2 through 15-hydroperoxy-PGE_2. This route is not well understood, although it may have modulatory implications [46]. The synthesis of PGE_2 is catalyzed by three different enzymes. Two of them are membrane-bound (mainly to the endoplasmic reticulum) and one is soluble. Interestingly, these enzymes are differentially coupled to the COXs, thus channeling released AA through constitutive COX-1 or through inducible COX-2, which has important regulatory consequences on the availability of PGE_2. mPGES-1 is a glutathione (GSH)-requiring perinuclear-enriched protein that belongs to the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) family. This enzyme is functionally coupled to COX-2 and is induced by proinflammatory stimuli, cytokines, growth factors or lipopolysaccharide, and its induction is downregulated by anti-inflammatory glucocorticoids. The second membrane-bound PGE synthase is mPGES-2, which, unlike the former, is constitutively expressed and may be coupled to COX-1 or COX-2. This enzyme has glutaredoxin–thioredoxin-like domains and is activated by thiol reagents. The cytosolic enzyme (cPGES) is coupled to COX-1 and allows rapid PGE_2 synthesis upon free AA release. It is a 23 kDa glutathione-requiring enzyme regulated by phosphorylation through casein kinase II and requiring the molecular chaperone HSP90 [47].

DIACYLGLYCEROL KINASES

The slow glutamate responses are mediated through metabotropic receptors coupled to G proteins. For review of metabotropic receptors (mGluRs) and G proteins, see Chapters 15 and 19 and reference [48]. One of the mGluR family, mGluR1, is linked to the inositol lipid-PLC pathway. Activation of neuronal inositol lipid signaling through mGluR1 and PKC_γ is involved in synaptic plasticity such as in learning, memory, LTP and long-term depression [49] and has been implicated in neurological and psychiatric diseases such as epilepsy, Alzheimer's disease and depression [24, 48, 50, 51]. Activation of mGluR1 triggers a short-lived signal with potent and sustained consequences in other signaling pathways. DAG-activated PKC contributes to feedback inhibition of the PLC pathway and, at the same time, may lead to PLD and PLA_2 activation. The termination of the DAG signal is mainly accomplished by DAG kinase $(DGK)_\varepsilon$, which selectively phosphorylates AA-DAG to generate AA-PA [52, 53]. Eight other mammalian DGKs have been identified, but only DGK_ε uses AA-DAG [54]. The central role played by DAG_ε in the inositol lipid-PLC pathway was revealed using mice with targeted disruption of the DGKε gene [55]. *DGKε*$^{-/-}$ mice display higher resistance to seizures and attenuation of LTP in the hippocampus. Interestingly, not only the inositol lipid-PLC pathway was greatly affected by the mutation, but also the $cPLA_2$-AA and PLD-DAG pathways, reflecting the impact of the former in modulating multiple pathways that participate in synaptic activity and neuronal plasticity.

The DAG signal itself can also be terminated by DAG lipases with the sequential release of 2-AA glycerol (2-AG) and then free AA and glycerol. As the primary second messengers are removed to terminate their functions, other bioactive lipids are generated; 2-AG is an endogenous ligand for the CA1 cannabinoid receptor with neuromodulatory functions [56], while PA and its PLA_2 product lyso-PA are potent intra- and intercellular messengers. Lyso-PA is released from the cells and interacts with cell surface G-protein-coupled receptors. Lyso-PA inhibits neurite growth and neuroblastome differentiation, stimulates neurotransmitter release in PC12 cells and has been implicated in Alzheimer's disease.

LIPID SIGNALING PATHWAYS AND NEUROINFLAMMATION

A platelet-activating-factor-stimulated signal-transduction pathway is a major component of the kainic-acid-induced cyclooxygenase-2 expression in hippocampus. Both PAF [57] and COX-2 are potent mediators of the injury/inflammatory response (Fig. 33-5). PAF and COX-2 are also interrelated in neuronal plasticity. The PAF transcriptional activation of COX-2 may provide clues about novel neuronal cell-death pathways. In fact, the delayed induction of COX-2 by kainic acid precedes selective neuronal apoptosis by this agonist in the hippocampus [42, 58].

In cerebrovascular diseases, the phospholipase-A_2-related signaling triggered by ischemia–reperfusion may be part of a delicate balance between neuroprotection and neuronal cell death. Events that would tilt the balance toward neuroprotection are possible therapeutic targets. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase [59], is a long-term signal with consequences for neurons [60] through sustained expression of COX-2 (Fig. 33-6). Overexpression of hippocampal COX-2 during cerebral ischemia [61] and seizures may in turn lead to the formation of neurotoxic metabolites, such as ROS. The regulatory subunit of

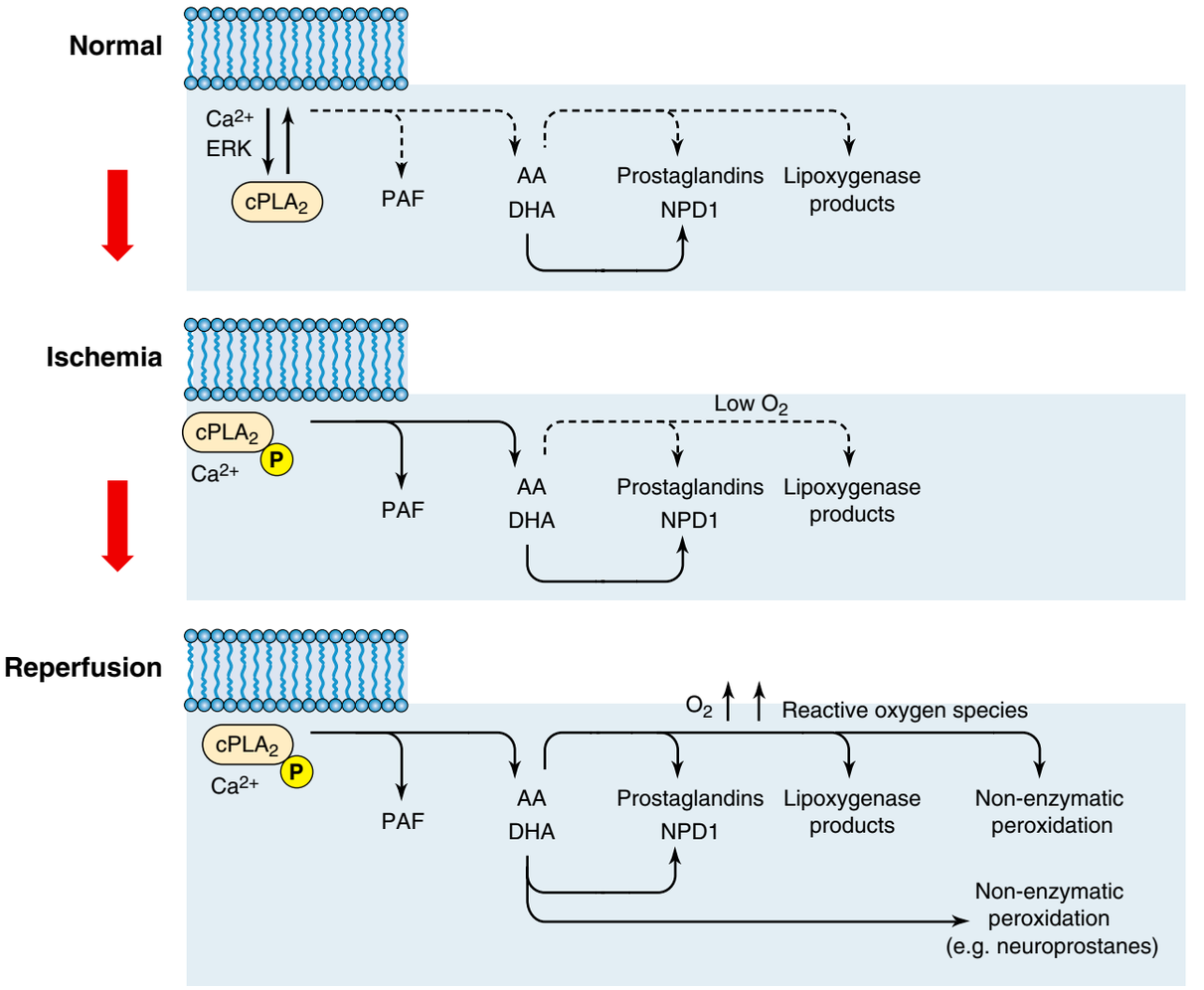

FIGURE 33-6 Phospholipases A_2 (*cPLA*$_2$) in the generation of eicosanoids, docosanoids, and lipid peroxidation products during brain, retina, or spinal cord ischemia and reperfusion. During the ischemic phase, phospholipase overactivation and the downregulation of oxidative and energy metabolism, and of fatty acid reacylation, promote the accumulation of free arachidonic acid (*AA*), free docosahexaenoic acid (*DHA*) and lysophospholipids such as lyso-PAF. The reperfusion phase facilitates eicosanoid and docosanoid synthesis. Reactive oxygen species are generated at rates that can overload the antioxidant and free radical scavenger systems of the brain, thus promoting peroxidation of polyunsaturated fatty acids. *ERK*, extracellular signal-regulated kinase; *NPD1*, neuroprotectin D1.

PAF-acetylhydrolase (intracellular) is the *lis-1* gene, mutated in Miller–Diecker syndrome, a neuronal disease characterized by the absence of gyri and sulci in the cerebral cortex (lissencephaly). A defect in neuronal migration during brain development may underlie the smooth cerebrum of people afflicted with the syndrome, and PAF and PAF-acetylhydrolase may be involved in neuronal migration [62]. Current investigations aim at determining whether other messengers, such as nitric oxide, cooperate to enhance neuronal damage and to what extent astrocytes and microglial cells are involved. Further understanding of these potentially neurotoxic events involving lipid messengers and COX-2 will contribute to the identification of new therapeutic strategies for the management of cerebrovascular diseases, neurodegenerative diseases and other pathologic conditions involving neuroinflammation.

Free arachidonic acid, along with diacylglycerols and free docosahexaenoic acid, is a product of membrane lipid breakdown at the onset of cerebral ischemia, seizures and other forms of brain trauma. Because polyunsaturated fatty acids are the predominant FFA pool components that accumulate under these conditions, this further supports the notion that fatty acids released from the C2 position of membrane phospholipids are major contributors to the FFA pool, implicating PLA_2 activation as the critical step in FFA release [1, 2] (Fig. 33-6).

Free fatty acid release during cerebral ischemia is a complex process that includes the activation of signaling cascades. In addition to $cPLA_2$, these cascades probably involve the simultaneous and/or sequential activation of other phospholipid degradation pathways and the

successive recruitment of different phospholipid pools. This is alluded to by the fact that, in focal and global cerebral ischemia, FFA accumulation is preceded by a lag of a few seconds after the start of ischemia (Fig. 33-6). Moreover, the initial release of FFA is, at least partially, derived from the activity of phospholipase C on inositol lipids (mainly phosphatidylinositol 4,5-bisphosphate), with FFAs being subsequently released from DAG by DAG lipase and monoacylglycerol lipase. FFAs and DAGs enriched in stearic and AAs accumulate during cerebral ischemia and seizures, concomitant with a rapid decrease in the inositol lipid pool. A sequential activation of PLC and PLA_2 is suggested as a mechanism for the time-dependent metabolism of different phospholipid pools during brain ischemia. The inositol lipid pool is initially a contributor to release of FFAs that are further metabolized via the PLC–DAG lipase pathway. However, as the ischemia progresses or after seizures, the calcium-dependent PLA_2 pathway prevails, with the FFA now derived from other phospholipids. A mechanism could be envisaged to explain these observations in which PI-PLC activation leads to the release of DAG and inositol trisphosphate (IP3). DAG activates protein kinase C, which in turn may modulate PI-PLC. At the same time, IP3 mobilizes intracellular calcium and thus stimulates $cPLA_2$ translocation to the membrane.

The rate of free fatty acid production in the mammalian brain correlates to the extent of resistance to ischemia. FFA production rate is much lower in the brains of neonatal mammals and poikilothermic animals, organisms that display a greater resistance to cerebral ischemic insults than mature mammals [63]. In addition, within the mammalian brain, FFA release is higher in the gray matter compared with white matter, and there is a greater accumulation of AA in areas of the brain, such as the hippocampus, selectively vulnerable to cerebral ischemic damage.

Activation of the arachidonic acid cascade during ischemia–reperfusion is a multistage process. During the ischemic phase, activation of phospholipases, inhibition of energy-dependent reacylation of membrane lipids and the lowering of oxygen tension in the brain promote the accumulation of free AA. When, or if, blood flow is restored, the rise in oxygen tension, coupled with a large pool of free AA, stimulates oxygenation-dependent reactions, some of them enzyme-mediated (e.g. for the synthesis of eicosanoids; Figs 33-2, 33-3) and others non-enzyme-mediated, which promote the formation of lipid hydroperoxides. Platelet-activating factor (PAF) synthesis is also enhanced under these conditions. Accordingly, eicosanoids accumulate during reperfusion.

Cyclooxygenase and lipoxygenase products accumulate during reperfusion following cerebral ischemia. The levels of lipoxygenase products (Fig. 33-3) increase more slowly than COX products but remain raised for longer periods of time. The cellular sites of synthesis of ischemia–reperfusion-induced eicosanoids within the brain have not yet been fully assessed. Glia and microglia comprise major inflammatory cells of the brain, as are leukocytes that infiltrate the brain during reperfusion or traumatic injury [4]. In the unstimulated brain, both 5-lipoxygenase and COX-1 and -2 have been localized to neurons. The rapid (2–3 min) activation of 5-lipoxygenase through membrane translocation is in response to reperfusion following transient (5 min) forebrain ischemia. In this study postischemic LTC4 accumulation was found to be threefold greater in the hippocampus than in the cerebral cortex and thus correlated with the high vulnerability of this region to ischemia. COX-1 activity is governed by the availability of substrate (AA and oxygen), and thus activation of the COX pathway during ischemia–reperfusion is triggered by AA release in the presence of sufficient oxygen. Induction of COX-2 transcription is rapid in ischemia and in seizures [47]. Given the involvement of glutamatergic neurotransmission in the induction of neuronal COX-2, it may be predicted that the release of glutamate triggered by cerebral ischemia would provide a trigger for COX-2 induction. There are several early-response genes that encode transcription factors that are rapidly induced under these conditions, such as hypoxia-inducible factor (HIF)-1α and NF-κB. The COX-2 promoter contains consensus sequences for these transcription factors. Nonetheless, brain displays 'constitutive' inducible COX-2 under physiological conditions. This is explained by the involvement of COX-2 in synaptic plasticity and long-term potentiation [64].

The cerebrovasculature is also an abundant source of eicosanoids. Platelets, leukocytes and vascular endothelium are all capable of synthesizing eicosanoids (see above). Brain microvessels isolated from ischemic rat brain demonstrate enhanced synthesis of eicosanoids, and leukocytes and platelets may account for much of the LTD4 produced during ischemia–reperfusion in the gerbil.

DOCOSAHEXAENOIC ACID

Brain and retina are the tissues containing the highest contents of docosahexaenoic acid. DHA is used continuously for the biogenesis and maintenance of neuronal and photoreceptor membranes (see also Ch. 49). This system is supported by the liver, which supplies DHA incorporated into plasma lipoproteins [65]. The uptake of DHA by the retina involves the retinal pigment epithelium (RPE) and its subsequent delivery to photoreceptors. During the daily photoreceptor renewal, an active recycling of phagosome-derived DHA by RPE via the interphotoreceptor matrix retains DHA within photoreceptors. A signal generated in brain or retina to control DHA

delivery from the liver has been postulated [65]; this would allow activation of DHA export during development, when DHA is required for active synapse and photoreceptor outer segment formation. This activation may also occur when injury or neurodegenerations result in loss of DHA from excitable membranes and replenishment of DHA is needed [65].

Rhodopsin in photoreceptors is immersed in a lipid environment highly enriched in phospholipids containing docosahexaenoic acid, which is essential for rhodopsin function. Moreover, retinal DHA, like brain DHA, is very resistant to n-3 fatty acid dietary deprivation [65]. Because of this constant systemic flow of DHA via a 'long loop', it is highly relevant that retinitis pigmentosa is associated with alterations in lipoprotein metabolism. The most frequently reported phenotype is low plasma and red blood cell levels of DHA [66–69]. In Usher's syndrome, an autosomal recessive retinitis pigmentosa that is associated with hearing loss, lower plasma levels of 22:6- and 20:4-phospholipids are also found [70]. Interestingly, changes show a direct correlation to the severity of disease and are more accentuated in patients with Usher's type I than in those with the less severe type II form.

Low plasma levels of 22:6n-3 in retinitis pigmentosa patients have also been interpreted as a mechanism to protect the retina from oxidative damage resulting from photochemical function of rhodopsin [71]. Stress signals originating from retinas undergoing degeneration (e.g. retinitis pigmentosa) may shut off the communication between the retina and the liver [65], reducing the systemic liver supply of 22:6n-3. In dogs with progressive rod–cone degeneration (prcd; [72]) as well as in other retinal degeneration models [71], low levels of DHA in photoreceptor phospholipids occur. Moreover, the synthesis of DHA from 20:5n-3 in RPE cultures from prcd is not affected by the retinal degeneration [73], and dietary supplementation with DHA failed to prevent the loss of photoreceptor DHA and the prcd phenotype [72]. Moreover, cultured hepatocytes from prcd dogs display higher accumulation of 22:6-phospholipids in the liver and impaired hepatocyte secretion of DHA-containing very low-density lipoproteins [74], supporting the hypothesis that in retinal degenerations a systemic DHA metabolic defect occurs that leads to reduced liver DHA supply to the retina. Whether DHA dietary supplementation protects the retina of retinitis pigmentosa patients is not known.

LIPID PEROXIDATION AND OXIDATIVE STRESS

Docosahexaenoic-acid-containing phospholipids are targets for lipid peroxidation. As a result of free-radical-catalyzed peroxidation, F4-isoprostanes are formed [75, 76]. F2-isoprostanes are also derived from free-radical-catalyzed peroxidation, although from AA instead [77]. F4-isoprostanes are found esterified in phospholipids and it has been reported that their content is increased in the brains of patients with Alzheimer's disease [78].

NEUROPROTECTIN D1: A DOCOSAHEXAENOIC-ACID-DERIVED MEDIATOR

Docosanoids, enzyme-derived docosahexaenoic acid metabolites, were identified initially in the retina. These have been proposed to elicit neuroprotective actions [14, 79]. Very recently it was ascertained by tandem LC-PDA-ESI-MS-MS-based lipidomic analysis [4] that the synthesis of DHA-oxygenation messengers occurs during brain ischemia–reperfusion (Fig. 33-6). Two DHA-oxygenation pathways operate: one that gives rise to 10,17*S*-docosatriene and one that results in the synthesis of resolvin-type messengers (17*R*-DHA). Resolvins were elegantly identified outside the nervous system as a response to aspirin treatment [80]. The brain resolvins are also made upon treatment with aspirin [4]. Both DHA-oxygenation pathways generate messengers that are counter-proinflammatory signals [80–82]. The docosanoid neuroprotectin D1 (10,17*S*-docosatriene) is a potent inhibitor of brain ischemia–reperfusion-induced polymorphonuclear infiltration, as well as of NF-κB and COX-2 expression [4]. Moreover, marked attenuation of the stroke volume was observed when neuroprotectin D1 was infused into the third ventricle during ischemia–reperfusion [4]. Discovery of these DHA-oxygenation messengers sheds new light on how the brain modulates its response to inflammatory injury and raises the exciting potential for designing new drugs to treat neurologic disorders that have a neuroinflammatory component, such as stroke, traumatic brain injury or spinal cord injury. In particular, the very high biological activity of neuroprotectin D1 marks it a potent modulator of neuronal survival.

THE FUTURE OF LIPID SIGNALING IN THE NERVOUS SYSTEM

The significance of lipid signaling in the nervous system will be greatly expanded by newer experimental approaches. Following the genomics era, we are now in 'proteomics times', when not only is the proteome being defined but metabolomics are emerging. Among the powerful new tools available are lipidomic analyses. Lipidomics is beginning to allow us to precisely define lipid organization, metabolism, synthesis of stereospecific mediators (e.g. neuroprotectin D1) and signal transduction in a given cell or part of a cell (e.g. dendrites). This is mainly due to the power, ease and versatility of what has evolved from the development of electrospray ionization mass spectrometry (ESI/MS) [83]. The detailed composition of

lipid classes and molecular species can now be approached more accurately. Moreover, the detailed identification of changes in the lipidome during the development, function, aging and dysfunctions of the nervous system will be tackled. These studies will be integrated with nutritional approaches and clinical neurosciences.

An evolving area is the understanding of the fundamental inner workings of the dendrites, which contain complex intracellular membranes rich in polyunsaturated phospholipids. Dendrites undergo profound changes during neuronal function, including the membrane vesicular transport of neurotransmitter receptors, ion channels and other proteins destined to the dendritic spine, where critical postsynaptic elements of neurotransmission are located. Definition of the dendritic lipidome will also define the participation of lipid signaling in dendritic development and in the establishment of synaptic contacts as well as overall dendritic plasticity.

Although arachidonic acid is widely implicated in signaling in brain, there are several gaps in our understanding of the release of this fatty acid from membrane reservoirs. Reports that AA is released primarily by G-protein-mediated PLA_2 activation remain to be confirmed [84, 85]. In addition, modulation of PLA_2 by Ca^{2+} and protein kinase needs to be better defined. It is clear that NMDA receptor activation promotes the release of AA [86], and that a variety of eicosanoids are then generated (Fig 33-2, 33-3). The modulatory events that channel AA towards specific eicosanoids are not understood. The endocannabinoid family of lipid messengers will remain an active focus of interest because of the growing evidence of their actions in synaptic function, learning, memory, and other forms of behavior [56, 87].

The essential fatty acid of the linolenic acid family, DHA, is most highly concentrated in brain and retina. The significance of this polyunsaturated fatty acid, in addition to being a target for nonenzymatic peroxidation under various pathologic conditions, will be a focus of continued research. Clearly, DHA-containing molecular species of phospholipids confer a unique environment for ion channels, receptors, transporters and protein–protein interactions critical in signaling. How these events are regulated, including the synthesis and remodeling of these highly unsaturated phospholipids, is not clearly understood. The enzyme-mediated synthesis of docosanoids raises the possibility of exploring the specific generation of novel DHA-oxygenation messengers that are neuroprotective [4]. How these docosanoid messengers are synthesized, through which receptors they elicit their actions, how they are affected by pharmacologic agents and how the docosanoids themselves may become a template for drug design are questions for the near future. The use of tandem LC-PDA-ESI-MS-MS-based lipidomic analysis in combination with other experimental approaches will greatly contribute to our understanding of the significance of DHA in health and in pathologic conditions.

The knowledge evolving from lipidomic neurobiology will also be potentiated by multidisciplinary approaches such as multiphoton confocal analysis. Structural neurobiology will also come into play, because the lipidome will provide new insights into the precise stereochemical structure of lipids of excitable membranes, as well as of intracellular membranes. There is also growing evidence of the exquisite signaling interplay among neurons, astrocytes, oligodendrocytes and microglia. Prostaglandins are among the lipid messengers explored to date as modulators of astrocyte release of glutamate [15]. Microglia also actively make prostaglandins in response to injury [88–90], although prostaglandin E2 may be engaged in neuroprotection [91]. Moreover, the renewed interest in defining the significance of non-neuronal cells in the nervous system [92–94] will be greatly enhanced by lipidomic approaches. Overall, the near future will witness breakthroughs using lipidomic neurobiology to more precisely define synaptic lipid signaling that will contribute to a renewed vista of the function of the nervous system as well as of the neurobiology of diseases.

REFERENCES

1. Horrocks, L. A. and Farooqui, A. A. NMDA receptor-stimulated release of arachidonic acid: mechanisms for the Bazan effect. In Municio, A. M. and Miras-Portugal, M. T. (eds), *Cell Signal Transduction, Second Messengers, and Protein Phosphorylation in Health and Disease*. New York: Plenum Press, 1994, pp. 113–128.
2. Sun, G. Y., Xu, J., Jensen, M. D. and Simonyi, A. Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J. Lipid Res.* 45: 205–213, 2004.
3. Niu, S. L., Mitchell, D. C., Lim, S. Y. *et al.* Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *J. Biol. Chem.* 23, 279: 31098–31104, 2004.
4. Marcheselli, V. L., Hong, S., Lukiw, W. J. *et al.* Novel docosanoids inhibit brain ischema-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J. Biol. Chem.* 278, 43807–43817, 2003.
5. Mukherjee, P. K., Marcheselli, V. L., Serhan, C. N. and Bazan, N. G. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl Acad. Sci. U.S.A.* 101, 8491–8496, 2004.
6. Giulian, D. Microglia and diseases of the nervous system. *Curr. Neurol.* 12: 23–27, 1992.
7. Kato, K., Clark, G. D., Bazan, N. G. and Zorumski, C. F. Platelet activating factor as a potential retrograde messenger in CA1 hippocampal long-term potentiation. *Nature* 367, 175–179, 1994.
8. Matsuzawa, A., Makoto, M., Atsumi, G. *et al.* Release of secretory phospholipase A2 from rat neuronal cells and its possible function in the regulation of catecholamine secretion. *Biochem. J.* 318, 701–709, 1996.
9. Lambeau, G., Barhanin, J., Schweitz, H., Qar, J. and Lazdunski, M. Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the taipan venom. *J. Biol. Chem.* 264, 11503–11510, 1989.

10. Lambeau, G., Schmid-Alliana, A., Lazdunski, M. and Barhanin, J. Identification and purification of a very high affinity binding protein for toxic phospholipases A2 in skeletal muscle. *J. Biol. Chem.* 265: 9526–9532, 1990.
11. Lambeau, G., Ancian, P., Barhanin, J. and Lazdunski, M. Cloning and expression of a membrane receptor for secretory phospholipases A2. *J. Biol. Chem.* 269: 1575–1578, 1994.
12. Lambeau, G., Ancian, P., Nicolas, J.-P. *et al.* Structural elements of secretory phospholipases A2 involved in the binding to M-type receptors. *J. Biol. Chem.* 270: 5534–5540, 1995.
13. Kolko, M., DeCoster, M. A., Rodriguez de Turco, E. B. and Bazan, N. G. Synergy by secretory phospholipase A2 and glutamate on inducing cell death and sustained arachidonic acid metabolic changes in primary cortical neuronal cultures. *J. Biol. Chem.* 271: 32722–32728, 1996.
14. Bazan, N. G., Birkle, D. L. and Reddy, T. S. Docosahexaenoic acid (22:6, n 3) is metabolized to lipoxygenase reaction products in the retina. *Biochem. Biophys. Res. Commun.* 125: 741–747, 1984.
15. Bezzi, P., Carmignoto, G., Pasti, L. *et al.* Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* 391: 281–285, 1998.
16. Hecht, J. H., Weiner, J. A., Post, S. R. and Chun. J. Ventricular zone gene-1 (*vzg-1*) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell Biol.* 135: 1071–1083, 1996.
17. Guo, Z., Liliom, K., Fischer, D. J. *et al.* Molecular cloning of a high-affinity receptor for the growth factor-like mediator lysophosphatidic acid from *Xenopus* oocytes. *Proc. Natl Acad. Sci. U.S.A.* 93: 14367–14372, 1996.
18. An, S., Dickens, M. A., Bleu, T., Hallmark, O. G. and Goetzl, E. J. Molecular cloning of the human EDG2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem. Biophys. Res. Commun.* 231: 619–622, 1997.
19. Dennis, E. A. Diversity of group types, regulation and function of phospholipase A2 *J. Biol. Chem.* 269: 13057–13060, 1994.
20. Tay, A., Maxwell, P., Li, Z. G., Goldberg, H. and Skorecki, K. Cytosolic phospholipse A2 gene expression in rat mesangial cells is regulated post-transcriptionally *Biochem. J.* 304: 417–422, 1994.
21. Clark, J. D., Lin, L.-L., Kriz, R. W. *et al.* A novel arachidonic acid-selective cytosolic PLA2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP *Cell* 65: 1043–1051, 1991.
22. Herschman, R. W. Prostaglandin synthase 2. *Biochim. Biophys. Acta.* 1299: 125–140, 1996.
23. Brock, T. G., McGish, R. W. and Peters-Golden, M. Translocation and leukotriene synthetic capacity of nuclear 5-lipoxygenase in rat basophilic leukemia cells and alveolar macrophages. *J. Biol. Chem.* 270: 21652–21658, 1995.
24. Bazan, N. G., Rodriguez de Turco, E. B. and Allan, G. Mediators of injury in neurotrauma: Intracellular signal transduction and gene expression. *J. Neurotrauma* 12: 791–814, 1995.
25. Bonventre, J. V., Huang, Z., Taheri, M. R. *et al.* Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 390: 622–625, 1997.
26. Flower, R. J. and Rothwell, N. J. Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol. Sci.* 15: 71–76, 1994.
27. Clark, G. D., Happel, L. T., Zorumski, C. F. and Bazan, N. G. Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor *Neuron* 9: 1211–1216, 1992.
28. Birkle, D. L. and Bazan, N. G. Effect of bicuculline-induced status epilepticus on prostaglandins and hydroxyeicosatetraenoic acids in rat brain subcellular fractions *J. Neurochem.* 48: 1768–1778. 1987.
29. Bazan, N. G. Inflammation. A signal terminator. *Nature* 374: 501–502, 1995.
30. Wieraszko, A., Li, G., Kornecki, E., Hogan, M. V. and Ehrlich, Y. H. Long-term potentiation in the hippocampus induced by platelet-activating factor. *Neuron* 10: 553–557, 1993.
31. Izquierdo, I., Fin, C., Schmitz, P. K. *et al.* Memory enhancement by intrahippocampal, intraamygdala, or intraentorhinal infusion of platelet-activating factor measured in an inhibitory avoidance task. *Proc. Natl Acad. Sci. U.S.A.* 92: 5047–5051, 1995.
32. Packard, M. G., Teather, L. and Bazan, N. G. Effects of intrastriatal injections of platelet-activating factor and the PAF antagonist BN 52021 on memory. *Neurobiol. Learn. Mem.* 66: 176–182, 1996.
33. Kumar, R., Harvey, K., Kester, M., Hanahan, D. and Olsen, M. Production and effects of platelet-activating factor in the rat brain. *Biochim. Biophys. Acta* 963: 375–390, 1988.
34. Bito, H., Nakamura, M., Honda, Z. *et al.* Platelet-activating factor (PAF) receptor in rat brain: PAF mobilizes intracellular Ca^{2+} in hippocampal neurons. *Neuron* 9: 285–294, 1992.
35. Panetta, T., Marcheselli, V. L., Braquet, P., Spinnewyn, B. and Bazan, N. G. Effects of a platelet-activating factor antagonist (BN52021) on free fatty acids, diacylglycerols, polyphosphoinositides and blood flow in the gerbil brain: Inhibition of ischemia–reperfusion induced cerebral injury. *Biochem. Biophys. Res. Commun.* 149: 580–587, 1987.
36. Kaufmann, W. E., Worley, P. F., Pegg, J., Bremer, M. and Isakson, P. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. *Proc. Natl Acad. Sci. U.S.A.* 93: 2317–2321, 1996.
37. Chandrasekharan, N. V., Dai, H., Roos, K. L. *et al.* COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl Acad. Sci. U.S.A.* 99: 13926–13931, 2002.
38. Cui, J. G., Kuroda, H., Chandrasekharan, N. V. *et al.* Cyclooxygenase-3 gene expression in Alzheimer hippocampus and in stressed human neural cells. *Neurochem. Res.* 29: 1731–1737, 2004.
39. Bazan, N. G. and Flower, R .J. Lipid signals in pain control. *Nature* 420: 135–138, 2002.
40. Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A. and Worley, P. F. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* 11: 371–386, 1993.
41. Bazan, N. G., Fletcher, B. S., Herschman, H. R. and Mukherjee, P. K. Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene. *Proc. Natl Acad. Sci. U.S.A.* 91: 5252–5256, 1994.
42. Marcheselli, V. L. and Bazan, N. G. Sustained induction of prostaglandin endoperoxide synthase-2 by seizures in hippocampus. Inhibition by a platelet-activating factor antagonist. *J. Biol. Chem.* 271: 24794–24799, 1996.

43. Lothman, E. W. and Williamson, J. M. Rapid kindling with recurrent hippocampal seizures. *Epilepsy Res.* 14: 209–220, 1993.
44. Tu, B. and Bazan, N. G. Hippocampal kindling epileptogenesis upregulates neuronal cyclooxygenase-2 expression in neocortex. *Exp. Neurol.* 179: 167–175, 2003.
45. Bazan, N. G., Packard, M. G., Teather, L. and Allan, G. Bioactive lipids in excitatory neurotransmission and neuronal plasticity. *Neurochem. Int.* 30: 225–231, 1997.
46. Thoren, S., Weinander, R., Saha, S. *et al.* Human microsomal prostaglandin E synthase-1: purification, functional characterization, and projection structure determination. *J. Biol. Chem.* 278: 22199–22209, 2003.
47. Kobayashi, T., Nakatani, Y., Tanioka, T. *et al.* Regulation of cytosolic prostaglandin E synthase by phosphorylation. *Biochem. J.* 381: 59–69, 2004.
48. Conn, P. J. and Pin, J. P. Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* 37: 205–237, 1997.
49. Aiba, A., Kano, M., Chen, C. *et al.* Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell* 79: 377–388, 1994.
50. Pacheco, M. A. and Jope, R. S. Phosphoinositide signaling in human brain. *Prog. Neurobiol.* 50: 255–273, 1996.
51. Bordi, F. and Ugolini, A. Group I metabotropic glutamate receptors: implications for brain diseases. *Prog. Neurobiol.* 59: 55–79, 1999.
52. Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M. and Prescott S. M. Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. *J. Biol. Chem.* 271: 10237–10241, 1996.
53. Pettitt, T. R. and Wakelam, M. J. O. Diacylglycerol kinase ε, but not ζ, selectively removes polyunsaturated diacylglycerol, inducing altered protein kinase C distribution in vivo. *J. Biol. Chem.* 274: 36181–36186, 1999.
54. Topham, M. K. and Prescott, S. M. Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* 274: 11447–11450, 1999.
55. Rodriguez de Turco, E. B., Tan, W., Topham, M. K., Sakane, F., Marcheselli, V. L., Chen, C., Taketomi, A., Prescott, S. and Bazan N. G. Diacylglycerol kinase epsilon regulates seizures susceptibility and long-term potentiation through inositol lipid signaling. *Proc. Natl Acad. Sci. U.S.A.* 98: 4740–4745, 2001.
56. Piomelli, D. The molecular logic of endocannabinoid signaling. *Nat. Rev. Neurosci.* 4: 873–884, 2003.
57. Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. Platelet-activating factor antagonist. *J. Biol. Chem.* 265: 17381–17384, 1990.
58. Chen, J., Marsh, T., Zhang, J. S. and Graham, S. H. Expression of cyclooxygenase 2 in rat brain following kainate treatment. *Neuroreport* 6: 245–248, 1995.
59. Stafforini, D. M., McIntyre, T. M., Zimmerman, G. A. and Prescott, S. M. Platelet-activating factor acetylhydrolases. *J. Biol. Chem.* 272: 17895–17898, 1997.
60. Bazan, N. G. and Allan, G. Platelet-activating factor and other bioactive lipids. In M. D. Ginsberg and J. Bogousslavasky (eds), *Cerebrovascular Disease, Pathophysiology, Diagnosis and Management.* Malden, MA: Blackwell Science, 1998, pp. 532–555.
61. Nogawa, S., Zhang, F., Ross, M. E. and Iadecola, C. Cyclooxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J. Neurosci.* 17: 2746–2755, 1997.
62. Hattori, M., Adachi, H., Tsujimoto, M., Arai, H. and Inoue, K. Miller–Diecker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. *Nature* 370: 216–218, 1994.
63. Aveldaño, M. I. and Bazan, N. G. Differential lipid deacylation during brain ischemia in a homeotherm and a poikilotherm. Content and composition of free fatty acids and triacylglycerol. *Brain Res.* 100: 99–110, 1975.
64. Chen, C., Magee, J. C. and Bazan, N. G. Cyclooxygenase-2 regulates prostaglandin E2 signaling in hippocampal long-term synaptic plasticity. *J. Neurophysiol.* 87: 2851–2857, 2002.
65. Scott, B. L. and Bazan, N. G. Membrane docosahexaenoate is supplied to the developing brain and retina by the liver. *Proc. Natl Acad. Sci. U.S.A.* 86: 2903–2907, 1989.
66. Converse, C. A., Hammer, H. M., Packard, C. J. and Shephard, J. Plasma lipid abnormalities in retinitis pigmentosa and related conditions. *Trans. Ophthalmol. Soc. U.K.* 103: 508–512, 1983.
67. Anderson, R. E., Maude, M. B., Lewis, R. S., Newsome, D. A. and Fishman, G. A. Abnormal plasma levels of polyunsaturated fatty acid in autosomal dominant retinitis pigmentosa. *Exp. Eye Res.* 44: 155–159, 1987.
68. Gong, J., Rosner, B., Rees, D. G. *et al.* Plasma docosahexaenoic acid levels in various genetic forms of retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 33: 2596–2602, 1992.
69. Hoffman, D. R. and Birch, D. G. Omega 3 fatty acid status in patients with retinitis pigmentosa. *World Rev. Nutr. Diet* 83: 52–60, 1998.
70. Bazan, N. G., Scott, B. L., Reddy, T. S. and Pelias, M. Z. Decreased content of docosahexaenoate and arachidonate in plasma phospholipids in Usher's syndrome. *Biochem. Biophys. Res. Commun.* 141: 600–604, 1986.
71. Anderson, R. E., Maude, M. B., Alvarez, R. A., Acland, G. and Aguirre, G. D. A hypothesis to explain the reduced blood levels of docosahexaenoic acid in inherited retinal degenerations caused by mutations in genes encoding retina-specific proteins. *Lipids* 34, S235–S237, 1999.
72. Aguirre, G. D., Acland, G. M., Maude, M. B. and Anderson, R. E. Diets enriched in docosahexaenoic acid fail to correct progressive rod-cone degeneration (prcd) phenotype. *Invest. Ophthalmol. Vis. Sci.* 38: 2387–2407, 1997.
73. Chen, H., Ray, J., Scarpino, V., Acland, G. M., Aguirre, G. D. and Anderson, R. E. Synthesis and release of docosahexaenoic acid by the RPE cells of prcd-affected dogs. *Invest. Ophthalmol. Vis. Sci.* 40: 2418–2422, 1999.
74. Bazan, N. G. and Rodriguez de Turco, E. B. Alterations in plasma lipoproteins and DHA transport in progressive rod-cone degeneration (prcd). In S. Kato, N. N. Osborne and M. Tamai (eds), *Retinal Degeneration and Regeneration, Proceedings of an International Symposium in Kanazawa, Japan, July 8–9, 1995.* Amsterdam: Kugler, 1996, pp. 89–97.
75. Nourooz-Zadeh, J., Liu, E. H. C., Anggard, E. E. and Halliwell, B. F4-isoprostanes: a novel class of prostanoids formed during peroxidation of docosahexaenoic acid. *Biochem. Biophys. Res. Commun.* 242: 338–344, 1998.
76. Roberts, L. J. II, Montine, T. J., Markesbery, W. R. *et al.* Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J. Biol. Chem.* 273: 13605–13612, 1998.
77. Morrow, J. D., Hill, K. E., Burk, R. F. *et al.* A series of prostaglandin F2-like compounds are produced in vivo in human

by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl Acad. Sci. U.S.A.* 87: 9383–9387, 1990.
78. Nourooz-Zadeh, J., Liu, E. H. C., Yhlen, B., Anggard, E. E. and Halliwell, B. F4-isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. *J. Neurochem.* 72: 734–740, 1999.
79. Bazan, N. G. Supply of n-3 polyunsaturated fatty acids and their significance in the central nervous system. In R. J. Wurtman and J. J. Wurtman (eds), *Nutrition and the Brain.* New York: Raven Press, 1990, pp. 1–24.
80. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R. and Mirick, G. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J. Exp. Med.* 196: 1025–1037, 2002.
81. Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N. and Gronert, K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2: nonsteroidal antiinflammatory drugs and transcellular processing. *J. Exp. Med.* 192: 1197–1204, 2000.
82. Hong, S., Gronert, K., Devchand, P. R., Moussignac, R. L. and Serhan, C. N. Novel docosatrienes and 17*S*-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. *J. Biol. Chem.* 278: 14677–14687, 2003.
83. Han, X. and Gross, R. W. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J. Lipid Res.* 44: 1071–1079, 2003.
84. Axelrod, J., Burch, R. M. and Jelsema, C. L. Receptor-mediated activation of phospholipase A2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends Neurosci.* 11: 117–123, 1988.
85. Burch, R. M. G protein regulation of phospholipase A2. *Mol. Neurobiol.* 3: 155–171, 1989.
86. Dumuis, A., Sebben, M., Haynes, L., Pin, J. P. and Bockaert, J. NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature* 336: 68–70, 1988.
87. Carlson, G., Wang, Y. and Alger, B. E. Endocannabinoids facilitate the induction of LTP in the hippocampus. *Nat. Neurosci.* 5: 723–724, 2003.
88. Minghetti, L., Greco, A., Cardone, F., Puopolo, M., Ladogana, A. and Almonti, S. Increased brain synthesis of prostaglandin E2 and F2-isoprostane in human and experimental transmissible spongiform encephalopathies. *J. Neuropathol. Exp. Neurol.* 59: 866–871, 2000.
89. Minghetti, L. and Levi, G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog. Neurobiol.* 54: 99–125, 1998.
90. Fiebich, B. L., Schleicher, S., Spleiss, O., Czygan, M. and Hull, M. Mechanisms of prostaglandin E2-induced interleukin-6 release in astrocytes: possible involvement of EP4-like receptors, p38 mitogen-activated protein kinase and protein kinase C. *J. Neurochem.* 5: 950–958, 2001.
91. Kim, E. J., Kwon, K. J., Park, J. Y., Lee, S. H., Moon, C. H. and Baik, E. J. Neuroprotective effects of prostaglandin E2 or cAMP against microglial and neuronal free radical mediated toxicity associated with inflammation. *J. Neurosci. Res.* 70: 97–107, 2002.
92. Barres, B.A. What is a glial cell? *Glia* 43: 4–5, 2003.
93. Hansson, E. and Rönnbäck, L., Glial neuronal signaling in the central nervous system. *FASEB J.* 17: 341–348, 2003.
94. Villegas, S. N., Poletta, F. A. and Carr, N. G. Glia: a reassessment based on novel data on the developing and mature central nervous system. *Cell Biol. Int.* 27: 599–609, 2003.

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Metabolic encephalopathies result from alterations of brain chemistry at both neocortical and brainstem ARAS centers. Respiration may be diminished and pupils appear small but reactive. As encephalopathy progresses, asterixis, also termed 'flapping tremor', is encountered, particularly in hepatic disease, uremia and sedative intoxication. Asterixis results from the loss of postural tone in voluntary muscles of the limbs, trunk, head or tongue. More advanced stages of metabolic encephalopathy may be characterized by seizures (for example in hypoglycemia and acute liver failure) and, ultimately, Cheyne–Stokes pattern of respiration resulting from loss of brainstem respiratory control. In addition, many metabolic encephalopathies, including those caused by vitamin deficiencies and ingestion of toxic substances, are characterized by focal metabolic changes in basal ganglia and cerebellar structures, resulting in disorders of movement control and coordination.

BRAIN ENERGY METABOLISM

The brain has an absolute dependence on the blood for its immediate supply of oxygen and energy substrates. Interruption of oxygen or substrate supply by compromise of pulmonary or cardiovascular function or metabolic factors results in encephalopathy and, if prolonged, neuronal cell death. The brain uses approximately 20% of the total oxygen supply of the body. While glucose remains the primary energy substrate for the brain, alternative substrates may be used under certain circumstances (see Ch. 31).

Given the high dependency of cerebral energy production and neurotransmitter synthesis on glucose and oxygen, limitations in supply of these substrates results in metabolic encephalopathy.

METABOLIC ENCEPHALOPATHY DUE TO LACK OF ENERGY SUBSTRATE

Hypoglycemia results in depression of CNS function, with rostral brain regions being affected before more caudally situated regions. For example, in severe hypoglycemia associated with isoelectric EEG tracings, cerebral cortical activity is absent but medullary function persists, as indicated by the maintenance of effective respiratory and cardiovascular activity.

Early clinical signs in hypoglycemia include sweating, also termed diaphoresis, tachycardia and anxiety. If unheeded, these symptoms give way to a more serious CNS disorder progressing through confusion, lethargy and delirium followed by seizures and coma. Prolonged hypoglycemia may lead to neuronal cell death and irreversible brain damage.

During the progression of hypoglycemia, as blood glucose falls below 2.5 mmol/l, confusion and delirium occur and the cerebral metabolic rate for glucose (CMR_{glc}) falls more rapidly than does the cerebral metabolic rate for oxygen ($CMRO_2$), a finding that signifies the utilization of substrates other than glucose by the brain. Such substrates include tricarboxylic acid (TCA) cycle intermediates as well as amino acids, particularly glutamine and glutamate. However, these substrates are quickly used up and support cerebral energy requirements for only a few minutes in the absence of glucose. As blood glucose concentrations fall below 2 mmol/l, the electroencephalogram (EEG) initially shows increased amplitude and decreased frequency as blood glucose concentrations approach 1 mmol/l. Below 1 mmol/l blood glucose concentrations, brain ATP levels become depleted [1] (Fig. 34-1), the EEG becomes isoelectric and coma develops.

The initial neurological symptoms of hypoxia and hypoglycemia result from reduced synthesis of neurotransmitters rather than a global cerebral energy deficit. Glucose oxidation by the brain not only provides energy in the form of the anhydride bonds of ATP but also provides precursors for the synthesis of some key excitatory and inhibitory neurotransmitters, including acetylcholine (ACh), γ-amino-butyric acid (GABA) and glutamate.

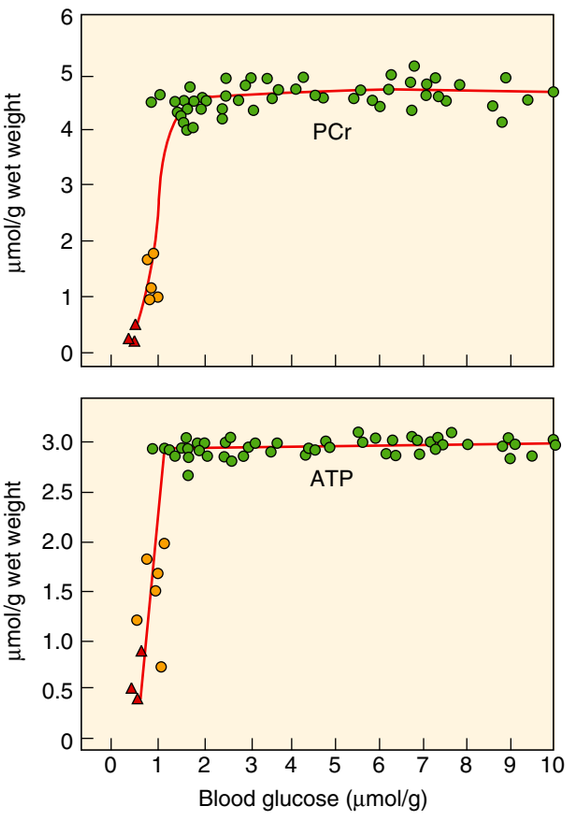

FIGURE 34-1 Neocortical concentrations of phosphocreatine (*PCr*) and ATP in rats subjected to graded hypoglycemia at various stages during the development of encephalopathy. *Green circles*, precoma; *orange circles*, coma; *red triangles*, isoelectric EEG. (With permission from reference [1].)

CHAPTER

34

Metabolic Encephalopathies

Roger F. Butterworth

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The metabolic encephalopathies comprise a series of neurological disorders not caused by primary structural abnormalities; rather, they result from systemic illness, such as diabetes, liver disease and renal failure. Metabolic encephalopathies usually develop acutely or subacutely and are reversible if the systemic disorder is treated. If left untreated, however, metabolic encephalopathies may result in secondary structural damage to the brain.

There are two major types of metabolic encephalopathy: those due to lack of glucose, oxygen or metabolic cofactors (vitamins, for example) and those due to peripheral organ dysfunction. This chapter will review the neurochemistry of the major metabolic encephalopathies, which are listed in Table 34-1.

Clinical signs and symptoms of metabolic encephalopathies consist of a generalized depression of cerebral function, including consciousness. The effects on consciousness may be a consequence of decreased integrative capacity of the neocortex. Arousal of the neocortex and other forebrain structures involved in cognition is mediated by specific brainstem nuclei and their projecting fiber tracts, which together constitute the ascending reticular activating system (ARAS). Activating pathways ascend from the ARAS via thalamic synaptic relays to the neocortex.

TABLE 34-1 Metabolic encephalopathies

Hyponatremia	
Hypernatremia	
Phosphate depletion	
Uremia	Uremic encephalopathy
	Dialysis dementia
Hepatic encephalopathy	
Hyperbilirubinemia	
Hypocalcemia	
Hypercalcemia	
Hyperparathyroidism	
Hypoparathyroidism	
Thiamine deficiency (Wernicke's) encephalopathy	
Diabetic ketoacidosis	
Nonketotic hyperosmolar coma	
Phosphate depletion	
Hypoglycemia	
Hypoxemia	
Hypercapnia	

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Most evidence suggests that the neurological symptoms characteristic of hypoglycemic encephalopathy prior to isoelectric EEG stages result from 'neurotransmission failure' involving one or more of these neurotransmitter systems. Pyruvate derived from glucose is the major precursor of the acetyl group of ACh. Inhibition of pyruvate oxidation results in reduced ACh synthesis both *in vitro* and *in vivo*. Incorporation of [^{14}C] choline into ACh in brain *in vivo* is decreased in rats with insulin-induced hypoglycemia. That hypoglycemic encephalopathy results in decreased synthesis of the neurotransmitter pool of ACh is supported by the observation that administration of the cholinesterase inhibitor physostigmine to hypoglycemic animals delays the onset of seizures and coma [2].

Similar findings of an adverse effect of hypoglycemia on the synthesis of the amino acid neurotransmitters GABA and glutamate have also been reported. Utilization of amino acids such as glutamate and glutamine as alternative energy substrates in moderate to severe hypoglycemia results in accumulation of aspartate and ammonia in the brain [3]. Hypoglycemia also produces a transient but substantial increase in extracellular concentrations of glutamate, GABA and dopamine, as measured using *in vivo* cerebral microdialysis. Alterations of neurotransmission mediated by ACh, glutamate, GABA and/or dopamine could contribute to the neurological signs and symptoms that characterize moderate hypoglycemia.

As hypoglycemia progresses below 1 mmol/l the EEG becomes isoelectric and neuronal cell death ensues. As is the case in other metabolic encephalopathies, cell death is not global in distribution; rather, certain brain structures, in particular hippocampal and cortical structures, are selectively vulnerable to hypoglycemic insult. Pathophysiological mechanisms responsible for neuronal cell death in hypoglycemia include glutamate excitotoxicity. Neuronal cell death mechanisms related to glutamate excitotoxicity are discussed in Chapter 32.

An uninterrupted supply of oxygen is vital for cerebral function. Normal human brain consumes 3.3 ml of oxygen per 100 g of brain per minute ($CMRO_2$), a value that remains relatively stable during periods of wakefulness.

Experimental studies demonstrate that cerebral energy metabolism remains normal when mild to moderate hypoxia (P_aO_2 = 25–40 mmHg) results in severe cognitive dysfunction in human volunteers [1]. In mild hypoxia, cerebral blood flow (CBF) increases in order to maintain oxygen delivery to the brain. However, CBF can increase only about twofold; beyond this $CMRO_2$ starts to fall and symptoms of hypoxia occur. In normal individuals exposed acutely to high altitudes (Table 34-2), difficulties with complex learning tasks and short-term memory appear at altitudes of 10,000 feet as P_aO_2 falls below 45 mmHg, and above 20,000 feet, where P_aO_2 = 30 mmHg, cognitive disturbances and problems with motor coordination start to appear. Acute hypoxia of less than P_aO_2 = 20 mmHg generally results in coma. In graded hypoxia in experimental animals, brain ATP levels remain normal at P_aO_2 = 20–25 mmHg despite EEG slowing and attenuated sensory evoked responses.

TABLE 34-2 Hypoxic thresholds for CNS dysfunction

Simulated altitude (feet)	F_iO_2 (%)	P_aO_2 (mmHg)	Neurological status
Sea level	21	90	Normal
500	17	80	Impaired dark vision
8,000–10,000	15–14	55–45	Impaired short-term memory, difficulty learning complex tasks
15,000–20,000	11–9	40–30	Loss of judgment, euphoria, obtundation
>20,000	<9	<25	Coma

Values derived from young volunteers subjected to acute (minutes) decompression hypoxia.
F_iO_2, fractional percentage of ambient oxygen.

Glycolysis is stimulated to maintain brain adenosine triphosphate levels in moderate hypoxia. Studies in experimental animals reveal that, at P_aO_2 = 50 mmHg, an oxygen tension well in excess of that necessary to cause impaired ATP synthesis, an increase in the lactate:pyruvate ratio and a decrease in brain tissue pH are observed. Increased lactate production is accompanied by a rise in CMR_{glc} [1], indicating accelerated glycolysis. The cytosolic redox potential measured by the NADH:NAD^+ ratio is shifted toward a more reduced state at P_aO_2 of 20 mmHg. This steep fall in phosphocreatine coincides with a decrease in ATP and reflects failure of mitochondrial respiration and oxidative phosphorylation.

Hypoxia results in alterations of neurotransmitter synthesis and release. As in hypoglycemia (above) neurotransmitter synthesis in brain is exquisitely sensitive to hypoxia. When rats are made to breathe 10% oxygen, ACh synthesis is decreased by 68% [4]. Hypoxia of a similar magnitude also results in altered synthesis and release of other neurotransmitters, including those derived from glucose. Arterial oxygen tension of less than 50 mmHg results in reductions in synthesis of glutamate and GABA in brain. Synthesis of the biogenic amines may also be susceptible to hypoxia since tyrosine hydroxylase and tryptophan hydroxylase, the rate-limiting enzymes in monoamine neurotransmitter synthesis, require molecular oxygen, for which the K_m (O_2) is 12 µmol/l, equivalent to 7 mmHg.

HYPONATREMIC ENCEPHALOPATHY

If uncontrolled, hyponatremia may cause increased intracranial pressure, brain herniation and death. The major cause of hyponatremic encephalopathy is an osmotic imbalance between extracellular fluid and neural cells leading to a net movement of water into the brain

and, consequently, brain edema. If uncontrolled, this process may lead to increased intracranial pressure and brain herniation. Symptoms of hyponatremia include lethargy and decreased responsiveness progressing to seizures and, if uncontrolled, death. Hyponatremia is commonly encountered in a wide range of disorders including liver cirrhosis, diabetes and acquired immune deficiency syndrome (AIDS).

Regulation of the fluid–electrolyte environment of the brain is influenced by a neuroendocrine system that affects the capillary endothelium, the choroid plexus and astroglial cells. These barrier system tissues express receptors for endogenous neuropeptides whose action affects water and ion movement. Brain responds to hyponatremia by extrusion of cations (Na^+, K^+, some amino acids) in order to lower its osmolarity towards that of plasma; water then enters brain, reducing the progression towards edema. Steroid hormones modulate these peptidergic systems and evidence suggests that the interplay between steroid hormones and endogenous vasoactive peptides is important in the pathogenesis of hyponatremic encephalopathy [5]. Circulating steroid hormones are lipophilic and readily cross the blood–brain barrier.

Ovarian hormones influence fluid intake by interaction with the brain renin–angiotensin system and it has been shown that gonadal steroids affect brain fluid–electrolyte balance by interactions with vasopressin. Both hyperosmolarity and increased intracranial pressure stimulate vasopressin release and intraperitoneal administration of vasopressin antagonists decrease brain volume.

Treatment of hyponatremia involves the use of hypertonic saline. However, care is required since correction at rates in excess of 1 mEq/l/h may result in central pontine myelinolysis (CPM) [5]. Symptoms of CPM include progressive weakness leading to quadriparesis, pseudobulbar palsy and altered mental status. CPM is often fatal and is characterized neuropathologically by demyelinating lesions in the central pons.

HYPERCAPNIC ENCEPHALOPATHY

Respiratory acidosis leads to decreased brain pH. Patients with chronic pulmonary disorders may exhibit lethargy, confusion, memory loss and stupor. The combined insults of hypoxia and hypercapnia, which result in CO_2 retention, contribute to the encephalopathy but neurological symptoms correlate best with the degree of CO_2 retention. Acute moderate hypercapnia associated with 5–10% CO_2 in the expired air, leads to arousal and excitability whereas higher CO_2 concentrations, >35% in the expired air, are anesthetic.

Although CO_2 is a normal metabolite, it is toxic at elevated levels. CO_2 exists in equilibrium with carbonic acid (H_2CO_3) and with bicarbonate (HCO_3^-), a major H^+ buffer. Renal conservation of HCO_3^- is generally sufficient to buffer hypercapnia; however, an added insult, such as an infection, fatigue or ingestion of a sedative, may further compromise pulmonary function, resulting in further CO_2 retention and disruption of the normal buffering mechanisms. The respiratory acidosis associated with CO_2 retention in blood leads to a proportional increase in brain tissue $[H^+]$.

The combination of hypoxia and hypercapnia in pulmonary insufficiency results in cerebral vasodilation and increased CBF and may lead to increased intracranial pressure. Arteriovenous differences for oxygen across the brain generally decrease as a function of increased CBF, leaving $CMRO_2$ unchanged [6].

Acute hypercapnic acidosis leads to an increase in concentrations of glycolytic intermediates above the phosphofructokinase step; a decrease below this step is probably due to inhibition of phosphofructokinase by $[H^+]$. Brain ATP concentrations are unchanged in hypercapnia and it is generally believed that decreased brain glucose utilization is the result of decreased neuronal activity and, hence, reduced fuel (glucose) requirements. As in hypoglycemia and hypoxia, neurotransmitter-related mechanisms that could contribute to hypercapnic encephalopathy include decreased neurotransmitter glutamate and GABA pools and decreased synthesis of acetylcholine.

METABOLIC ENCEPHALOPATHY DUE TO PERIPHERAL ORGAN FAILURE

Hepatic encephalopathy.

Hepatic encephalopathy is a neuropsychiatric disorder resulting from liver failure. Neurologically, hepatic encephalopathy evolves slowly. Early symptoms include altered sleep patterns and personality changes, followed by shortened attention span and asterixis progressing through stupor to coma as liver function deteriorates. Hepatic encephalopathy in acute liver failure progresses rapidly through altered mental status to stupor and coma within hours or days. Seizures are occasionally encountered and mortality rates are high. Death most frequently results from brainstem herniation caused by increased intracranial pressure as a consequence of astrocytic swelling and brain edema.

Hepatic encephalopathy in chronic liver failure, on the other hand, is characterized neuropathologically by astrocytic changes known as Alzheimer type II astrocytosis. The Alzheimer type II astrocyte has a characteristic swollen shape with a large, pale nucleus, prominent nucleolus and margination of chromatin (Fig. 34-2). In addition, astrocytes in chronic liver failure manifest reduced expression of key astrocytic proteins, such as glial fibrillary acidic protein (GFAP), glutamine synthetase and monoamine oxidase type B, and increased expression of astrocytic 'peripheral-type' benzodiazepine receptors (PTBRs) [7]. These changes suggest that normal astrocytic functions, such as ammonia removal and the uptake and metabolism of monoamine and amino acid neurotransmitters, are impaired; and it has been proposed that hepatic

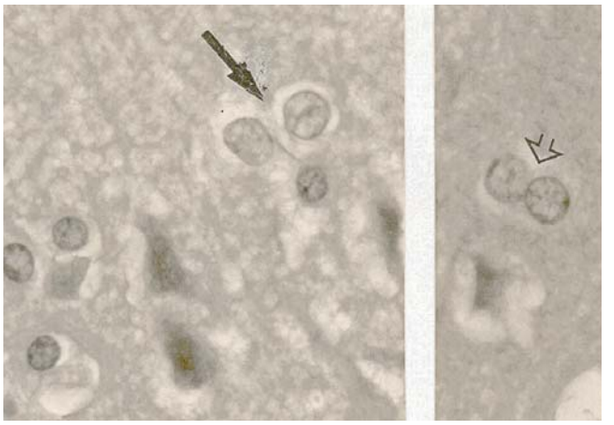


FIGURE 34-2 Typical pattern of Alzheimer type II astrocytosis in frontal cortex of a 51-year-old cirrhotic patient who died in hepatic coma. Note astrocytes with large pale nucleus and margination of chromatin (*large arrow*). Dodoublets of Alzheimer type II cells frequently appear (*open arrowhead*).

encephalopathy in chronic liver failure results from a disorder of 'neuron-astrocyte trafficking' of key substrates.

Liver failure leads to the accumulation of toxic substances in brain. Two such substances have been identified, namely ammonia and manganese. Magnetic resonance imaging (MRI) reveals increased signal intensity in pallidum on T1-weighted MRI in the majority of patients with chronic liver disease, suggesting the presence of a paramagnetic substance in this region of the brain. Evidence to date suggests that this substance is manganese. Blood manganese concentrations are increased in cirrhotic patients, and a positive correlation is observed between blood and brain manganese content and the magnitude of pallidal MRI signal hyperintensity in these cases [8]. Manganese deposition in the basal ganglia could explain the extrapyramidal symptoms frequently encountered in hepatic encephalopathy patients, such as tremor and rigidity (see Ch. 46).

Both ammonia and manganese may be responsible for the astrocytic morphological changes observed in hepatic encephalopathy. Exposure of cultured astrocytes to ammonia results in decreased expression of GFAP and decreased capacity of cells to take up glutamate, phenomena that are consistently observed in the brains of humans or experimental animals with hepatic encephalopathy. Similar reduced glutamate uptake is observed following exposure of cultured astrocytes to manganese and exposure to manganese causes Alzheimer type II astrocytosis.

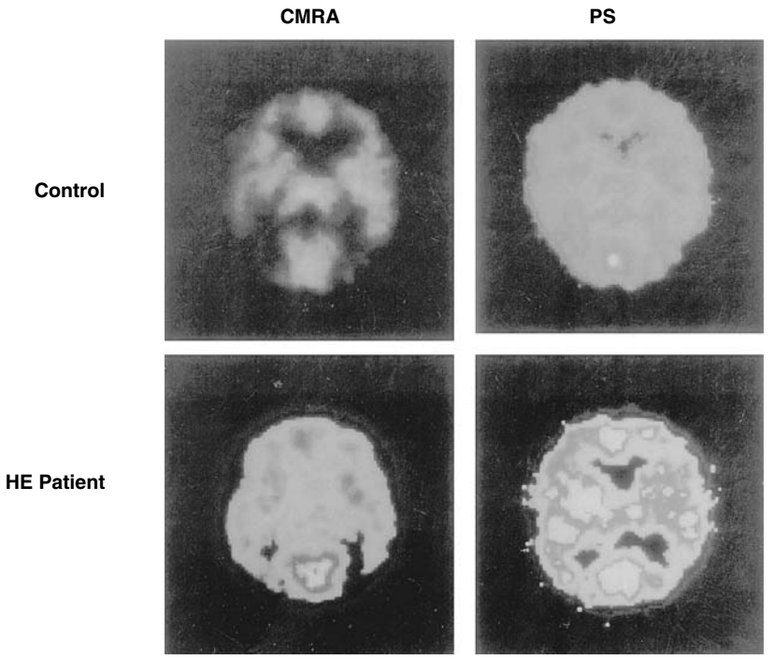
Ammonia concentrations in arterial blood of patients with liver failure rise to 0.5–1 mmol/l, in contrast to the normal range of 0.01–0.02 mmol/l. Using positron emission tomography (PET; see Ch. 58), increases of the cerebral metabolic rate for ammonia (CMRA), i.e. the rate at which ammonia is taken up and metabolized by the brain, have been reported in chronic liver failure [9]. Increased CMRA in chronic liver failure is accompanied by increases in the permeability/surface area product (PS), a measure of blood–brain barrier permeability to ammonia (Fig. 34-3).

Ammonia has deleterious effects on brain function by direct and indirect mechanisms. Concentrations of ammonia in the 1–2 mmol/l range, equivalent to those reported in the brain in liver failure, impair postsynaptic inhibition in cerebral cortex and brainstem by a direct effect on Cl^- extrusion from the postsynaptic neuron. Millimolar concentrations of ammonia also inhibit excitatory neurotransmission. Synaptic transmission from Schaffer collaterals to CA1 hippocampal neurons is reversibly depressed by 1 mmol/l ammonia, and the firing of CA1 neurons by iontophoretic application of glutamate is inhibited by 2 mmol/l ammonia [10].

Addition of millimolar concentrations of ammonia to brain preparations results in inhibition of α-ketoglutarate dehydrogenase (αKGDH). αKGDH is a rate limiting enzyme of the TCA cycle and, consistent with decreased oxidation of pyruvate (and α-ketoglutarate), both acute and chronic liver failure result in increased brain lactate concentrations. Ammonia-precipitated coma in portacaval-shunted rats results in decreased brain ATP, but these changes are not apparent until late (preterminal) stages associated with deep coma.

Hepatic encephalopathy is a disorder of multiple neurotransmitter systems. In common with hypoglycemic and hypoxic encephalopathies, early stages of hepatic encephalopathy do not result from a cerebral energy deficit; rather they are associated with alterations of multiple neurotransmitter systems in the brain. In most cases, these changes appear to be directly or indirectly the consequence of ammonia or manganese toxicity. Neuron-astrocyte trafficking of substrates is impaired results in glutamatergic synaptic dysregulation, a phenomenon that could contribute to the pathogenesis of hepatic encephalopathy. Glutamate uptake into cultured astrocytes is reduced by exposure to submillimolar concentrations of ammonia. Furthermore, consistent with diminished clearance of glutamate from the synaptic cleft in hepatic encephalopathy, microdialysis studies in animals with experimental acute liver failure reveal increased extracellular concentrations of glutamate as a function of the degree of neurological impairment, and reduction of expression of the astrocytic glutamate transporter (EAAT-2) has been reported in brain in experimental acute liver failure.

Many of the neuropsychiatric symptoms in early hepatic encephalopathy, such as altered sleep patterns, are signs that have classically been attributed to modifications of serotoninergic neurotransmission. Serotonin turnover, as indicated by the ratio of the concentrations of the metabolite 5-hydroxyindoleacetic acid to serotonin, is increased in brain in both human and experimental hepatic encephalopathy.



	Normal (5)	Hepatic encephalopathy Patients (5)
Arterial ammonia μmol/l	30 ± 7	62 ± 20*
Cerebral metabolic rate for ammonia (CMRA) (μmol/l100 g/min)	0.35 ± 0.15	0.91 ± 0.36*
Blood–brain barrier permeability (PS) to ammonia (ml/g/min)	0.13 ± 0.03	0.22 ± 0.07*

*$p < 0.01$, compared to normal values

FIGURE 34-3 Positron emission tomography using $^{13}NH_3$ showing increased brain ammonia uptake in a patient with liver cirrhosis and mild hepatic encephalopathy. *CMRA*, cerebral metabolic ratio for ammonia; *HE*, hepatic encephalopathy; *PS*, permeability/surface area product. (With permission from reference [9].)

The PTBR is expressed in increased amounts in hepatic encephalopathy. In the CNS, PTBRs are localized mainly on the mitochondrial membranes of astrocytes. Increases of expression and maximal binding site densities of PTBRs have consistently been described in brain and peripheral tissues in experimental and human hepatic encephalopathy brain. Increased PTBR expression also occurs in cultured astrocytes exposed to ammonia or manganese. Precisely how the increased expression of PTBRs in brain is related to the neuropsychiatric symptoms in hepatic encephalopathy is unclear, but it has been demonstrated that activation of the PTBR leads to the production of neurosteroids, such as 3-α-hydroxy-5α-pregnan-20-one (allopregnanolone), a compound that has a high affinity for the $GABA_A$ receptor and for which it is a positive allosteric modulator. These findings suggest that PTBR activation could contribute to 'increased GABAergic tone' in hepatic encephalopathy via the production of neurosteroids [7].

Finally, there is an emerging body of evidence to suggest that the endogenous opioid system of the brain may be involved in the mediation of some of the effects of chronic liver failure on cerebral function. Increased plasma concentrations of the endogenous opioid peptide met-enkephalin have been described in patients with primary biliary cirrhosis, and β-endorphin concentrations are increased in the brains of rats following portacaval shunting together with alterations of μ and δ opioid-binding sites [11].

Bilirubin encephalopathy.

Bilirubin, a product of hemoglobin metabolism, causes brain dysfunction and apoptotic cell death. In many premature neonatal infants, high levels of unconjugated bilirubin (UCB) occur in the blood, causing jaundice. In severe cases this can lead to bilirubin encephalopathy characterized by hypotonia and lethargy, which may progress to neuronal damage and death if not treated promptly. Surviving infants have an increased prevalence of cerebral palsy and mental retardation [12].

Both astrocytes and neurons are susceptible to UCB exposure. Isolated neurons treated with UCB show increased evidence of apoptosis with release of cytochrome

c and activation of caspase-3. In addition, UCB exposure decreases mitochondrial transmembrane potential and increases mitochondrial-associated BAX protein levels while concomitantly disrupting membrane lipid and protein structure. Apoptosis has also been reported in the cerebellum of jaundiced Gunn rat pups. The Gunn rat has an autosomal recessive deficiency of glucuronyl transferase resulting in hyperbilirubinemia.

Exposure of rat cortical astrocytes to UCB results in a significant reduction of high-affinity glutamate uptake which is time, concentration and pH-dependent leading to increased extracellular glutamate. Consequently, NMDA-receptor-mediated excitotoxicity may be implicated in the pathogenesis of neuronal cell death due to UCB exposure. In favor of this possibility, the NMDA receptor antagonist MK801 attenuates the severity of cerebellar neuronal loss in the Gunn rat [13].

Uremic and dialysis encephalopathies. Patients with renal failure continue to manifest neuropsychiatric symptoms despite significant advances in therapeutics and management. Patients with renal failure who are not yet on dialysis develop an array of symptoms, including clouding of consciousness, disturbed sleep patterns, tremor and asterixis that may progress to coma and death.

Uremia is characterized by retention in the blood of urea, phosphates, proteins, amines and a number of poorly defined low-molecular-weight compounds. Despite acidemia, the pH of brain, muscle and CSF are normal in humans with renal failure. Whereas brain water, K^+ and Mg^{2+} content are normal, Al^{3+} and Ca^{2+} concentrations are significantly increased [14].

Uremia leads to alterations of the blood–brain barrier. Uremia results in increased permeability of the blood–brain barrier to sucrose and insulin; K^+ transport is enhanced whereas Na^+ transport is impaired. There is an increase in brain osmolarity in acute renal failure due to the increase in urea concentrations. However, in contrast to acute renal failure, the increase in osmolarity in chronic renal failure results from the presence of idiogenic osmoles in addition to urea. CBF is increased in uremic patients but $CMRO_2$ and CMR_{glc} are decreased. In the brains of rats with acute renal failure, ATP, phosphocreatine and glucose are increased whereas AMP, ADP and lactate are decreased, most probably as a result of decreased energy demands.

Parathyroid hormone (PTH) produces CNS effects in normal subjects and neuropsychiatric symptoms are frequently encountered in patients with primary hyperparathyroidism, where EEG changes resemble those described in acute renal failure. Circulating PTH is not removed by hemodialysis. In uremic patients both EEG changes and neuropsychiatric symptoms are improved by either parathyroidectomy or medical suppression of PTH. The mechanism whereby PTH causes disturbances of CNS function is not well understood, but it has been suggested that increased PTH might facilitate the entry of Ca^{2+} into the cell resulting in cell death.

Aluminum neurotoxicity may play a role in dialysis encephalopathy. This condition, also termed 'dialysis dementia', is a progressive, frequently fatal neurological disorder that occurs in patients on chronic hemodialysis. Symptoms progress through personality changes, psychosis, seizures and dementia. Dialysis dementia may result from aluminum accumulation in brain: the aluminum content of cerebral gray matter from patients with dialysis dementia is 11 times higher than normal and three times higher than that observed in patients on chronic hemodialysis but without dialysis dementia. Aluminum is normally excreted by the kidney.

Epidemiological studies have shown a strong association between the aluminum content of the water used to prepare dialysates and the incidence of dialysis dementia. Reducing water aluminum content below 20 μg/l appears to prevent the onset of the disease in patients who have just started dialysis.

THIAMINE DEFICIENCY (WERNICKE'S) ENCEPHALOPATHY

Thiamine deficiency results in early decreases in activity of the mitochondrial enzyme α-ketoglutarate dehydrogenase in brain. Wernicke's encephalopathy, also known as the Wernicke–Korsakoff syndrome is a neuropsychiatric disorder characterized by ophthalmoplegia, ataxia and memory loss. Wernicke's encephalopathy is encountered in chronic alcoholism, in patients with HIV-AIDS and in other disorders associated with grossly impaired nutritional status. The condition results from thiamine deficiency.

In the 1930s, Peters and co-workers showed that thiamine deficiency in pigeons resulted in the accumulation of lactate in the brainstem [15]. Furthermore, they showed that the addition of small quantities of crystalline thiamine to the isolated brainstem tissue from thiamine-deficient birds *in vitro* resulted in normalization of lactate levels. These findings led to the formulation of the concept of 'the biochemical lesion' in thiamine deficiency. Subsequent studies showed that the enzyme defect responsible for the 'biochemical lesion' was α-KGDH rather than pyruvate dehydrogenase (PHDC), as had previously been presumed. α-KGDH and PHDC are major thiamine diphosphate (TDP)-dependent enzymes involved in brain glucose oxidation (Fig. 34-4).

In brain, as in most mammalian cells, thiamine occurs predominantly in the form of TDP, the remainder being made up of thiamine monophosphate (10%), thiamine triphosphate (5–10%) and trace amounts of free thiamine. Thiamine is transported into brain and phosphorylated by the action of thiamine pyrophosphokinase, and inhibition of this enzyme by thiamine antagonists such as pyrithiamine results in decrease synthesis of TDP. Treatment of experimental animals with pyrithiamine results in a generalized reduction of TDP concentrations and an early selective loss in activity of α-KGDH in regions

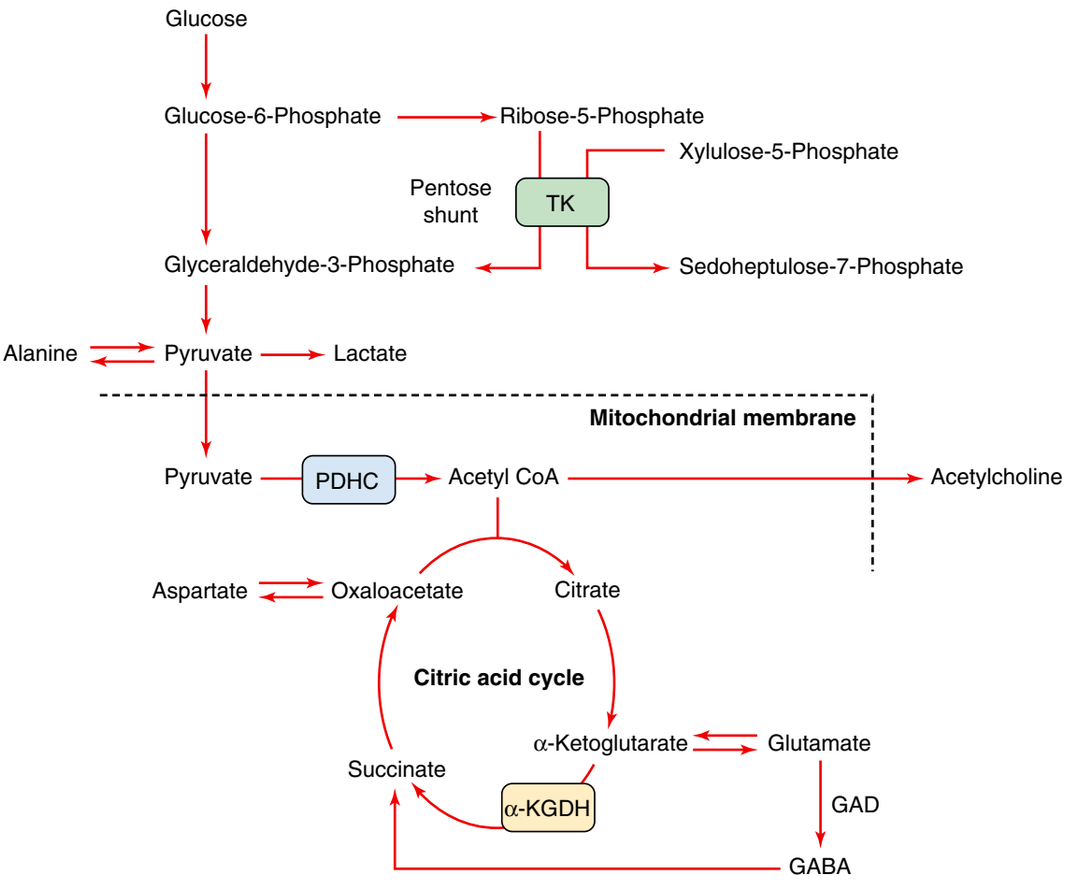


FIGURE 34-4 Thiamine-diphosphate-dependent enzymes associated with the tricarboxylic acid cycle and glucose oxidation. Thiamine deficiency results in an early loss of activity of α-KGDH in brain and reduced synthesis of acetylcholine, glutamate and GABA. *α-KGDH,* α-ketoglutarate dehydrogenase; *PDHC,* pyruvate dehydrogenase complex; *TK,* transketolase in the pentose shunt.

of the brain such as thalamus, which are destined to manifest selective neuronal cell loss. Decreased activities of α-KGDH following treatment with pyrithiamine are associated with decreased synthesis of glucose-derived excitatory and inhibitory amino acids including glutamate, aspartate and GABA with a concomitant increase in lactate and alanine consistent with decreased flux of carbon through the TCA cycle. Both the α-KGDH decreases and the changes in synthesis of amino acids are initially reversible following thiamine rehabilitation in pyrithiamine-treated animals [16], suggesting that these changes are an integral part of 'the biochemical lesion' originally proposed by Peters.

Oxidative decarboxylation of α-ketoglutarate (and pyruvate) are decreased in isolated mitochondria from the brains of thiamine deficient rats, consistent with reduction in activity of α-KGDH. Moreover, studies in mitochondrial preparations from thiamine-deficient animals revealed that state 3 respiration is significantly decreased using α-ketoglutarate as substrate but was unchanged using succinate (succinate is oxidized independently of TDP-dependent enzymes, Fig. 34-4). Cultured cerebellar granule cells exposed to pyrithiamine manifest significant reductions in TDP and activities of α-KGDH and increased lactate production indicative of decreased TCA cycle flux and a significant lowering of cellular pH [17]. More prolonged exposure to thiamine deficiency leads to depolarization, lactate accumulation, decreased ATP production and cell necrosis.

Oxidative stress contributes to selective neuronal cell death in thiamine-deficiency. Increasing evidence suggests a role for oxidative stress in the pathophysiology of selective neuronal cell death due to thiamine deficiency. Increased production of reactive oxygen species has been reported in the brains of pyrithiamine-treated rats [18] and increased peroxidase activity indicative of oxidative stress is related to the neuropathology in Wernicke's encephalopathy in humans. Other findings consistent with oxidative stress as a contributing factor to thiamine-deficiency-related neuropathology include reports of increase in heme oxygenase, early induction of endothelial NOS (eNOS) and microglial activation.

Neuropathologic studies in animals with experimental thiamine deficiency consistently show early damage to glial cells rather than neurons. Studies in human patients with Wernicke's encephalopathy likewise show changes in astroglia, together with microglial proliferation, which is

apparent even in regions of the brain showing little if any neuronal cell death. A systematic study of astroglial integrity in thiamine deficiency using cell-specific markers (ED-1 for microglial activation; GFAP for astrocytic proliferation) revealed early microglial activation in brains of thiamine-deficient animals [19]. Increased ED-1 immunolabeling was confined to vulnerable brain structures such as thalamus, inferior colliculus and vestibular nuclei and was found to precede neuronal cell loss, breakdown of the blood–brain barrier and reactive astrocytosis in the brains of these animals. These findings suggest that metabolically compromised neurons (due to thiamine deficiency) could initiate, by cell-to-cell signaling pathways, microglial activation as an initial cellular event.

A recent study showed significant increases of expression of eNOS in the brains of rats treated with pyrithiamine [20]. Increased eNOS expression was apparent prior to the onset of neurological symptoms and was restricted to vulnerable medial thalamus and inferior colliculus. Expression of inducible (iNOS) and neuronal (nNOS) isoforms were minimally altered in brain in thiamine deficiency and it has also been shown that targeted disruption of the eNOS (but not the iNOS or nNOS) gene results in reduced extent of neuropathological damage in thalamus of thiamine deficient animals [21] (Fig. 34-5).

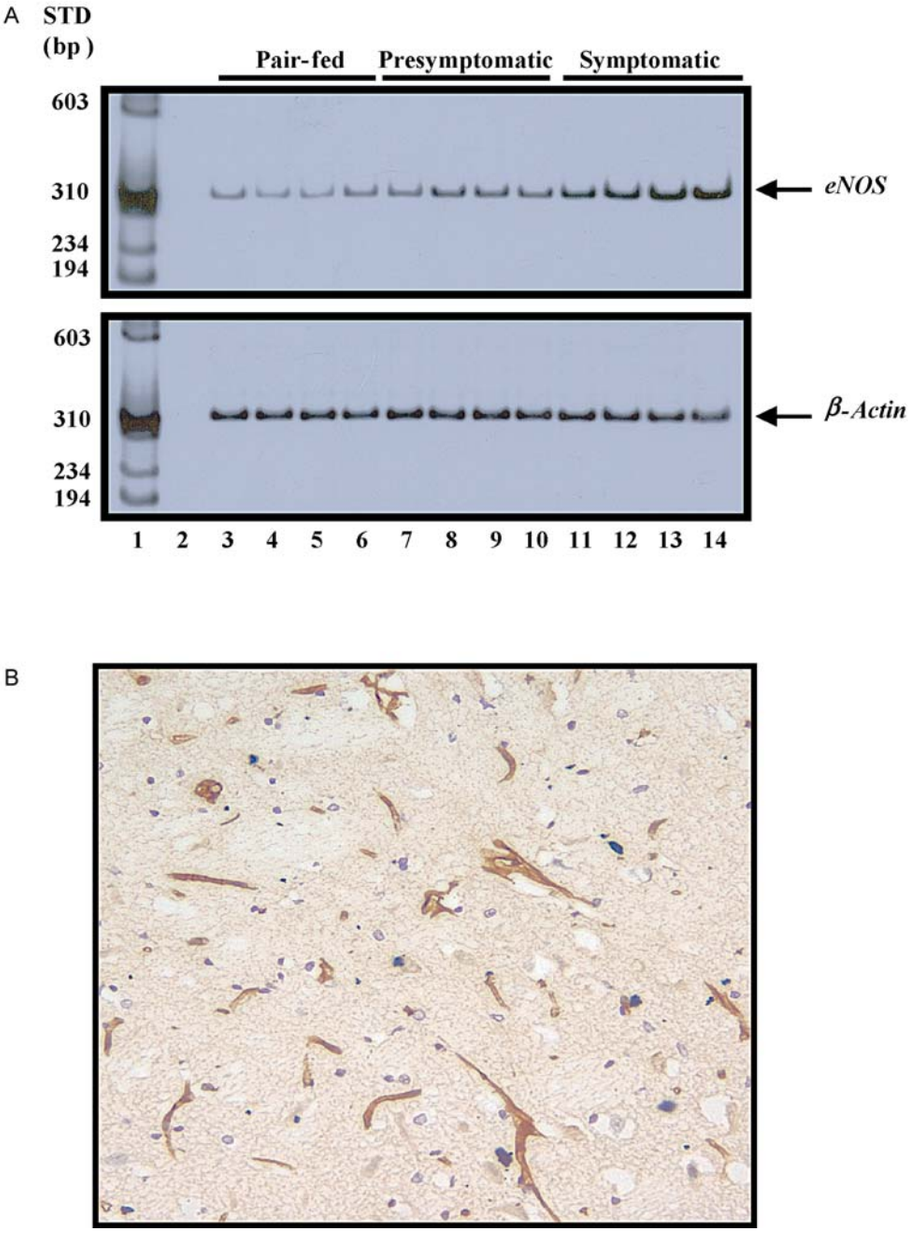


FIGURE 34-5 Induction of endothelial nitric oxide synthase (*eNOS*) in medial thalamus of thiamine-deficient rats. (A) Increased eNOS mRNA. (B) Increased eNOS immunolabeling of vascular endothelial cells (magnification ×200).

The suggestion that eNOS-derived NO is implicated in neuronal cell death mechanisms in thiamine deficiency contrasts with current views in cerebral ischemia in which increased eNOS-derived NO is thought to play a neuroprotective role by virtue of its vasodilatory potential.

PYRIDOXINE (VITAMIN B_6) DEFICIENCY

Pyridoxine, in its phosphorylated form, pyridoxal phosphate, is an important coenzyme in several enzymic reactions, some of which are involved in neurotransmitter synthesis (e.g. decarboxylation of glutamate to GABA–see Ch. 16). Developing brain is particularly sensitive to pyridoxine deficiency, resulting in impaired synaptogenesis and hypomyelination. Adult animals fed pyridoxine-deficient diets develop learning disabilities, irritability, gait ataxia and seizures; the latter develop in parallel with the fall in activity of glutamic acid decarboxylase and GABA synthesis.

COBALAMIN (VITAMIN B_{12}) DEFICIENCY

Cobalamins have two biochemical functions. Adenosylcobalamin is the cofactor for the mutase enzyme that converts methylmalonyl CoA to succinyl CoA. Methylcobalamin, on the other hand, is a cofactor for the enzyme that converts homocysteine to methionine. Cobalamin deficiency occurs relatively commonly in the clinic and involves both hematological and neurological changes. Neurologically, cobalamin deficiency results in paresthesias, loss of sensation and spastic weakness. Neuropathologically, a spongy demyelination of the long fiber tracts of spinal cord, particularly the posterior columns, is noted. Whether the CNS symptoms and neuropathology are the consequence of deficiency of the mutase or the methyltransferase enzymes is unresolved.

REFERENCES

1. Siesjo, B. K. (ed.) *Brain Energy Metabolism.* New York: John Wiley & Sons, 1978, pp. 380–446.
2. Gibson, G. E. and Blass, J. P. Impaired synthesis of acetylcholine in brain accompanying hypoglycemia and mild hypoxia. *J. Neurochem.* 27: 37–42, 1976.
3. Butterworth, R. F. Metabolism of gluatamate and related amino acids in insulin hypoglycaemia. In L. Hertz, E. Kvamme, E. G. McGeer and A. Schousboe (eds), *Glutamine, Glutamate and GABA in the Central Nervous System.* New York: Alan R. Liss, 1983, pp. 595 – 608.
4. Gibson, G. E., Pulsinelli, W., Blass, J. P. and Duffy, T. E. Brain dysfunction in mild to moderate hypoxia. *Am. J. Med.* 70: 1247–1254, 1981.
5. Kucharczyk, J., Fraser, C. L. and Arieff, A. I. Central nervous system manifestations or hyponatremia. In: A. I. Arieff and R. C. Griggs (eds) *Metabolic Brain Dysfunction in Systemic Disorders.* Boston: Little, Brown & Co, 1992, pp 55–86.
6. Miller, A. L. Carbon dioxide narcosis. In D. W. McCandless (ed.) *Cerebral Energy Metabolism and Metabolic Encephalopathy.* New York: Plenum Press, 1985, pp. 143–162.
7. Bélanger, M., Ahboucha, S., Desjardins, P. and Butterworth, R.F. Upregulation of peripheral-type (mitochondrial) benzodiazepine receptors in hyperammonemic syndromes: consequences for neuronal excitability. *Adv. Mol. Cell Biol.* 31: 983–997, 2004.
8. Spahr, L., Butterworth, R. F., Fontaine, S. *et al.* Increased blood manganese in cirrhotic patients: relationship to pallidal magnetic resonance signal hyperintensity and neurological symptoms. *Hepatology* 24: 1116–1120, 1996.
9. Lockwood, A. H., Yap, E. W. H. and Wong, W. H. Cerebral ammonia metabolism in patients with severe liver disease and minimal hepatic encephalopathy. *J. Cereb. Blood Flow Metab.* 11: 337–341, 1991.
10. Szerb, J. C. and Butterworth, R. F. Effect of ammonium ions on synaptic transmission in the mammalian central nervous system. *Prog. Neurobiol.* 39: 135–153, 1992.
11. De Waele, J. P., Audet, R. M., Leong, D. K. and Butterworth, R. F. Portacaval anastomosis induces region-selective alterations of the endogenous opioid system in the rat brain. *Hepatology* 24: 895–901, 1996.
12. Ostrow, J. D., Pascolo, L. and Tiribelli, C. Mechanisms of bilirubin neurotoxicity. *Hepatology* 35: 1277–1280, 2002.
13. McDonald, J. W., Shapiro, S. M., Silverstein, F. S. and Johnston, M. V. Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. *Exp Neurol.* 150: 21–29, 1998.
14. Fraser, C. L. Neurologic manifestations of the uremic state. In A. I. Arieff and R. C. Griggs (eds), *Metabolic Brain Dysfunction in Systemic Disorders.* Boston: Little, Brown & Co., 1992, pp. 139–166.
15. Peters, R. A. The biochemical lesion in vitamin B1 deficiency. Application of modern biochemical analysis in its diagnosis. *Lancet* 1: 1161–1164, 1936.
16. Butterworth, R. F. and Héroux, M. Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. *J. Neurochem.* 52, 1079–1084, 1989.
17. Pannunzio, P., Hazell, A. S., Pannunzio, M., Rama Rao, K. V. and Butterworth, R. F. Thiamine deficiency results in metabolic acidosis and energy failure in cerebellar granule cells: an *in vitro* model for the study of cell death mechanisms in Wernicke's encephalopathy. *J. Neurosci. Res.* 62: 286–292, 2000.
18. Gibson, G. E. and Zhang, H. Interactions of oxidative stress with thiamine homeostasis promote neurodegeneration. *Neurochem. Int.* 40: 493–504, 2002.
19. Todd, K. G. and Butterworth, R. F. Early microglial response in experimental thiamine deficiency: an immunohistochemical analysis. *Glia* 25, 190–198, 1999.
20. Kruse, M., Desjardins, P. and Butterworth, R. F. Increased brain endothelial nitric synthase expression in thiamine deficiency: relationship to selective vulnerability. *Neurochem. Int.* 43: 49–56, 2004.
21. Calingasan, N. Y., Huang, P. L., Chun, H. S., Fabian, A. and Gibson, G. E. Vascular factors are critical in selective neuronal loss in an animal model of impaired oxidative metabolism. *J. Neuropathol. Exp. Neurol.* 59: 207–217, 2000.

CHAPTER

35

Apoptosis and Necrosis

Mark P. Mattson

Nicolas G. Bazan

DISTINGUISHING FEATURES OF APOPTOSIS AND NECROSIS 603
During embryonic and postnatal development, and throughout adult life, many cells in the nervous system die 603
Many of the morphological and biochemical changes that occur in cells that die by necrosis are very different from those that occur in apoptosis 604

APOPTOSIS 604
Adaptive apoptosis occurs in the developing and adult nervous system 604
Apoptosis occurs in acute neurological insults 605
Apoptosis occurs in neurodegenerative disorders 607
There are many triggers of apoptosis 608
Once apoptosis is triggered, a stereotyped sequence of premitochondrial events occurs that executes the death process 609
Several different changes in mitochondria occur during apoptosis 610
The postmitochondrial events of apoptosis include activation of the caspases 610
A widely used criterion for identifying a cell as 'apoptotic' is nuclear chromatin condensation and fragmentation 611
Cells in the nervous system possess many different mechanisms to prevent apoptosis 611
The morphological and biochemical characteristics of apoptosis are not always manifest in cells undergoing programmed cell death 612
Apoptotic cascades can be triggered, and pre- and postmitochondrial events can occur, without the cell dying 613

NECROSIS 613
Necrosis is a dramatic and very rapid form of cell death in which essentially every compartment of the cell disintegrates 613
There are few cell death triggers that are only capable of inducing either apoptosis or necrosis 614

TARGETING APOPTOSIS AND NECROSIS IN NEUROLOGICAL DISORDERS 613

DISTINGUISHING FEATURES OF APOPTOSIS AND NECROSIS

During embryonic and postnatal development, and throughout adult life, many cells in the nervous system die. Such cell deaths are called 'programmed' because they are normal, being either adaptive or neutral with regards to their impact on the function of the nervous system. One particular form of programmed cell death (PCD) that has been extensively studied is called apoptosis. The details of the molecular cascades and morphological features of apoptosis vary somewhat depending on cell type, death stimulus and other factors [1]. However, there are several morphological and biochemical changes that are generally considered to be defining features of apoptosis (Table 35-1). Morphological changes include cell shrinkage, blebbing of the plasma membrane and nuclear chromatin condensation and fragmentation with preservation of the structure of mitochondria and the endoplasmic reticulum. Biochemical events that define apoptosis include: upregulation and/or mitochondrial translocation of proapoptotic proteins such as Bax and Par-4; formation of mitochondrial permeability transition pores and the release of cytochrome c and/or apoptosis-inducing factor (AIF) from the mitochondria; activation of effector caspases, which control the cell death process by cleaving a range of cytoplasmic, membrane-associated and nuclear protein substrates; exposure of phosphatidylserine on the plasma membrane surface, which serves as a signal for recognition and engulfment of the dying cell by macrophages and microglia. If the preceding events occur *and* if the death of the cells can be prevented by caspase inhibitors and inhibitors of protein synthesis, then the mode of cell death can be considered to be apoptosis.

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TABLE 35-1 Examples of caspase substrates

Cytoskeletal and associated proteins	Actin, spectrin, tau, vimentin, β-catenin, gelsolin, kinectin
Nuclear and DNA-associated proteins	Ataxia telangiectasia mutated (ATM), poly(ADP ribose) polymerase (PARP), DNA-dependent protein kinase, DNA replication factor C, DNA topoisomerase I, DNA fragmentation factor (DFF)45, inhibitor of caspase-activated DNAse (ICAD), lamins A, B1, and C
Signal transduction proteins	TRAF-1, Raf1, Ras, GAP, GDP dissociation inhibitor of Rho family GTPases, phospholipase A_2, Stat1
Kinases and phosphatases	Protein kinase Cd, Akt kinase, calcium/calmodulin-dependent protein kinase IV, mitogen-activated protein kinase kinase (MEKK-1), focal adhesion kinase (FAK), protein phosphatase (PP)2A, calcineurin
Transcription factors	NF-κB subunits p65 and p50, AP-2α, forkhead transcription factor FOXO3a, Max
Ion channels	IP3R1 and IP3R2, glutamate (AMPA) receptor subunits GluR1 and GluR4
Neurological-disorder-related proteins	Amyloid precursor protein (APP), presenilin-1, presenilin-2, parkin, tau, huntingtin, ataxin-3

Many of the morphological and biochemical changes that occur in cells that die by necrosis are very different from those that occur in apoptosis. During necrosis cells swell, mitochondria and endoplasmic reticulum lose their structure and become dysfunctional and the nuclear membrane becomes disrupted (Fig. 35-1). Necrotic death is independent of premitochondrial apoptotic proteins such as Bax, cytochrome c release and caspase activation. Necrosis is further distinguished from apoptosis by the fact that necrosis usually occurs as the result of a traumatic physical injury or stroke and cells die *en masse*, whereas apoptosis typically occurs in individual cells within a population of surviving neighbors.

The information presented in this chapter concerns the mammalian nervous system. However, it should be recognized that similar mechanisms may occur in other organisms and, indeed, many important aspects of the process of apoptosis were initially discovered in studies of the nematode *Caenorhabditis elegans* [2].

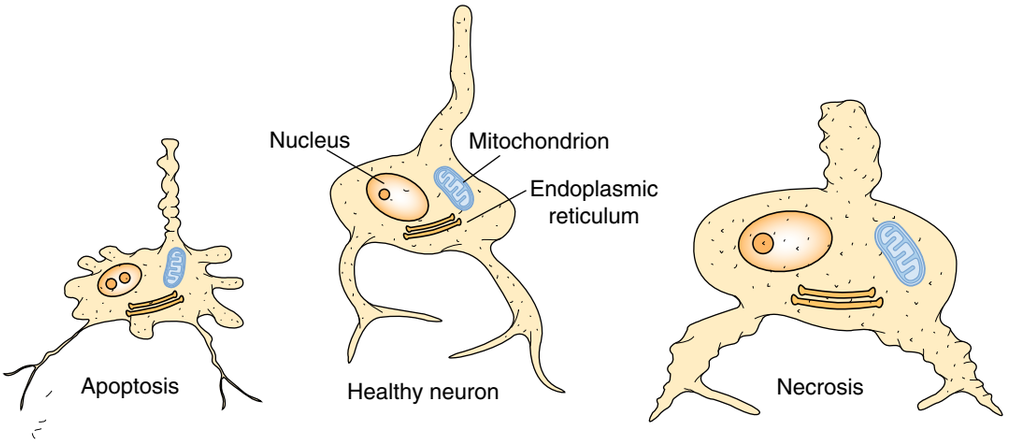
APOPTOSIS

The conditions and signaling pathways that trigger or prevent apoptosis, and the molecular control of the apoptotic process, are topics of intense interest in the field of neuroscience. Knowledge of the cellular and molecular mechanisms of apoptosis is necessary for a full understanding of how the brain and other regions of the nervous system develop. Evidence is increasing that apoptosis and related forms of cell death occur in a range of neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases and stroke; there is therefore intense interest in developing approaches directed toward apoptosis as a therapeutic means in these diseases (see respective chapters for details related to therapy).

Adaptive apoptosis occurs in the developing and adult nervous system. During embryonic and early postnatal development, many cells undergo apoptosis in most regions of mammalian nervous systems. Such developmental apoptosis is believed to be adaptive in that it eliminates cells that are not integrated into neuronal circuits and are therefore unnecessary for the proper function of the nervous system. While initially established in populations of neurons that innervate peripheral targets (dorsal root ganglia, sympathetic ganglia and motor neurons), developmental apoptosis has also been documented in several populations of neurons in the brain. There appear to be two major 'waves' of apoptosis, one occurring early and involving neuronal progenitor cells and another occurring later and involving neurons that are in the process of forming synaptic connections with target cells [3]. While neuronal apoptosis is well established, the extent to which glial cells (oligodendrocytes and astrocytes) undergo apoptosis during development is unclear (see Development in Ch. 25).

Developmental processes continue in at least some locations in the adult nervous system. For example, relatively large numbers of neural stem cells are located in the subventricular zone of the cerebral cortex and in the subgranular layer of the dentate gyrus of the hippocampus (see Stem Cells in Ch. 29). The subventricular zone stem cells give rise to interneurons in the olfactory bulb, while hippocampal stem cells can differentiate into dentate granule neurons. Although some newly generated neurons that arise from stem cells in the adult brain become integrated into neuronal circuits, many of these cells undergo apoptosis [4]. Neurogenesis increases in response to brain injury, which is probably an adaptive mechanism designed to replace damaged neurons (see Regeneration in Ch. 30).

The signals that determine whether neural stem cells and their progeny survive or undergo apoptosis include growth factors (Ch. 27), cytokines (Chs 30, 32 and 33) and cell adhesion molecules (Ch. 7). The discovery of nerve growth factor (NGF) heralded investigations into the roles of neurotrophic factor signaling in determining whether neurons live or die during development. It was shown by Levi-Montalcini and Hamburger in the 1950s that sensory and sympathetic neurons depend on a substance later identified as nerve growth factor (NGF) for their survival



Apoptosis	Necrosis
Cell shrinkage	Cell swelling
Maintenance of organellar integrity	Organellar swelling and damage
Maintenance of ATP levels	Depletion of ATP
Maintenance of ion homeostasis	Loss of ion homeostasis
Membrane surface blebbing	Membrane rupture
Nuclear chromatin condensation/fragmentation	Nuclear lysis
Requires synthesis of death effector proteins	Cessation of protein synthesis
Prevented by blocking steps in the death cascade	Irreversible
Does not adversely affect neighbor cells	Promotes death of neighbor cells

FIGURE 35-1 Distinguishing features of apoptosis and necrosis.

and maintenance. Neurons that do not receive sufficient trophic support undergo apoptosis. Other factors that can determine whether stem cells and/or neurons live or die include basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF) and epidermal growth factor [4, 5]. Examples of other signaling molecules that may regulate developmental apoptosis include nitric oxide, Par-4 and ceramide, PTEN (phosphatase and TENsin homolog) and the transcription factor E2F1.

Apoptosis occurs in acute neurological insults. Traumatic and ischemic injury to the nervous system are common events that result in considerable morbidity and mortality. Accordingly, there is intense interest in understanding the cellular and molecular mechanisms responsible for the death of neurons in such acute neurodegenerative conditions. Many neurons may undergo apoptosis in response to a traumatic injury to the brain, spinal cord or peripheral nerves, as indicated by activation of various proapoptotic proteins including p53 and caspases [6]. In particular, neurons adjacent to the necrotic region of severe trauma are prone to apoptosis. Drugs that attenuate apoptosis have been shown to be beneficial in animal models of traumatic brain and spinal cord injury, suggesting a potential for such an approach in human patients.

Ischemic stroke occurs when a blood vessel in the brain becomes occluded, usually as the result of atherosclerosis and clot formation (Ch. 32). Neurons in the brain supplied by the affected vessel may undergo apoptosis [7]. Indeed, biochemical, morphological and functional evidence of apoptosis has been documented in animal models of stroke. Triggers of ischemic apoptosis include oxygen free radicals, glutamate receptor activation, calcium influx and release from intracellular stores, and lipid mediators. DNA damage and activation of poly(ADP ribose) polymerase (PARP) and p53 are also implicated in stroke-induced neuronal apoptosis. Mediators of ischemic neuronal apoptosis may include Bax, Par-4, cytochrome c, apoptosis-inducing factor and caspases. Activation of glutamate receptors may play a particularly important role [8].

Phospholipids of cellular membranes in the nervous system are endowed with the highest content of ω-3 docosahexaenoic acid (DHA). So far, the interpretation

of the significance of this high degree of unsaturation in excitable membranes has been the resulting relatively high-membrane-fluidity environment, where specific proteins (e.g. receptors, transporters, ion channels) perform their functions. Oxidative stress targets these highly unsaturated phospholipids, resulting in phospholipids containing peroxidated acyl chains that in turn impair membrane function. Thus, DHA-containing phospholipids are a target for free radical-catalyzed peroxidation (Fig. 35-2) and, as a result, F4-isoprostanes are formed [9, 10]. F2-isoprostanes are also derived from free-radical-catalyzed peroxidation, although from arachidonic acid instead [11]. F4-isoprostanes (neuroprostanes) are found esterified in phospholipids, and it has been reported that their content is increased in the brains of patients with Alzheimer's disease [12]. Excessive accumulation of free-radical-mediated lipid peroxidation products promotes cellular damage and death.

As an example, rhodopsin in photoreceptors is immersed in a lipid environment highly enriched in phospholipids containing DHA, and DHA is essential for rhodopsin function [13–15]. Moreover, retinal DHA, like brain DHA, is very resistant to n-3 fatty acid dietary deprivation. On the other hand, DHA was recently shown to be the precursor of a retinal and brain neuroprotective signaling, the docosanoids. These are enzyme-derived, stereospecific compounds, identified initially in the retina and proposed to elicit neuroprotection, which were detected by lipidomic analysis. The docosanoid 10,17*S*-docosatriene is a potent inhibitor of brain ischemia–reperfusion-induced polymorphonuclear leukocyte infiltration, as well as of NF-κB and cyclooxygenase (COX)-2 expression [16]. Moreover, marked attenuation of the stroke volume was observed when 10,17*S*-docosatriene was infused into the third ventricle during ischemia–reperfusion [16]. The elucidation of this DHA-oxygenation messenger sheds new light on how the brain modulates its response to oxidative stress and inflammatory injury, and raises the exciting potential for designing new drugs to treat neurologic disorders that have a neuroinflammatory component, such as stroke, traumatic brain injury, spinal cord injury, or Alzheimer's disease. In particular, the very high biological activity of the docosanoid 10,17*S*-docosatriene marks it as a potential effector of neuroprotection.

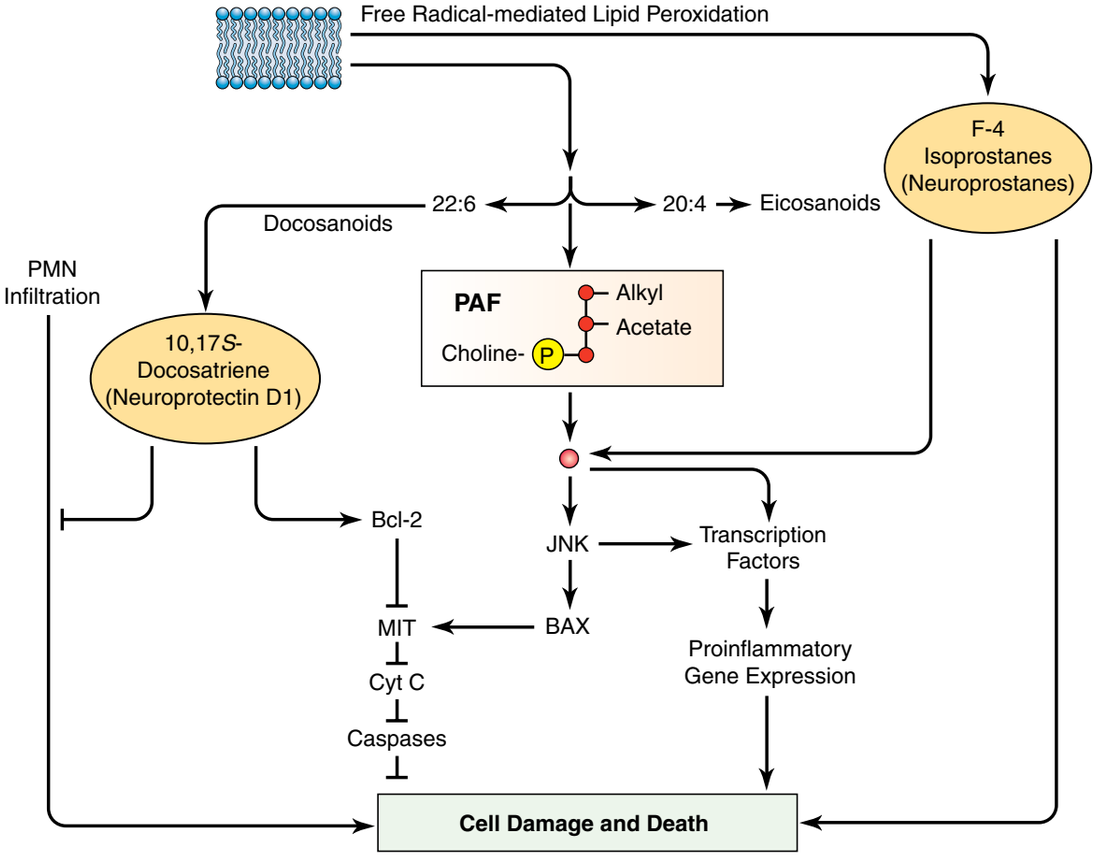


FIGURE 35-2 Phospholipids of cellular membranes can be peroxidized by free-radical-catalyzed reactions. Excessive accumulation of lipid peroxides contributes to cell damage and death. In the nervous system, these phospholipids contain the largest quantities of docosahexaenoic acid (22:6), which was recently demonstrated to be the precursor of the neuroprotective docosanoid 10,17*S*-docosatriene (neuroprotectin D1, NPD1). NPD1 counteracts proinflammatory cellular signaling and decreases polymorphonuclear leukocyte (PMN) infiltration in ischemic brain [16], and inactivates proapoptotic signaling and upregulates antiapoptotic signaling in oxidatively stressed retinal pigment epithelial cells [17].

The newly isolated dihydroxy-containing DHA derivative is called 'neuroprotectin D1' (NPD1) (1) because of its neuroprotective properties in brain ischemia–reperfusion [16] and in oxidative-stress-challenged retinal pigment epithelial cells [17]; (2) because of its potent ability to inactivate proapoptotic signaling (Bad and Bid) and to upregulate Bcl-2 and Bcl-xl; and (3) because it is the first identified neuroprotective mediator of DHA.

Apoptosis occurs in neurodegenerative disorders. Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) are four prominent fatal neurodegenerative disorders that involve the death of specific populations of neurons (see details in respective chapters). Studies of patients and animal and culture models have provided considerable insight in the cellular and molecular mechanisms responsible for synaptic dysfunction and neuronal degeneration in each disorder [18]. In Alzheimer's disease, abnormalities in proteolytic processing of the amyloid precursor protein, due to gene mutations (in the amyloid precursor protein or in the presenilins) or age-related factors, result in accumulation of neurotoxic forms of amyloid beta peptide (Fig. 35-3). In Parkinson's disease, age-related increases in oxidative stress and possibly environmental neurotoxins cause selective degeneration of dopaminergic neurons in the substantia nigra. In Huntington's disease, inheritance of a mutant huntingtin gene that encodes a huntingtin protein with increased numbers of polyglutamine repeats causes degeneration of striatal neurons. ALS, which is characterized by selective degeneration of motor neurons, can result from mutations in Cu/Zn-superoxide dismutase or from other unknown genetic and environmental causes. While the causes of these neurodegenerative disorders may differ, they each involve increased oxidative stress, metabolic compromise and disruption of cellular calcium ion homeostasis as important contributors to the cell death process [18].

There is considerable evidence that many of the neurons that die in Alzheimer's disease, Parkinson's disease,

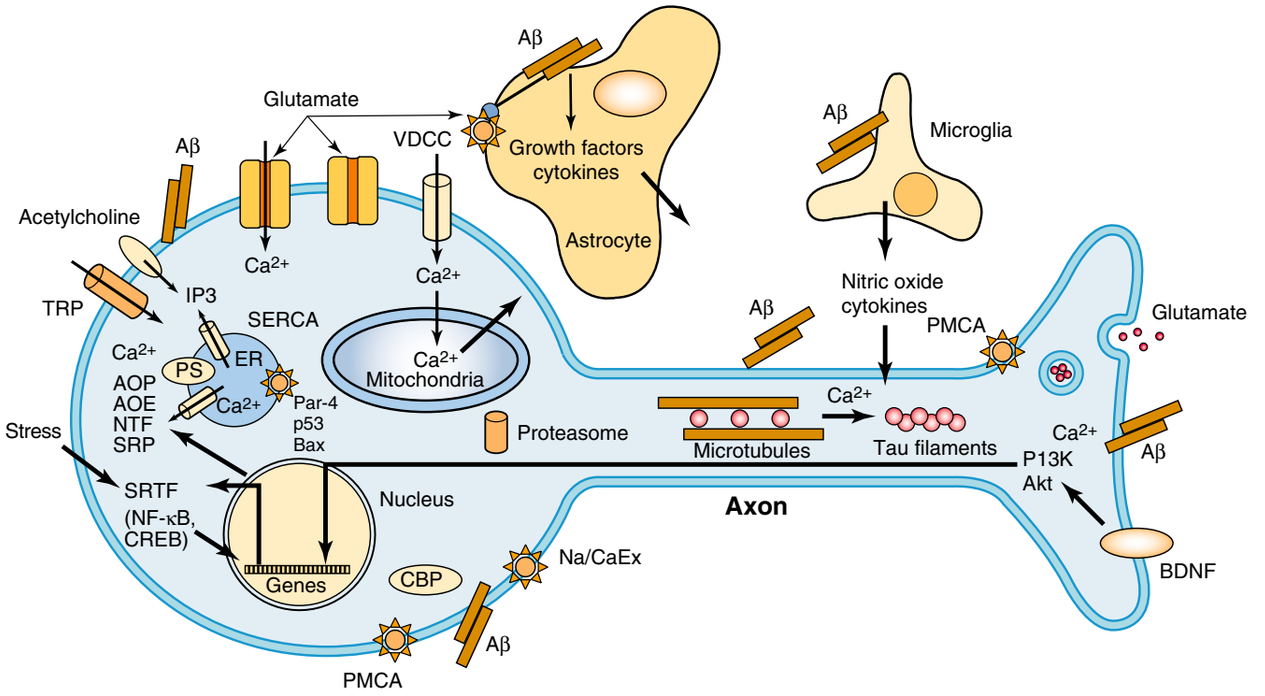


FIGURE 35-3 Examples of inter- and intracellular signaling mechanisms and biochemical cascades that can determine whether a neuron lives or dies in physiological and pathological settings. Neurons respond to a wide array of extracellular signals that can either prevent or promote cell death; examples include neuroprotective growth factors and neurotoxic amyloid β-peptide (*Aβ*). The calcium ion (Ca^{2+}) often plays important roles in determining whether neurons live or die. Excessive influx of Ca^{2+} through glutamate receptor channels, voltage-dependent Ca^{2+} channels and capacitative Ca^{2+} entry channels (*TRP*) in the plasma membrane, and/or excessive release of Ca^{2+} from endoplasmic reticulum (*ER*) and mitochondrial stores, can trigger apoptosis. On the other hand, effective removal of Ca^{2+} from the cytoplasm via the activities of plasma membrane (*PMCA*) and ER (*SERCA*) Ca^{2+}-ATPases, the plasma membrane Na^+/Ca^{2+} exchanger (*Na/CaEx*) or sequestration by Ca^{2+}-binding proteins (*CBP*) can prevent apoptosis. Cell death may also be triggered by oxidative stress, insufficient trophic factor support and abnormalities in the cytoskeleton, such as occur, for example, in Alzheimer's disease where microtubules depolymerize and the microtubule-associated protein tau forms abnormal aggregates. By activating transcription factors that induce the expression of cytoprotective genes, some signaling pathways can prevent cell death. For example, stress-responsive transcription factors such as NF-κB and CREB induce the expression of antiapoptotic proteins (*AOP;* Bcl-2 and IAPs, for example), antioxidant enzymes (*AOE*), neurotrophic factors (*NTF*) and stress resistance proteins (*SRP;* HSP-70 and GRP-78, for example). Microglia may facilitate neuronal death by producing neurotoxic substances such as nitric oxide and proinflammatory cytokines.

Huntington's disease and ALS undergo apoptosis or a related form of PCD. The spatial and temporal patterns of neuronal death in these neurodegenerative disorders are consistent with apoptosis in that neurons in vulnerable brain regions do not die in unison; instead, individual neurons die on a progressive basis. Examinations of the brains of Alzheimer's disease, Parkinson's disease and Huntington's disease patients and of the spinal cords of ALS patients have revealed evidence for activation of caspases and upregulation of apoptotic proteins such as Bax, p53 and Par-4. Perhaps the strongest evidence that apoptosis is a major mode of cell death comes from studies in which mutant genes that cause disease (amyloid precursor protein and presenilin mutations in Alzheimer's disease, alpha-synuclein mutations in Parkinson's disease, huntingtin mutations in Huntington's disease and Cu/Zn-SOD mutations in ALS) have been shown to increase the vulnerability of neurons to apoptosis [19, 20]. Dietary modifications such as dietary restriction and folate supplementation, and/or drugs that block apoptotic pathways have proved to be effective in reducing neuronal degeneration and improving functional outcome in animal models of Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS. When taken together with epidemiological studies that have identified risk factors for Alzheimer's disease and Parkinson's disease, findings from animal studies support a role for apoptosis in these disorders. For example, dietary restriction can protect hippocampal and dopaminergic neurons in models of Alzheimer's disease and Parkinson's disease by a mechanism involving upregulation of BDNF and stress resistance proteins, and epidemiological data suggest that individuals with low calorie intakes have a reduced risk of Alzheimer's disease and Parkinson's disease [21].

There are many triggers of apoptosis. Hundreds of factors that can trigger apoptosis of neural cells have been described, and there are surely many others that remain to be discovered. Examples of five prominent types of apoptotic trigger are described below.

Insufficient trophic support. When developing neurons are deprived of neurotrophic support, either by withdrawal of the neurotrophic factor in cell culture or by removal of the target cells for the neurons *in vivo*, they undergo apoptosis. For example, when sympathetic neurons in culture are deprived of NGF they upregulate Bax, release cytochrome c from their mitochondria, activate caspases and exhibit cell body shrinkage and nuclear chromatin condensation and fragmentation [22]. Depletion of neurotrophic factors may also contribute to the deaths of neurons that occur during aging and in various neurodegenerative conditions and, indeed, there is evidence that this is the case in Alzheimer's disease, Parkinson's disease and Huntington's disease.

Death receptor activation. Several different ligands can induce apoptosis of neural cells including certain cytokines (Fas ligand and interleukin-1β), the neurotransmitter glutamate and thrombin. Like tumor necrosis factor (TNF) receptors, Fas is coupled to downstream death effector proteins that ultimately induce caspase activation (Ch. 22). Fas and TNF receptors recruit proteins called FADD and TRADD respectively; FADD and TRADD then activate caspase-8, which, in turn, activates caspase-3 (Fig. 35-4). Calcium ion influx mediates neuronal apoptosis induced by glutamate receptor activation; calcium induces mitochondrial membrane permeability transition pore opening, release of cytochrome c and caspase activation. Interestingly, in the absence of neurotrophic factors some neurotrophic factor receptors can activate apoptotic cascades, the low-affinity NGF receptor being one example of such a death receptor mechanism [23].

DNA damage. Considerable evidence suggests that DNA damage may be a pivotal trigger of apoptosis in both physiological and pathological settings. Neurons undergoing developmental apoptosis exhibit DNA damage and upregulation of DNA-damage-responsive proteins. The causes of DNA damage during development have not been clearly established but may include increased oxyradical production, reduced trophic support and impaired DNA repair mechanisms. DNA damage can induce the upregulation of proapoptotic proteins including ATM kinase, p53 and PARP. Damage to telomeric DNA (located at the ends of chromosomes) may be a particularly potent trigger of apoptosis in neural progenitor cells and newly generated neurons. DNA damage has been documented in vulnerable neuronal populations in patients suffering from traumatic brain injury, stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS. An increase in the amounts of oxidatively modified DNA bases in these disorders suggests a major role for oxidative stress. In addition, abnormalities in one carbon metabolism that may be induced by homocysteine, for example, may contribute to accumulation of damaged DNA during aging and in neurodegenerative disorders.

Oxidative and metabolic stress. Studies of cell culture and animal models have clearly shown that oxidative stress and impaired energy metabolism can trigger neuronal apoptosis. For example, exposure of cultured neurons to hydrogen peroxide, Fe^{2+} or amyloid β-peptide induces membrane-associated oxidative stress and activates a mitochondrial-dependent apoptotic cascade. Oxidative stress may trigger apoptosis by activating membrane-associated apoptotic signaling cascades; for example, ceramide generated from membrane sphingomyelin in response to oxidative stress can be a potent inducer of apoptosis [24]. Oxidative damage to various membrane transporters, mitochondrial proteins and membranes and nuclear DNA can also trigger apoptosis. The ability of metabolic compromise to induce apoptosis is evident from the fact that various mitochondrial toxins (e.g. rotenone, MPP^+, cyanide and 3-nitropropionic acid) can

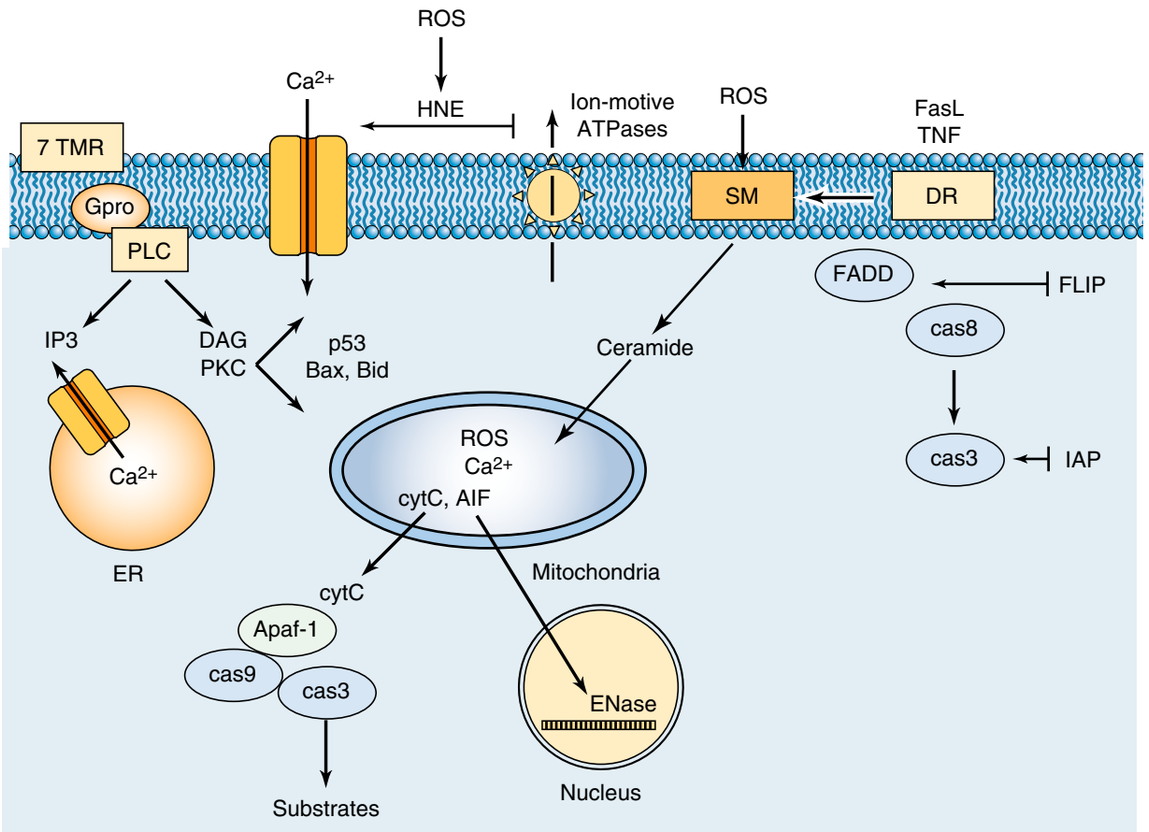


FIGURE 35-4 Examples of plasma-membrane-initiated cell death cascades. Many cells, including neurons, express so-called death receptors (*DR*). In the example shown, a receptor for Fas ligand (*FasL*) or tumor necrosis factor (*TNF*) binds a protein called FADD (Fas-associated death domain), which then recruits and activates caspase 8 (*cas8*). Caspase 8 then activates caspase 3, which plays a major role in executing the cell death process. The latter death receptor pathway can be blocked by the activities of FLIP (FLICE inhibitory protein) and IAPs (inhibitor of apoptosis proteins). Cell death can also be triggered by reactive oxygen species (*ROS*) that induce membrane-associated oxidative stress. Membrane lipid peroxidation generates the aldehyde 4-hydroxynonenal (*HNE*) which can induce apoptosis by covalently modifying various membrane proteins including ion-motive ATPases and calcium channel proteins. Membrane oxidative stress also activates sphingomyelinases, which cleave sphingomyelin (*SM*), resulting in the production of ceramide, which can trigger apoptosis by inducing mitochondrial membrane permeability transition. Receptors with seven transmembrane domains (*7TMR*) coupled to GTP-binding proteins (*Gpro*) and activation of phospholipase C (*PLC*) can trigger cell death by inducing calcium release from IP3-sensitive ER stores. Once initiated, such cell death cascades often involve proapoptotic proteins acting at mitochondrial membranes (p53, Bax and Bid, for example), proteins released from mitochondria (cytochrome C and AIF), caspases and endonucleases (*ENase*).

trigger apoptosis. While impaired energy metabolism clearly plays a role in many neurodegenerative disorders, its role in physiological cell death remains to be established.

Once apoptosis is triggered, a stereotyped sequence of premitochondrial events occurs that executes the cell death process. In many cases proteins and/or lipid mediators that induce changes in mitochondrial membrane permeability and calcium regulation are produced or activated. For example, the pro-apoptotic Bcl-2 family members Bax, Bad and Bid may associate with the mitochondrial membrane and modify its permeability. Membrane-derived lipid mediators such as ceramide and 4-hydroxynonenal can also induce mitochondrial membrane alterations that are critical for the execution of apoptosis.

Numerous mechanisms have been described that are involved in early, premitochondrial steps in apoptosis (Figs 35-3, 35-4). For example, the polymerization state of actin filaments and microtubules can determine whether or not apoptosis is triggered by glutamate because these cytoskeletal proteins modulate the activity of ionotropic glutamate receptors and voltage-dependent calcium ion channels [8]. Similarly, the presence and amounts of calcium-binding proteins and antioxidants such as glutathione and vitamin E can shift the threshold for activation of the cell death cascade by different apoptotic triggers. Events occurring in the endoplasmic reticulum (ER) have been shown to induce, prevent or modify apoptotic cascades at a premitochondrial step; calcium ion release and uptake by the ER appears to be particularly important in this regard (see Chs 5 and 22).

Apoptosis requires macromolecular synthesis. In this regard, several transcription factors and target genes have been identified as playing pivotal roles in apoptosis. For example, stress-activated protein kinases (SAPK) and c-Jun N-terminal kinase (JNK) mediate apoptotic responses to various triggers and affect cell death by activating the transcription factors AP-1 and GADD153. Another prominent example is the transcription factor p53, which is activated in response to DNA damage and which induces the transcription of proapoptotic genes including those encoding Bax and p21. Another protein whose upregulation is required for at least some cases of apoptosis is Par-4, which can be induced at the translational level.

Several different changes in mitochondria occur during apoptosis. These include a change in membrane potential (usually depolarization), increased production of reactive oxygen species, potassium channel activation, calcium ion uptake, increased membrane permeability and release of cytochrome c and apoptosis inducing factor (AIF) [25]. Increased permeability of the mitochondrial membranes is a pivotal event in apoptosis and appears to result from the formation of pores in the membrane; the proteins that form such permeability transition pores (PTP) may include a voltage-dependent anion channel (VDAC), the adenine nucleotide translocator, cyclophilin D, the peripheral benzodiazepine receptor, hexokinase and creatine kinase (Fig. 35-5). Pro-apoptotic Bcl-2 family members, such as Bax and Bid, promote PTP formation. Permeabilization of the mitochondrial membranes provides a route for the release of cytochrome c into the cytoplasm where it binds to a protein called Apaf-1. The synchronous release of cytochrome c from all mitochondria in a cell is thought to be orchestrated by calcium ion release from IP3-sensitive ER stores [26]. Smac/DIABLO and Omi/HtrA2 are two other mitochondrial intermembrane proteins that are released from mitochondria during apoptosis; they then bind to and neutralize cytosolic inhibitors of apoptosis proteins (IAPs). Because IAPs normally bind and inhibit caspases 9 and 3, the binding of IAPs by Smac/DIABLO and Omi/HtrA2 enhance the activation of these caspases. Two DNA-degrading enzymes (AIF and endonuclease G) are also released from mitochondria and then translocate to the nucleus where they cleave DNA into nucleosomal fragments. AIF is a flavoprotein with an oxidoreductase domain and may, in addition to its pro-apoptotic function, modulate mitochondrial redox state.

The postmitochondrial events of apoptosis include activation of the caspases. Cytochrome c binds to the protein Apaf-1 in the cytosol, resulting in the recruitment and activation of caspase-9, which in turn activates caspase-3 (Figs 35-4, 35-5). Fourteen different mammalian caspases have been identified and each may play a key role

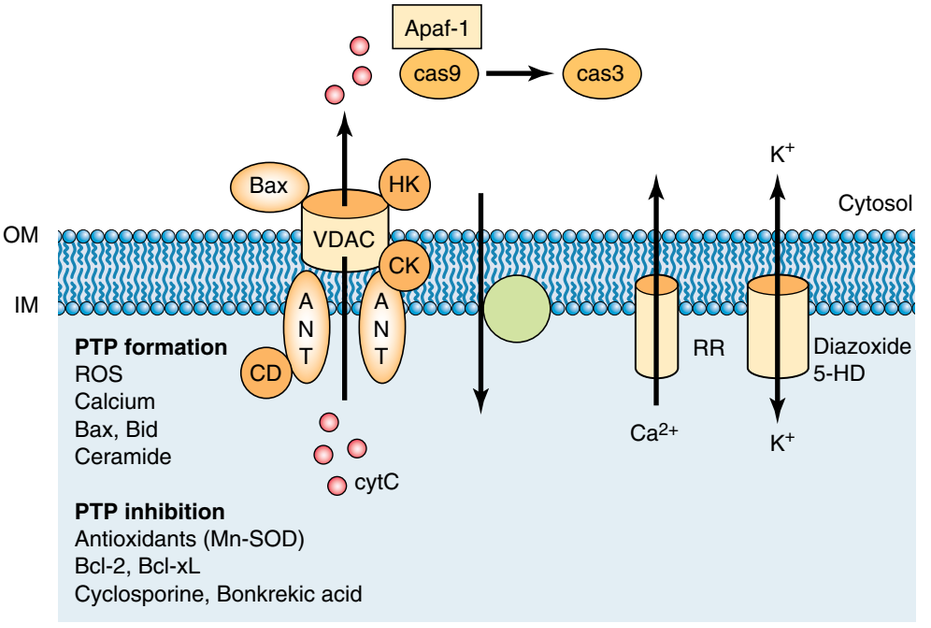


FIGURE 35-5 The presumptive mitochondrial permeability transition pore complex and its regulation by Ca^{2+} and K^+ fluxes. The membrane permeability transition pore is believed to consist of VDAC (voltage-dependent anion channel) and ANT (adenine nucleotide translocator) and associated proteins that modulate its opening including hexokinase (*HK*), creatine kinase (CK), cyclophilin D (*CD*) and Bax. Formation and opening of the channel results in the release of cytochrome C (*cytC*), which then binds to Apaf-1, resulting in the sequential activation of caspases 9 and 3. Mitochondrial membrane permeability pore formation is subject to regulation by fluxes of Ca^{2+} and K^+. Agents that suppress Ca^{2+} flux (*RR*, ruthenium red) and K^+ flux (*5-HD*, 5-hydroxydecanoate) can prevent pore formation. Interestingly, activation of mitochondrial K^+ channels with diazoxide can also prevent apoptosis by inducing a preconditioning response.

in apoptosis depending upon the cell type and the nature of the specific cell death stimulus [27]. For example, caspases 8 and 10 can be activated by death receptor engagement, independently of mitochondria, and caspase-12 is activated in response to ER stress. Numerous caspase substrates have been identified (Table 35-1), and in several cases the consequences of cleavage of the substrate in the context of the cell death process is known. Cleavage of a protein by a caspase may disable the protein or, in some cases, may activate the protein. Indeed, effector caspases themselves (caspases 3, 6 and 7) can be activated by cleavage of their procaspase forms by initiator caspases (caspases 8, 9 and 10). Cellular processes modified by caspase-mediated proteolysis include inactivation of antiapoptotic proteins, impairment of homeostatic and repair processes (DNA repair enzymes, for example), disruption of the cell cytoskeleton, nuclear and plasma membrane morphological changes, and marking of cells for phagocytosis.

A widely used criterion for identifying a cell as 'apoptotic' is nuclear chromatin condensation and fragmentation. The nature of the molecular alterations that occur in the nucleus and the proteins responsible for inducing these changes are in the process of being identified [28]. Some caspases (caspase-6, for example) enter the nucleus and cleave nuclear matrix proteins including lamins and NuMA. Other caspases that cleave nuclear proteins involved in regulating DNA replication and repair processes include topoisomerases, PARP, DNA-dependent protein kinase and Rad51. Caspases may also activate endonucleases such as ICAD and DNAses I and II, resulting in DNA fragmentation. Caspase-3 can cleave acinus resulting in chromatin condensation. Granzyme B, cathepsin B and histone-associated protease also cleave nuclear substrates during apoptosis. Other apoptotic proteins, such as DEDD and Daxx, that act in the nucleus may influence the apoptotic process by altering gene transcription.

Dramatic morphological changes in the plasma membrane occur during apoptosis; as the cell shrinks, membrane blebs form. Individual apoptotic cells are removed from tissues via phagocytosis by macrophages/microglia. The phagocytic cells recognize the apoptotic cell because during the process of apoptosis, phosphatidylserine, which is normally present in the inner leaflet of the plasma membrane bilayer, translocates to the cell surface (see Chs 2 and 20). The phosphatidylserine binds to a specific phosphatidylserine receptor (PSR) on the surface of the phagocytic cells [29]. In this way, apoptotic cells are removed when their membrane is still intact, thereby preventing release of potentially damaging intracellular contents. Indeed, mice lacking the PSR accumulate dead cells, particularly in the brain and lungs, which process results in damage to adjacent living cells and ultimately these animals cannot survive.

Cells in the nervous system possess many different mechanisms to prevent apoptosis. These include intercellular signals and the pathways activated by those signals, intrinsic antiapoptotic proteins, antioxidants, protein chaperones and systems that regulate ion homeostasis [30] (Fig. 35-6).

Neurotrophic factors, cytokines and cell adhesion molecules. A remarkable number of growth factors and cytokines have been identified that can prevent neuronal apoptosis [30]. A short list includes NGF, bFGF, BDNF, neurotrophin-4/5, glial-cell-line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor (IGF)-1, transforming growth factor (TGF)β, tumor necrosis factor (TNF)α, interleukin-6 and secreted forms of amyloid precursor protein. In addition to such soluble ligands, extracellular matrix (laminin, fibronectin, etc.) or cell-surface-bound ligands (NCAM, cadherins) bind to receptors such that are involved in cell–substratum adhesion. The latter receptors are coupled to intracellular signaling pathways similar to those employed by growth factors. For example, engagement of integrins by laminin activates the PI3-kinase–Akt pathway, a signaling pathway also activated by BDNF and IGF-1. In general, signaling by growth factors and cell adhesion molecules results in activation of transcription factors, which activate genes that encode antiapoptotic proteins such as Bcl-2, IAPs, antioxidant enzymes and calcium-regulating proteins. However, these signaling pathways can also antagonize apoptosis by modifying the phosphorylation state of apoptosis-related proteins such as Bcl-2 family members.

Antiapoptotic proteins. There are many different intracellular proteins that can prevent apoptosis by inhibiting specific steps in the cell death process. These include Bcl-2 family members such as Bcl-2 and Bcl-xL which can stabilize (mitochondrial, ER and plasma) membranes (Bcl-2 may also have intrinsic antioxidant activity). Other proteins, IAPs such as XIAP (X-linked) and NIAP (neuronal), which can directly inhibit caspases [31]. Additional examples of antiapoptotic proteins include protease inhibitors such as calpastatin, and protein chaperones such as GRP-78 and heat shock protein (HSP)-70.

Hormesis-based mechanisms. When cells are exposed to sublethal levels of stress (heat, oxidative stress, metabolic stress, or other stresses) they often become resistant to being killed by more severe stress. This process, which is a form of hormesis or 'preconditioning', typically involves the activation of stress signaling pathways that activate transcription factors that induce the expression of genes encoding cytoprotective proteins. Examples of antiapoptotic proteins upregulated in response to a preconditioning stress are neurotrophic factors such as BDNF and bFGF, protein chaperones such as HSP-70 and glucose-regulated protein-78, Bcl-2 and antioxidant enzymes such as Mn-superoxide dismutase (Mn-SOD). Interestingly, hormesis appears to be a major mechanism whereby mild

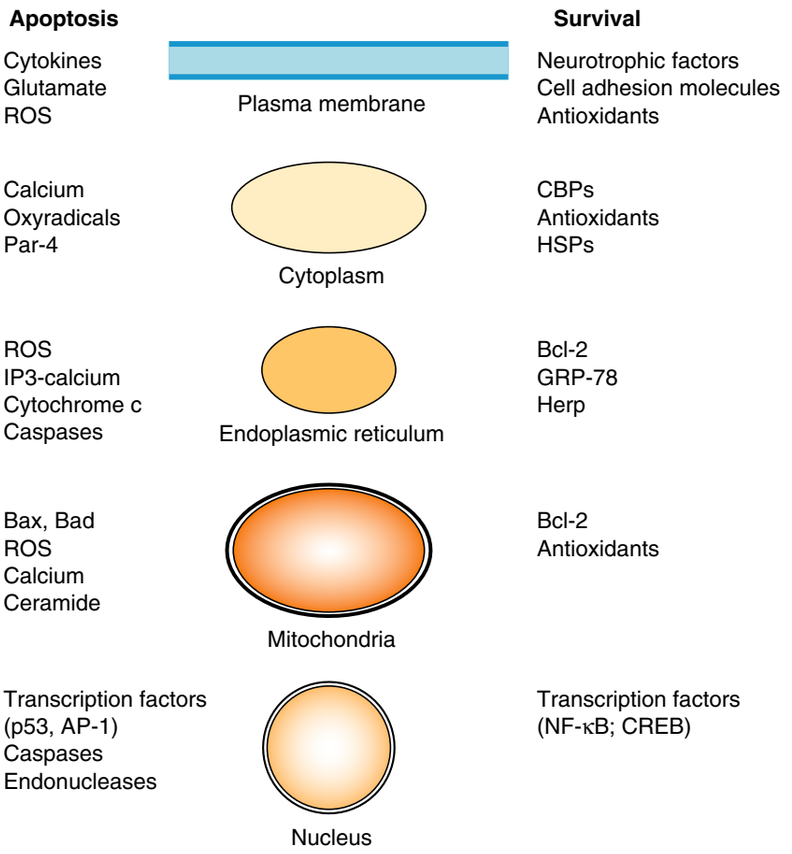


FIGURE 35-6 Examples of apoptotic and antiapoptotic mechanisms that act on or within different subcellular compartments. CBP, Ca^{2+} binding protein; CREB, cyclic AMP response element binding protein; HSP, heat shock protein; IP3, inositol 1,4,5-trisphosphate; ROS, reactive oxygen species.

stresses such as dietary calorie restriction, physical exercise and environmental enrichment (mental gymnastics) protect neurons against apoptosis [21].

Antioxidants and calcium-stabilizing proteins. Oxidative stress and perturbed cellular calcium homeostasis have been shown to play pivotal roles in the apoptosis of neural cells in physiological and pathological settings. Accordingly, cells possess multiple mechanisms to reduce levels of oxidative stress and to maintain and restore calcium homeostasis. Oxidative stress is suppressed by antioxidant enzymes (Cu/Zn-SOD, Mn-SOD, catalase, glutathione peroxidase) and molecules with intrinsic radical-scavenging ability such as glutathione, bilirubin, vitamins E and C, uric acid and creatine. Proteins that stabilize calcium homeostasis include calcium-binding proteins (calbindin, calreticulin and parvalbumin, for example), calcium pumps in the plasma and ER membranes, and sodium/calcium exchangers. Interestingly, antiapoptotic proteins such as Bcl-2 may act, in part, by suppressing oxidative stress and enhancing calcium homeostasis.

The morphological and biochemical characteristics of apoptosis are not always manifest in cells undergoing programmed cell death. Studies have clearly shown that there are several, and perhaps many, variant forms of PCD and that there can be a continuum of cell death cascades that is related to the specific cell death trigger involved and its intensity and duration. In some cases cells exhibit characteristics of both apoptosis and necrosis [1]. A form of PCD involving autophagy occurs in many instances, including developmental apoptosis of neurons in sympathetic and isthmo-optic nuclei. Autophagy is a lysosome-mediated mechanism by which cells degrade and recycle damaged proteins and organelles (see Lysosomes in Ch. 41). Autophagy is characterized by the formation of cytoplasmic vacuoles with double membranes that enclose fragments of mitochondria and ER and contain high concentrations of lysosomal hydrolases. Autophagic cell death is often a variant of apoptosis in which caspase activation plays a pivotal role. Proteins that may play a role in autophagic PCD include Beclin1, PI3 kinase, TOR (target of rapamycin), p70S6 kinase, AMPA receptors and Bcl-2. There is evidence that at least some neurons undergo autophagic cell death in Parkinson's, Huntington's and Alzheimer's diseases.

Neuronal PCD with features of necrosis was reported in studies of naturally occurring motor neuron death in which the cells exhibited dilation of ER, Golgi and nuclear membranes but not condensation of chromatin. A similar

form of necrotic PCD may occur in response to Fas activation; such cell deaths involve swelling of ER and mitochondria but cytochrome c is not released. Perturbed ER calcium homeostasis, sustained increases in cytoplasmic calcium ion concentrations and activation of calpains can trigger necrotic PCD in some types of cells.

Apoptotic cascades can be triggered, and pre- and postmitochondrial events can occur, without the cell dying. For example, studies have shown that apoptotic cascades involving Par-4, p53, mitochondrial membrane permeabilization and caspase activation can be induced locally in synaptosomes and in growth cones and neurites of cultured neurons [32]. Such cascades can be triggered locally by activation of glutamate receptors and can occur in a reversible manner. Interestingly, the cleavage of certain synaptic substrate proteins by caspases (AMPA receptor subunits and cytoskeletal proteins, for example) may play roles in synaptic plasticity (learning and memory) and perhaps in remodeling of synaptic structure and the regulation of growth cone motility (Fig. 35–7).

NECROSIS

Necrosis is a dramatic and very rapid form of cell death in which essentially every compartment of the cell disintegrates. Necrosis is characterized by marked dysregulation of ion homeostasis resulting in cell swelling, dilation of mitochondria and the ER and the formation of vacuoles in the cytoplasm [33]. Proteases play important roles in the degradation of cells during necrosis. In contrast to apoptosis, where caspases are the key death proteases, calpains and lysosomal proteases (cathepsins B and D, in particular) are major players in necrosis. Caspases may be activated in response to mitochondrial damage and

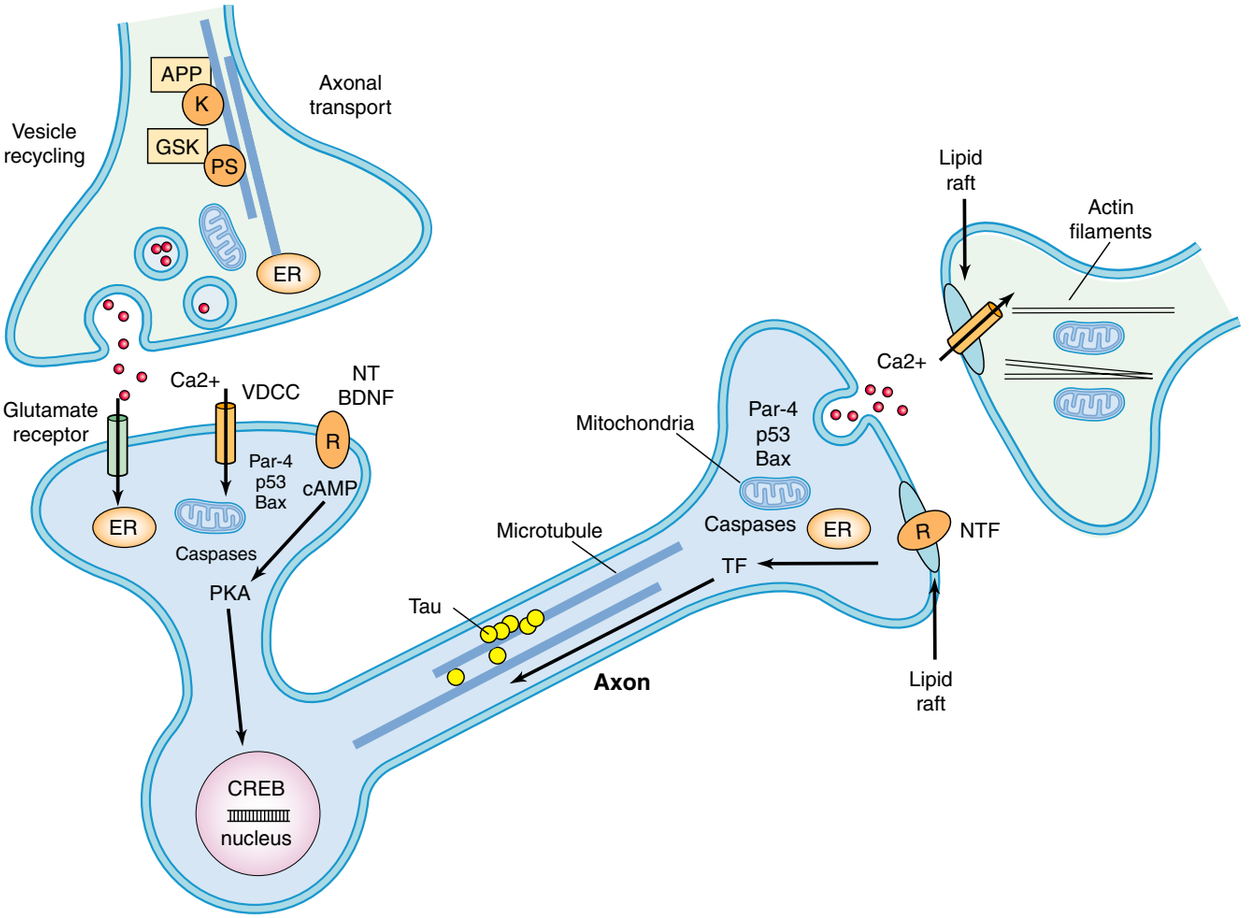


FIGURE 35-7 Apoptotic and anti-apoptotic synaptic signaling mechanisms. Synapses are sites where various signal transduction pathways are activated including those of neurotransmitters (*NT*; glutamate receptors linked to calcium influx and receptors coupled to cAMP production are shown), and neurotrophic factors (*NTF*). Synapses contain all the major organelles (except the nucleus) and proteins involved in apoptosis including *Bcl-2* family members, p53, Par-4, mitochondria and ER, and caspases. Alterations in axonal transport may also trigger apoptosis. *APP*, amyloid precursor protein; *CREB*, cyclic AMP response element binding protein; *ER*, endoplasmic reticulum; *GSK*, glycogen synthase kinase; *K*, kinesin; *PKA*, protein kinase A; *PS*, presenilin; *R*, receptor; *TF*, transcription factor; *VDCC*, voltage-dependent calcium channel.

cytochrome c release during necrosis but appear not to be essential for cell death. During the cell death process the chromatin clumps and the nuclear membrane is disrupted. Finally, the cell lyses, releasing its contents into the extracellular compartment, where the contents may damage neighboring cells and induce an inflammatory response. Transcription of genes and protein synthesis stop and ATP is rapidly depleted in cells undergoing necrosis.

There are few cell death triggers that are only capable of inducing either apoptosis or necrosis. Instead, whether a cell undergoes apoptosis or necrosis is usually determined by the intensity and/or duration of the death-inducing stimulus. In general, severe and/or sustained insults trigger necrosis whereas less severe transient stresses induce apoptosis. For example, moderate overactivation of glutamate receptors may trigger apoptosis while more intense and sustained activation of glutamate receptors induces excitotoxic necrosis (Chs 15 and 32). The environment of the cell at the time it is subjected to the death stimulus can also determine the mode of death. Thus, a certain level of glutamate receptor activation may induce apoptosis in a cell receiving normal amounts of oxygen and glucose but may cause necrosis when that same cell is subjected to ischemia.

Trauma. Acute trauma as in head injury (e.g. automobile accidents, sports injuries) can induce necrosis of the tissue at and surrounding the site of the trauma. Physical damage to cellular membranes may induce necrosis in the traumatized tissue, while disruption of ion homeostasis and energy depletion may trigger necrosis in cells adjacent to the directly damaged cells. Traumatized cells release the contents of their organelles into the extracellular space, resulting in exposure of adjacent cells to various lysosomal proteases and to acidosis. Marked changes in pH can also trigger necrosis; interestingly, intracellular acidification may contribute to necrotic cell death induced by extensive DNA damage.

Energy failure/ischemia. Cellular energy failure is one defining feature of necrosis, and severe reduction in glucose and/or oxygen availability is sufficient to trigger necrosis. Mitochondrial toxins can trigger apoptosis at low levels but at higher levels they induce necrosis as the result of severe depletion of ATP.

Excitotoxicity. Overactivation of glutamate receptors, particularly when neurons are subjected to metabolic and oxidative stress, can trigger excitotoxic necrosis. Sodium ion influx through AMPA/kainate receptors and voltage-dependent sodium channels induces cell swelling, and calcium ion influx through NMDA receptors and voltage-dependent calcium channels activates various proteases (calpains, for example) that degrade various structural and metabolic proteins.

Calcium ion release from the ER and the accumulation of misfolded proteins in the ER can trigger necrosis, and agents that inhibit such calcium release can protect cells against necrosis.

TARGETING APOPTOSIS AND NECROSIS IN NEUROLOGICAL DISORDERS

Studies of cell culture and animal models of neurodegenerative conditions that involve apoptosis have established the preventative and therapeutic potential of antiapoptotic approaches [18, 30, 34]. The most effective means of preventing apoptosis is to abolish the triggering event. Examples include: dietary and drug treatments that suppress atherosclerosis and blood clot formation to prevent a stroke from occurring; eliminating exposure to neurotoxins that may cause Parkinson's disease; blocking the production of amyloid β-peptide to prevent Alzheimer's disease; and wearing a safety helmet when riding a motorcycle to prevent traumatic brain injury. Once the initiating events in the neurodegenerative process have been triggered, specific early events in the apoptotic cascade can be targeted. Examples include: antagonists of glutamate receptor or calcium channels to prevent excitotoxic apoptosis; drugs that block production of, or receptors for, apoptotic cytokines; antioxidants such as vitamin E and glutathione; and agents that block DNA damage response pathways such as PARP and p53 inhibitors. Drugs that modulate the cytoskeleton, such as cytochalasins and taxol, have also proved to be effective in experimental models of neuronal apoptosis. Targets further downstream of the triggering events have also been successfully blocked to prevent apoptosis. Among such agents are cyclosporin, which blocks mitochondrial membrane permeability transition pores, dantrolene, which blocks ER calcium channels, and caspase inhibitors.

An alternative (or complementary) strategy for preventing apoptosis is to activate antiapoptotic pathways. This might be accomplished by administering a growth factor (bFGF, BDNF, GDNF, or others) that activates a signaling pathway which induces the expression of Bcl-2, antioxidant enzymes or IAPs, for example. Mild preconditioning stimuli which upregulate stress resistance proteins and neurotrophic factor signaling can prevent neuronal apoptosis in models of Alzheimer's disease, Parkinson's disease, Huntington's disease and stroke. Examples of such hormetic stimuli include dietary calorie restriction, environmental enrichment and physical exercise [21].

In regards to necrosis, it is clear that the old adage 'an ounce of prevention is worth a pound of cure' applies. Agents that stabilize ion homeostasis have proved to be effective in preventing necrosis in cell culture studies. For example, drugs that activate plasma membrane potassium ion channels or chloride ion channels can prevent membrane depolarization and so inhibit sodium and calcium ion influx. Agents that prevent large sustained increases in intracellular free calcium levels can also prevent neuronal

necrosis; examples include the calcium chelator BAPTA and dantrolene, which blocks calcium release from the ER. Calpain inhibitors can prevent necrosis, as can inhibitors of lysosomal proteases. Treatments that promote maintenance of cellular ATP levels, such as pyruvate and uridine, and creatine can also prevent necrosis of neurons under some conditions.

REFERENCES

1. Yuan, Y., Lipinski, M., and Degterev, A. Diversity in the mechanisms of neuronal cell death. *Neuron* 40: 401–413, 2003.
2. Fraser, A. G. Programmed cell death in *C. elegans*. *Cancer Metastasis Rev.* 18: 285–294, 1999.
3. De la Rosa, E. J. and de Pablo, F. Cell death in early neural development: beyond the neurotrophic theory. *Trends Neurosci.* 23: 454–458, 2000.
4. Lee, J., Duan, W. and Mattson, M. P. Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. *J. Neurochem.* 82: 1367–1375, 2000.
5. Dragunow, M., MacGibbon, G. A., Lawlor, P. *et al.* Apoptosis, neurotrophic factors and neurodegeneration. *Rev. Neurosci.* 8: 223–265, 1997.
6. Eldadah, B. A. and Faden, A. I. Caspase pathways, neuronal apoptosis, and CNS injury. *J. Neurotrauma.* 17: 811–829, 2000.
7. Endres, M. and Dirnagl, U. Ischemia and stroke. *Adv. Exp. Med. Biol.* 513: 455–473, 2002.
8. Mattson, M. P. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med.* 3: 65–94, 2003.
9. Nourooz-Zadeh, J., Liu, E. H. C., Anggard, E. E. and Halliwell, B. F4-isoprostanes: a novel class of prostanoids formed during peroxidation of docosahexaenoic acid. *Biochem. Biophys. Res. Commun.* 242: 338–344, 1998.
10. Roberts, L. J. II, Montine, T. J., Markesbery, W. R. *et al.* Formation of isoprostane-like compounds (neuroprostanes) *in vivo* from docosahexaenoic acid. *J. Biol. Chem.* 273: 13605–13612, 1998.
11. Morrow, J. D., Hill, K. E., Burk, R. F. *et al.* A series of prostaglandin F2-like compounds are produced *in vivo* in human by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl Acad. Sci. U.S.A.* 87: 9383–9387, 1990.
12. Nourooz-Zadeh, J., Liu, E. H. C., Yhlen, B., Anggard, E. E. and Halliwell, B. F4-isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. *J. Neurochem.* 72: 734–740, 1999.
13. Bazan, N. G. Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *J. Lipid Res.* 44: 2221–2233, 2003.
14. Bush, R. A., Malnoë, A., Remé, C. E. and Williams, T. P. Dietary deficiency of N-3 fatty acids alters rhodopsin content and function in the rat retina. *Invest. Ophthalmol. Vis. Sci.* 35: 91–100, 1994.
15. Niu, S.-L., Mitchell, D. C., Lim, S.-Y. *et al.* Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *J. Biol. Chem.* 279: 31098–31104, 2004.
16. Bazan, N. G. Neuroprotectin D1 (NPD1): a DHA-derived mediator that protects brain and retina against cell injury-induced oxidative stress. *Brain Pathol.* 15: 159–166, 2005.
17. Mukherjee, P. K., Marcheselli, V. L., Serhan, C. N. and Bazan, N. G. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. *Proc. Natl Acad. Sci. U.S.A.* 101: 8491–8496, 2004.
18. Mattson, M. P. Apoptosis in neurodegenerative disorders. *Nat. Rev. Mol. Cell Biol.* 1: 120–129, 2000.
19. Riess, O., Berg, D., Kruger, R. and Schulz, J. B. Therapeutic strategies for Parkinson's disease based on data derived from genetic research. *J. Neurol.* 250: I3–10, 2003.
20. Vila, M. and Przedborski, S. Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* 4: 365–375, 2003.
21. Mattson, M. P., Chan, S. L. and Duan, W. Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. *Physiol. Rev.* 82: 637–672, 2002.
22. Deshmukh, M. and Johnson, E.M. Jr Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. *Mol. Pharmacol.* 51: 897–906, 1997.
23. Bredesen, D.E. Apoptosis: overview and signal transduction pathways. *J. Neurotrauma.* 17: 801–810, 2000.
24. Cutler, R. G., Kelly, J., Storie, K. *et al.* Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc. Natl Acad. Sci. U.S.A.* 101: 2070–2075, 2004.
25. Mattson, M. P. and Kroemer, G. Mitochondria in cell death: novel targets for neuro- and cardio-protection. *Trends Mol. Med.* 9: 196–205, 2003.
26. Boehning, D., Patterson, R. L., Sedaghat, L. *et al.* Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat. Cell Biol.* 5: 1051–1061, 2003.
27. Salvesen, G. S. Caspases and apoptosis. *Essays Biochem.* 38: 9–19, 2002.
28. Robertson, J. D., Orrenius, S. and Zhivotovsky, B. Review: nuclear events in apoptosis. *J. Struct. Biol.* 129: 346–358, 2000.
29. Li, M. O., Sarkisian, M. R., Mehal, W. Z. *et al.* Phosphatidylserine receptor is required for clearance of apoptotic cells. *Science* 302: 1560–1563, 2003.
30. Mattson, M. P., Culmsee, C. and Yu, Z. F. Apoptotic and anti-apoptotic mechanisms in stroke. *Cell Tiss. Res.* 301: 173–187, 2000.
31. Robertson, G. S., Crocker, S. J., Nicholson, D. W. *et al.* Neuroprotection by the inhibition of apoptosis. *Brain Pathol.* 10: 283–292, 2000.
32. Gilman, C. P. and Mattson, M. P. Do apoptotic mechanisms regulate synaptic plasticity and growth cone motility. *Neuromolecular Med.* 2: 197–214, 2002.
33. Syntichaki, P. and Tavernarakis, N. The biochemistry of neuronal necrosis: rogue biology? *Nat. Rev. Neurosci.* 4: 672–684, 2003.
34. Waldmeier, P. C. Prospects for antiapoptotic drug therapy of neurodegenerative disorders. Prog. Neuropsychopharmacol. *Biol. Psychiatry.* 27: 303–321, 2003.

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CHAPTER

36

Peripheral Neuropathy

David Pleasure

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INTRODUCTION: CLINICAL FEATURES OF NEUROPATHIES

This chapter provides a short review of peripheral nerve diseases. Diseases that involve motor neurons, the presynaptic compartment of neuromuscular junctions, or the enteric nervous system are also discussed.

Peripheral neuropathies may be widely disseminated or focal. Patients with disseminated polyneuropathy, whether demyelinative or axonal, usually demonstrate distal sensory and/or motor impairment. Multifocal neuropathy, also referred to as mononeuropathy multiplex, is often a consequence of lesions affecting the vasa nervorum, the blood vessels that supply peripheral nerves. The most common diseases to compromise the vasa nervorum and cause infarction of nerve fascicles are diabetes mellitus and periarteritis nodosa. Other frequent causes of mononeuropathy multiplex include infection (e.g. Lyme disease and leprosy) and multiple compression injury (e.g. bilateral carpal tunnel syndrome). When mononeuropathy multiplex progresses to affect myriad peripheral nervous system (PNS) sites, distal motor and sensory deficits in all limbs clinically indistinguishable from those in polyneuropathy may result.

Diseases selectively targeting spinal cord and brainstem motor neurons (e.g. amyotrophic lateral sclerosis and the familial spinal muscular atrophies) or the presynaptic component of neuromuscular junctions (e.g. Lambert–Eaton syndrome, botulism and *Ixodes* tick paralysis) cause weakness without sensory impairment. Disorders involving the enteric nervous system (e.g. Chagas' disease and Hirschsprung's disease) impair bowel motility.

Electromyography and nerve conduction studies are helpful in evaluating PNS disease [1]. Needle electromyography in patients in whom motor neurons or their axons are damaged will often demonstrate fibrillations (spontaneous action potentials of single denervated muscle fibers) and fasciculations (spontaneous firings of motor units, which include all muscle fibers innervated by a motor neuron) in muscle at rest, and recruitment of lesser numbers than normal of motor units with volitional muscle contraction. These motor units become progressively larger than normal with chronic muscle denervation, because of collateral reinnervation of denervated muscle fibers by surviving axons.

Determining the rate of propagation of action potentials elicited by transcutaneous nerve electrical stimulation helps to discriminate between primarily demyelinative and primarily axonal neuropathies. The electrophysiological hallmark of demyelination is a diminution in nerve conduction velocity of 25% or more. However, the severity of slowing of nerve conduction in demyelinative or dysmyelinative neuropathies often fails to correlate with the extent of clinical neurological deficits. For example, patients in the early stages of subacute idiopathic demyelinative polyneuropathy (Guillain–Barré syndrome) can become paralyzed, yet have normal nerve conduction velocities. This discrepancy between clinical and electrophysiological severity may be attributable to a

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combination of demyelination confined at this early stage to nerve segments not sampled by the electrophysiological study (i.e. nerve roots), and to failure of the conduction studies to detect subpopulations of axons in which conduction has been blocked because multiple contiguous segments of myelin have been stripped from them. Conversely, during recovery from segmental demyelination, the thin new myelin sheaths that are first formed are sufficient to permit restoration of conduction of action potentials and recovery of normal neurological function but not to restore normal conduction velocities, which require reconstitution of full thickness myelin.

In axonal neuropathies, the velocity of action potential propagation in surviving axons is well maintained but the number of axons capable of conducting action potentials is diminished. Transcutaneous nerve stimulation and recording, the method routinely used for studying nerve conduction in the clinic, does not permit evaluation of the function of autonomic or unmyelinated sensory axons. These smaller, slow-conducting axons can be analyzed, in research studies, by intraneural recording with needle electrodes.

Measuring muscle-evoked responses to repetitive motor nerve electrical stimulation permits detection of presynaptic neuromuscular junction dysfunction. In botulism and the Lambert–Eaton syndrome, repetitive stimulation elicits a smaller than normal skeletal muscle response at the beginning of the stimulus train, due to impaired initial release of acetylcholine-containing vesicles from presynaptic terminals of motor neurons followed by a normal or accentuated incremental muscle response during repeated stimulation. This incremental response to repetitive stimulation in presynaptic neuromuscular disorders can be distinguished from the decremental response that characterizes autoimmune myasthenia gravis, which affects the postsynaptic component of neuromuscular junctions.

Diagnostic indications for nerve biopsy have become less frequent with improvements in EMG and nerve conduction techniques and the advent of specific molecular and biochemical tests for many inherited and acquired neuropathies. However, nerve biopsy does remain useful for diagnosis of vasculitis and amyloidosis, and plays an important role in research.

FEATURES COMMON TO THE PERIPHERAL AND CENTRAL NERVOUS SYSTEM IMPORTANT IN THE PATHOGENESIS AND PATHOPHYSIOLOGY OF NEUROPATHIES

Somatic motor and sensory neurons that give rise to PNS axons maintain large fractions of their total protoplasmic bulk within the CNS. Many of the extracellular matrix components and axonal guidance molecules involved in CNS development are employed for the same purpose in the PNS. PNS microglia-like cells, like microglia in the CNS, are bone-marrow-derived and have a similar repertoire of responses to activation [2]. Both oligodendroglia and Schwann cells speed axonal action potential propagation by assembling and maintaining myelin. Capillary endothelial cells linked by tight junctions restrict entry of polar molecules into the PNS, as into the CNS [3].

As might be predicted from these similarities between PNS and CNS, many disease entities can affect both these tissues. It should be noted, however, that the clinical expression of such diseases is variable and is sometimes restricted to the PNS. For example, patients with thiamine deficiency may display symmetrical distal sensorimotor polyneuropathy without accompanying CNS degeneration. Untreated infection with human immunodeficiency virus (HIV) may cause early polyneuropathy, with dementia appearing months or years later. Similarly, patients with sulfatidase deficiency or adrenoleukodystrophy may present initially with polyneuropathy, while their CNS dysfunction remains clinically undetectable.

FEATURES OF PERIPHERAL NERVES THAT INFLUENCE THEIR VULNERABILITY TO DISEASE AND CAPACITY FOR REGENERATION

Table 36-1 lists some of the mechanisms important in governing the susceptibility of the PNS to disease and injury. Peripheral nerves, although toughened by their high content of collagen, are prone to injury to myelin by compression (e.g. carpal tunnel syndrome and tardy ulnar palsy) and to axons by excessive stretch (e.g. brachial plexopathy in newborn infants following a difficult delivery). Subcutaneous nerves, because of their exposed position, are also vulnerable to cold or heat injury.

Neuropathies can result from mutations that alter the structure or level of expression of PNS myelin proteins (e.g. overexpression of PMP22 in Charcot–Marie–Tooth syndrome (CMT) type 1A), the metabolism of myelin lipids (e.g. metachromatic leukodystrophy), or the capacity of PNS neurons to support their axons in patients with CMT caused by mutations of *KIF1B* [4] or *NF-L* [5, 6]. Both acquired and inherited amyloid neuropathies can result from the deposition of poorly soluble proteins, for example cryoglobulins or mutant transthyretins, in and around endoneurial blood vessels [7–9].

Schwann cells are responsible for PNS trophic functions that, in CNS, are carried out by both oligodendroglia and astroglia. Schwann cell diseases usually present as disorders of myelination. Axonal degeneration and diminution in axonal diameter may also occur in primary disorders of Schwann cells, as a consequence of loss of Schwann cell trophic support for axons. (See also Chs 4 and 38.)

TABLE 36-1 Mechanisms of pathogenesis of PNS-specific disease

Mechanism	PNS-specific disease
Mutation affecting gene expressed exclusively in PNS (e.g. *PMP22*)	Charcot–Marie–Tooth type 1A
Toxin penetrates PNS, not CNS	Botulism, cisplatin neuropathy
Immune attack on antigen accessible in PNS, not CNS (e.g. gangliosides GQ1b and GT1a)	Miller–Fisher syndrome
Mechanical injury to exposed segment PNS	Nerve compression and stretch injuries
Superficial nerves maintained at lower than normal core body temperature	Cryoglobulinemic neuropathy; lepromatous neuropathy

Early in the course of Wallerian degeneration in the PNS, mononuclear cells from the bloodstream enter the injured nerve and assist resident Schwann cells, microglia-like cells and fibroblasts to clear fragmented myelin [10, 11]. Activated macrophages and fibroblasts in the injured nerve secrete a variety of cytokines that enhance Schwann cell proliferation and axonal regrowth [12, 13]. Axonal regeneration is more robust in PNS than in CNS. This is attributable, at least in part, to the stimulatory effect of Schwann cells on neurite growth, versus the inhibition of neurite outgrowth by oligodendroglia in CNS. Schwann cells in the injured nerve aggregate to form longitudinally oriented tubes ('bands of Bungner') through which regenerating axons penetrate through the injured nerve segment to reach their end-organs [14]. Perineurium, flattened, basal lamina-lined cells derived from fibroblasts [15], may also help to guide regenerating axons.

PATHOGENESIS OF NEUROPATHIES

Infections can damage nerves directly, or via exotoxins.

The lepromatous form of leprosy is characterized by loss of cutaneous sensibility. Hansen's bacillus (*Mycobacterium leprae*), which proliferates only in environments cooler than the core temperature maintained by most mammals, is capable of infecting Schwann cells in subcutaneous nerves because the basal lamina of these cells contains α-dystroglycan, to which this mycobacterium binds, and because subcutaneous nerves are often cooler than deeper tissues. Lepromatous neuropathy is a common cause of sensory mononeuropathy multiplex in the developing World [16, 17].

Neuropathy in human immunodeficiency virus infection has many causes. Multiple mechanisms cause neuropathy in patients with HIV. An immune-mediated, Guillain–Barré-like syndrome (see below) may occur at the time of HIV seroconversion. Later in the course of infection, patients may present with mononeuropathy multiplex, sometimes as a consequence of vasculitis associated with coinfection with hepatitis C. Distal sensory–autonomic axonal polyneuropathy may develop in patients with more advanced HIV, either as a consequence of high titers of HIV itself or of the neurotoxicity of antiretroviral drugs [18, 19].

Diphtheria causes a demyelinative neuropathy. *Corynebacterium diphtheriae* colonizes the pharynx or open wounds, and secretes a protein exotoxin. The B subunit of this exotoxin binds to plasma membranes and facilitates entry into cytosol of the A subunit, which catalyzes ADP-ribosylation, and inactivation of an elongation factor required for protein synthesis. Cardiac muscle and Schwann cells are particularly susceptible to this toxin, and hence patients with diphtheria develop cardiomyopathy and demyelinative polyneuropathy [20]. While diphtheria is now uncommon because of childhood immunization against *C. diphtheriae*, the disruption in preventative medicine programs caused by disintegration of the Soviet Union was followed by a substantial incidence of diphtheritic polyneuropathy in Russia.

Botulinum exotoxin impedes release of neurotransmitter vesicles from cholinergic terminals at neuromuscular junctions. Botulinum exotoxin is ingested with food or, in infants, synthesized *in situ* by anaerobic bacteria that colonize the gut. A characteristic feature of botulinum paralysis is that the maximal force of muscle contraction increases when motor nerve electrical stimulation is repeated at low frequency, a phenomenon attributable to the recruitment of additional cholinergic vesicles with repetitive depolarization of neuromuscular presynaptic terminals. Local administration of *Clostridium botulinum* exotoxin is now in vogue for its cosmetic effects and is used for relief of spasticity in dystonia and cerebral palsy [21].

Antibody- and cell-mediated mechanisms of neuropathy.

Both antibody-mediated (Table 36-2) and cell-mediated mechanisms contribute to the pathogenesis of this diverse group of neuropathies.

Acute inflammatory demyelinating polyneuropathy is a common cause of reversible paralysis. Acute inflammatory demyelinating polyneuropathy (AIDP), the classic form of the Guillain–Barré syndrome, often begins a week or two after recovery from cytomegalovirus, Epstein–Barr virus or *Mycoplasma* infection. Patients present with rapidly advancing symmetrical weakness, loss of deep tendon reflexes, often with distal numbness, and limb or back pain. Cerebrospinal fluid (CSF) protein concentration is elevated, but in most cases there is little or no increase in number of inflammatory cells in the CSF. This 'albuminocytologic dissociation' contrasts with the elevation of both

TABLE 36-2 Antibody-mediated PNS disorders

Neuropathic syndrome	Antigenic specificity
Paraneoplastic sensory neuropathy	HuB, HuC, HuD
Lambert–Eaton myasthenic syndrome	Presynaptic voltage-gated calcium channel
Neuromyotonia (Isaacs' syndrome)	Voltage-gated potassium channel
Idiopathic autonomic neuropathy	Ganglionic acetylcholine receptor
Chagas' disease	M2 muscarinic acetylcholine receptors
Anti-MAG IgM paraproteinemic sensory neuropathy	Myelin-associated glycoprotein (MAG), sulfated glucuronyl paragloboside, sulfated glucuronyl lactosaminyl paragloboside
Vasculitic mononeuritis multiplex	Myeloperoxidase (MPO), proteinase 3 (PR3)
Multifocal motor neuropathy	GM1, GD1b, asialo-GM1 gangliosides
Miller–Fisher syndrome, Bickerstaff's brainstem encephalitis	GQ1b, GT1a gangliosides
Acute motor axonal neuropathy	GM1, GM1b, GD1a, GalNac-GD1a gangliosides
Immunoglobulin light-chain amyloid neuropathy	Unknown
Cryoglobulinemic neuropathy	Unknown

protein and cell count in the CSF of patients with paralysis caused by assault on primary motor neurons by neurotropic viruses (e.g. poliovirus and West Nile virus). Histological examination of peripheral nerves in AIDP demonstrates segmental demyelination and infiltration by lymphocytes. Most patients recover full neurological function within months, particularly if treated early by plasmapheresis or immunoglobulin infusion [22].

Acute motor axonal neuropathy also causes reversible paralysis. Acute motor axonal neuropathy (AMAN) resembles AIDP in clinical features and favorable long-term prognosis but differs in that axons, rather than myelin sheaths, are primarily injured. AMAN occurs sporadically in the USA but has sometimes reached epidemic proportions in China. Immunogenetic studies in China have demonstrated that there are also marked distinctions in the frequency of HLA class II antigens between those patients with AIDP and those with AMAN [23]. In many patients with AMAN, axonal injury appears to be a consequence of complement-fixing antiganglioside antibodies generated in response to an antecedent *Campylobacter jejuni* infection [24]. Plasmapheresis or immunoglobulin infusion is recommended for AMAN, and prognosis is similar to that for AIDP [22].

Miller–Fisher syndrome is mediated by pathogenic antibodies. Patients with the Miller–Fisher syndrome present subacutely with paralysis of extraocular muscles (ophthalmoplegia), ataxia and loss of deep tendon reflexes, sometimes after an antecedent *C. jejuni* infection. Most of these patients express serum antibodies against GQ1b ganglioside [24]. These antibodies bind complement and form membrane attack complexes on motor axons. Infusion of immunoglobulins accelerates recovery of normal neurological function in these patients, as in AIDP and AMAN, probably by inhibiting binding of the pathological antibodies to neural targets [25].

Autoimmune autonomic neuropathy is also associated with autoimmune antibodies. The most common presentation of autoimmune autonomic neuropathy is the acute onset of orthostatic hypotension, impaired bowel motility, and loss of sweating, often following a nonspecific viral infection. Both sympathetic and parasympathetic dysfunction are present in most patients. More than 50% of the patients with this syndrome demonstrate high titers of nicotinic acetylcholine receptor autoantibodies [26].

Experimental autoimmune neuritis and experimental autoimmune autonomic neuropathy are robust models for human autoimmune neuropathies. The most widely accepted hypothesis about the pathogenesis of AIDP, AMAN, the Miller–Fisher syndrome and autoimmune autonomic neuropathy is that they are initiated by sensitization to epitopes shared by an infecting microorganism and a component of the PNS ('molecular mimicry') [24]. Both cellular and antibody-mediated effector mechanisms then contribute to nerve damage [27, 28]. Which form of autoimmune neuropathy affects a given patient depends both upon the characteristics of the infecting microorganism and the immunogenetic determinants expressed by the patient [23].

Experimental autoimmune neuritis (EAN) mirrors AIDP in clinical, electrophysiological, CSF and pathological features. It is induced in susceptible strains of rats by sensitization to whole PNS myelin or to P2 or P0 peptide epitopes, and in rabbits by immunization with the myelin glycolipid galactocerebroside (galC) [29–32]. A similar syndrome has been recognized in humans immunized with a rabies vaccine prepared in brain tissues [33]. Like AIDP, EAN is typically a monophasic illness, although recurrent attacks can be elicited in Lewis rats if they are subjected to low-level immunosuppression. P2-EAN and P0-EAN are mediated primarily by sensitized T lymphocytes, whereas galC-EAN appears to be largely antibody-mediated. The PNS selectivity of P2- and P0-EAN is due to the restricted distribution of P2 and P0 to PNS myelin. GalC, on the other hand, is a constituent of CNS myelin, as well as PNS myelin, and the PNS selectivity of galC-EAN may be a consequence of more ready ingress of pathogenic complement-fixing galC antibodies to the PNS than to the CNS.

Experimental autoimmune autonomic neuropathy can be induced in rabbits by immunization with a neuronal

acetylcholine receptor α3 subunit fusion protein. These rabbits develop severe panautonomic failure closely resembling that seen in patients with autoimmune autonomic neuropathy [34].

Immune-mediated neuropathies, often vasculitis, are associated with systemic inflammatory disorders. Systemic inflammatory disorders (e.g. periarteritis nodosa, rheumatoid arthritis and Sjögren's syndrome) are often associated with mononeuropathy multiplex. In general, neuropathy in these diseases is caused by damage to vasa nervorum by inflammatory cells that infiltrate the blood vessel walls [9, 35], sometimes in conjunction with cryoglobulins. Many patients with vasculitic mononeuropathy multiplex express antibodies against myeloperoxidase and proteinase 3, demonstration of which is helpful in diagnosis [36].

Immune mechanisms contribute to paraneoplastic neuropathies.

Paraneoplastic neuropathies often occur in patients with carcinoma. Subacute sensory or sensorimotor axonal polyneuropathy, often with associated limbic encephalitis and cerebellar degeneration, is a common complication of small-cell lung cancer and other carcinomas, occasionally presenting even prior to diagnosis of the underlying neoplasm. Some patients with paraneoplastic neuropathy express anti-Hu antibodies, which recognize epitopes associated with the HuD neuronal RNA binding protein [37, 38].

Lambert–Eaton syndrome is an antibody-mediated neuromuscular junction disorder. This disorder, which is most frequently encountered in patients with small-cell lung carcinoma, is characterized clinically by weakness and hyporeflexia. The impaired release of acetylcholine vesicles from presynaptic terminals at neuromuscular junctions that causes the weakness is a consequence of autoantibodies against small cell carcinoma epitopes that cross-react with and downregulate the expression of motor nerve terminal Ca^{2+} channels [39]. (See in Ch. 43.)

Neuromyotonia (Isaacs' syndrome) is an immune-mediated hyperexcitability syndrome often associated with tumors. Neuromyotonia is a neuromuscular hyperexcitability syndrome characterized clinically by frequent muscle twitching, muscle enlargement, and excessive sweating. Electromyography shows repetitive high frequency discharges of motor units. Some patients with this disorder have associated thymoma, small cell lung carcinoma, or myasthenia gravis. More than a third of the patients with neuromyotonia have antibodies against voltage-gated potassium channels, with lesser numbers expressing antibodies against ganglionic or muscle acetylcholine receptors [40, 41].

Several forms of neuropathy occur in patients with plasma cell dyscrasias. In light chain amyloid neuropathy, insoluble aggregates of immunoglobulin light chains accumulate in the nerves of patients with multiple myeloma or other plasma cell dyscrasias. Clinical features of this acquired form of amyloid polyneuropathy include sensory, autonomic and motor deficits. Serum and/or urine protein electrophoresis in such patients demonstrates the presence of a monoclonal light chain protein, and amyloid is demonstrable in nerve biopsy specimens [7].

The syndrome of IgM_k paraproteinemia and sensory ataxia is usually encountered in elderly men. Nerve biopsy in these patients shows focal abnormalities in compaction of PNS myelin lamellae, and their IgM paraprotein recognizes epitopes of myelin-associated glycoprotein (MAG) and sulfate-3-glucuronyl paragloboside [24, 42].

POEMS is an eponym applied to patients with a variety of plasma cell dyscrasias who present with *p*olyneuropathy, *o*rganomegaly, *e*ndocrinopathy, an *M* protein and *s*kin changes; this disorder is also referred to as Crow–Fukase syndrome. Additional manifestations of this disorder are pulmonary hypertension, renal failure, a predisposition to thrombosis and congestive heart failure; some of these features are likely to be attributable to vascular endothelial growth factor (VEGF) and matrix metalloproteinases, which are often elevated in the plasma of these patients [43].

Toxins and hormone deficiency states are frequent causes of neuropathy.

Toxins and medications that affect the axonal cytoskeleton cause neuropathy. Axonal polyneuropathy is a common complication of the administration to cancer patients of the microtubule depolymerizing drug vincristine and a rare complication of therapy with another microtubule-interacting agent, colchicine [44, 45]. Exposure to *N*-hexane or its metabolite 2,5-hexanedione, to carbon disulfide or to β,β′-iminodiproprionitrile results in cross-linking of neurofilaments and produces a giant axonal neuropathy [46]. Secondary demyelination of enlarged axonal segments causes a multifocal nerve conduction block more suggestive of demyelinative than of axonal polyneuropathy. Many other frequently used drugs, such as the statin HMG-CoA reductase inhibitors, also cause distal axonal degeneration in occasional patients [47].

Megadosage vitamin B6 (pyridoxine) and cisplatin cause sensory neuronopathy. Pyridoxine toxicity, usually the result of inappropriate self-overmedication, kills dorsal root ganglion neurons. Affected individuals become severely ataxic because of loss of proprioceptive afferent input to the cerebellum. A similar syndrome is elicited by cisplatin chemotherapy. Neurotrophin-3 treatment is effective in protecting dorsal root ganglion neurons in experimental animals against these toxins [48, 49].

Neuropathy can result from deficiency of vitamins or hormones. Alcoholics often obtain a large proportion of their caloric needs from ethanol, and hence become thiamine-deficient. Alcoholic neuropathy results from a combination of thiamine deficiency, which impairs

carbohydrate metabolism, and a direct toxic effect of ethanol on the PNS [50]. More than a third of patients newly diagnosed with hypothyroidism demonstrate clinical and electrophysiological evidences of sensorimotor axonal polyneuropathy, which resolves with thyroid hormone replacement therapy [51]. Diabetic neuropathy, a late consequence of insulin deficiency, is discussed below.

Many genetically determined neuropathies are now recognized.

Diabetes mellitus is the most common cause of peripheral neuropathy in the United States. Approximately half of all diabetics demonstrate evidences of neuropathy. The usual clinical pattern is that of a slowly progressive, mixed sensorimotor and autonomic polyneuropathy. More acute, asymmetrical motor neuropathies are also seen, usually affecting the lumbosacral plexus, particularly in older persons with type 2 (non-insulin-dependent) diabetes mellitus. Patients with diabetes mellitus are also prone to develop isolated palsies of cranial nerve III or VII, and there is a high incidence of asymptomatic focal demyelination in the distal median nerve.

Among the pathogenetic mechanisms that have been proposed for diabetic neuropathy are excess glycation of neural proteins, alteration in nerve polyol metabolism induced by hyperglycemia, deficiency of a trophic factor necessary for PNS survival (e.g. sonic hedgehog) and nerve ischemia [52, 53]. The presence of small-vessel disease in human diabetic nerves suggests that diminished endoneurial blood flow plays a role in human diabetic neuropathy, particularly with respect to the scattered infarctions of the proximal regions of peripheral nerves attributable to vasa nervorum inflammatory vasculopathy [54].

Familial demyelinative/dysmyelinative neuropathies may be caused by impaired peroxisomal lipid metabolism. Refsum's disease is an autosomal recessive peroxisomal disorder characterized by accumulation of phytanic acid and other 3-alkyl-branched fatty acids [55]. Patients develop hypertrophic demyelinative polyneuropathy, retinitis pigmentosa, ichthyosis and deafness. The disorder can be successfully treated by institution of a diet poor in phytanic acid, in conjunction with plasmapheresis to remove circulating phytanic acid.

Adrenoleukodystrophy is an X-linked dysmyelinative disorder caused by mutations in the *ABCD1* gene, which encodes the peroxisomal integral membrane ALD protein, a member of the ATP binding cassette transporter family. These mutations result in impaired clearance of plasma very-long-chain fatty acids. Affected males may present with symmetrical distal axonal polyneuropathy, adrenocortical insufficiency or CNS demyelination, while occasional heterozygous women demonstrate deficits suggestive of multiple sclerosis [56]. Manipulation of dietary fatty acid intake has some minimal therapeutic effect, while bone marrow transplantation has diminished deficits in a few patients. (See in Ch. 41.)

Familial demyelinative/dysmyelinative and axonal neuropathies may also be caused by impaired lysosomal lipid metabolism. Metachromatic leukodystrophy (sulfatide lipidosis) results from mutations of the arylsulfatase A gene, which encodes a lysosomal enzyme required for sulfatide turnover. Myelin is affected in both CNS and PNS, though dysfunction is restricted to the PNS in some patients, and the onset of symptoms can occur at any time between infancy and adulthood. Bone marrow transplantation can slow disease progression and improve nerve conduction velocities [57]. (See in Ch. 41.)

Fabry's disease results from mutations of lysosomal α-galactosidase-A, which causes accumulation of α-D-galactosyl-conjugated glycosphingolipids. Neuropathy in this disorder affects small but not large axons and presents with pain and hypohidrosis. Another major feature of this disease is vasculopathy affecting the kidneys and CNS. Periodic infusion of patients with Fabry's disease with recombinant human α-galactosidase-A diminishes neuropathic pain and improves sweating function [58].

Acute intermittent porphyria is a dominantly inherited partial deficiency of porphobilinogen deaminase, and causes axonal polyneuropathy. Acute intermittent porphyria is caused by partial deficiency of porphobilinogen deaminase, an enzyme required for heme biosynthesis. Patients may present with acute abdominal pain, rapidly progressive sensorimotor axonal polyneuropathy or psychosis, and have elevated concentrations of the heme precursor δ-amino-levulinic acid in their urine. Symptoms may be precipitated by treatment with barbiturates or other drugs and are suppressed by treatment with hematin [59].

Several familial forms of amyloid neuropathy are now recognized. The dominantly inherited amyloid neuropathies are caused by mutations of the transthyretin, gelsolin or apolipoprotein A1 genes that diminish the solubility of these proteins, resulting in the formation of protein aggregates in vasa nervorum and perineurium in peripheral nerves. The familial amyloid neuropathies present with autonomic dysfunction, often together with progressive distal sensory or sensorimotor neuropathy [7]. Liver transplantation may prevent progression of amyloid neuropathy caused by transthyretin mutations.

There are multiple forms of hereditary motor and sensory neuropathy. Charcot–Marie–Tooth syndrome is a diverse group of demyelinative and axonal polyneuropathies caused by mutations of myelin-associated proteins (PMP22, MPZ and Cx32) [60] or other genes expressed by Schwann cells or PNS neurons (e.g. *GDAP1* and *NF-L*). Collectively, the CMTs rival neurofibromatosis type 1 as the most common genetic disorder affecting the PNS. Demyelinative forms of CMT are characterized by reduced velocity of nerve action potentials, prominent segmental demyelination and Schwann cell proliferation, sometimes

with onion-bulb formation. There is typically also a paucity of large axons in patients with these primarily demyelinative polyneuropathies, probably resulting from deficient provision by Schwann cells of trophic influences necessary for maintenance of a normal axonal cytoskeleton. Axonal forms of CMT manifest distal Wallerian degeneration.

CMT1A, the most common form of CMT [61], results from a 1.5 Mb duplication in chromosome 17p11.2–12. Among the genes encoded in this duplicated segment is PMP22, a component of PNS myelin, and patients with CMT1A therefore have three copies of this gene. Mice transgenic for PMP22 also develop demyelinative polyneuropathy. It is of considerable interest that treatment of these transgenic mice with ascorbic acid results in PNS remyelination and enhanced life expectancy, probably because ascorbic acid represses PMP22 expression [62].

A deletion of the same 1.5 Mb segment of chromosome 17 that is duplicated in CMT1A is the cause of hereditary neuropathy with predisposition to pressure palsies (HNPP). Patients with HNPP, who have only one copy of the *PMP22* gene, develop repeated focal demyelinative mononeuropathy or mononeuropathy multiplex, and many also eventually manifest demyelinative polyneuropathy as well. Histological examination of their nerves demonstrates segmental demyelination and scattered sausage-shaped myelin sheath swellings, hence the alternative name for HNPP, tomaculous neuropathy [63]. A point mutation of the *PMP22* gene is responsible for recessively inherited demyelinative polyneuropathy in the *trembler* mouse [64]. Both recessive and dominant forms of hereditary motor and sensory neuropathy result from mutations in the gene encoding PNS myelin P0 glycoprotein, and these may be primarily demyelinative or primarily axonal. An X-linked form of CMT (CMT1X) is typically a mixed demyelinative and axonal polyneuropathy caused by mutations of a gene encoding the gap-junction protein connexin32 [65]. Gap junctions containing connexin32 are expressed in Schmidt–Lantermann incisures and in paranodal myelin of myelinating Schwann cells. Recessively inherited forms of CMT, caused by *GDAP1*, *ER2*, *MTMR2*, *NDRG1*, *PRX* or *A/C* gene mutations and manifested as either demyelinating or axonal polyneuropathies, are relatively frequent in regions where consanguineous marriages are common [66]. (See also Ch. 38.)

Familial motor neuron disease can result from mutations of a variety of genes. Inherited diseases targeting motor neurons include familial amyotrophic lateral sclerosis (FALS) associated with superoxide dismutase (SOD)1 mutations, X-linked familial bulbospinal muscle atrophy, infantile and adult forms of glycogen-storage disease type IV caused by mutations of the gene encoding the glycogen branching enzyme, and familial infantile, juvenile and adult forms of spinal muscular atrophy caused by mutations of the survival motor neuron (*SMN*) gene, which plays an essential role in pre-mRNA processing [67, 68].

X-lined bulbospinal muscular atrophy, also termed Kennedy's syndrome, is caused by an unstable expanded CAG repeat in the first exon of the androgen receptor gene. Affected men demonstrate progressive proximal limb and bulbar weakness together with gynecomastia. As in Friedreich's ataxia and other neurological disorders associated with unstable triplet expansions, there is a tendency toward earlier onset and greater severity of symptoms with each succeeding generation in a family, which correlates with increasing repeat number [69]. Mutations of SOD1 that result in a toxic gain of function are the most common cause of FALS [70]. (See also Ch. 44.)

Friedreich's ataxia is caused by an intronic triplet repeat expansion. Friedreich's ataxia is an autosomal recessive disorder characterized by progressive ataxia, nystagmus, distal sensory polyneuropathy and corticospinal tract degeneration. It is caused by an unstable expanded GAA repeat in intron 1 of the frataxin gene on chromosome 9q13. This diminishes expression of frataxin, a mitochondrial iron-storage protein that participates in free radical metabolism [71].

Neurofibromatosis is a frequent cause for peripheral nerve tumors.

Neurofibromatosis type 1 is a common cause of PNS tumors. Neurofibromatosis type 1 (NF1, von Recklinghausen's disease) is the most frequent dominantly inherited disorder of the PNS, with an incidence of about 1 in 2,000 persons. Among its diagnostic features are benign Schwann cell tumors arising from small cutaneous nerves, termed subcutaneous neurofibromas, or within larger nerves, plexiform neurofibromas; cutaneous accumulations of melanocytes, termed *café-au-lait spots*; focal accumulations of pigmentary cells in the iris, termed Lisch nodules; and a variety of orthopaedic deformities, including absence of a segment of the sphenoid bone, causing pulsating exophthalmos; kyphosis; and tibial non-union. While all these features can be attributed to abnormalities of neural-crest- or placode-derived tissues, patients with NF1 may also manifest various CNS abnormalities, including mental retardation and gliomas, particularly of the optic nerves or optic chiasm. Patients with NF1 are prone to develop Schwann cell malignancies, termed neurofibrosarcomas, as well as other forms of cancer. NF1 is caused by mutations in the neurofibromin gene, situated on chromosome 17, which encodes a large tumor-suppressor protein with a GTPase-activating domain and many other domains of unknown function. It has been demonstrated that NF1-deficient Schwann cells contain elevated levels of Ras-GTP [72], and that the second copy of the neurofibromin gene is inactivated or lost in benign neurofibromas [73]. Studies in mouse models of NF1 have demonstrated that other cellular components of neurofibromas (e.g. mast cells) contribute to tumor growth in NF1 [74].

Neurofibromatosis type 2 affects the acoustic nerves and CNS glia. Neurofibromatosis type 2, a much rarer disorder than NF1, is characterized by bilateral acoustic schwannomas and CNS glial tumors, and is caused by mutations of a tumor suppressor gene on chromosome 22q that encodes the protein merlin. Merlin, closely homologous to the ERM proteins, ezrin, radixin and moesin, inhibits Rac signaling, thus favoring Schwann cell tumorigenesis [75].

HEREDITARY AND ACQUIRED DISEASES INVOLVING THE ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) is derived from the neural crest. Neurons in enteric ganglia resemble various classes of CNS neurons in neurotransmitter specificity and growth factor requirements [76]. Enteric neuroglia resemble astroglia in expressing GFAP and glutamine synthetase [77]. Many diseases that affect the PNS also involve the ENS. Among these are infections such as Chagas' disease [78] and metabolic disorders such as diabetes mellitus and amyloidosis. Some disorders selectively involve the ENS. Among these is Hirschsprung's disease, which presents typically in infancy with massive segmental dilatation of the colon. The dilatation is the result of segmental absence of colonic enteric ganglia, among the causes of which are mutations of the RET receptor tyrosine kinase and of the endothelin B receptor [79].

REFERENCES

1. Pleasure, D., Bird, S., Scherer, S., Sladky, J. and Schotland, D. Neuromuscular disease. In R. N. Rosenberg (ed.), *Atlas of Clinical Neurology*. Oxford: Butterworth-Heinemann, pp. 11.1–11.116, 1998.
2. Corti, S., Locatelli, F., Donadoni, C. *et al.* Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sensory ganglia. *J. Neurosci. Res.* 70: 721–733, 2003.
3. Hirakawa, H., Okajima, S., Nagaoka, T., Takamatsu, T. and Oyamada, M. Loss and recovery of the blood-nerve barrier in the rat sciatic nerve after crush injury are associated with expression of intercellular junctional proteins. *Exp. Cell Res.* 284: 196–210, 2003.
4. Zhao, C., Takita, J., Tanaka, Y. *et al.* Charcot–Marie–Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβ. *Cell* 105: 587–597, 2001.
5. Brownlees, J., Ackerley, S., Grierson, A. J. *et al.* Charcot–Marie–Tooth disease neurofilament mutations disrupt neurofilament assembly and axonal transport. *Hum. Mol. Genet.* 11: 2837–2844, 2002.
6. Jordanova, A., De Jonghe, P., Boerkoel, C. F. *et al.* Mutations in the neurofilament light chain gene (*NEFL*) cause early onset severe Charcot–Marie–Tooth disease. *Brain* 126: 590–597, 2003.
7. Rajkumar, S. V., Gertz, M. A. and Kyle, R. A. Prognosis of patients with primary systemic amyloidosis who present with dominant neuropathy. *Am. J. Med.* 104: 232–237, 1998.
8. Low, P. A., Vernino, S. and Suarez, G. Autonomic dysfunction in peripheral nerve disease. *Muscle Nerve* 27: 646–661, 2003.
9. Ramos-Casals, M., Anaya, J. M., Garcia-Carrasco, M. *et al.* Cutaneous vasculitis in primary Sjogren syndrome: classification and clinical significance of 52 patients. *Medicine (Baltimore)* 83: 96–106, 2004.
10. Stoll, G., Griffin, J. W., Li, C. Y. and Trapp, B. D. Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. *J. Neurocytol.* 18: 671–683, 1989.
11. Avellino, A. M., Hart, D., Dailey, A. T., MacKinnon, M., Ellegala, D. and Kliot, M. Differential macrophage responses in the peripheral and central nervous system during wallerian degeneration of axons. *Exp. Neurol.* 136: 183–198, 1995.
12. Lindholm, D., Heumann, R., Meyer, M. and Thoenen, H. Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. *Nature* 330: 658–659, 1987.
13. Shamash, S., Reichert, F. and Rotshenker, S. The cytokine network of Wallerian degeneration: tumor necrosis factor-α, interleukin-1α, and interleukin-1β. *J. Neurosci.* 22: 3052–3060, 2002.
14. Tetzlaff, W. Tight junction contact events and temporary gap junctions in the sciatic nerve fibres of the chicken during Wallerian degeneration and subsequent regeneration. *J. Neurocytol.* 11: 839–858, 1982.
15. Bunge, M. B., Wood, P. M., Tynan, L. B., Bates, M. L. and Sanes, J. R. Perineurium originates from fibroblasts: demonstration in vitro with a retroviral marker. *Science* 243: 229–231, 1989.
16. Rambukkana, A., Yamada, H., Zanazzi, G. *et al.* Role of alpha-dystroglycan as a Schwann cell receptor for *Mycobacterium lepra*e. *Science* 282: 2076–2070, 1998.
17. Britton, W. J. and Lockwood, D. N. J. Leprosy. *Lancet* 363: 1209–1219, 2004.
18. Cacoub, P., Renou, C., Rosenthal, E., Cohen, P. *et al.* Extrahepatic manifestations associated with hepatitis C virus infection. *Medicine* 79: 47–56, 2000.
19. Brew, B. J. The peripheral nerve complications of human immunodeficiency virus (HIV) infection. *Muscle Nerve* 28: 542–552, 2003.
20. Pleasure, D. and Messing, A. Diphtheritic polyneuropathy. In P. J. Dyck and P. K. Thomas (ed.), *Peripheral Neuropathy*, 4th edn. Philadelphia: WB Saunders, in press.
21. Coffield, J. A., Bakry, N., Zhang, R. D., Carlson, J., Gomella, L. G. and Simpson, L. L. In vitro characterization of botulinum toxin types A, C and D action on human tissues: combined electrophysiologic, pharmacologic and molecular biologic approaches. *J. Pharmacol. Exp. Ther.* 280: 1489–1498, 1997.
22. Donofrio, P. D. Immunotherapy of idiopathic inflammatory neuropathies. *Muscle Nerve* 28: 273–292, 2003.
23. Magira, E. E., Papaioakim, M., Nachamkin, I. *et al.* Differential distribution of HLA-DQβ/DRβ epitopes in the two forms of Guillain–Barré syndrome, acute motor axonal neuropathy and acute inflammatory demyelinating

polyneuropathy (AIDP): identification of DQβ epitopes associated with susceptibility to and protection from AIDP. *J. Immunol.* 170: 3074–3080, 2003.
24. Willison, H. J. and Yuki, N. Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125: 2591–2625, 2002.
25. Jacobs, B. C., O'Hanlon, G. M., Bullens, R. W. M., Veitch, J., Plomp, J. J. and Willison, H. J. Immunoglobulins inhibit pathophysiological effects of anti-CQ1b-positive sera at motor nerve terminals through inhibition of antibody binding. *Brain* 126: 2220–2234, 2003.
26. Sandroni, P., Vernino, S., Klein, C. M. *et al.* Idiopathic autonomic neuropathy: comparison of cases seropositive and seronegative for ganglionic acetylcholine receptor antibody. *Arch. Neurol.* 61: 44–48, 2004.
27. Hughes, R. A. C., Hadden, R. D. M., Gregson, N. A. and Smith, K. J. Pathogenesis of Guillain–Barré syndrome. *J. Neuroimmunol.* 100: 74–97, 1999.
28. Wanschitz, J., Maier, H., Lassmann, H., Budka, H. and Berger, T. Distinct time pattern of complement activation and cytotoxic T cell response in Guillain–Barré syndrome. *Brain* 126: 2034–2042, 2003.
29. Saida, T., Saida, K., Silberberg, D. H. and Brown, M. J. Experimental allergic neuritis induced by galactocerebroside. *Ann. Neurol.* 9(Suppl.): 87–101, 1981.
30. Rostami, A., Gregorian, S. K., Brown, M. J. and Pleasure, D. E. Induction of severe experimental autoimmune neuritis with a synthetic peptide corresponding to the 53–78 amino acid sequence of the myelin P2 protein. *J. Neuroimmunol.* 30: 145–151, 1990.
31. Gabriel, C. M., Hughes, R. A., Moore, S. E., Smith, J. J. and Walsh, F. S. Induction of experimental autoimmune neuritis with peripheral myelin protein-22. *Brain* 121: 1895–1902, 1998.
32. Zhu, J., Pelidou, S. H., Deretzi, G. *et al.* P0 glycoprotein peptides 56–71 and 180–199 dose-dependently induce aucte and chronic experimental autoimmune neuritis in Lewis rats associated with epitope spreading. *J. Neuroimmunol.* 114: 99–106, 2001.
33. Laouini, D., Kennou, M. F., Khoufi, S. and Dellagi, K. Antibodies to human myelin proteins and gangliosides in patients with acute neuroparalytic accidents induced by brain-derived rabies vaccine. *J. Neuroimmunol.* 91: 63–72, 1998.
34. Vernino, S., Low, P. A. and Lennon, V. A. Experimental autoimmune autonomic neuropathy. *J. Neurophysiol.* 90: 2053–2059, 2003.
35. Hattori, N., Ichimura, M., Nagamatsu, M. *et al.* Clinicopathological features of Churg–Strauss syndrome-associated neuropathy. *Brain* 122: 427–439, 1999,
36. Aslam, A., Newman, T. L. and Misbah, S. A. Audit of the clinical usefulness of a rapid qualitative ELISA screen for antimyeloperoxidase and antiproteinase 3 antibodies in the assessment of patients with suspected vasculitis. *J. Clin. Pathol.* 56: 775–777, 2003.
37. Sillevis Smitt, P., Grefkens, J., de Leeuw, B. *et al.* Survival and outcome in 73 anti-Hu positive patients with paraneoplastic encephalomyelitis/sensory neuronopathy. *J. Neurol.* 249: 745–753, 2002.
38. Camdessanche, J-P., Antoine, J-C., Honnorat, J. *et al.* Paraneoplastic peripheral neuropathy associated with anti-Hu antibodies. A clinical and electrophysiological study of 20 patients. *Brain* 125: 166–175, 2002.
39. Pinto, A., Iwasa, K., Newland, C., Newsom-Davis, J. and Lang, B. The action of Lambert–Eaton myasthenic syndrome immunoglobulin G on cloned human voltage-gated calcium channels. *Muscle Nerve* 25: 715–724, 2002.
40. Vernino, S. and Lennon, V. A. Ion channel and striational antibodies define a continuum of autoimmune neuromuscular hyperexcitability. *Muscle Nerve* 28: 702–707, 2002.
41. Hart, I. K., Maddison, P., Newsom-Davis, J., Vincent, A. and Mills, K. R. Phenotypic variants of autoimmune peripheral nerve hyperexcitability. *Brain* 125: 1887–1895, 2002.
42. Nobile-Orazio, E., Meucci, N., Baldini, L., Troia, A. D. and Scarlato, G. Long-term prognosis of neuropathy associated with anti-MAG IgM M-proteins and its relationship to immune therapies. *Brain* 123: 710–717, 2000.
43. Dispenzieri, A., Kyle, R. A., Lacy, M. Q. *et al.* POEMS syndrome: definitions and long-term outcome. *Blood* 101: 2496–2506, 2003.
44. Sahenk, Z., Brady, S. T. and Mendell, J. R. Studies on the pathogenesis of vincristine-induced neuropathy. *Muscle Nerve* 10: 80–84, 1987.
45. Ravelli, R. B., Gigant, B., Curmi, P. A. *et al.* Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. *Nature* 428: 198–202, 2004.
46. Griffin, J. W. and Price, D. L. Demyelination in experimental beta, beta'-iminodipropionitrile and hexacarbon neuropathies. Evidence for an axonal influence. *Lab. Invest.* 45: 130–141, 1981.
47. Jeppesen, U., Gaist, D., Smith, T. and Sindrup, S. H. Statins and peripheral neuropathy. *Eur. J. Pharmacol.* 54: 835–838, 1999.
48. Gao, W. Q., Dybdal, N., Shinsky, N. *et al.* Neurotrophin-3 reverses experimental cisplatin-induced peripheral sensory neuropathy. *Ann. Neurol.* 38: 30–37, 1995.
49. Helgren, M. E., Cliffer, K. D., Torrento, K. *et al.* Neurotrophin-3 administration attenuates deficits of pyridoxine-induced large-fiber sensory neuropathy. *J. Neurosci.* 17: 372–382, 1997.
50. Koike, H., Iijima, M., Sugiura, M. *et al.* Alcoholic neuropathy is clinicopathologically distinct from thiamine-deficiency neuropathy. *Ann. Neurol.* 54: 19–29, 2003.
51. Duyff, R. F., Van den Bosch, J., Laman, D. M., van Loon, B. J. and Linssen, W. H. Neuromuscular findings in thyroid dysfunction: a prospective clinical and electrodiagnostic study. *J. Neurol. Neurosurg. Psychiat.* 68: 750–755, 2000.
52. Sheetz, M. J. and King, G. L. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 288: 2579–2588, 2002.
53. Calcutt, N. A., Allendoerfer, K. L., Mizisin, A. P. *et al.* Therapeutic efficacy of sonic hedgehog protein in experimental diabetic neuropathy. *J. Clin. Invest.* 111: 507–514, 2003.
54. Said, G., Lacroix, C., Lozeron, P., Ropert, A., Plante, V. and Adams, D. Inflammatory vasculopathy in multifocal diabetic neuropathy. *Brain* 126: 376–385, 2003.
55. Foulon, V., Asselberghs, S., Geens, W., Mannaerts, G. P., Casteels, M. and Van Veldhoven, P. P. Further studies on the substrate spectrum of phytanoyl-CoA hydroxylase: implications for Refsum disease? *J. Lipid Res.* 44: 2349–2355, 2003.
56. Kemp, S., Pujol, A., Waterham, H. R. *et al. ABCD1* mutations and the X-linked adrenoleukodystrophy muation database: role in diagnosis and clinical correlations. *Hum. Mutat.* 18: 499–515, 2001.

57. Gieselmann, V., Matzner, U., Hess, B. *et al.* Metachromatic leukodystrophy: molecular genetics and an animal model. *J. Inherit. Metab. Dis.* 21: 564–574, 1998.
58. Schiffmann, R., Floeter, M. K., Dambrosia, J. M. *et al.* Enzyme replacement therapy improves peripheral nerve and sweat function in Fabry disease. *Muscle Nerve* 28: 703–710, 2003.
59. Floderus, Y., Shoolingin-Jordan, P. M. and Harper, P. Acute intermittent porphyria in Sweden. Molecular, functional and clinical consequences of some new mutations found in the porphobilinogen deaminase gene. *Clin. Genet.* 62: 288–297, 2002.
60. Hattori, N., Yamamoto, M., Yoshihara, T. *et al.* Study Group for Hereditary Neuropathy in Japan. Demyelinating and axonal features of Charcot–Marie–Tooth disease with mutations of myelin-related proteins (PMP22, MPZ and Cx32): a clinicopathological study of 205 Japanese patients. *Brain* 126: 134–151, 2003.
61. Boerkoel, C. F., Takashima, H., Garcia, C. A. *et al.* Charcot–Marie–Tooth disease and related neuropathies. Mutation distribution and genotype-phenotype correlation. *Ann. Neurol.* 51: 190–201, 2002.
62. Passage, E., Norreel, J. C., Noack-Fraissignes, P. *et al.* Ascorbic acid treatment corrects the phenotype of a mouse model of Charcot–Marie–Tooth disease. *Nat. Med.* 10: 396–405, 2004.
63. Chance, P. F., Abbas, N., Lensch, M. W. *et al.* Two autosomal dominant neuropathies result from reciprocal DNA duplication/deletion of a region on chromosome 17. *Hum. Mol. Genet.* 3: 223–228, 1994.
64. Suter, U., Moskow, J. J., Welcher, A. A. *et al.* A leucine-to-proline mutation in the putative first transmembrane domain of the 22-kDa peripheral myelin protein in the trembler-J mouse. *Proc. Natl Acad. Sci. U.S.A.* 89: 4382–4386, 1992.
65. Bergoffen, J., Scherer, S. S., Wang, S. *et al.* Connexin mutations in X-linked Charcot–Marie–Tooth disease. *Science* 262: 2039–2042, 1993.
66. Birouk, N., Azzedine, H., Dubourg, O. *et al.* Phenotypical features of a Moroccan family with autosomal recessive Charcot–Marie–Tooth disease associated with the S194X mutation in the GDAP1 gene. *Arch. Neurol.* 60: 598–604, 2003.
67. Pellizzoni, L., Kataoka, N., Charroux, B. and Dreyfuss, G. A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell* 95: 615–624, 1998.
68. Tay, S. K., Akman, H. O., Chung, W. K. *et al.* Fatal infantile neuromuscular presentation of glycogen storage disease type IV. *Neuromusc. Dis.* 14: 253–260, 2004.
69. La Spada, A. R., Roling, D. B., Harding, A. E. *et al.* Meiotic stability and genotype–phenotype correlation of the trinucleotide repeat in X-linked spinal and bulbar muscular atrophy. *Nat. Genet.* 2: 301–304, 1992.
70. Stieber, A., Gonatas, J. O., Moore, J. S. *et al.* Disruption of the structure of the Golgi apparatus and the function of the secretory pathway by mutants G93A and G85R of Cu, Zn superoxide dismutase (SOD1) of familial amyotrophic lateral sclerosis. *J. Neurol. Sci.* 219: 45–53, 2004.
71. Puccio, H. and Koenig, M. Friedreich ataxia: a paradigm for mitochondrial diseases. *Curr. Opin. Genet. Dev.* 12: 272–277, 2002.
72. Kim, H. A., Rosenbaum, T., Marchionni, M. A., Ratner, N. and DeClue, J. E. Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. *Oncogene* 11: 325–335, 1995.
73. Eisenbarth, I., Beyer, K., Krone, W. and Assum, G. Toward a survey of somatic mutation of the *NF1* gene in benign neurofibromas of patients with neurofibromatosis type 1. *Am. J. Hum. Genet.* 66: 393–401, 2000.
74. Yang, F. C., Ingram, D. A., Chen, S. *et al.* Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for Nf1+/- mast cells. *J. Clin. Invest.* 112: 1851–1861, 2003.
75. Kissil, J. L., Wilker, E. W., Johnson, K. C., Eckman, M. S., Yaffe, M. B. and Jacks, T. Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1. *Mol. Cell* 12: 841–849, 2003.
76. Bondurand, N., Natarajan, D., Thapar, N., Atkins, C. and Pachnis, V. Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from fetal and postnatal gut cultures. *Development* 130: 6387–6400, 2003.
77. Broussard, D. L., Bannerman, P. G., Tang, C. M., Hardy, M. and Pleasure, D. Electrophysiologic and molecular properties of cultured enteric glia. *J. Neurosci. Res.* 34: 24–31, 1993.
78. Perez Leiros, C., Sterin-Borda, L., Borda, E. S., Goin, J. C. and Marlene Hosey, M. Desensitization and sequestration of human m2 muscarinic acetylcholine receptors by autoantibodies from patients with Chagas' disease. *J. Biol. Chem.* 272: 12989–12998, 1997.
79. Goyal, R. K. and Hirano, I. The enteric nervous system. *N. Engl. J. Med.* 334: 1106–1115, 1996.

CHAPTER

37

The Epilepsies: Phenotype and Mechanisms

John W. Gibbs

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EPILEPSY IS A COMMON NEUROLOGICAL DISORDER

The epilepsies constitute a common, serious neurological disorder in humans, affecting approximately 60 million people worldwide. Well in excess of 40 distinct epileptic syndromes have been identified to date. Current treatment is only symptomatic except in uncommon instances when surgical treatment is possible. While available antiseizure medications target ion channels such as the γ-amino-butyric acid $(GABA)_A$ receptor and voltage activated sodium (Na^+) channels, current research seeks to elucidate the cellular and molecular mechanisms by which a normal brain becomes epileptic. Hopefully, this research will lead to the identification of new targets for which small molecules can be identified and used for prevention or cure of epilepsy.

The term 'seizure' denotes a fleeting change of behavior caused by the disordered, synchronous and rhythmic firing of populations of neurons. The term 'epilepsy' denotes a disorder of brain function expressed as the periodic and unpredictable occurrence of seizures. Pharmacological agents in current clinical use inhibit epileptic seizures and thus are referred to as *antiseizure* drugs. It is currently unknown, however, whether any of these agents has any prophylactic value in preventing the development of epilepsy (*epileptogenesis*). Other commonly used terms include electroencephalographic (EEG) descriptors such as *ictal* (seizure-like) and *interictal*

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TABLE 37-1 Classification of partial epileptic seizures

Partial Seizure	Features	Antiseizure Drugs
Simple partial	Phenotype determined by cortical region activated (e.g. if motor cortex representing left thumb is activated, then left thumb jerking results). Consciousness is preserved	Carbamazepine, phenytoin, valproate, gabapentin, lamotrigine, levetiracetam, tiagabine, topiramate, zonisamide
Complex partial	Impaired consciousness lasting seconds to minutes, often associated with automatisms such as lip smacking	Carbamazepine, phenytoin, valproate, gabapentin, lamotrigine, levetiracetam, tiagabine, topiramate, zonisamide
Partial with secondary generalization	Simple or partial complex seizure evolves into a tonic–clonic seizure with loss of consciousness. There are sustained muscular contractions (tonic) followed by periods of relaxation (clonic) lasting 1–2 min	Carbamazepine, phenytoin, phenobarbital, valproate, gabapentin, lamotrigine, levetiracetam, tiagabine, topiramate, zonisamide

(between seizures), as used by clinicians to describe individual EEG patterns.

Seizures are thought to arise from the cerebral cortex, and not from other CNS structures such as the thalamus, brainstem or cerebellum. Epileptic seizures have been classified as *partial* seizures, which are those beginning focally in a cortical site, and *generalized* seizures, those that involve discharges from both hemispheres widely from the outset [1]. The behavioral manifestations of a seizure are determined by the functions normally served by the cortical site at which the seizure arises. For example, a seizure involving motor cortex is associated with the clonic jerking of the body part controlled by this region of cortex. A *simple* partial seizure is associated with preservation of consciousness whereas a *complex* partial seizure is associated with impairment of consciousness. The majority of complex partial seizures originate from the temporal lobe (Table 37-1). Examples of *generalized* seizures include absence, myoclonic, and tonic–clonic seizures (Table 37-2). The type of epileptic seizure, diagnosed both by clinical and EEG methodology, is one determinant of the drug selected for therapy as different antiseizure medications often demonstrate varying abilities to control different seizure phenotypes.

Apart from this epileptic seizure classification, an additional classification specifies *epileptic syndromes,* which refer to a cluster of symptoms frequently occurring together and include seizure type, etiology, age of onset and other factors [2]. The epileptic syndromes have been categorized into partial *versus* generalized epilepsies. The partial epilepsies account for roughly 60% of all epilepsies. The etiology commonly consists of a lesion in some part of the cortex, such as a tumor, developmental malformation, damage due to trauma or stroke, etc. Such lesions often are evident on brain imaging studies such as magnetic resonance imaging. Alternatively, the etiology may be genetic (Table 37-3). The generalized epilepsies are characterized by one or more generalized seizure types (listed in Table 37-2). These account for approximately 40% of all epilepsies and are thought to have a genetic etiology. The most common generalized epilepsy is juvenile myoclonic epilepsy (JME), accounting for approximately 10% of all epileptic syndromes. The age of onset is in the early teens and the condition is characterized typically by myoclonic, tonic–clonic and often absence seizures. Like most generalized epilepsies, JME is usually due to the inheritance of multiple susceptibility genes; often, there is a familial clustering of cases but the pattern of inheritance is not mendelian.

Disrupting the delicate balance of inhibitory and excitatory synaptic transmission can trigger the disordered, synchronous firing of neurons that underlies a seizure. More than a century ago, John Hughlings Jackson, an extraordinarily insightful clinician, proposed that seizures

TABLE 37-2 Classification of generalized epileptic seizures

Generalized Seizure	Features	Antiseizure Drugs
Absence	Abrupt loss of consciousness associated with staring and cessation of activities normally lasting less than 30 seconds	Ethosuximide, valproate, lamotrigine
Myoclonic	Brief muscular contraction, either focal or generalized in nature	Valproate
Tonic–clonic	Sustained muscular contractions (tonic) followed by periods of relaxation (clonic) lasting 1–2 min	Carbamazepine, phenobarbital, phenytoin, primidone, valproate, topiramate

TABLE 37-3 Idiopathic human epilepsies: genes and syndromes

Syndrome	Gene
Autosomal dominant nocturnal frontal lobe epilepsy, 1	Nicotinic receptor subunit: α_4
Autosomal dominant nocturnal frontal lobe epilepsy, 3	Nicotinic receptor subunit: β_2
Benign neonatal epilepsy, type 1	K^+ channel subunit: KCNQ2
Benign neonatal epilepsy, type 2	K^+ channel subunit: KCNQ3
Generalized epilepsy with febrile seizures plus ($GEFS^+$), severe myoclonic epilepsy of infancy (SMEI)	Na^+ channel subunit: $Na_V1.1$
Febrile and afebrile seizures, benign familial neonatal-infantile seizures	Na^+ channel subunit: $Na_V1.2$
$GEFS^+$	Na^+ channel subunit: β_1
$GEFS^+$, childhood absence epilepsy	$GABA_A$ receptor: γ_2
Juvenile myoclonic epilepsy	$GABA_A$ receptor: α_1
Partial epilepsy with auditory features	Leucine-rich, glioma-inactivated 1

were caused by 'occasional, sudden, excessive, rapid and local discharges of gray matter', and a generalized convulsion resulted when normal brain tissue was invaded by the seizure activity initiated in the abnormal focus. In fact, this prediction provided a valuable framework for current thinking about mechanisms of partial epilepsy. The advent of the EEG in the 1930s permitted the recording of electrical activity from the scalp of human beings and demonstrated that the epilepsies are disorders of neuronal excitability.

The pivotal role of synapses in mediating communication among neurons in the mammalian brain suggested that defective synaptic function might lead to an epileptic seizure, with a delicate synaptic balance necessary to maintain the normal state of neurons. In other words, a reduction of inhibitory synaptic activity or enhancement of excitatory synaptic activity might be expected to trigger a seizure. Indeed, pharmacological studies of seizures have supported this hypothesis. The neurotransmitters mediating the bulk of synaptic transmission in the mammalian brain are amino acids, GABA and glutamate being the principal inhibitory and excitatory neurotransmitters, respectively (see Chs 15 and 16). Pharmacological studies in normal animals disclosed that injection of antagonists of the $GABA_A$ receptor or of agonists of different glutamate-receptor subtypes (NMDA, AMPA or kainic acid) triggered seizures in otherwise normal animals *in vivo*. In contrast, pharmacological agents that enhance GABA-mediated synaptic inhibition, such as benzodiazepines, reduce seizure activity. Likewise, glutamate receptor antagonists inhibit seizures in diverse models, including seizures evoked by electroshock or chemical convulsants.

These findings were confirmed and extended by *in vitro* electrophysiological studies of slices from normal animals that revealed that subtle (e.g. 20%) reductions of inhibitory synaptic function could lead to epileptiform activity. Importantly, activation of excitatory synapses is often pivotal in the expression of a seizure in distinct models *in vitro*. In addition to these important pharmacological observations, electrophysiological analyses of individual neurons during a partial seizure revealed that neurons undergo a massive depolarization and fire action potentials at high frequencies (Fig. 37-1) [3]. This pattern of

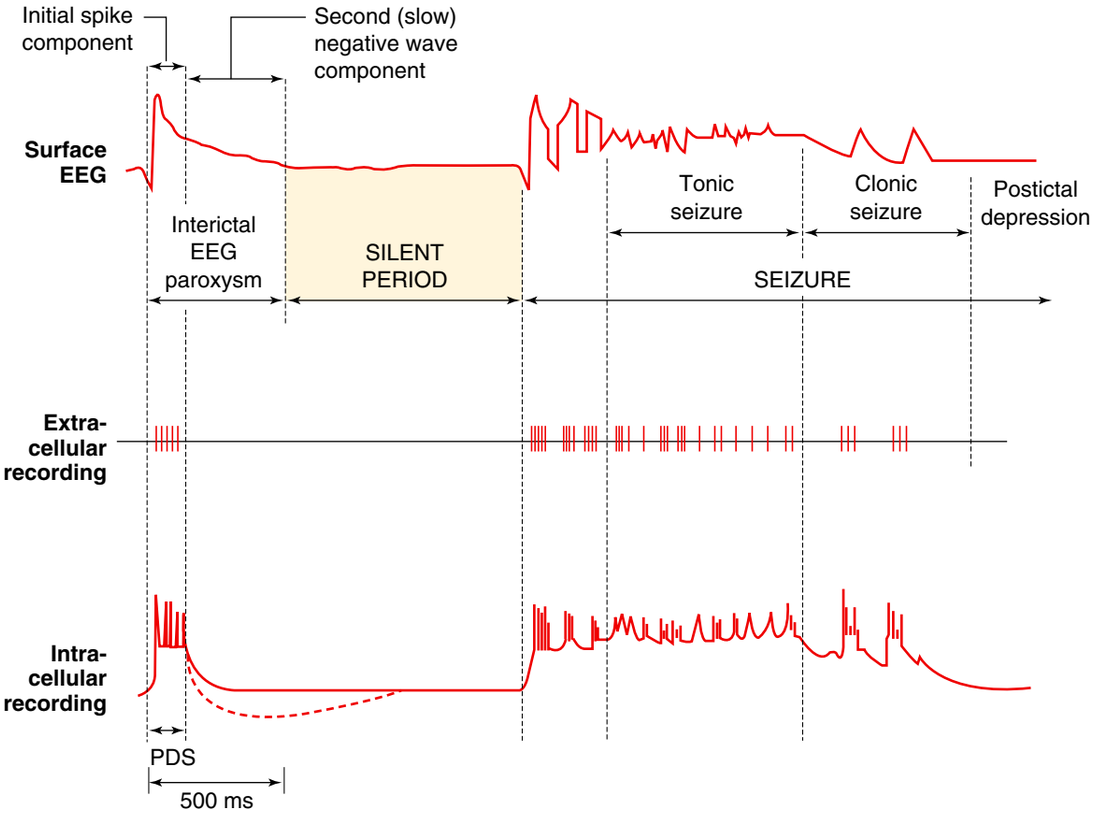

FIGURE 37-1 Relationship among cortical EEG, extracellular and intracellular recordings in a seizure focus exposed to a convulsant agent in cortex. Note the high-frequency firing of the neuron in both intracellular and extracellular recordings during the paroxysmal depolarization shift (*PDS*) [3]. Cellular electrophysiological studies of epilepsy over roughly two decades beginning in the mid-1960s were focused on elucidating the mechanisms underlying the DS, the intracellular correlate of the 'interictal spike'. The interictal spike is a sharp waveform recorded in the EEG of patients with epilepsy; it is asymptomatic in that it is accompanied by no detectable change in a patient's behavior. However, the location of the interictal spike helps to localize the brain region from which seizures originate in a given patient. The DS consists of a large depolarization of the neuronal membrane associated with a burst of action potentials. In most cortical neurons, a large excitatory synaptic current that can be enhanced by activation of voltage-regulated intrinsic membrane currents generates the DS.

neuronal firing is characteristic of a seizure and is uncommon during physiological neuronal activity, a finding that provides a plausible explanation for the ability of some antiseizure drugs to inhibit seizures with minimal effect on normal functions of mammalian brain. While synapses are important, the initiation and expression of a seizure can involve a diversity of additional mechanisms including nonsynaptic mechanisms such as the volume of the extracellular space and intrinsic properties of a neuron such as voltage-regulated ion channels, including those gating K^+, Na^+ and Ca^{2+} ions [4]. Identification of these diverse synaptic and nonsynaptic factors controlling seizures *in vitro* provides potentially valuable pharmacological targets for regulating seizure susceptibility *in vivo* in humans.

Cellular mechanisms underlying hyperexcitability have been analyzed by electrophysiological studies of hippocampal slices isolated from animals with epilepsy. The pharmacological evidence implicating GABA and glutamatergic synapses in the expression of seizures both *in vivo* and *in vitro* led to hypotheses as to the mechanism of the enduring hyperexcitability of the epileptic brain. That is, scientists hypothesized that one mechanism accounting for the hyperexcitability of the epileptic brain might be an impaired function of inhibitory synapses and/or enhanced function of excitatory synapses. The availability of animal models of epilepsy provided a powerful tool to test these hypotheses, particularly when *ex vivo* study of hippocampal slices isolated from epileptic animals permitted analysis of synaptic function. Also, the anatomical focus on the properties of an identified population of neurons thought to be important for epilepsy was critical, with the dentate granule cells of hippocampus providing one such anatomic locale. Consideration of the animal models and the rationale for study of the dentate granule cells will be followed by review of the analyses of the synaptic properties of these neurons in epilepsy models.

A diversity of animal models of epilepsy have been identified [5]. Two of the most commonly studied models are 'kindling' and 'status epilepticus' models. Kindling is a model of temporal lobe epilepsy (TLE) that is induced by the periodic administration of brief, low-intensity electrical stimulation of the amygdala or other limbic structures. Described by Graham Goddard and his colleagues [6], initial stimulations evoke a brief electrical seizure recorded on EEG without behavioral change, but repeated (e.g. 10–20) stimulations result in progressive intensification of seizures, culminating in tonic–clonic seizures. Once established, the enhanced sensitivity to electrical stimulation persists for the life of the animal. Despite the lifelong propensity to express intense seizures in response to low intensity stimulation, spontaneous seizures or a truly epileptic condition do not occur until approximately 100 stimulation-evoked seizures have occurred. The ease of control of kindling induction (i.e. stimulations administered at the investigator's convenience), its graded onset and the ease of quantifying epileptogenesis (number of stimulations required to evoke tonic–clonic seizures) simplify study of early stages of epileptogenesis with this model.

A variety of models exist in which epilepsy arises weeks after an episode of status epilepticus, a state of continuous seizures lasting hours. Whether triggered by chemoconvulsants (e.g. pilocarpine or kainic acid) or continuous electrical stimulation, the fleeting episode of status epilepticus is followed in the coming weeks by the onset of spontaneous seizures [7, 8], an intriguing parallel to the scenario of complicated febrile seizures in young children followed by the emergence of spontaneous seizures years later. In contrast to the limited or absent neuronal loss characteristic of the kindling model, overt destruction of hippocampal neurons occurs in both the pilocarpine and kainate models, mimicking characteristics of hippocampal sclerosis observed in many humans with medically refractory TLE. Indeed, the discovery that complicated febrile seizures are followed by and thus almost certainly one cause of hippocampal sclerosis in young children establishes yet another commonality between these models and the human condition [9].

Normally the dentate granule cells of hippocampus limit excessive activation of their targets, the CA3 pyramidal cells. Insight into the structural and functional properties of diverse populations of principal neurons of the hippocampus and its connections have provided a context for considering how the function of these neurons underlie normal behaviors such as learning and memory as well as the hyperexcitability of TLE. The extensive recurrent excitatory synaptic connections among CA3 pyramidal neurons together with the propensity of individual CA3 pyramidal neurons to fire action potentials in a bursting pattern explained the propensity of these neurons to exhibit seizures [10]. Given this propensity, it became clear that the principal afferents of the CA3 pyramidal cells, the dentate granule cells, must function as a gatekeeper to limit the activation of the CA3 pyramidal cells and their subsequent explosion into seizure activity [11]. The uniqueness of the innervation of their targets by the granule cells underscores their function as gatekeeper. That is, excitatory principal neurons (e.g. CA3 pyramidal cell innervation of its targets in CA1) of mammalian forebrain typically innervate other excitatory neurons directly in great quantitative preference to inhibitory interneurons [12]. In stark contrast, the dentate granule cells innervate approximately 10 inhibitory interneurons for every one CA3 pyramidal neuron; these inhibitory interneurons mediate feedforward inhibition of the CA3 pyramidal neurons, thus underlying the net inhibitory and tight control of CA3 pyramidal cell firing by the dentate granule cells [13]. Using deoxyglucose autoradiography studies, the dentate granule cells did indeed appear to function as a barrier for invasion of hippocampus by seizure activity *in vivo* [14]. These findings led to the hypothesis that

compromise of the normal barrier function of the granule cells permits activation of the CA3 pyramidal cells and recruitment of the hippocampal circuit into seizure activity, thereby contributing to the hyperexcitability of TLE.

Analyses of afferents of dentate granule cells from epileptic animals reveal abnormal inhibitory and excitatory synaptic input. Enhanced function of excitatory synapses selectively using NMDA receptors has been identified in afferents of the dentate granule cells in hippocampal slices isolated from kindled animals [15]. In the pilocarpine model, net reductions of inhibitory synaptic transmission mediated by $GABA_A$ receptors have been demonstrated in dentate granule cells; this may be due in part to altered responses to GABA by $GABA_A$ receptors, which in turn may be due to altered subunit composition of the $GABA_A$ receptor [16]. Not surprisingly, the reality is substantially more complex than described here. Paradoxically, enhanced function of $GABA_A$ synaptic input has been identified in the dentate granule cells in slices isolated from kindled animals, most probably underlying a compensatory response aimed at preventing emergence of spontaneous seizures in these animals [17].

Axonal and dendritic sprouting lead to abnormal recurrent excitatory synaptic circuits among the dentate granule cells in epileptic brain. Repeated seizures have been demonstrated to result in a structural reorganization of hippocampal circuitry, a reorganization that increases substantially in the presence of cell death as occurs often in temporal lobe epilepsy. The best described structural reorganization is that in which axons of the excitatory granule cells sprout and reinnervate themselves and/or their neighbors through recurrent collaterals forming a feed-forward excitatory loop termed 'mossy fiber sprouting' [18]. More recently, sprouting of basilar dendrites of the granule cells has also been identified and these provide additional targets for the sprouted axons [19]. The recurrent axonal collaterals are readily identified experimentally in various seizure models and the human condition using the Timm's stain. The fact that networks of recurrent excitatory synapses represent an efficient substrate for initiation and propagation of seizure activity underscores the appeal of the idea that this reorganized granule cell synaptic network may contribute to the hyperexcitability of TLE. Functional evidence for the presence of recurrent excitatory synapses has emerged from synaptic physiological studies of slices in the pilocarpine model. Nonetheless, the extent to which this reorganized network of dentate granule cells contributes to the hyperexcitability of the epileptic brain is uncertain at present.

While alterations in dentate granule synaptic physiology and anatomy provide a snapshot to begin to understand how a normal brain changes to an 'epileptic' brain, the dentate granule cells represent only one small piece of the complex puzzle of how a normal brain becomes epileptic. A myriad of changes have been reported to occur in neurons elsewhere in the hippocampus and other regions of the brain. Not all of these changes involve excitatory or inhibitory synapses. For example, the defective inactivation of voltage-gated sodium channels has been identified in CA1 pyramidal neurons in slices isolated from the kindling model [20]. Therefore, when one begins to assess the hyperexcitability of the epileptic brain, it is imperative to view these alterations on a global platform and not just localized to a single population of neurons, in order to appreciate the vast complexities that are involved.

Epileptogenesis is the process by which a normal brain becomes epileptic. The very complexity of understanding mechanisms underlying the hyperexcitability of the epileptic brain has contributed to enhanced emphasis in attempts to prevent development of epilepsy, i.e. epileptogenesis.

Interestingly, many forms of partial epilepsy are characterized by a seizure-free interval lasting months to years between the occurrence of the causative insult and the emergence of epilepsy; termed the 'latent period', this provides a valuable window of opportunity during which pharmacologic intervention might be implemented in high-risk individuals so that development of epilepsy could be prevented.

Identifying molecular mechanisms of epileptogenesis will provide new targets for developing small molecules to prevent epilepsy. Understanding the cellular mechanisms of epileptogenesis in molecular terms will hopefully lead to identification of molecular targets for which small molecules might be identified for the prevention of epilepsy. Studies of the kindling model established the critical role of pathological activity in the pathogenesis of partial epilepsy. This led to the question as to what molecular consequences of pathologic activity might mediate the transformation of a normal brain to an epileptic brain. In turn, this led to focus on neurotrophins such as nerve growth factor and brain derived neurotrophic factor (BDNF) that have been implicated in transducing fleeting experiences into lasting changes in phenotypes (see Growth Factors, Ch. 27). In fact, seizures produce striking increases in the expression of BDNF. Indeed enhanced activation of the tyrosine kinase receptor of BDNF, TrkB, occurs in multiple models of epileptogenesis [21]. BDNF somehow contributes to epileptogenesis in the kindling model because epileptogenesis is partially inhibited in mice in which one or both alleles of the BDNF gene have been eliminated. By contrast, epileptogenesis in the kindling model is prevented altogether in mice in which both alleles of the TrkB gene are eliminated [21], providing the first genetic or pharmacological perturbation that is required for epileptogenesis in this model. These findings focus the search for mechanisms of epileptogenesis on structural and functional consequences of TrkB activation. They also implicate TrkB and its downstream signaling pathways as highly attractive targets for

development of small molecule inhibitors for the prevention of epilepsy.

MECHANISMS OF ANTISEIZURE DRUGS

Insights into the mechanisms of action of medications effective against partial seizures [22] emerged from electrophysiological studies of reduced preparations, such as CNS neurons maintained in primary culture. The experimental control and accessibility available in these models combined with use of clinically relevant concentrations led to clarification of the mechanisms of various antiseizure medications. Although difficult to prove that a given antiseizure drug effect observed *in vitro* is the mechanism by which a drug acts *in vivo* to inhibit a seizure, there is a strong likelihood that the putative mechanisms identified in the laboratory underlie actions *in vivo* in humans. To date, most antiseizure drugs target voltage-gated sodium channels or synapses using $GABA_A$ receptors.

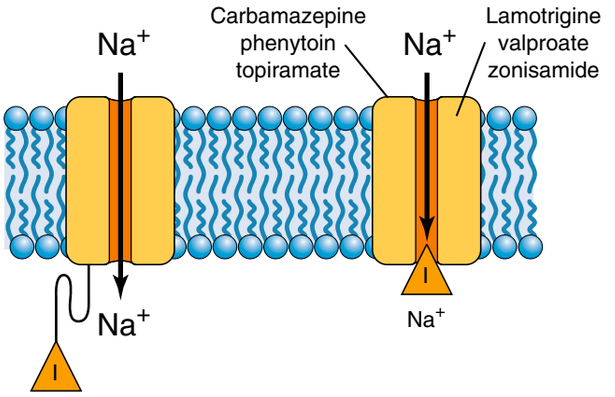

FIGURE 37-2 Antiseizure Na^+ channel inactivation. Some antiseizure drugs work mechanistically by prolonging the inactivation of the Na^+ channel, thereby reducing the ability of neurons to fire at high frequencies. Antiseizure drugs known to promote inactivation of this channel include carbamazepine, phenytoin, topiramate, lamotrigine, valproate and zonisamide. Note that the inactivated channel appears to remain open but is blocked by the inactivation gate (I) at the pore.

Many antiseizure drugs act on voltage-gated sodium channels to limit high-frequency but not low-frequency firing of neurons. As described earlier, electrophysiological analyses of individual neurons during a partial seizure demonstrate that neurons undergo depolarization and fire action potentials at high frequencies (Fig. 37-1). This pattern of neuronal firing is the hallmark of a seizure and is rare during physiological activity. Therefore, the selective inhibition of this high-frequency firing pattern would be expected to reduce seizures, hopefully with minimal unwanted effects. Carbamazepine, lamotrigine, phenytoin and valproic acid modulate high-frequency firing at concentrations known to be effective in the limitation of seizures in humans [22]. The mechanism by which the drugs limit high-frequency firing involves slowing the recovery of voltage-gated Na^+ channels from inactivation, also termed use-dependent blockade (Fig. 37-2).

Stated differently, depolarization-triggered opening of the Na^+ channels in the axonal membrane of a neuron is required for an action potential; after opening, the channels spontaneously close, a process termed *inactivation* (see Ch. 6). This inactivation is thought to mediate the refractory period, the brief period following an action potential during which it is not possible to evoke another action potential. Upon recovery from inactivation, the Na^+ channels are poised to participate in generation of another action potential. Because firing at a slow rate permits sufficient time for Na^+ channels to recover from inactivation, inactivation has little or no effect on low-frequency firing. However, reducing the rate of recovery of Na^+ channels from inactivation would limit the ability of a neuron to fire at high frequencies, an effect that most probably underlies the effects of carbamazepine, lamotrigine, phenytoin, topiramate, valproic acid and zonisamide against partial seizures.

Other antiseizure drugs enhance GABA-mediated synaptic inhibition. Insights into mechanisms of seizures suggest that enhancing GABA-mediated synaptic inhibition, particularly $GABA_A$ inhibition, would reduce neuronal excitability and therefore raise the seizure threshold. Several drugs are thought to inhibit seizures by regulating GABA-mediated synaptic inhibition through an action at distinct sites of the synapse [22]. The principal postsynaptic receptor of synaptically released GABA is the $GABA_A$ receptor. Activation of the $GABA_A$ receptor effects inhibition of the postsynaptic cell by increasing the flow of Cl^- ions into the cell, which tends to hyperpolarize the neuron (see Ch. 16). Clinically relevant concentrations of both benzodiazepines and barbiturates can enhance $GABA_A$-receptor-mediated inhibition through distinct actions on the $GABA_A$ receptor (Fig. 37-3) [22]. This mechanism probably underlies the effectiveness of these compounds against partial and tonic–clonic seizures in humans. At higher concentrations, such as might be used for status epilepticus, these drugs also can inhibit high-frequency firing of action potentials. γ-vinyl GABA (vigabatrin) is thought to exert its antiseizure action by irreversibly inhibiting an enzyme that degrades GABA, GABA transaminase; this probably leads to increased amounts of GABA available for synaptic release [22]. A third mechanism of enhancing GABA-mediated synaptic inhibition is thought to underlie the antiseizure mechanism of tiagabine; tiagabine inhibits the GABA transporter, GAT-1, and reduces neuronal and glial uptake of GABA [22] (Fig. 37-3).

Other antiseizure drugs regulate a subset of voltage-gated calcium currents. In contrast to partial seizures, which arise from localized regions of the cerebral cortex, the 'absence' or 'petit mal' form of generalized-onset seizures arise from the reciprocal firing of the thalamus and

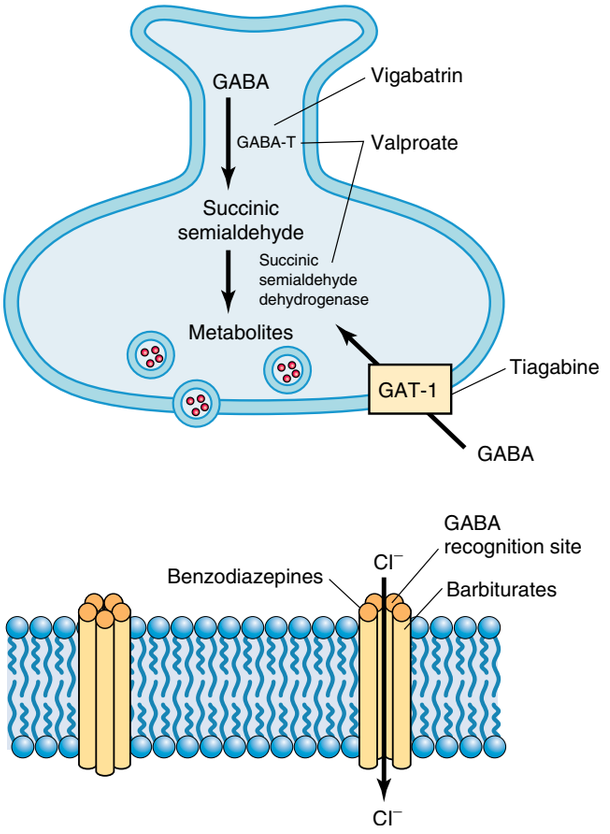

FIGURE 37-3 Increased GABAergic transmission mediated by antiseizure drugs. Some antiseizure drugs mediate the increased opening of $GABA_A$ receptors in turn increasing membrane hyperpolarization like benzodiazepines and barbiturates. Others inhibit GABA reuptake (tiagabine) or the degradation of synaptically released GABA (vigabatrin).

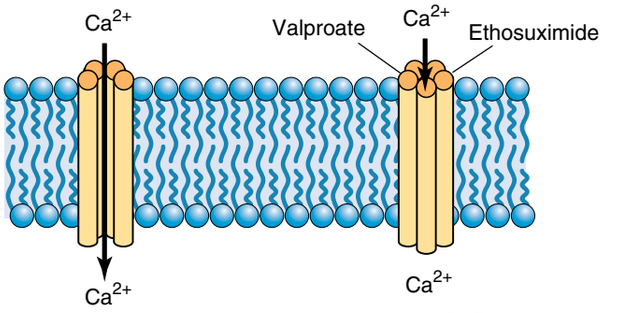

FIGURE 37-4 Antiseizure mediated reduction of I_T. Certain antiseizure drugs reduce the flow of calcium through T-type Ca^{2+} channels (ethosuximide, valproate), thereby reducing the pacemaker current that underlies spike-wave discharges of generalized absence epilepsy.

cerebral cortex [23]. Although a detailed consideration of the mechanisms is beyond the scope of this chapter, many of the structural and functional properties of thalamus and cortex that underlie the generalized spike-and-wave discharges of petit mal or absence seizures have been clarified in the past decade [23]. The EEG hallmark of an absence seizure is generalized spike-and-wave discharges at a frequency of 3 Hz. These bilaterally synchronous spike-and-wave discharges, recorded locally from electrodes in both the thalamus and the neocortex, represent oscillations between thalamus and cortex. Correlative EEG and intracellular recordings reveal that the EEG spikes are associated with the firing of action potentials and the following slow wave with prolonged inhibition. These reverberatory, low-frequency rhythms are made possible by a combination of factors, including reciprocal excitatory synaptic connections between the cortex and thalamus as well as intrinsic properties of neurons in the thalamus, most importantly the low-threshold calcium current or I_T [23]. The expression of I_T in thalamic neurons is critical to the generation of the 3 Hz spike and waves. In contrast to its small size in most cortical and hippocampal pyramidal neurons, the thalamic I_T is of large amplitude with bursts of action potentials superimposed on this current. Thus the I_T serves an amplifying role in thalamic oscillations, with each oscillation being the three-per-second spike and wave of the absence seizure. Importantly, the principal mechanism by which some antiabsence seizure drugs (ethosuximide, valproic acid) are thought to act is by inhibition of the I_T [24] (Fig. 37-4).

GENETICS OF EPILEPSY

Many forms of epilepsy have genetic determinants. Progress in molecular biology and molecular genetics has fueled discovery of genes causing epilepsy in humans and mice in the past 10–15 years [25]. Overall perhaps 50–60% of all human epilepsies have genetic determinants. A small fraction (less than 5%) of the epilepsies are inherited in a mendelian pattern in which the cause can be traced to a single mutant gene. The gene(s) causing a mendelian form are typically found to be responsible for a single or a few pedigrees but do not cause common forms of human epilepsy such as most cases of JME or childhood absence epilepsy (CAE). Disorders such as JME and CAE appear to be caused by simultaneous inheritance of two or more susceptibility genes interacting with environmental factors [26], a scenario similar to late-onset Alzheimer's disease in which apolipoprotein E isoforms powerfully influence risk but alone are not sufficient to produce Alzheimer's disease (see Ch. 47]. By analogy with the rare pedigrees of Alzheimer's disease that are inherited in a mendelian pattern (such as the mutation in amyloid precursor protein in a Swedish pedigree), a single JME pedigree was identified in which a mutation of the α_1 subunit of a $GABA_A$ receptor was causal [27]. In contrast to Alzheimer's disease, the gene(s) conferring susceptibility to the common forms of epilepsy like JME are unknown.

Tables 37-3 and 37-4 list the genes responsible for idiopathic and symptomatic epilepsies in humans, respectively. Table 37-5 lists genes identified as causing epilepsy

TABLE 37-4 Symptomatic human epilepsies: genes and syndromes

Syndrome	Inheritance Pattern	Gene
Miller–Dieker Syndrome	*De novo*	Platelet-activating factor acetylhydrolase
Unverricht–Lungborg disease	Autosomal recessive	Cystatin B
Myoclonic epilepsy with ragged red fibers (MERRF)	Mitochondrial	$tRNA^{lys}$
Batten disease	Autosomal recessive	*CLN3*
Angelman syndrome	Mostly *de novo*	*UBE3A*
Lafora' disease	Autosomal recessive	*EPM2A*, *EPM2B*
Subcortical band heterotopia	X-linked	Doublecortin
Periventricular nodular heteropia	X-linked	Filamin
Lissencephalic cortical neuronal migration defect	X-linked	Reelin
Rett's syndrome	X-linked	*MECP2*
Cerebral cavernous malformations	Autosomal dominant	*KRIT1*
Infantile neuronal ceroid lipofuscinosis (CLN1)	Autosomal recessive	Palmitooylprotein thioesterase
Late infantile neuronal ceroid lipofuscinosis (CLN2)	Autosomal recessive	Pepstatin-insensitive lysosomal peptidase
Juvenile Gaucher's disease	Autosomal recessive	Glucocerebrosidase
Sialidosis I	Autosomal recessive	α-neurominidase
Sialidosis II	Autosomal recessive	Stabilizing protein of the α-neurominidase-β-galactosidase complex
Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke like episodes (MELAS)	Mitochondrial	tRNA (leu)
Dentatorubalpallidoluysian atrophy (DRPLA)	Autosomal dominant	CAG trinucleotide repeat
Acanthocytosis	Autosomal recessive	Chorein

in mice. Several themes emerge from this immense information. As noted above in descriptions of epilepsy syndromes, the idiopathic epilepsies affect individuals who are otherwise normal and no structural cause has been identified. Perhaps the most remarkable feature of the genetic causes of the idiopathic epilepsies is that all but one of these genes encodes an ion channel gated by voltage or a neurotransmitter. This is of particular interest because several other episodic disorders involving other organs also are caused by mutations of genes encoding ion channels. For example, episodic disorders of the heart (cardiac arrhythmias), skeletal muscle (periodic paralyses), cerebellum (episodic ataxia), vasculature (familial hemiplegic migraine) and other organs all have been linked to mutant genes encoding a component of an ion channel [28]. In none of these episodic disorders is it understood what triggers an event or what terminates the event. The sole gene that does not encode an ion channel is partial epilepsy with auditory features in which the gene encoding 'leucine-rich glioma-inactivated' is responsible; loss of both of these alleles is associated with enhanced progression of glial tumors but the function of the protein encoded by this gene is obscure [29]. Whether this gene is somehow related to localization or degradation of an ion channel remains to be determined.

Except for JME, each of these syndromes is rare and, together, these forms probably account for considerably less than 1% of all human epilepsies. The *GABRA1* mutation identified in JME involved a single pedigree and was not evident in multiple sporadic cases of JME. In no instance is it clear how the genotype leads to the phenotype of epilepsy. Identification of the genes permits the engineering of mutant mice that hopefully will exhibit epilepsy. To date, however, no mouse exhibiting a 'knocked-in' human mutation or even a transgenic mouse overexpressing a human mutation has been demonstrated to exhibit a well documented form of epilepsy similar to the human form. Arguably the closest mimic at present is the *K_v1.1*-null mutant mouse, which exhibits complex partial and secondarily generalized tonic–clonic seizures, forms of seizures similar to some humans carrying mutations that are probably haploinsufficient [30]. Such mice will provide tools vital to determining how the genotype leads to the phenotype.

Apart from determining steps, if any, between expression of a mutant ion channel and emergence of epileptic seizures, in some instances the mutant channels suggest some intriguing molecular targets for development of antiseizure drugs acting by novel mechanisms. For example, the mutations of the K^+ channel (KCNQ2 and KCNQ3) underlie a slowly inactivating K^+ current and mediate spike frequency adaptation. These channels provide a novel molecular target for development of antiseizure drugs.

Table 37-4 lists a number of symptomatic epilepsies of humans for which mutant genes have been identified. As suggested by the terminology, these epilepsies are symptomatic of some overt underlying disease, some of which begin at various times in the first decade of life or later and are progressive whereas others exhibit catastrophic neurological abnormalities at birth and do not progress further. These diseases include epileptic seizures as a prominent feature but are also associated with additional prominent abnormalities of CNS function and often structure. In contrast to the striking homogeneity with respect to genes encoding ion channels in the idiopathic epilepsies, the genes causing the symptomatic epilepsies are striking in their diversity. The syndromes include

TABLE 37-5 Mouse epilepsy genes

Gene	Protein	Human Gene (and Location)
Induced mutations		
npy	Neuropeptide Y	*NPY* (7pter-q22)
syn-1	Synapsin 1	*SYN1* (Xp 11.2)
syn-2	Synapsin 2	*SYN2* (3p)
slc-1a (*glt-1*)	Glutamate transporter	*SLC1A2* (11p13-p12)
akp-2	Tissue-nonspecific alkaline phosphatase	*ALPL* (1p36.1-p34)
gad-2	Glutamic acid decarboxylase	*GAD2* (10p21.3)
gabrβ3	$GABA_A$ receptor subunit	*GABRB3* (15q11.2-q12)
gabrδ	$GABA_A$ receptor subunit	*GABRD* (1p)
gria-2	Glutamate receptor subunit 2	*GRIA2* (4q32-q33)
htr-2c	Hydroxytryptamine receptor 2c	*HTR2C* (Xq24)
kcna-1	Voltage-gated K^+ channel	*KCNA1* (12p13)
scn-2a	Sodium channel type 2a	*SCN2A1* (2q23-q24.3)
kcnj-6 (*girk-2*)	Inward-rectifying K^+ channel	*KCNJ6* (21q22.2)
camka	Calcium calmodulin kinase α subunit	*CAMKA* (5q31.3-q32)
itrp-1	Inositol 1,4,5 triphosphate receptor	*ITPR1* (3p26–25)
gap-43	Growth-associated protein	*GAP43* (3q21-qter)
cdk-5r	Neuronal specific activator of cyclin kinase	*CDK5R* (17p)
otx-1	Orthodenticle homolog	*OTX1* (p13.3)
jrk	Jerky	*JH8* (8q24.3)
pcmt-1	Protein L-isoaspartate (D-aspartate) *O*-methyltransferase	*PCMT1* (6q22.3–6q24)
hex-a	α subunit of β-hexosaminase	*HEX-A* (15q23-q24)
hex-b	β subunit of β-hexosaminase	*HEX-B* (5q13)
stfb (*cstb*)	Cystatin-B	*STFB* (21q22.3) (*CSTB*)
psap	Sphingolipid activator protein	*PSAP* (10q22.1)
Il-6	Cytokine, interleukin 6	*IL6* (7p21)
app	Amyloid precursor protein	*APP* (21q21.2)
hdh	Huntingtin's amino-terminal polyglutamine sequence	*HD* (4p16.3)
Polyglutamine repeat	146 unit CAG repeat inserted in *hprt* gene	–
Spontaneous mutations		
ccha-1a	Calcium channel α 1a subunit (tottering)	*CACNA1A* (19p13.1)
ccha-2a	Calcium channel α 1a subunit (tottering leaner)	*CACNA1A* (19p13.1)
cch-b4	Calcium channel β4 subunit (tottering leaner)	*CACNB4* (2q22-q23)
cacng-2	Calcium channel γ subunit (tottering leaner)	*CACNG2* (22q)
slc-9a1	Na^+/H^+ antiporter (slow wave epilepsy)	*SLC9A1* (1p36.1-p35)
kcnj-6 (*girk-2*)	Inwardly rectifying K^+ channel (Weaver)	*KCNJ6* (21q22.2)
itpr-1	Inositol 1,4,5 triphosphate receptor 1 (opisthotonus)	*ITPR1* (3p26–25)
cacna-2d2	Calcium channel α2δ2 subunit (ducky)	*CACNA2D2*

genetically determined abnormalities of neuronal migration, segmentation and patterning as seen in periventricular nodular heteropia, as well as dysregulation of transcription factors as observed in diseases such as Rett's syndrome.

Some spontaneous and some engineered mutations of mice result in epilepsy. In parallel to identifying the genes causing human epilepsies, a diversity of genes causing epilepsy in mice have been identified (Table 37-5). In most instances, the genes were overexpressed or eliminated by homologous recombination and the epileptic phenotype was an unexpected consequence. Genes encoding proteins subserving a component of the synaptic function constitute the single most common subset of mutations in this category (Table 37-5). This is no surprise given the role of synapses in the CNS and the delicate balance that exists between excitation and inhibition. For example, the fact that deletion of a gene encoding a subunit found in the $GABA_A$ receptor or a K^+ channel leads to epilepsy is not surprising. However, the occurrence of an epileptic phenotype in mice carrying null mutations of synapsin 1 was unexpected until this mutation was shown to preferentially compromise the efficacy of inhibitory synaptic transmission [31].

Finally, linkage analysis followed by sequencing led to identification of genes in spontaneously arising mutations that had been found to cause epilepsy in mice. In most of these, the nature of the epilepsy was a generalized spike and wave, mimicking the generalized-onset epilepsies such as absence. In contrast to absence epilepsy in humans, many of these mutant mouse strains exhibit cerebellar ataxia and often degeneration. Like the idiopathic epilepsies of humans, many of these genes encode ion channels. Interestingly, none of the mutant genes in these mouse strains have been identified to cause a form of absence epilepsy in humans.

REFERENCES

1. Commission on Classification and Terminology of the International League Against Epilepsy. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. *Epilepsia* 22: 489–501, 1981.
2. Commission on Classification and Terminology of the International League Against Epilepsy. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. *Epilepsia* 30: 389–399, 1989.
3. Ayala, G. F., Dichter, M., Gumnit, R. J., Matsumoto, H. and Spencer, W. A. Genesis of epileptic interictal spikes. New knowledge of cortical feedback systems suggests a neurophysiological explanation of brief paroxysms. *Brain Res.* 52: 1–17, 1973.
4. Traynelis, S. F. and Dingledine, R. Potassium-induced spontaneous electrographic seizures in a rat hippocampal slice. *J. Neurophysiol.* 59: 259–276, 1988.
5. Schwartzkroin, P. A. *Epilepsy: Models, mechanisms and concepts.* Cambridge: Cambridge University Press, 1993.
6. Goddard, G. V., McIntyre, D. C. and Leech, C. K. A permanent change in brain function resulting from daily electrical stimulation. *Exp. Neurol.* 25: 295–330, 1969.
7. Lemos, T. and Cavalheiro, E. A. Suppression of pilocarpine-induced status epilepticus and the late development of epilepsy in rats. *Exp. Brain Res.* 102: 423–428, 1995.
8. Longo, B. M. and Mello, L. E. Supragranular mossy fiber sprouting is not necessary for spontaneous seizures in the intrahippocampal kainate model of epilepsy in the rat. *Epilepsy Res.* 32: 172–182, 1998.
9. VanLandingham, K. E., Heinz, E. R., Cavazos, J. E. and Lewis, D. V. Magnetic resonance imaging evidence of hippocampal injury after prolonged focal febrile convulsions. *Ann. Neurol.* 43: 413–426, 1998.
10. Traub, R. D., Miles, R. and Wong, R. K. Model of the origin of rhythmic population oscillations in the hippocampal slice. *Science* 243: 1319–1325, 1989.
11. Lothman, E. W., Stringer, J. L. and Bertram, E. H. The dentate gyrus as a control point for seizures in the hippocampus and beyond. *Epilepsy Res. Suppl.* 7: 301–313, 1992.
12. Henze, D. A., Wittner, L. and Buzsaki, G. Single granule cells reliably discharge targets in the hippocampal CA3 network in vivo. *Nat. Neurosci.* 5: 790–795, 2002.
13. Buzsaki, G., Geisler, C., Henze, D. A. and Wang, X. J. Interneuron diversity series: circuit complexity and axon wiring economy of cortical interneurons. *Trends Neurosci.* 27: 186–193, 2004.
14. Collins, R. C., Tearse, R. G. and Lothman, E. W. Functional anatomy of limbic seizures: focal discharges from medial entorhinal cortex in rat. *Brain Res.* 280: 25–40, 1983.
15. Kohr, G. and Mody, I. Kindling increases *N*-methyl-D-aspartate potency at single *N*-methyl-D-aspartate channels in dentate gyrus granule cells. *Neuroscience* 62: 975–981, 1994.
16. Gibbs J. W. III, Shumate, M. D. and Coulter, D. A. Differential epilepsy-associated alterations in postsynaptic $GABA_A$ receptor function in dentate granule and CA1 neurons. *J. Neurophysiol.* 77: 1924–1938, 1997.
17. Buhl, E. H., Otis, T. S. and Mody, I. Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. *Science* 271: 369–373, 1996.
18. Nadler, J. V. The recurrent mossy fiber pathway of the epileptic brain. *Neurochem. Res.* 28: 1649–1658, 2003.
19. Ribak, C. E., Korn, M. J., Shan, Z. and Obenaus, A. Dendritic growth cones and recurrent basal dendrites are typical features of newly generated dentate granule cells in the adult hippocampus. *Brain Res.* 1000: 195–199, 2004.
20. Gorter, J. A., Borgdorff, A. J., van Vliet, E. A., Lopes da Silva, F. H. and Wadman, W. J. Differential and long-lasting alterations of high-voltage activated calcium currents in CA1 and dentate granule neurons after status epilepticus. *Eur J Neurosci.* 16: 701–712, 2002.
21. He, X. P., Kotloski, R., Nef, S., Luikart, B. W., Parada, L. F. and McNamara, J. O. Conditional deletion of TrkB but not BDNF prevents epileptogenesis in the kindling model. *Neuron* 43: 1–20, 2004.
22. Macdonald, R. L. and Greenfield L. J. Jr. Mechanisms of action of new antiepileptic drugs. *Curr. Opin. Neurol.* 10: 121–128, 1997.
23. Huguenard, J. R. Neuronal circuitry of thalamocortical epilepsy and mechanisms of antiabsence drug action. *Adv. Neurol.* 79: 991–999, 1999.
24. Kelly, K. M., Gross, R. A. and Macdonald, R. L. Valproic acid selectively reduces the low-threshold (T) calcium current in rat nodose neurons. *Neurosci. Lett.* 116: 233–238, 1990.
25. McNamara, J. O. Emerging insights into the genesis of epilepsy. *Nature* 399: A15–A22, 1999.
26. Noebels, J. L. The biology of epilepsy genes. *Annu. Rev. Neurosci.* 26: 599–625, 2003.
27. Cossette, P., Liu, L., Brisebois, K. *et al.* Mutation of *GABRA1* in an autosomal dominant form of juvenile myoclonic epilepsy. *Nat. Genet.* 31: 184–189, 2002.
28. Ptacek, L. J. Channelopathies: ion channel disorders of muscle as a paradigm for paroxysmal disorders of the nervous system. *Neuromusc. Disord.* 7: 250–255, 1997.
29. Kalachikov, S., Evgrafov, O., Ross, B. *et al.* Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nat. Genet.* 30: 335–341, 2002.
30. Rho, J. M., Szot, P., Tempel, B. L. and Schwartzkroin, P. A. Developmental seizure susceptibility of kv1.1 potassium channel knockout mice. *Dev. Neurosci.* 21: 320–327, 1999.
31. Terada, S., Tsujimoto, T., Takei, Y., Takahashi, T. and Hirokawa, N. Impairment of inhibitory synaptic transmission in mice lacking synapsin I. *J. Cell Biol.* 145: 1039–1048, 1999.

CHAPTER

38

Diseases Involving Myelin

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GENERAL CLASSIFICATION 639
Myelin deficiency can result from failure of synthesis during development or from myelin breakdown after its formation 639
Many of the biochemical changes associated with central nervous system demyelination are similar regardless of etiology 640

ACQUIRED ALLERGIC AND/OR INFECTIOUS DISEASES OF MYELIN 640
Nervous system damage in acquired demyelinating diseases is directed rather selectively against myelin or myelin-forming cells, but axons also are often affected 640
Experimental allergic encephalomyelitis is an animal model of autoimmune demyelination 640
A number of animal diseases caused by viruses involve primary demyelination and often are associated with inflammation 641
Multiple sclerosis is the most common demyelinating disease of the central nervous system in humans 641
Some human peripheral neuropathies involving demyelination are immune-mediated 645
Other acquired disorders affecting myelin in humans may be secondary to viral infections, neoplasias or immunosuppressive therapy 646

GENETICALLY DETERMINED DISORDERS OF MYELIN 647
The human leukodystrophies are inherited disorders of central nervous system white matter 647
Deficiencies of peripheral nerve myelin in common inherited human neuropathies are caused by mutations of genes for sheath proteins 648

TOXIC AND NUTRITIONAL DISORDERS OF MYELIN 649
A variety of biological and chemical toxins can impair myelin formation or cause its breakdown 649
General undernourishment or dietary deficiencies of specific substances can lead to a preferential reduction in myelin formation 649

DISORDERS PRIMARILY AFFECTING NEURONS WITH SECONDARY INVOLVEMENT OF MYELIN 649

REPAIR IN DEMYELINATING DISEASES 650
The capacity for remyelination is generally greater in the peripheral than in the central nervous system 650
Remyelination in the CNS can be promoted by various treatments 650
Measures to enhance axonal survival and regeneration also are critical in demyelinating conditions 650

†Pierre Morell tragically passed away early in the preparation of this chapter. A remembrance of his life and work appears on p. xxiii

The integrity of myelin sheaths is dependent upon the normal functioning of myelin-forming oligodendrocytes in the CNS and Schwann cells in the PNS as well as on the viability of the axons that they ensheathe. Neuronal death inevitably leads to subsequent degeneration of both axons and the myelin surrounding them. The title of this chapter was chosen to emphasize that myelin cannot be considered an isolated entity in the context of disease processes. Deficiencies of myelin can result from a multitude of causes, including autoimmunity, viral infections, genetic defects, toxic agents, malnutrition and mechanical trauma, that affect myelin, myelin-forming cells or myelinated neurons. More comprehensive clinical descriptions of most of the diseases described in this chapter are available in specialized books about myelin [1, 2].

GENERAL CLASSIFICATION

Myelin deficiency can result from failure of synthesis during development or from myelin breakdown after its formation. An impediment of normal myelin formation is referred to as *hypomyelination* or, in some cases, *dysmyelination*. According to the definition of Poser, dysmyelination is a genetically determined disorder of myelinogenesis in which 'myelin initially formed was abnormally constituted, thus inherently unstable, vulnerable, and liable to degeneration' [1]. Diseases involving loss of normal myelin after it is formed, i.e. demyelination, can be subdivided into primary and secondary categories

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on the basis of morphological observations. *Primary demyelination* involves the early destruction of myelin with relative sparing of axons; subsequently, other structures may be affected. However, in recent years it has become increasingly apparent that some diseases that had been classified as primary demyelination may involve more injury to axons than originally thought. This axonal damage could be caused by inflammation in autoimmune and viral disorders or loss of the influence of myelin proteins on axonal structure. It is now known from transgenic knockout mice that the absence of some proteins localized in myelin sheaths, including proteolipid protein (PLP), 2′3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and myelin-associated glycoprotein (MAG) results in pathological axonal changes *in vivo* (see Ch. 4). *Secondary demyelination* includes those disorders in which myelin is involved only after damage to neurons and axons. The classification for myelin disorders used in this chapter is based on etiology as well as on comparative neuropathology. Disorders causing hypomyelination and demyelination are both included in four categories: (1) acquired allergic and infectious diseases; (2) genetic disorders; (3) toxic and nutritional disorders; and (4) disorders primarily affecting neurons with secondary involvement of myelin.

Many of the biochemical changes associated with central nervous system demyelination are similar regardless of etiology. The most pronounced changes occur in white matter where there is a marked increase in water content, a decrease of myelin proteins and lipids and, in many demyelinating diseases, the appearance of cholesterol esters and/or glial fibrillary acidic protein (GFAP) [1]. Particularly noteworthy with regard to lipids are dramatic decreases in galactocerebroside, ethanolamine plasmalogens and cholesterol, all of which are enriched in myelin membranes (see Ch. 4). The major structural proteins of CNS myelin, myelin basic protein (MBP) and proteolipid protein (PLP), are also invariably decreased. These changes can be explained by the breakdown and gradual loss of myelin (which is relatively rich in solids) and its replacement by extracellular fluid, astrocytes and inflammatory cells (which are more hydrated, relatively lipid-poor and free of myelin-specific constituents). The frequent appearance of cholesterol esters in demyelinating diseases is related to the fact that cholesterol is not readily degraded and is esterified by phagocytes that often remain at the site of the lesion for some time. Since cholesterol esters are essentially absent from normal mature brain, their presence in myelin disorders is indicative of inflammation and recent demyelination. Such compounds are also responsible for the neutral fat staining, or *sudanophilia*, demonstrated histochemically in many demyelinating diseases. In the CNS, GFAP is specific to astrocytes, and an increase of this protein during demyelination is due to reactive astrocytes associated with the pathology (see Ch. 1). The magnitudes of the changes mentioned above vary considerably from disease to disease and from specimen to specimen in the same disease, depending on the severity, location, duration and activity of the pathological processes.

ACQUIRED ALLERGIC AND/OR INFECTIOUS DISEASES OF MYELIN

Nervous system damage in acquired demyelinating diseases is directed rather selectively against myelin or myelin-forming cells, but axons also are often affected. In most of these disorders (except where noted otherwise), the lesions are disseminated and characterized by perivenular demyelination and inflammation, macrophage activity, sudanophilic deposits consisting of myelin degradation products and relative sparing of axons. The extent to which these criteria are fulfilled depends on the particular type and phase of disease. Also, as mentioned above, axonal pathology may be more frequent than previously thought. Furthermore, it is not always clear whether the immunological activity is autoimmune in nature or whether it is related primarily to an antecedent viral infection, nor is the amount of damage directly caused by the virus always clear. The acquired diseases discussed here are reviewed in more detail elsewhere [1, 2].

Experimental allergic encephalomyelitis is an animal model of autoimmune demyelination. The animal model thought to most clearly resemble multiple sclerosis (MS) is experimental allergic encephalomyelitis (EAE). EAE is an acute or chronic demyelinating disease of the CNS; the disease is induced by immunization of susceptible animals with either myelin or various myelin components that are encephalitogenic. The EAE model was initially identified through efforts to understand the cause of encephalomyelitis developing after inoculation of patients with the Pasteur rabies vaccine, which was prepared from virus-infected nervous tissue. It was noted that even control experimental animals inoculated with uninfected neural tissue developed an encephalomyelitis, and EAE has been carefully studied since then as a model of human diseases such as MS. A detailed examination of the EAE model and the extensive immunological data that have been obtained are beyond the scope of this chapter, and readers are referred to recent reviews for more detail [3, 4].

In brief, EAE can be induced by immunization with several encephalitogenic proteins from myelin, most notably MBP, PLP and myelin-oligodendrocyte glycoprotein (MOG). MBP was the first protein shown to cause EAE, and this classical form of the disease is mediated primarily by $CD4^+$, MHC class II restricted T cells, because it can be transferred from an immunized animal to a naive animal using these cells. These data document the importance of $CD4^+$ lymphocytes for EAE induction, but additional pathogenic roles for other T cell populations are possible, especially in later stages of the disease. Also, there is

considerable variation in susceptibility of various animals, and this variation is largely due to the genetic background, especially that of MHC genes. It is now understood that elements of the three components of antigen recognition, the T cell receptor (TCR), antigen and the MHC molecule that presents antigen, together known as the trimolecular complex, are central to disease induction. The encephalitogenic immunodominant part of MBP that causes the disease varies between different strains of mice and other species. The exquisite specificity seen for the trimolecular complex of encephalitogenic T cells has resulted in the development of numerous therapies that target the trimolecular complex and can modify disease. These include generating an immune response to the TCR or administration of a peptide that can bind to MHC but without activating T cells.

The most characteristic component of the EAE lesion is perivascular inflammation, and the extent of demyelination varies between species and the identity of the immunogen used to induce the disease. Evidence from studying EAE in the rat or mouse indicates that activated T cells, regardless of specificity, can cross the blood–brain barrier and enter the nervous system. If the T cell encounters its antigen, the cell will be further activated and begin production of proinflammatory cytokines such as gamma-interferon (INFγ) and tumor necrosis factor (TNF)α, which in turn activate the endothelial cells with upregulation of cell adhesion molecules (see Ch. 7) promoting an inflammatory response. The outcome of this process, at least in some animals, is myelin damage. The actual effector mechanisms for myelin damage include a direct toxic effect of TNFα or nitric oxide on oligodendrocytes or damage mediated by macrophages and microglial cells. The later possibility can be enhanced by the presence of antibodies, which bind to myelin and provide a ligand for activated monocytes. Indeed, demyelination associated with EAE in several animals including the rat, mouse and marmoset, a nonhuman primate, is increased by the presence of antibodies to surface components of myelin, such as galactocerebroside or MOG. In fact, immunization of rodents with MOG alone has been shown to induce a relapsing–remitting demyelinating disease with both cellular and humoral immunity to this glycoprotein [3].

A number of animal diseases caused by viruses involve primary demyelination and often are associated with inflammation. These diseases are studied as animal models, which may provide clues about how a viral infection could lead to immune-mediated demyelination in humans [1, 5, 6]. Canine distemper virus causes a demyelinating disease, and the lesions in dog brain show a strong inflammatory response with some similarities to acute disseminated encephalomyelitis in man [1]. Visna is a slowly progressive demyelinating disease of sheep caused by a retrovirus [1].

Two neurotropic viruses of mice are of particular interest with regard to the way in which immune-mediated demyelination of the CNS can be induced by a viral infection [5, 6]. JHM virus, which infects oligodendrocytes and produces acute and chronic inflammatory demyelination in rodents, is a neurotropic strain of mouse hepatitis virus in the coronavirus family. Theiler's virus is a picornavirus that infects many neural cell types and induces a chronic CNS encephalomyelitis. Investigation of animal models such as these has revealed several mechanisms by which viral infections can cause demyelination including: (1) a direct infection of oligodendrocytes resulting in cell death; (2) a virus-specific inflammatory response in which cytokines or other immune mediators damage oligodendrocytes/myelin indirectly as a 'bystander' effect; and (3) activation of an immune response directed at components of oligodendrocytes/myelin by molecular mimicry or epitope spreading. Molecular mimicry occurs when a viral antigen has structural homology to a myelin/oligodendrocyte component, resulting in cross-reactivity, and epitope spreading results from host immune cell processing of myelin/oligodendrocyte antigens released following tissue injury.

Multiple sclerosis is the most common demyelinating disease of the central nervous system in humans. Multiple sclerosis is a chronic demyelinating disease affecting the CNS [1, 2, 7, 8]. The disease primarily affects young adults and is characterized by a highly variable course. Most often the disease begins in the third or fourth decade with neurological dysfunction, such as decreased vision, dysarthria, weakness in limbs, diplopia, nystagmus, sensory deficits and paresthesias. The initial course is characterized by acute episodes of one or more of these symptoms followed by subsequent recovery, and this course is known as relapsing–remitting MS. Over time, the improvement after attacks may be incomplete and the relapsing–remitting course may evolve into one of increasing progression of disability, termed secondary progressive MS. A small number of patients develop a form of disease that begins as a slowly progressive process, without acute episodes of worsening followed by improvement, which has been termed primary progressive MS. A few patients will have a very aggressive course, which can even lead to death over a short period.

The diagnosis of MS is dependent on clinical evidence of central nervous system involvement separated in both time and space. Thus, evidence that two areas of the CNS are involved and a history that this involvement occurred at two different times and without any other identifiable cause, is necessary to establish a diagnosis of clinically definite MS. While there is no diagnostic test that is specific for MS, the appearance of MBP, oligoclonal protein bands or elevated IgG/protein ratios in the CSF, as well as abnormalities in electrophysiological tests of visual, auditory and somatosensory evoked potentials and magnetic resonance imaging (MRI), can help to identify subclinical lesions and assist in establishing the diagnosis. Of all of these tests, the use of MRI has probably resulted in the greatest change in our understanding of MS. T2-weighted MRI images are extremely effective in identifying MS lesions.

The areas of increased signal seen on T2-weighted MRI reflect increased water and therefore are neither specific for MS nor able to distinguish new from old lesions. This is because both inflammation and tissue loss associated with demyelination or gliosis after ischemic events will result in increased signal. Therefore, the interpretation must include the clinical context. A second technique, T1-weighted imaging after the administration of gadolinium-DTPA, which does not normally cross the blood–brain barrier, can identify new lesions in which the blood–brain barrier has been compromised. Serial studies of patients with early relapsing–remitting MS have shown that the first event in the development of a new MS lesion as seen on a T2-weighted MRI is disruption of the blood–brain barrier seen on a gadolinium-enhanced MRI. Thus, postcontrast MRI provides a very sensitive technique for following at least one aspect of disease activity. Using this approach, several groups of investigators have shown that MS is an active and progressive disease even in the early relapsing–remitting phase and during periods of clinical stability in many patients. Newer imaging techniques now hold promise for providing greater specificity for the various stages of lesion development (see Ch. 58).

MS lesions or plaques can be identified grossly at autopsy (Fig. 38-1) and are sharply demarcated from the surrounding tissue. Plaques occur throughout the white matter, but areas of predilection such as the periventricular white matter are well known. Microscopic examination characteristically shows loss of myelin with preservation of axons (primary demyelination). However, although the most prominent pathology in MS is demyelination, there are recent indications also for axonal and cortical pathology. Now techniques of confocal microscopy and immunocytochemistry have clearly demonstrated that transected axons are common in MS lesions [9].

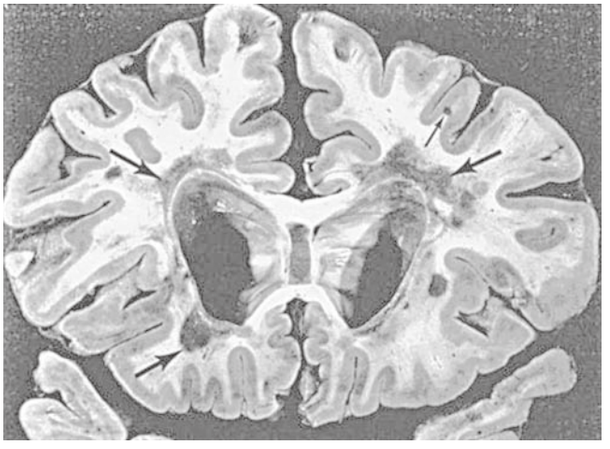

FIGURE 38-1 Coronal slice of brain from a patient who died with MS. Demyelinated plaques are clearly visible in white matter (*large arrows*). Small plaques are also observed at the boundaries between gray and white matter (*small arrows*). (Reproduced with permission from Raine, C. S. The neuropathology of myelin diseases. In P. Morell (ed.) *Myelin*. New York: Plenum Press, 1984, ch. 8.)

It has become generally accepted that axonal loss is an important aspect of MS pathology and may account for much of the irreversible neurological impairment in this disease. Plaques commonly develop around venules, and 'early lesions' contain many lymphocytes, plasma cells and macrophages. The greatest cellularity is found at the margins of acute lesions, which are believed to be the locations at which some of the earliest changes associated with myelin loss are occurring.

For many years there has been discussion about whether the primary pathological effect in this disease is directed at myelin sheaths themselves or at the myelin-forming oligodendrocytes. Investigators utilizing sophisticated neuropathological and immunocytochemical methods for examining lesions have now reported that there are actually several subtypes of MS patients [10], and myelin sheaths are the primary target in some whereas oligodendrocytes are targeted in others. Most common were cases that resembled EAE in which myelin was the primary target. The pathology was characterized by macrophage/T-cell-mediated or antibody/complement-mediated demyelination. However, other MS patients had lesions characterized by oligodendrocyte dystrophy, which is reminiscent of viral or toxin-induced demyelination rather than autoimmunity. These patients were less frequent, and lesions of this type were generally observed in patients with a shorter disease course than those with EAE-like lesions. These findings indicate that the demyelinated plaque may be a common endpoint resulting from a variety of different immunological and pathological mechanisms. The pathogenic heterogeneity in different patients may have fundamental implications for the diagnosis and therapy of this disease. Generally, chronic lesions are sharply defined containing bare nonmyelinated axons and many fibrous astrocytes.

The biochemistry of demyelination in MS was reviewed in detail [11]. Affected areas of MS white matter exhibit the expected decrease of myelin constituents and a buildup of cholesterol esters. For example, polyacrylamide gel electrophoresis of homogenates of macroscopically normal appearing white matter, outer periplaque, inner periplaque and plaque shows the expected decline of MBP and PLP in going from the normal appearing white matter to the center of the plaque in both chronic and acute lesions (Fig. 38-2). There is a virtual absence of these myelin proteins in the center of the chronic plaque and an accumulation of glial fibrillary acidic protein (GFAP) indicative of astrogliosis (Fig. 38-2B). A plaque from a more acute lesion is not completely demyelinated, as indicated by the presence of some MBP and PLP, and there is no accumulation of GFAP (Fig. 38-2C). The more acute plaque contains albumin as a result of breakdown of the blood–brain barrier. Immunocytochemical and quantitative biochemical analyses of myelin proteins have revealed that MAG is often decreased more than other myelin proteins at the periphery of plaques (reviewed in [12]), and this may be indicative of early pathological events in the

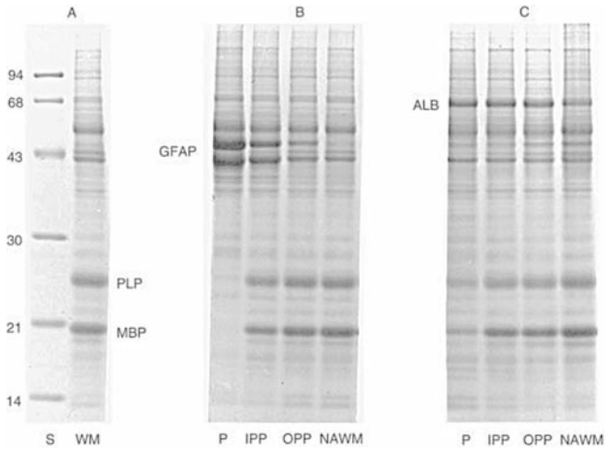

FIGURE 38-2 Polyacrylamide gel electrophoresis of proteins in control and MS tissue. Total proteins of the tissue samples were solubilized with a detergent (sodium dodecyl sulfate) and electrophoresed on a polyacrylamide gel system that separates proteins according to their size. After electrophoresis, the proteins were stained with Coomassie brilliant blue dye. **(A)** Molecular weight standards (*S*) labeled according to their molecular mass (kDa) and the proteins of control white matter (WM). **(B)** Samples from the region of a chronic MS plaque. **(C)** Samples from the region of an acute MS plaque. *ALB*, serum albumin; *GFAP*, glial fibrillary acidic protein; *IPP*, inner periplaque; *MBP*, myelin basic protein; *NAWM*, macroscopically normal appearing white matter near the plaque; *OPP*, outer periplaque; *P*, plaque center; *PLP*, proteolipid protein. (With permission from reference [39].)

distal periaxonal regions of oligodendroglial processes where MAG is selectively localized (see Ch. 4). Evidence suggests that this selective loss of MAG is characteristic of the subset of MS patients who exhibit oligodendroglial dystrophy, and the MAG loss may reflect a dying-back oligodendrogliopathy [10]. A number of biochemical studies have indicated that myelin constituents are significantly reduced even in some areas of macroscopically normal-appearing white matter of MS brain in comparison to control white matter [11], and this is most probably explained by the presence of microlesions throughout the affected brain. Although the yield of myelin from MS tissue is reduced, most studies indicate that there is no compositional difference between isolated MS and control myelin, suggesting that the etiology of MS is unlikely to be related to an underlying defect in myelin composition. On the other hand, comparisons of post-translational modifications of MBP from MS brain with those from controls have demonstrated an increase of the least cationic, deaminated citrulline-rich isoform, increased methylation at arginine 107 and decreased phosphorylation [13]. These changes could alter the stability of myelin, but it is not clear whether the modifications reflect inborn abnormalities of myelin or result from the disease process.

The cause of MS is not known, but most evidence points to an immunologically mediated disease with important genetic and environmental risk factors [1, 2, 7, 8]. The evidence for an autoimmune process is largely circumstantial and based in part on evidence derived from animal models of autoimmune demyelinating disease such as EAE. In addition to providing insights into the natural history of the disease and a tool that can be used to monitor new treatments, MRI is also providing insights into the nature of the MS lesion. As indicated previously, blood–brain barrier disruption seems to be the initial event in lesions, and pathological studies suggest that enhancement is often characterized by acute inflammation. From findings derived from studies of EAE, it is likely that migration of activated T cells represents the initial step in lesion development.

What is not clear is the specificity of the T cells. There are numerous studies of T cell reactivity to myelin antigens such as MBP, PLP and MOG, all of which are encephalitogenic in animals. However, the evidence taken as a whole has failed to demonstrate a substantially increased response to these antigens in most patients with MS compared to healthy controls. Nevertheless, at this time, MOG appears to be a leading candidate among myelin proteins to be an important target in the autoimmune aspects in many patients because of: (1) its accessibility to immune attack due to its surface localization on oligodendrocytes; (2) its capacity to produce a relapsing–remitting form of EAE resembling MS when used as an immunogen; and (3) the detection of immunoreactivity to MOG in a relatively high proportion of MS patients [3, 14]. It may be that several myelin antigens play a role in MS, and that the relative importance of different antigens varies among individuals depending on their immunogenetic background and environmental factors encountered during their life. Further, studies of TCR usage have generally failed to provide evidence for a restricted or limited TCR usage in myelin-antigen-specific T cells. What has emerged from studies of the T cell response to antigens such as MBP is that some portions of the molecule are immunodominant (e.g. MBP (83–99)), and these portions are regions that can be encephalitogenic in some animal models. Also, the HLA types that most readily present MBP to MBP-specific T cells, namely, the DR2 haplotype, are those that are over-represented in Caucasian patients with MS. Thus, it can be postulated that the T cell response to myelin antigens could represent one component of lesion development but one that, alone, is not sufficient for disease. Other factors could influence the integrity of the blood–brain barrier and regulation of cytokines.

Once a T cell enters the CNS and encounters antigen, it is postulated that a proinflammatory cytokine cascade is initiated that produces further compromise of the blood–brain barrier and amplifies the inflammatory response, which eventually results in demyelination. The cause of myelin damage is not known. Possibilities include direct toxic effects of some cytokines such as TNFα and NO on oligodendrocytes, or macrophage-mediated damage either through production of proteases or through ligand-mediated interaction with the myelin membrane. Electron microscopy of active MS lesions indicates that one

mechanism for myelin destruction is the direct removal, by macrophages, of myelin lamellae from the surface of intact sheaths. This involves the attachment of superficial lamellae to coated pits at the macrophage surface, implying the presence of receptors that bind to a ligand on the myelin. The Fc receptors on the macrophages may bind to immunoglobulins attached to the myelin and, as mentioned previously, studies of EAE have shown that antibodies to galactocerebroside, MOG or other surface molecules of myelin can augment demyelination.

There is much evidence indicating that proteases and other catabolic enzymes are involved in the myelin loss in MS [11]. MBP is highly susceptible to proteases even when present in myelin membranes. A neutral protease released by stimulated macrophages catalyzes the conversion of plasminogen to plasmin, which rapidly degrades MBP. Acid proteinase and other degradative enzymes, presumably lysosomal and of macrophage origin, are elevated in affected MS tissue and are likely to be involved in breakdown of myelin proteins and lipids. In addition, protease activities intrinsic to myelin sheaths (such as Ca^{2+}-activated neutral protease) may facilitate myelin destruction. Immunologically reactive, proteolytic fragments of MBP appear in the cerebrospinal fluid (CSF) and urine of MS patients during exacerbations of the disease, presumably reflecting myelin breakdown. However, the presence of this material is not specific for MS since it occurs in other conditions with myelin damage such as stroke and encephalitis. Matrix metalloproteases (MMPs) have attracted interest in recent years with regard to MS, because they participate in degradation of extracellular matrix, thereby allowing immune cells to move from the blood into the white matter.

In addition to immunological elements, it is likely that both genetic and environmental factors contribute to disease susceptibility [1, 2, 7, 8]. Two types of study have provided data that favor a genetic component in MS. First, the prevalence of MS varies substantially among ethnic groups, with Caucasian populations showing a higher frequency than some other groups. For example, MS occurs at low frequency both in Japan and in Japanese Americans living in high-prevalence regions of the United States. Secondly, there is increased risk among first-degree relatives of individuals with the disease, especially in monozygotic twins where the rate of concordance for the disease is 20–30% and even higher with careful examination by techniques such as MRI. Several possible genetic loci have been examined in MS and, as mentioned above, a fairly consistent association, although weak, with HLA makeup has been found in most studies. Investigators have attempted to examine the entire genome in patients with MS. While some areas of possible significance have been found, it is certain that the effect of any single genetic locus is relatively small. It is almost certain that multiple genes are involved, each with a small influence on susceptibility.

Several types of evidence point to an environmental influence in MS. These include unequal geographic distribution of the disease, effect of migration between low- and high- risk areas on susceptibility, and evidence for epidemics or clusters. Each of these sets of observations is open to variable interpretation and in many cases the genetic influence is difficult to separate from that of the environment. However, the bulk of evidence suggests that environment contributes to risk. The nature of an environmental influence is not known, but an infectious agent has long been suspected [1, 2]. There are a number of naturally occurring and experimental disorders in animals caused by viruses that have long incubation periods and involve primary demyelination with relative sparing of axons (see above). A possible viral etiology for MS has stimulated an extensive amount of research over the years, leading to many reports of viruses associated with MS. One way in which a viral infection could set off an autoimmune attack on myelin would be by molecular mimicry, in which a foreign peptide has sufficient sequence similarity to a neural antigen to set off an autoimmune attack. There is currently a substantial amount of interest in a possible involvement of human herpes virus 6 [15]. However, at this time a definite causative infectious agent has not been established. Nevertheless, a viral infection occurring before puberty and possibly modifying immune function, rather than resulting in a persistent infection, seems likely. Thus, although it is widely thought that MS is an autoimmune disease and associated with an infectious agent, the putative antigen(s) and virus(es) remain elusive. Furthermore, as stated above there is growing evidence that all cases of MS may not be associated with the same virus or antigen but rather the disease process may be set in motion by a variety of infectious agents leading to a spectrum of immune cascades varying from patient to patient depending on their genetic background, but leading to a similar final pathology as represented by demyelinated plaques.

Therapies that are thought to modify immunopathogenic aspects of the disease have been approved for use in treating patients with relapsing–remitting MS, including interferon beta and copolymer-1 (Cop-1, glatiramer acetate) [8, 16]. The mechanism of action of the beta interferons is not entirely clear, but appears to involve modulation of the expression of adhesion molecules and matrix metalloproteases, resulting in a decrease of blood–brain barrier breakdown, as well as suppressing T cell activation and inhibiting proinflammatory cytokine production. Cop-1 is a synthetic random polymer, composed of the amino acids L-alanine, L-glutamic acid, L-lysine and L-tyrosine, which is believed to selectively bind to HLA class II molecules and to block antigen presentation. It also induces a shift in cytokine production and the generation of Th2 cells, which cross-react with myelin components and mediate bystander suppression. Although these therapies are moderately effective, they only reduce disease exacerbations by about 30% or delay disease progression. Therefore, research aimed at interfering with the autoimmune process at different levels is under way

in many laboratories [7, 8, 16], and MRI is providing a powerful tool for monitoring the initial testing of experimental therapies. One example is mitoxantrone, approved for treatment of secondary progressive and progressive-relapsing MS, a synthetic antineoplastic anthrocenedione that inhibits proliferation of B and T cells and macrophages, impairs antigen presentation and secretion of INFγ, TNFα and interleukin (IL)-2 *in vitro* [17]. Another pharmacologic approach is to inhibit migration of T cells across the blood–brain barrier by antibody blocking of α4-integrin. An example is natalizumab, a humanized monoclonal antibody presently under clinical trials [18]. Other examples of therapeutic approaches under investigation are inhibitors of metallic proteinases, which also alter cell migration across blood–brain barrier; vaccination with pathogenic T cells specific for candidate autoantigens in myelin; and treatment with altered peptide ligands that block T cell activation or induce bystander suppression. Increasing use of powerful technologies such as gene microarray analysis of MS tissues and proteonomics should help identify more disease markers and effectors that can be targeted and ultimately lead to therapies tailored to different stages of disease or subsets of MS patients. Alternative approaches to therapy involve repair both by stimulating remyelination and promoting the survival and regeneration of axons. The latter is likely to be a major emphasis in the future, now that it is known that much of the irreversible neurological damage in MS is caused by axonal loss. However, a serious problem to the use of methodologies to stimulate repair in MS is that the lesions of MS develop at different times and locations in optic nerve, cerebrum, cerebellum, brainstem and spinal cord, each of which may have its own unique requirements. The various approaches to repair tissue damage in myelin diseases is discussed in greater detail at the end of this chapter, because they also apply to other myelin disorders with differing etiologies.

Some human peripheral neuropathies involving demyelination are immune-mediated. Guillain–Barré syndrome (GBS) is an acute inflammatory demyelinating polyneuropathy that is monophasic, self limiting and frequently occurs following an antecedent bacterial or viral infection [1, 2, 19] (see also Ch. 36). It is generally characterized by primary demyelination, although variants with severe axonal involvement also exist. Chronic inflammatory demyelinating polyneuropathy (CIDP) is similar but is progressive or relapsing–remitting with a duration of many months or years [1, 2, 20]. With regard to potential target antigens for autoimmune diseases of the PNS, it should be kept in mind that, although PNS and CNS myelin are morphologically similar, they differ significantly in chemical composition, especially in protein constituents (see Ch. 4). Cumulative evidence suggests that nerve injury in GBS and CIDP is mediated by immunological mechanisms but, as in MS, the roles of the patients' cell mediated and humoral responses in causing the demyelination have not been fully defined. An important role of humoral immunity in these diseases is strongly suggested by findings that sera from GBS patients cause demyelination in appropriate test systems and that plasmapheresis and intravenous administration of immunoglobulin are effective therapies in many of these patients. Neuropathies also occur in association with monoclonal gammopathy (also called paraproteinemia) in which there is expansion of a clone of plasma cells leading to large amounts of a monoclonal antibody in the patient's serum. It is thought that the neuropathies in these patients are likely to be caused by binding of the monoclonal antibodies to neural antigens [21].

Although experimental allergic neuritis (EAN) is often considered to be a good animal model for GBS, and the P2 myelin protein is implicated as an important antigen in this model (see Ch. 36), neither cellular nor humoral immunity to P2 or other myelin proteins has been detected consistently in GBS. Similarly, although antibodies to galactocerebroside have been shown to cause peripheral demyelination in experimental animals, evidence for significant levels of antibodies to this glycolipid in GBS is lacking. However, a substantial body of research now indicates that antibodies to more complex glycolipids, such as gangliosides (see Ch. 3), are involved in the pathogenesis in many of the human immune-mediated neuropathies described above [21]. The first indications of this came from patients with demyelinating sensorimotor neuropathy in association with IgM gammopathy. The monoclonal antibodies in these patients were shown to react with a carbohydrate epitope in MAG that is shared with complex glycolipids and other glycoproteins, including P0 and peripheral myelin protein-22 (PMP-22) of compact PNS myelin. The principal antigenic glycolipid, which is present in much larger amounts in the mature PNS than the CNS, is sulfate-3-glucuronyl paragloboside (SGPG). The specificity of these human antibodies is very similar to that of the HNK-1 antibody, which reacts with a number of adhesion proteins in the nervous system (see Ch. 4). Several animal models involving administration of the human anti-MAG/SGPG antibodies or immunization with SGPG strongly suggest that this disease is caused by the antibodies, but the relative importance of the potential glycolipid and glycoprotein target antigens in contributing to the pathology remains to be established. About half of patients with neuropathy in association with IgM gammopathy have antibodies of this specificity. It is also noteworthy that monoclonal IgM antibodies in patients with neuropathy who are MAG/SGPG-negative frequently react with gangliosides, suggesting that complex glycolipids may be important target antigens in immune-mediated neuropathies.

Once the monoclonal antibodies reacting with complex glycolipids were shown to occur frequently in patients with neuropathy in association with gammopathy, antibodies were demonstrated in some patients with GBS and other forms of inflammatory demyelinating neuropathy [21].

For example, antibodies to GM1 ganglioside are strongly associated with a subset of GBS that is particularly severe, has a high degree of axonal degeneration and presents with predominantly motor symptoms. Antibodies to GM1 ganglioside are also found frequently in a distinct chronic progressive neuropathy defined by multifocal motor conduction blocks. This condition, known as multifocal motor neuropathy (MMN), is characterized by weakness, muscle atrophy and motor nerve demyelination, while sensory function is normal or only slightly affected. Generally, there appears to be a strong correlation between antibodies to GM1 ganglioside and motor nerve disorders, including GBS, MMN and the neuropathies in association with monoclonal gammopathy described below. Another correlation of clinical presentation with antibody specificity involves patients with ataxic neuropathies and antibodies to disialosyl epitopes in gangliosides such as GD1b, GT1b and GQ1b. This correlation is found both in patients with monoclonal gammopathy and those with the Miller–Fisher variant of GBS.

Research has resulted in major advances in animal modeling of anti-glycolipid antibody-associated neuropathies *in vitro* and *in vivo*, which make a strong case that the antibodies cause much of the neurological impairment in these patients [21]. Furthermore, molecular mimicry between immunogens expressed by antecedent infectious agents and neural gangliosides appears to account for the presence of the glycolipid antibodies in some cases. Thus, carbohydrate configurations very similar or identical to those of gangliosides occur in the lipopolysaccharide (LPS) of strains of *Campylobacter jejuni* that are associated with these subsets of GBS (Fig. 38-3). Although it is now well established that anti-glycolipid antibodies are important pathogenic agents in many patients, not all patients with acquired acute or chronic peripheral neuropathies express these antibodies. Therefore, much remains to be learned about the pathogenic roles of antibody and T cell responses to other neural antigens, the importance of cytotoxic immune mediators and other factors in the acquired immune-mediated neuropathies.

Other acquired disorders affecting myelin in humans may be secondary to viral infections, neoplasias or immunosuppressive therapy. Acute disseminated encephalomyelitis, also called postinfectious or postimmunization encephalitis, represents a group of disorders usually of mixed viral–immunological etiology. The condition is most commonly related to a spontaneous viral infection, of which major examples are measles, smallpox or chickenpox [1, 2].

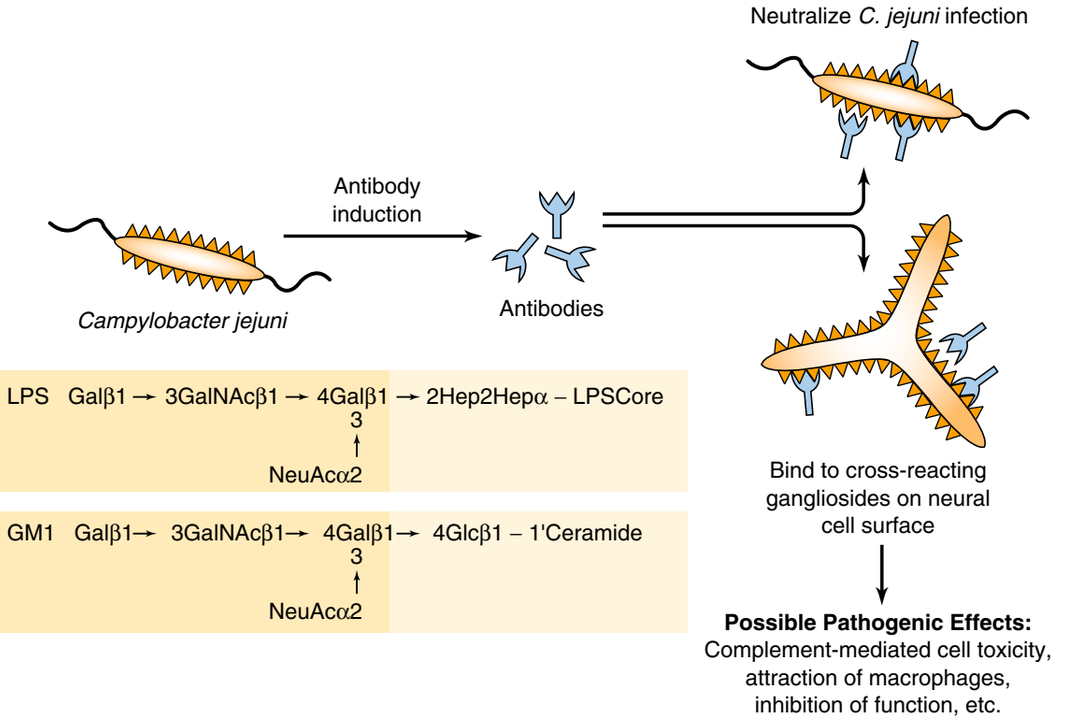

FIGURE 38-3 Molecular mimicry in subsets of patients with Guillain–Barré syndrome. Carbohydrate configurations (*dark orange triangles*) in the lipopolysaccharide (*LPS*) on some strains of *Campylobacter jejuni* are the same as in GM1 ganglioside on neural membranes. The chemical structures of LPS and GM1 are shown in the inset with the shared terminal tetrasaccharide in *dark orange*. Antibodies produced in response to LPS during a *C. jejuni* infection are intended to fight the infection but may cross-react with GM1 on neural cells, causing pathogenic effects in Guillain–Barré syndrome by mechanisms such as those listed in the lower right of the figure. Similarly, the LPS in other strains of *C. jejuni* have carbohydrate structures similar to those in GQ1b ganglioside, and these strains are associated with the Miller–Fisher variant of Guillain–Barré syndrome with prominent cranial nerve involvement, ataxia and areflexia. Antibodies to GQ1b are found in essentially all patients with this variant.

The demyelination occurring in these conditions is likely to be mediated at least in part by immune mechanisms since T cells sensitized to MBP are detected in many of these patients.

The nervous system is affected in a high proportion of patients with acquired immunodeficiency syndrome (AIDS) [2, 22]. Abnormalities related to myelin include a generalized myelin pallor observed histologically, vacuolar myelopathy and focal demyelination. Although it was originally thought that the widespread myelin pallor could be indicative of substantial demyelination, it now appears that the reduced staining is related to accumulation of fluid resulting from breakdown of the blood–brain barrier and not to extensive myelin loss. It is generally accepted that HIV infection in the nervous system *in vivo* is largely restricted to macrophages and microglia, and that the myelin and oligodendrocyte damage associated with HIV may be mediated by cytokines released by infected macrophages (see section on toxins, below).

Progressive multifocal leukoencephalopathy (PML) is historically a rare demyelinating disease that is usually associated with disorders of the reticuloendothelial system, neoplasias and immunosuppressive therapy [1, 2]. However, it has become more important in clinical medicine because it is frequently seen as an opportunistic secondary infection in immunocompromised persons with AIDS. PML is characterized by focal lesions that are noninflammatory and caused by infection of oligodendrocytes with the JC papovavirus.

GENETICALLY DETERMINED DISORDERS OF MYELIN

The human leukodystrophies are inherited disorders of central nervous system white matter. These disorders are characterized by a diffuse deficiency of myelin caused by a variety of genetic lesions and often manifest before 10 years of age (Table 38-1). Some are caused by mutations in the *PLP* gene and resemble the *PLP* animal mutants described in Chapter 4 [1, 23]. As with the animal models, depending on the nature of the mutation, they vary from a severe form in connatal Pelizaeus–Merzbacher disease (PMD) through an intermediate phenotype in classical PMD to a mild phenotype in spastic paraplegia. It is noteworthy that some mutations of the *PLP* gene also cause a peripheral neuropathy [24], very probably related to the expression of low levels of PLP in peripheral nerve (see Ch. 4).

Other leukodystrophies are associated with the lysosomal and peroxisomal disorders in which specific lipids or other substances accumulate due to a deficiency in a catabolic enzyme – for example Krabbe's disease, metachromatic leukodystrophy (MLD) and adrenoleukodystrophy (ALD) [1, 2]. (These are discussed in detail in Ch. 40.) Similarly, disorders of amino acid metabolism can lead to hypomyelination – for example phenylketonuria and Canavan's disease (spongy degeneration) [1, 2, 25] (Ch. 40). The composition of myelin in the genetically

TABLE 38-1 Genetically determined disorders affecting myelin in humans

Disorder	Inheritance	Genetic lesion	Comments	References
Adrenoleukodystrophy	X-linked	Peroxisomal membrane protein in the ABC transporter family	Decreased peroxidation of saturated, very-long-chain fatty acids, causing their accumulation in brain, adrenals and other tissues; variable phenotypes with regard to hypomyelination; see text	1, 26, Ch. 40
Canavan's disease (spongy degeneration)	AR	Aspartoacylase	Widespread white matter edema with diminished myelin; *N*-acetylaspartic aciduria; see text	1, 25, Ch. 40
Charcot–Marie–Tooth disease and other inherited neuropathies	AD, AR or X-linked	PMP-22, P0, connexin-32 and other genes	Variable degrees of myelin deficiency specific for the PNS; see text	1, 28–30
Krabbe's leukodystrophy	AR	Galactocerebroside-β-galactosidase	Globoid cells contain galactocerebroside; see text	1, 2, Ch. 40
Metachromatic leukodystrophy	AR	Aryl sulfatase A	Accumulation of sulfatide in brain; see text	1, 2, Ch. 40
Pelizaeus–Merzbacher disease (classical and connatal forms) and spastic paraplegia	X-linked	PLP	Variable hypomyelination due to different mutations in the major structural protein of CNS myelin; similar to rodent mutants such as the *jimpy* mouse	1, 23
Phenylketonuria	AR	Phenylalanine hydroxylase	White matter is up to 40% deficient in myelin; hypomyelination may be caused by inhibition of amino acid transport and/or protein synthesis by the high level of phenylalanine that accumulates	1, Ch. 40
Refsum's disease	AR	Oxidation of branched chain fatty acids; phytan-olyl CoA 2-hydroxylase in some cases	Increase of branched-chain phytanic acid, especially in PNS myelin	1, 38, Ch. 40
Vanishing white matter disease	AR	Eukaryotic initiation factor 2B (eIF2B)	Also referred to as childhood ataxia with central hypomyelination (CACH); see text	27

AD, autosomal dominant; AR, autosomal recessive

determined disorders can be normal, have a specific alteration reflecting the genetic lesion or show a nonspecific pathological composition that is found in many myelin disorders. The composition of the small amount of myelin isolated from Krabbe's disease is normal, and the hypomyelination is believed to be caused by the accumulation of galactosylsphingosine (psychosine) which has a cytotoxic effect on myelin-forming cells. By contrast, myelin isolated from postmortem metachromatic leukodystrophy (MLD) brain is highly enriched in sulfatide, but it is not known for certain if the myelin membrane contains more sulfatide or if the excess is due to sulfatide-enriched micelles that are co-isolated during the myelin purification. In some genetic disorders such as ALD and Canavan's disease, as well as in a wide variety of disorders involving secondary demyelination, the myelin preparations have similar abnormal chemical compositions. In each case, the isolated pathological myelin has a generally normal ultrastructural appearance but has much more cholesterol, less cerebroside and less phosphatidal ethanolamine than does normal myelin. Certain experimental disorders induced in animals by toxic agents show the same type of abnormal myelin. Myelin with this abnormal composition is referred to as the 'nonspecific pathological type' and probably represents a partially degraded form.

X-linked ALD has long been known to be a peroxisomal disease that involves a defect in the β-oxidation of very-long-chain fatty acids (VLCFAs), leading to their accumulation in complex lipids of myelin (Ch. 40) [1, 26] and it was thought that the genetic defect would turn out be in an enzyme such as VLCFA-CoA synthetase. However, the positional cloning approach showed that the defective gene is a 70 kDa peroxisomal membrane protein (ALD/ABCD1) in the ATP binding cassette transporter protein family (Ch. 5). An interesting aspect of ALD is that the phenotype can vary from a major neurological problem in the childhood form with a severe deficiency of myelin in the brain, through a milder form known as adrenomyeloneuropathy that occurs in young men and affects primarily spinal cord and peripheral nerve, to less common phenotypes without neurological involvement.

The function of the ALD protein is not fully understood, and knockout mice lacking it do not exhibit the severe CNS neurological deficits commonly associated with the human disease despite a similar accumulation of VLCFAs [26]. Furthermore, the clinical variability in human patients cannot be accounted for by the severity of the biochemical abnormality or the nature of the gene defect. These observations, plus other data from mice with defects in VLCFA metabolism, raise the issue of whether the accumulation of VLCFAs in myelin is crucial to the pathological mechanisms or is an epiphenomenon. Unlike most other lipid-storage diseases, active ALD brain lesions are characterized by perivascular accumulation of lymphocytes. For this reason, it has been hypothesized that the severity of CNS pathology may relate to an autoimmune reaction that varies from patient to patient and is minimal or missing in the ALD-deficient mice. Because of the inflammation, it is not surprising that substantial amounts of cholesterol ester accumulate in the ALD brain. Similar to other disorders in which myelin debris is phagocytosed, most of the cholesterol esters are in an abnormal floating fraction of lower density than the density of isolated myelin.

MRI has led to the frequent identification of inherited leukodystrophies of unknown cause, many of which exhibit a syndrome now called childhood ataxia with central nervous system hypomyelination (CACH) or vanishing white matter disease (VWMD) [27]. The syndrome is usually characterized by the onset of ataxia in early childhood followed by progressive neurological deterioration. Also, histological examination frequently reveals novel foamy-appearing oligodendrocytes with numerous round inclusions in these patients. Most patients with this syndrome have mutations in the mRNA translation initiation factor eIF2B.

Deficiencies of peripheral nerve myelin in common inherited human neuropathies are caused by mutations of genes for sheath proteins. Remarkable progress in elucidating an expanding number of gene loci and pathogenic mechanisms for inherited neuropathies has been achieved, based on our increasing knowledge of the biology of Schwann cells, axons and their interactions [28–30]. From a historical perspective, the most common inherited neuropathies were divided into two forms of Charcot–Marie–Tooth (CMT) disease largely on the basis of electrophysiology – type 1 (CMT1), which primarily affects myelin, and type 2 (CMT2), which primarily affects axons. In addition, there are milder (hereditary neuropathy with liability to pressure palsies), more severe (Dejerine–Sottas syndrome), and congenital (congenital hypomyelinating neuropathy) inherited neuropathies. Mutations affecting the same gene can cause neuropathies falling into more than one of these clinical categories depending on the exact nature of the mutation, with gain-of-function phenotypes usually being more severe than those associated with loss of function. Common forms of CMT1 are caused by dominant mutations of genes for compact myelin proteins, PMP-22 in CMT1A and P0 protein in CMT1B. CMT1A is usually due to duplication of the PMP-22 gene with onset in the second or third decade of life and characterized by segmental demyelination, remyelination and onion bulb formation. Hereditary neuropathy with liability to pressure palsies is a milder neuropathy brought on by pressure or trauma to an affected nerve and is caused by a heterozygous deletion of the PMP-22 gene. These human disorders, caused by over and under expression of PMP-22, respectively, illustrate the importance of dosage of some myelin proteins for myelin stability (see Ch. 4). Some more severe forms of CMT1A and Dejerine–Sottas syndrome have missense mutations in transmembrane regions of PMP-22 similar to the murine *trembler* mutations (see Ch. 4). CMT1B and some forms of Dejerine–Sottas

syndrome are caused by a variety of mutations in the major P0 glycoprotein of compact myelin, presumably impairing its capacity to stabilize the intraperiod and/or major dense lines or interfering with a function in signal transduction (see Ch. 4).

There are other forms of inherited neuropathy affecting myelin that are caused by mutations of genes that alter Schwann cell proteins not localized in compact myelin [28–30]. Some examples include: (1) an X-linked form (CMT1X) caused by mutations of the gap junction protein connexin 32 (CX32), which is localized in paranodal loops and incisures where it probably forms gap junctions between adjacent layers of myelin sheaths; (2) a recessive form (CMT4F) caused by mutations of periaxin, which is part of a protein complex linking the extracellular basal lamina to the actin cytoskeleton of Schwann cells; and (3) a dominant form of CMT1 caused by mutations of a zinc finger transcription factor (EGR2, also called Krox 20). It is clear that genetic defects in a variety of Schwann cell functions can cause myelin deficiencies, but discussion of the likely pathogenic mechanisms is beyond the scope of this chapter. Furthermore, as is the case for disorders of CNS myelin, it is becoming increasingly apparent that axonal pathology and degeneration are often responsible for much of the neurological impairment in these inherited disorders caused by mutations expressed in Schwann cell genes, possibly due to defects in Schwann cell–axon interactions.

TOXIC AND NUTRITIONAL DISORDERS OF MYELIN

A variety of biological and chemical toxins can impair myelin formation or cause its breakdown. Biological toxins affecting myelin-forming cells can be produced by exogenous infectious agents in diseases such as diphtheria or endogenously by lymphocytes and macrophages. For example, diphtheritic neuropathy [1] is a possible complication of *Corynebacterium diphtheriae* infection and is characterized by vacuolation and fragmentation of myelin sheaths in the PNS. Also, cytokines, such as tumor necrosis factor and lymphotoxins, released by lymphocytes and macrophages can be toxic to oligodendrocytes *in vitro*, but the nature of their involvement in the pathogenic mechanisms of autoimmune diseases *in vivo* may be quite complex [31]. Activated immune cells also release oxygen radicals and nitric oxide, which are harmful to oligodendrocytes. These toxins could lead to loss of myelin- forming cells and myelin independently of whether the immune reaction is directed against myelin or an exogenous infectious agent. Damage to myelin by an immune reaction directed to an unrelated antigen is referred to as a 'bystander effect'.

Lead is a common environmental pollutant that is well known to cause hypomyelination and demyelination. Other chemical toxins affecting myelin-forming cells or myelin and that have been investigated experimentally include cuprizone, lysolecithin, triethyltin, hexachlorophene and tellurium. The effects of these and other chemical toxins on the biochemistry of myelin is beyond the scope of this chapter but have been covered in more detail in earlier editions of this book or in other sources [1].

General undernourishment or dietary deficiencies of specific substances can lead to a preferential reduction in myelin formation. Much of the CNS myelin in mammals is formed during a relatively restricted time period of rapid development. During this period, a large portion of the brain's metabolic activity and synthetic capacity are involved in myelinogenesis. Therefore, any metabolic insult during this 'vulnerable period' may lead to a preferential reduction in myelin formation [1]. The most vulnerable period appears to be the time of oligodendroglia proliferation, since animals deprived of food in this period have an irreversible deficit of myelin-forming cells and hypomyelination (see also Ch. 25). However, animals deprived at a later age can often demonstrate significant catch-up with regard to the amount of myelin when nutritional rehabilitation is instigated after a period of underfeeding. Failure to myelinate properly and demyelination are also associated with deficiencies of dietary protein, essential fatty acids, copper and several vitamins, including thiamine, B_{12} and B_6 [1].

DISORDERS PRIMARILY AFFECTING NEURONS WITH SECONDARY INVOLVEMENT OF MYELIN

Many insults to the nervous system initially cause damage to neurons but eventually result in regions of demyelination as a consequence of neuronal degeneration. The archetypical model for secondary demyelination is Wallerian degeneration (see Ch. 30). When a nerve (in the PNS) or a myelinated tract (in the CNS) is cut or crushed, the proximal segment often survives and regenerates [1]. In the distal segment, Wallerian degeneration occurs with disappearance of both axons and myelin and phagocytosis of the debris. From such experiments, it is clear that the integrity of the myelin sheath depends on continued contact with a viable axon. Any disease that causes injury to neurons may result in axonal degeneration and lead to the onset of myelin breakdown secondary to the neuronal damage. During Wallerian degeneration in the PNS, there is a rapid loss of myelin-specific lipids and proteins within a week or two with a concomitant increase in lysosomal enzymes. Between the second and fourth week after nerve section most of the myelin debris has been removed by Schwann cells and macrophages, and remyelination of regenerating axons begins. Degeneration of CNS myelin is a much slower process than in the PNS and takes place primarily within macrophages/microglia (not within the myelin-synthesizing cells, as in the PNS).

Secondary demyelination occurs in a variety of acquired and genetic CNS disorders that initially affect neurons, including mechanical trauma, infarcts, tumors, motor neuron disease, viral diseases such as subacute sclerosing panencephalitis, and sphingolipidoses such as Tay–Sachs disease, generalized gangliosidosis and Niemann–Pick disease (see Ch. 40). The isolated myelin from these diseases is usually of the 'nonspecific pathological type' that was discussed earlier in the context of some inherited primary demyelinating disorders. No cholesterol esters are found in the myelin, although they are abundant in the white matter, indicative of phagocytosis by macrophages.

REPAIR IN DEMYELINATING DISEASES

The capacity for remyelination is generally greater in the peripheral than in the central nervous system. Much of this chapter has considered biochemical mechanisms of myelin loss, but the capacity of nervous tissue to repair the damage by remyelination is also an important factor in the eventual clinical outcome of disorders affecting myelin. Following transection of peripheral nerve, as described in the previous section, myelination of the regenerating axons occurs soon after the final clean-up of myelin debris by Schwann cells [1]. The Schwann cells that form the new myelin are probably not the same ones that phagocytose the debris but appear to arise by cell division. The smaller capacity for remyelination in the CNS in comparison to the PNS may be caused in part by the nature of the myelin-forming cells, i.e. in the requirement for oligodendrocytes to myelinate many axons in contrast to the single segments of myelin formed by Schwann cells. Under some circumstances, such as in EAE and MS, it has been demonstrated that Schwann cells will migrate into the spinal cord and remyelinate demyelinated CNS axons.

Remyelination in the CNS can be promoted by various treatments. Substantial remyelination can occur in the CNS depending on the nature of the pathogenic insult, and it is likely that therapeutic procedures that enhance remyelination are feasible [2, 32, 33] (discussed in detail in Ch. 30). For example, rapid remyelination by oligodendrocytes does occur following acute demyelination caused by toxins such as cuprizone or lysolecithin, although the myelin sheaths are thinner than normal. The failure of significant remyelination in other circumstances may relate to the continuing effect of pathogenic processes. However, even in MS, careful observations of acute lesions have demonstrated the presence of healthy oligodendrocytes and regeneration of myelin in the presence of myelin breakdown. Although the remyelination attempt eventually fails and oligodendrocytes are lost from lesions that progress to advanced demyelinated plaques, such observations raise the possibility that management of patients with demyelinating diseases of the CNS may eventually involve treatments that take advantage of the capacity of oligodendrocytes for remyelination.

Research on experimental animals as well as human diseases indicates that remyelination can occur by proliferation and differentiation of host progenitor cells, which are quiescent in normal adult tissue (Ch. 29). Several approaches are being used to attempt to enhance the natural capacity of oligodendrocytes or their progenitors to remyelinate. Because a large number of growth factors (Ch. 27), such as epidermal growth factor, basic fibroblast growth factor, platelet derived growth factor, ciliary neurotrophic factor and insulin-like growth factor, are known to affect the survival, proliferation, migration and differentiation of oligodendrocyte progenitors, it is feasible that remyelination might be promoted in patients by treatment with one or more of these factors. An interesting finding in MS is that transforming factor-β produced by inflammatory cells induces expression in hypertrophic astrocytes of *jagged*, which activates the Notch signaling pathway in oligodendroglial precursors [34]. This appears to inhibit their capacity for remyelination and may provide a specific site for therapeutic intervention. Another observation that has relevance for enhancing remyelination is that various immunoglobulin preparations containing antibodies reacting with surface antigens of oligodendrocytes have the capacity to promote remyelination in experimental animals by activating a calcium ion signaling system [35]. An approach actively being pursued at this time in many laboratories involves the capacity of transplants of myelin-forming glial cells to repair demyelinated lesions [2, 35]. Much of this research involves the transplantation of Schwann cells, oligodendrocytes, oligodendrocyte progenitors, glial cell lines or stem cells into hypomyelinated mutants or focally demyelinated lesions and has clearly demonstrated that transplanted cells have the capacity to remyelinate. A promising report shows that xenografting isolated fetal or adult human oligodendroglial progenitors into dysmyelinated *shiverer* mouse brain results in widespread and robust myelination [36]. A serious problem in MS is that, if there is a window of time after an insult during which a particular stimulus is effective for regeneration, in MS there are multiple windows of time for multiple locations. Finally, it is worth noting that a combination of these different therapies may provide the best results, and the optimal treatment may vary from one myelin disorder to another. Further research is needed to evaluate the success of these approaches in the presence of astrocytic gliosis and inflammation (Ch. 30).

Measures to enhance axonal survival and regeneration also are critical in demyelinating conditions. With the increasing awareness that substantial axonal degeneration occurs in many diseases that had previously been thought to affect primarily myelin and that this may account for much of the permanent neurological deficit, increasing attention is being given to therapies that promote axonal

survival and regeneration. Various growth factors are likely to be beneficial in this regard. However, a complicating factor is that white matter is well known to contain a number of inhibitors of axonal regeneration, including Nogo, MAG and OMgp [37] (see Ch. 30). Nevertheless, it is promising that each of these inhibitory proteins appears to act through the same Nogo receptor signaling system, so that it may be feasible to block the effects of all three simultaneously. In conclusion, therapy for myelin disorders in the future may involve a combination of approaches aimed at preventing myelin loss, while at the same time promoting the survival, regeneration and remyelination of axons.

REFERENCES

1. Morell, P. Myelin. New York: Plenum Press, 1984.
2. Lazzarini, R. A. *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004.
3. Iglesias, A., Bauer, J., Litzenburger, T. *et al.* T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia* 36: 220–234, 2001.
4. Lassmann, H. Experimental autoimmune encephalomyelitis. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 1039–1071.
5. Fazakerley, J. K. and Walker, R. Virus demyelination. *J. Neurovirol.* 9: 148–164, 2003.
6. Bieber, A. J. and Rodriguez, M. Experimental models of virus-induced demyelination. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 1073–1100.
7. Steinman, L., Martin, R., Bernard, C. *et al.* Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu. Rev. Neurosci.* 25: 491–505, 2002.
8. Blevins, G. and Martin, R. Future immunotherapies in multiple sclerosis. *Semin. Neurol.* 23: 147–158, 2003.
9. Trapp, B. D., Peterson, J., Ransohoff, R. M. *et al.* Axonal transection in the lesions of multiple sclerosis. *N. Engl J. Med.* 338: 278–285, 1998.
10. Lucchinetti, C., Bruck, W., Parisi, J. *et al.* Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* 47: 707–717, 2000.
11. Cuzner, M. L. and Norton, W. T. Biochemistry of demyelination. *Brain Pathol.* 6: 231–242, 1996.
12. Quarles, R. H. Myelin sheaths: glycoproteins involved in their formation, maintenance and degeneration. *Cell. Mol. Life Sci.* 59: 1851–1871, 2002.
13. Kim, J. K., Mastronardi, F. G., Wood, D. D. *et al.* Multiple sclerosis: an important role for post-translational modifications of myelin basic protein in pathogenesis. *Mol. Cell. Proteomics* 2: 453–462, 2003.
14. Berger, T., Rubner, P., Schautzer, F. *et al.* Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. *N. Engl J. Med.* 349: 139–145, 2003.
15. Fogdell-Hahn, A., Soldan, S. S. and Jacobson, S. Association of chronic progressive neurological disease and ubiquitous viral agents: lessons from human herpesvirus 6 and multiple sclerosis. *Mol. Psychiatry* 7 Suppl 2, S29–31, 2002.
16. Antel, J. and Bar-Or, A. Multiple slerosis therapy. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 791–806.
17. Stuve, O., Kita, M., Pelletier, D. *et al.* Mitoxantrone as a potential therapy for primary progressive multiple sclerosis. *Mult. Scler.* 10(Suppl 1): S58–S61, 2004.
18. Miller, D. H., Khan, O. A., Sheremata, W. A. *et al.*, International Natalizumab Multiple Sclerosis Trial Group. A controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl J. Med.* 348: 15–23, 2003.
19. Hartung, H. P., Willison, H. J. and Kieseier, B. C. Acute immunoinflammatory neuropathy: update on Guillain–Barré syndrome. *Curr. Opin. Neurol.* 15: 571–577, 2002.
20. Kieseier, B. C., Dalakas, M. C. and Hartung, H. P. Immune mechanisms in chronic inflammatory demyelinating neuropathy. *Neurology* 59, S7–S12, 2002.
21. Willison, H. J. and Yuki, N. Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125: 2591–2625, 2002.
22. Albright, A. V., Soldan, S. S. and Gonzalez-Scarano, F. Pathogenesis of human immunodeficiency virus-induced neurological disease. *J. Neurovirol.* 9: 222–227, 2003.
23. Hudson, L. D., Garbern, J. Y. and Kamholz, J. A. Pelizaeus–Merzbacher disease. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 867–886.
24. Shy, M. E., Hobson, G., Jain, M. *et al.* Schwann cell expression of PLP1 but not DM20 is necessary to prevent neuropathy. *Ann. Neurol.* 53: 354–365, 2003.
25. Surendran, S., Matalon, K. M., Tyring, S. K. *et al.* Molecular basis of Canavan's disease: from human to mouse. *J. Child Neurol.* 18: 604–610, 2003.
26. Moser, H. W. Adrenoleukodystrophies. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 807–840.
27. Schiffmann, R. and van der Knaap, M. S. The latest on leukodystrophies. *Curr. Opin. Neurol.* 17: 187–192, 2004.
28. Shy, M. E., Garbern, J. Y. and Kamholz, J. Hereditary motor and sensory neuropathies: a biological perspective. *Lancet Neurol.* 1: 110–118, 2002.
29. Suter, U. and Scherer, S. S. Disease mechanisms in inherited neuropathies. *Nat. Rev. Neurosci.* 4: 714–726, 2003.
30. Wrabetz, L., Feltri, M. L., Kleopa, K. A. *et al.* Inherited neuropathies: clinical, genetic and biological features. In R. A. Lazzarini (ed.), *Myelin Biology and Disorders*. San Diego, CA: Elsevier Academic Press, 2004, pp. 905–952.
31. Merrill, J. E. and Scolding, N. J. Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. *Neuropathol. Appl. Neurobiol.* 25: 435–458, 1999.
32. Franklin, R. J. Why does remyelination fail in multiple sclerosis? *Nat. Rev. Neurosci.* 3: 705–714, 2002.
33. Colman, D., Lubetzki, C. and Reingold, S. Multiple paths towards repair in multiple sclerosis. *Trends Neurosci.* 26: 59–61, 2003.
34. John, G. R., Shankar, S. L., Shafit-Zagardo, B. *et al.* Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat. Med.* 8: 1115–1121, 2002.
35. Paz Soldan, M. M. and Rodriguez, M. Heterogeneity of pathogenesis in multiple sclerosis: implications for promotion of remyelination. *J. Infect. Dis.* 186(Suppl 2): S248–S253, 2002.
36. Windrem, M. S., Nunes, M. C., Rashbaum, W. K. *et al.* Fetal and adult human oligodendrocyte progenitor cell isolates

myelinate the congenitally dysmyelinated brain. *Nat. Med.* 10: 93–97, 2004.
37. Filbin, M. T. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat. Rev. Neurosci.* 4: 703–713, 2003.
38. Wierzbicki, A. S., Lloyd, M. D., Schofield, C. J. *et al.* Refsum's disease: a peroxisomal disorder affecting phytanic acid alpha-oxidation. *J. Neurochem.* 80: 727–735, 2002.
39. Johnson, D., Sato, S., Quarles, R. H. *et al.* Quantitation of the myelin-associated glycoprotein in human nervous tissue from controls and multiple sclerosis patients. *J. Neurochem.* 46: 1086–1093, 1986.

CHAPTER

39

Genetics of Neurodegenerative Diseases

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Rudolph E. Tanzi

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GENETIC ASPECTS OF COMMON NEURODEGENERATIVE DISEASES

Introduction to the genetics of neurodegenerative disease. For most of the neurodegenerative conditions outlined in this chapter, familial aggregation had already been recognized as a salient feature decades before any of the underlying molecular genetic and biochemical properties came to light. As a matter of fact, it was often only the identification of specific, disease-segregating mutations in previously unknown genes that directed the attention of molecular biologists to particular proteins and pathways (via 'positional cloning', see caption to Fig. 39-1) that are now considered to be crucial in the development of the various diseases. These include the discovery of mutations in the β-amyloid precursor protein causing Alzheimer's disease (AD), mutations in α-synuclein causing Parkinson's disease, or more recently mutations in the microtubule-associated protein tau causing frontotemporal dementia with parkinsonism. Another feature observed in most common neurodegenerative diseases – as well as in other common conditions like diabetes or certain forms of cancer – is the prominent dichotomy of familial (rare) *versus* seemingly nonfamilial (common) forms. The latter are also frequently described as 'sporadic' or 'idiopathic' forms, although this terminology has proven overly simplistic and would best be abandoned. This is based on the growing body of evidence suggesting that a large proportion of nominally 'sporadic' cases are significantly influenced by genetic factors, e.g. reflected by a significant increase in disease risk in first-degree relatives of index cases. In most cases of neurodegenerative disease, the rule of thumb is that the earlier the age-at-onset, the greater the impact of genetic factors on causation/susceptibility.

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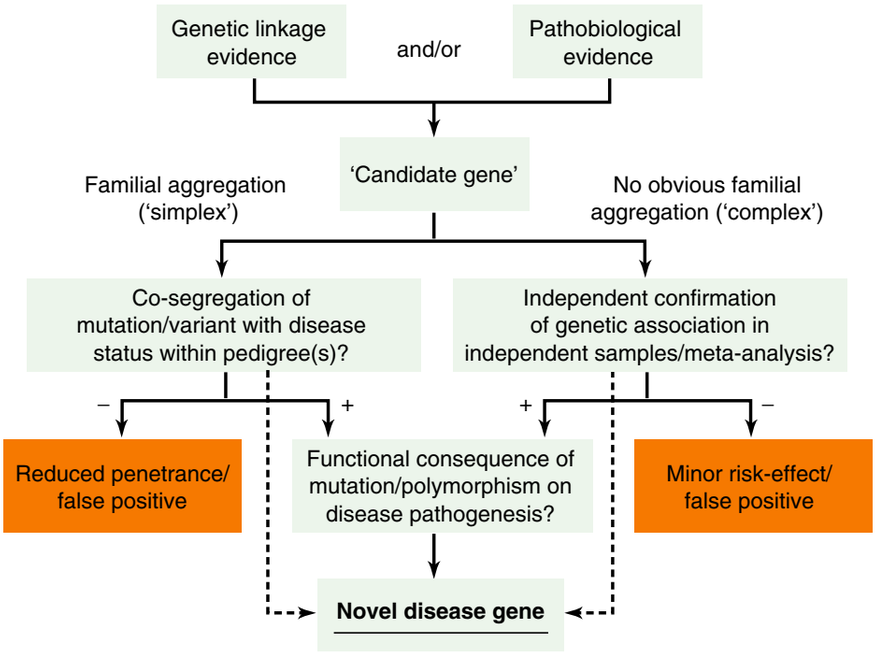

FIGURE 39-1 Simplified flow-chart of the strategies used to identify novel disease genes. This schema outlines one popular strategy to identify mutations and polymorphisms causing or predisposing to disease. Candidate genes are chosen on the basis of genetic linkage data and/or known or hypothesized pathobiological relevance to disease mechanisms. This procedure is typically referred to as 'candidate gene approach' (and contributed to the identification of *APOE* as a risk factor for AD). An alternative and inherently similar strategy is based on the detection of formerly unknown genes/proteins purely driven by genetic linkage data and is referred to as 'positional cloning' (examples include the identification of *PSEN1* mutations in AD). Broken lines indicate 'shortcuts' allowing for the elucidation of a novel disease gene based on the genetic evidence alone, e.g. *APOE*-ε4, of which the precise functional consequences still remain unknown. Note, that there are examples of genes/mutations with reduced penetrance or minor risk effects (*red boxes*) which are considered to be *bona fide* disease genes (e.g. certain mutations in *PSEN1* in Alzheimer's disease); however, it is usually much more difficult to conclusively prove their genetic and biochemical roles, which is why they are depicted separately.

Methods to find neurodegenerative disease genes. Despite the previous successes and recent advances in molecular and analytical techniques, the identification of genuine risk factors for the diseases outlined in this chapter – genetic and nongenetic – is aggravated by several circumstances. First, while diagnostic criteria have been proposed for all syndromes, these are usually based on clinical and/or neuropathological observations, which are never 100% specific and may even be assessed differently from one research center to the next. Thus, a sample described to consist of probable (i.e. clinically diagnosed) late-onset Alzheimer's disease cases, may actually be a conglomerate of patients with Alzheimer's, Lewy body dementia and frontotemporal dementia. A second, and related issue is that most neurodegenerative diseases manifest in old age, i.e. beyond 60 or 70 years. This makes the assessment of reliable familial histories – a prerequisite for genetic analyses – very difficult because a number of relatives may not have lived through the typical onset age, or may suffer from other conditions, which can mask or mimic the phenotype of interest. Third, common diseases typically display a large degree of genetic and phenotypic heterogeneity. This means that the same phenotype can be caused or modified by a number of different genetic loci and alleles but also that mutations or polymorphisms in the same gene may lead to clinically distinct syndromes. Further, certain combinations of genetic and nongenetic risk factors may significantly increase the odds of developing a disease in one ethnic group or geographical area, while another set of factors may be acting together elsewhere. The complexities observed in a variety of late-onset, multifactorial diseases are schematically displayed in Figure 39-2, using familial versus nonfamilial Alzheimer's disease as an example.

Collectively, these and other issues have led to the accumulation of a large number of proposed susceptibility genes and environmental risk factors in all the diseases covered in this chapter. However, these have received inconsistent support at best from subsequent and independent studies. Novel, more sophisticated analytical methods are needed to more efficiently filter genuine signals from background noise (i.e. false-positive findings) and to elucidate the complex interaction patterns between variables. Meta-analysis, i.e. the estimation of summary effect sizes and significances across a variety of independent studies, is one such technique, which is becoming

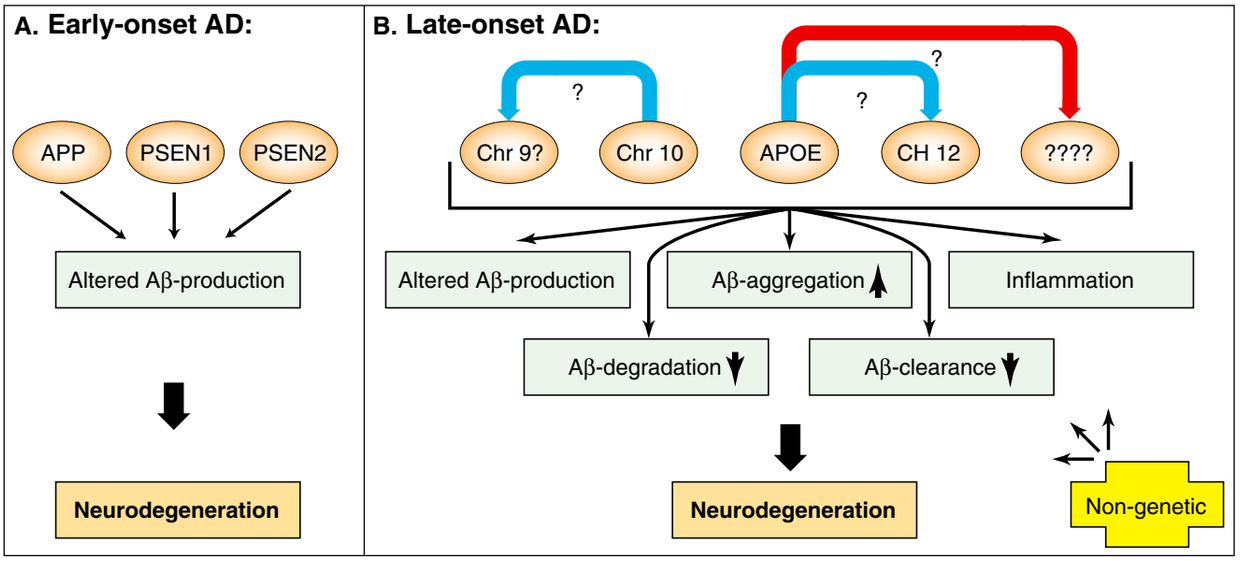

FIGURE 39-2 Scheme of contribution and interaction pattern of known and putative Alzheimer's disease (AD) genes. (**A**) Mutations in the familial, early-onset AD genes *APP*, *PSEN1*, *PSEN2* lead to an increase in Aβ-production and neurodegeneration without any other known essential factors. (**B**) Simplified scheme of the interaction pattern of known and proposed nonMendelian, late-onset AD genes. Probably, these risk-factor genes each affect one or more of the known or postulated pathogenetic mechanisms leading to neurodegeneration in AD. Their effects are further influenced by gene–gene interactions and the contribution of nongenetic risk factors. Note that none of the interaction patterns outlined here for didactic purposes has actually been established. (With permission from reference [97].)

increasingly more popular in the field of neurodegenerative diseases. Figure 39-1 outlines a simplified scheme of experimental strategies typically involved in the identification of novel disease genes.

Notwithstanding these difficulties, genetic analyses have laid the foundation to advance our understanding of a wide variety of disease mechanisms leading to neurodegeneration and associated symptoms. Likewise, a detailed understanding of the genetic basis of neurodegeneration will be essential for the development of effective strategies aimed at the early-prediction and early-prevention/treatment of these devastating diseases. In the following sections, we outline both the peculiarities and the similarities of genetics findings across a number of common neurodegenerative conditions. Note that more details on the molecular genetic consequences as well as neuropathogenetic mechanisms influenced by the various disease genes discussed here can be found in subsequent chapters of this book.

ALZHEIMER'S DISEASE

Alzheimer's disease is the most common form of age-related dementia and one of the most serious health problems in the industrialized world. AD is an insidious and progressive neurodegenerative disorder that accounts for the vast majority of dementia and is characterized by global cognitive decline and the accumulation of β-amyloid deposits and neurofibrillary tangles in the brain. Family history is the second greatest risk factor for the disease after age. The growing understanding of AD genetics over the last two decades has been central to the explosion in knowledge of the biology of the disease from the neuropathological to molecular levels. Genetically, AD is complex, heterogeneous and appears to follow an age-related dichotomy in which rare and highly penetrant early-onset (<60) familial AD (EOFAD) mutations are transmitted in an autosomal dominant fashion, while increased risk for late-onset AD without clear Mendelian inheritance (LOAD) is mainly conferred by common polymorphisms with relatively low penetrance but high prevalence [1]. Adding to the complexity and heterogeneity of AD genetics – as well as to the genetics of other neurodegenerative disorders – are currently ill-defined and difficult to model gene-gene and gene-environment interactions.

Early-onset familial Alzheimer's disease. Early-onset familial AD (EOFAD) represents only a small fraction of all AD cases (<5%) and typically presents with onset ages prior to the completion of the sixth decade, showing Mendelian, autosomal dominant disease transmission within affected families. To date, more than 160 mutations in three genes have been reported to cause EOFAD (Table 39-1). These include the β-amyloid precursor protein (*APP*) on chromosome 21 [2], presenilin 1 (*PSEN1*) on chromosome 14 [3] and presenilin 2 (*PSEN2*) on chromosome 1 [4, 5]. The most frequently mutated gene, *PSEN1*, generally leads to AD with onset ages in the 40s, and accounts for the majority of AD cases with onset prior to age 50 [6].

TABLE 39-1 Established genes causing Alzheimer's disease

Gene	Protein	Location	Inheritance	Relevance to Alzheimer's disease pathogenesis
APP	β-amyloid precursor protein	21q21	Dominant	Aβ production (Aβ↑; $A\beta_{42}$:$A\beta_{40}$ ratio↑)/aggregation
APOE	Apolipoprotein E	19q13	Risk factor	Unknown (Aβ clearance? Lipid metabolism?)
PSEN1	Presenilin 1	14q24	Dominant	Aβ production ($A\beta_{42}$:$A\beta_{40}$-ratio↑)
PSEN2	Presenilin 2	1q31	Dominant	Aβ production ($A\beta_{42}$:$A\beta_{40}$-ratio ↑)

While these AD-causing mutations occur in three different genes located on three different chromosomes, they all share a common biochemical pathway, i.e. the altered production of Aβ most frequently leading to a relative overabundance of the $A\beta_{42}$ species, eventually resulting in neuronal cell death and dementia. Aβ is produced by the sequential cleavage of *APP* by two enzymatic events, β- and γ-secretase cleavage. In contrast, the more common α-secretase cleavage (e.g. by ADAM10 or ADAM17) results in the production of a nonamyloidogenic soluble APPα fragment. Interestingly, all currently known AD-causing mutations in the APP gene are located in the vicinity of the secretase cleavage sites, encoded by exons 16 and 17. Moreover, the presenilins, together with three other proteins (Pen-2, Aph-1a and nicastrin) are essential components of the enzymatic complex responsible for γ-secretase cleavage of *APP* [7]. These discoveries have provided the essential connection between the occurrence of disease-causing mutations in *APP*, *PSEN1* and *PSEN2* and the increase in Aβ (particularly $A\beta_{42}$) production in the brains of AD patients.

***APOE* is the best established late-onset Alzheimer's disease gene.** Late-onset AD (LOAD) is classically defined as AD with onset ≥65 years and represents the vast majority of all AD cases. While segregation and twin-studies conclusively suggest a major role of genetic factors in this form of AD [8], to date, only one such gene has been firmly established for LOAD, the ε4 variant of the apolipoprotein E gene (*APOE*; Table 39-1) [9, 10]. In contrast to all other association-based findings in AD, the risk effect of *APOE*-ε4 has been consistently replicated in a large number of studies across many ethnic groups with odds ratios ranging from 1 to 6 for heterozygous and from 2 to 33 for homozygous carriers of the ε4 allele (for meta-analysis, see [11]). The three major alleles of the *APOE* locus, ε2, ε3 and ε4, correspond to combinations of two amino acid changes at residues 112 and 158 (ε2: Cys/Cys; ε3: Cys/Arg; ε4: Arg/Arg). In addition to the increased risk for AD exerted by the ε4-allele, several studies have also reported a weak, albeit significant, protective effect for the minor allele, ε2. Unlike the mutations in the known EOFAD genes, the *APOE*-ε4 allele is not sufficient to cause AD but instead operates as a genetic risk-modifier by decreasing the age of onset in a dose-dependent manner.

Despite its long-known and well-established genetic association with AD, the biochemical consequences of *APOE*-ε4 on AD pathogenesis are not yet fully understood. Current hypotheses are based on the observation that Aβ accumulation is clearly enhanced in the brains of carriers as well as in transgenic mice expressing the human ε4 allele and mutant APP (reviewed in [12]). Furthermore, the apoE protein normally plays a role in cholesterol transport and lipid metabolism; *APOE*-ε4 predisposes to heart disease as a result of its association with increased plasma cholesterol levels. High plasma cholesterol, in turn, has been correlated with increased β-amyloid deposition in the brain. Interestingly, cholesterol has also been shown both to increase Aβ production and to stabilize the peptide in the brains of transgenic AD mice. Thus, it is possible that *APOE*-ε4 confers risk for AD via a mechanism that is shared in common with its effect on cardiovascular disease by increasing a carrier's risk for hypercholesterolemia, as this would be expected to elevate accumulation of Aβ.

Other late-onset Alzheimer's disease loci. Several lines of evidence suggest that numerous additional LOAD and EOFAD loci still remain to be identified, since the four known AD genes together account for less than 30% of the genetic variance of AD [13]. There is also direct evidence for such genes based on simulation as well as empirical data. For instance, a recent simulation study predicted the existence of four to seven additional AD genes when searching for age of onset modifiers simulating a variety of different disease and inheritance models [13]. This number corresponds well with empirical data obtained in several full genome screens for novel AD genes performed to date, which indicate overlap on 11 chromosomes. Five different chromosomes reveal 'significant' results in at least one study in addition to the *APOE* region on chromosome 19 (i.e. chromosomes 6q, 9p, 10q, 12p, 21q) [14]. These disease-specific characteristics, together with the advent of relatively inexpensive and powerful high-throughput genotyping technologies and the completion of the human genome sequence have led to a steep increase in the number of laboratories investigating the genetics of AD worldwide.

Currently, more than three dozen genes that have been tested on positional (chromosomal location) and/or functional (biological) grounds have been reported to be associated with AD [15, 16]. Despite these vast efforts, however, no single gene has yet emerged with the degree of replication and consistency that has been observed by literally hundreds of laboratories that have addressed the association of *APOE*-ε4 and AD. While no firm conclusions can be drawn at this time, it is noteworthy that several of the more extensively studied (and replicated) putative novel AD candidates are – similar to the proposed role for *APOE* – directly involved in the clearance and/or degradation of extracellular Aβ (e.g. *LRP1* (lipoprotein

receptor related protein-1), *A2M* (α2-macroglobulin), *IDE* (insulin degrading enzyme)), lending further support to the hypothesis put forward by several authors that LOAD may be caused by deficits in the removal of Aβ, in contrast to the EOFAD mutations, which lead to increased *production* of the peptide (Fig. 39-2). Other physiological pathways that have been genetically associated with AD include inflammation (e.g. *IL1A* and *IL1B* (interleukin-1α and 1β), *IL6* (interleukin-6)), cholesterol metabolism (e.g. *SOAT1* (acyl-coenzyme A:cholesterol acyltransferase, or ACAT), *CYP46* (cholesterol 24-hydroxylase) and *CH25H* (cholesterol 25-hydroxylase), as well as the production of Aβ, itself (e.g. *PSEN1* and *NCSTN* (nicastrin, see above)). An up-to-date overview on the status of these and other potential AD candidate genes, including meta-analyses across published genetic association studies, can be found in the Alzheimer's Research Forum genetic database at www.alzgene.org.

PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disease of adult onset and, like almost all the other diseases outlined in this chapter, exhibits an increased prevalence with age but with an average age of onset between 50 and 60 years. Histopathologically, it is characterized by a severe loss of dopaminergic neurons in the substantia nigra and cytoplasmic inclusions consisting of insoluble protein aggregates (Lewy bodies) leading to a progressive movement disorder including the classic triad of tremor, bradykinesia and rigidity. Although the heritability – and thus the contribution of genetic factors to the overall prevalence of PD – is probably smaller than that of AD, genetics has played a major role in elucidating the potential causes of nigrostriatal neuronal loss across a wide spectrum of clinically and histopathologically heterogeneous cases of PD. As in AD, there appears to be an age-dependent dichotomy, the majority of cases with an early or even juvenile onset showing typical Mendelian inheritance. However, unlike AD, these cases show a predominantly autosomal recessive mode of inheritance and there is an ongoing debate as to whether genetic factors play any substantial role in contributing to disease risk in cases with onset beyond ≈50 years [17–19]. Notwithstanding these complexities, there are a plethora of genetic studies for both forms of the disease, and mutations in at least five genes have now been shown to cause familial early-onset PD (Table 39-2), with several other linkage regions pending characterization and/or replication. However, no single genetic susceptibility factor for late-onset PD has yet received anything like the same degree of support as *APOE*-ε4 in late-onset AD (see above).

Early-onset familial Parkinson's disease. Early-onset familial PD represents only a minority of all PD cases. While currently nearly a dozen chromosomal regions have been reported to potentially harbor disease-causing PD loci, only mutations in five of these have been shown to actually segregate with the disease beyond the initial study (Table 39-2). Similar to AD, the first locus to be characterized – *PARK1*, on chromosome 4q21 – involves the protein that is the major constituent of one of the classic neuropathological hallmarks of PD, α-synuclein (*SNCA*) [20]. Aggregated α-synuclein is at the core of Lewy bodies, round eosinophilic intracellular inclusion bodies that are found in affected brain regions. To date, three missense mutations in *SNCA* (Ala53Thr, Ala30Pro and E46K) have been described in a handful of families worldwide (reviewed in [21]). While the exact mechanisms underlying α-synuclein toxicity currently remain only incompletely understood, recent evidence suggests that *SNCA* mutations may change normal protein function quantitatively rather than qualitatively [22]. This is based on the observation that triplication of the α-synuclein gene causes PD in a large early-onset autosomal dominant pedigree [23]. Again, this bears a striking similarity to AD, where triplication of the *APP*-containing chromosome 21 causes an AD-like neurodegeneration in middle-aged patients with trisomy 21 (Down's syndrome). Subsequently, other autosomal-dominant PD pedigrees have been identified showing either triplication [24] or duplication of *SNCA* [22, 25], suggesting that genetic variants in this gene may play a more prominent role in PD pathogenesis than was previously assumed. Furthermore, in the few families studied to date, there appears a correlation between gene dose and clinical phenotype: while *SNCA* duplications apparently lead to a more 'typical' PD syndrome with onset ages around 50 years, triplication of the gene leads to a less typical clinical picture with earlier onset (in the 30s) and signs of Lewy body dementia (LBD, see below) in one of the pedigrees [23]. In addition to these findings in familial PD, there is some – albeit less clearly established – support for a potential role of *SNCA* polymorphisms on risk of late-onset PD [26]. Recently, mutations in a second gene with dominant inheritance have been identified by several different laboratories (*LRRK2* [26a, 26b]). While the functional consequences of *LRRK2* mutations are still unknown, it was suggested that at least some mutations could interfere with the protein's kinase activity [26c].

TABLE 39-2 Established genes causing Parkinson's disease

Gene	Protein	Location	Inheritance	Relevance to Parkinson's disease pathogenesis
SNCA	α-synuclein	4q21	Dominant	Neurotoxicity by aggregation of α-synuclein (?)
PARK2	Parkin	6q25	Recessive	Impaired protein degradation via proteasome
PARK7	DJ1	1p36	Recessive	Impaired oxidative stress response (?)
PINK1	PTEN-induced putative kinase 1	1p36	Recessive	Mitochondrial dysfunction (?)
LRRK2	Leucine-rich repeat kinase; dardarin	12q12	Dominant	Unknown

Parkin accounts for the majority of early-onset Parkinsonism. While *SNCA* and *LRRK2* mutations are the leading cause of autosomal dominant familial forms of PD, the majority of affected pedigrees actually exhibit a recessive mode of inheritance. To date, mutations in three genes have been reported to cause this latter form of familial parkinsonism (Table 39-2). The most frequently involved gene is *parkin* [27], formerly designated as *PARK2*, on chromosome 6q25 [28], which probably causes nearly half of all early-onset cases. Parkin is an ubiquitin ligase that is involved in the ubiquitination of proteins targeted for degradation by the proteasomal system. Because the spectrum of parkin variants ranges from amino-acid-changing single-point mutations to complex genomic rearrangements and exon deletions, it is likely that *parkin* mutations confer their pathogenetic effects via a loss of function. More specifically, it has been speculated that a loss of parkin activity may trigger cell death by rendering neurons more vulnerable to cytotoxic insults, e.g. the accumulation of glycosylated α-synuclein [29]. However, as none of the various parkin substrates identified to date show a specific enrichment in dopaminergic neurons, further research is required to elucidate the exact role of parkin dysfuntion in PD neuropathogenesis. It is noteworthy, that – unlike α-synuclein-related PD – Lewy bodies are typically not found in brains of cases bearing *parkin* mutations, potentially suggesting the existence of additional, α-synuclein-independent disease mechanisms.

***DJ1* and *PINK1* are recessively acting Parkinson's disease genes.** Two recessively acting early-onset PD genes, *DJ1* and *PINK1*, have been discovered more recently. Thus, only a little evidence has been accrued on their potential functional involvement in PD neuropathogenesis. In 2003, genetic analyses of two early-onset PD pedigrees from the Netherlands and Italy revealed two independent, homozygous mutations in the *DJ1* gene [30] on chromosome 1p36 (previously designated as *PARK7*) [31]. Both result in a loss of function of DJ1, a ubiquitously expressed protein that is suggested to be involved in oxidative stress response. While several subsequent studies in independent early-onset PD samples have confirmed the presence of additional DJ1 mutations, the frequency of disease-causing variants in this gene is estimated to be low, i.e. in the order of ≈1% [32]. Less than 13 Mb away on the same chromosome, two PD-causing mutations were discovered in *PINK1*, encoding PTEN-induced putative kinase I [33], previously designated as *PARK6* [34]. This enzyme is ubiquitously expressed with particularly high levels in brain, and the first two identified mutations (G309D and W437ter) were predicted to lead to a loss of function that – similar to the effects of *parkin* mutations – may render neurons more vulnerable to cellular stress. Interestingly, the predominant subcellular localization of the PINK1 protein is mitochondrial, which is also the primary target of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent neurotoxin that produces a syndrome almost indistinguishable from sporadic PD [35]. In only 4 months after its original discovery, there have been reports of at least a dozen additional disease-causing *PINK1* mutations [36, 37], rendering this locus the second most common cause of early-onset familial PD. With regard to pathology, it remains unclear whether or not mutations in *DJ1* and *PINK1* lead to the formation of Lewy bodies in the brains of affected patients.

Other proposed early-onset Parkinson's disease loci. In addition to the confirmed early-onset familial PD genes, at least six other candidate PD loci, including mutations in two genes, have been described. The putative disease-causing latter are located in the ubiquitin carboxy-terminal hydrolase L1 (*UCHL1*) [38] on chromosome 4p14 (also designated *PARK5*), and in a nuclear receptor of subfamily 4 (*NR4A2* or *NURR1*) [39] located on 2q22. However, in contrast to the previously outlined PD genes, neither of these genes maps to a known PD linkage region, and no studies beyond the initial reports were able to confirm the existence of either the reported or additional mutations in screens of independent samples. In fact, missense mutations in *NR4A2* were also found in patients with schizophrenia and bipolar affective disorder, arguing against a specific role of variants in this gene in neurodegeneration [40]. Furthermore, polymorphisms in both genes have been – albeit inconsistently – associated with PD in some case-control studies. In this context, it is noteworthy that a recent meta-analysis on all currently available association studies for the S18Y polymorphism in *UCHL1* showed a modest, but significant protective effect of the Y-allele [41], suggesting that this gene may actually be a susceptibility factor rather than a causal PD gene.

Late-onset Parkinson's disease. Late-onset PD is caused by a variety of different factors, to which genes may only contribute to a relatively minor extent in comparison with early-onset forms of the disease [17] (see above). As outlined above, the heritability of this form of parkinsonism is probably low, which attenuates the ability to find consistent gene signals. Despite these concerns, a number of whole-genome screens across several independent later-onset PD family samples have been performed. As might be expected, the results have varied considerably, with only few overlapping genomic intervals. One of the more extensively studied chromosomal regions is located on 17q21, near the gene encoding the microtubule-associated protein tau (*MAPT*) [42]. Previously, it had been shown that rare missense mutations in *MAPT* lead to a syndrome of frontotemporal dementia with parkinsonism (FTDP-17, see below). However, to date, no mutations have been identified to cause parkinsonism without frontotemporal degeneration. Meanwhile, haplotype analyses of the tau gene have revealed some evidence of genetic association of the H1-haplotype with both PD [43] and earlier with a related syndrome, progressive supranuclear palsy [44]. Interestingly, these findings were recently corroborated in a meta-analysis [45]. This study estimated an increase of ≈30–60% in PD risk in carriers of the H1 haplotype as

compared to noncarriers, and suggested that up to 25% of the population-attributable risk for PD may be caused by this variant. However, there is typically no evidence of tau-related pathology in classic PD brains, so it remains to be determined whether this genetic link can be corroborated by biochemical analyses. Several other putative late-onset PD regions have been reported, although the overall evidence supporting the presence of genuine PD loci in these regions is still weak.

Since the discovery of the genetic association between *APOE*-ε4 and AD, variants in this gene have also been tested for a role in PD and related syndromes, despite the lack of genetic linkage evidence to chromosome 19q13. To date, nearly three dozen studies have investigated the potential role of *APOE* in PD, but no obvious or consistent pattern of association has emerged. While some authors report a significant risk effect of ε4 on PD risk in general, others only see association with PD and concurrent dementia, or age of onset, but not risk. Some have even observed correlations opposite to those found in AD, i.e. an increase in risk related to the ε2 allele, which has been reported to be protective in AD (see above). It is noteworthy that a recent meta-analysis on the putative *APOE* effects in PD concludes that it is only this latter finding that remains significant when all published studies are considered jointly [46]. The authors report a modest but significant 20% increase in risk in carriers of the ε2 allele *versus* noncarriers, while no significant effects where detected for the ε3 or ε4 alleles. Although this result clearly warrants more research regarding the possible connection between *APOE* and PD, it appears unlikely that this gene plays nearly as pronounced a role in PD as in AD.

However, there is some preliminary evidence that other genetic risk factors may be shared across the two diseases. For example, recent linkage studies for AD and PD overlap – to some degree – in at least three instances: on chromosomes 9q [47, 48], 10q [49, 50] and 12p [51, 52]. These observations could indicate the presence of common risk or age-at-onset modifiers across different neurodegenerative diseases and potentially shed more light on the pathogenesis of neurodegeneration in general.

LEWY BODY DEMENTIA

According to some studies, Lewy body dementia (LBD) is the second most common type of degenerative dementia in the elderly, possibly accounting for up to 15% of all dementia cases in autopsy samples [53]. Clinically, LBD is characterized by progressive cognitive impairment with fluctuating course, recurrent visual hallucinations and parkinsonism. Although formal clinical criteria have been proposed [53], there is a pronounced overlap with AD as well as with PD with dementia (PDD), on both clinical and neuropathological grounds. The predominant histological feature of LBD is the presence of cortical and subcortical Lewy bodies, but many patients with LBD also have AD pathology, i.e. cortical amyloid plaques and neurofibrillary tangles. Conversely, Lewy bodies are also frequently observed in cases of classic AD, including patients with mutations in *APP*, *PSEN1* and *PSEN2* [54]. While a familial aggregation of LBD has been described [55], the identification of specific LBD genetic factors is aggravated by the still uncertain phenotypic classification of LBD, in particular its distinction from AD and PDD.

The genetics of Lewy body dementia remains largely uncertain. In contrast to studies of AD and PD, to date, no systematic full-genome screen has been performed in LBD-only samples. However, there exists some evidence arguing for the presence of potentially genuine genetic risk factors. For instance, in follow-up analyses to the original AD full-genome screen, which led to the identification of linkage to chromosome 12 [51], evidence for substantial heterogeneity in the investigated sample was detected [56]. Specifically, the initial AD linkage assigned to chromosome 12q13 near 50 Mb was found to be largely caused by a subset of families (eight of 54 families) that actually fulfilled the neuropathological criteria for LBD. In these families, the linkage evidence was shifted to an interval on 12p11, with a maximum signal at the position of 27 Mb distal of the telomere of the short arm. Interestingly, this same region was also implicated in a large Japanese pedigree with autosomal dominant PD (*PARK8*) [52], although none of the four patients that came to autopsy in this latter family, displayed any clinical signs of LBD or evidence for the presence of cortical or subcortical Lewy bodies. The underlying genetic defect in these families has now been shown to lie within *LRRK2* [52a], and it remains to be seen whether or not mutations in this gene also cause cases of LBD. The only susceptibility gene from this chromosomal region studied in LBD is the five-base-pair insertion/deletion polymorphism in *A2M* [57] that was found associated with the disease in one study of a population isolate in the Netherlands [58] but not in another [59]. Finally, and not surprisingly, a potential association of LBD with *APOE*-ε4 has been observed, but only inconsistently. However, a recent meta-analysis on all case-control studies from 2000–2004 investigating the possibility of an *APOE*-ε4 risk-effect suggested that this allele does lead to a significantly increased risk for LBD, while the ε2-allele, which may lead to an increase in risk for PD (see above), does not [60]. Furthermore, the very recent observation that the occurrence of cortical Lewy bodies and dementia in PD may be dependent on the gene dose of α-synuclein [22, 23] strongly suggests a potential role of this gene in LBD pathogenesis as well.

FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia (FTD) is a heterogenous group of syndromes defined clinically by a gradual and progressive change in behavior and personal conduct and/or a gradual and progressive language dysfunction [61]. The initial symptoms typically occur without affecting other

cognitive domains, such as memory, and rarely present with an onset age beyond 75 years. In some cases, behavioral and language deficits are accompanied by parkinsonism or progressive motor neuron disease. The major neuropathological finding consists of frontal and/or temporal lobe atrophy caused by neuronal degeneration. Frequently, affected neurons display intracellular, tau-positive inclusions that characteristically are distinct from the neurofibrillary tangles observed in AD [62].

There is an ongoing debate regarding which of the various clinical syndromes to include under the 'superordinate concept' of FTD. Recent guidelines on FTD [61] have suggested embracing entities such as Pick's disease, FTD with parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration, progressive supranuclear palsy, neurofibrillary tangle dementia, frontotemporal lobar degeneration and FTD with motor neuron disease. Other authors use a very similar classification scheme but are in favor of the term 'Pick's complex' [63].

While there is clear evidence for a familial aggregation of most of these syndromes (25–40% of all cases are believed to be familial [64]), their clinical and neuropathological variability suggests the existence of several distinct genetic factors underlying these disorders. On the other hand, recent advances in the genetics of FTDP-17 have shown that different mutations in the same gene (or even exon) can lead to a diverse spectrum of FTD-type syndromes, providing genetic support for merging the apparently diverse clinical entities into one overarching category.

Frontotemporal dementia with parkinsonism linked to chromosome 17. FTDP-17 was the first FTD-syndrome found to harbor mutations in the tau gene (*MAPT*; Table 39-3) [65], following linkage evidence pointing to the chromosomal region on 17q21 near tau [66, 67]. Currently, there are over 30 known *MAPT* mutations in more than 100 families worldwide, the majority of which are located in exon 10 and the intron immediately downstream [6]. The phenotype observed with *MAPT* mutations ranges from classic Pick's disease, to corticobasal degeneration, frontotemporal lobar degeneration, and progressive supranuclear palsy [64]. Although to date no case of pathologically proven AD has been reported to harbor mutations in *MAPT*, there have been sporadic observations of early progressive memory loss reminiscent of AD, e.g. segregating with the R406W mutation in exon 13 [68, 69]. However, the same mutation was found also in families segregating more typical FTD [65]. Molecular genetic studies could show that the biochemical consequences of the various mutations on the protein level are quite diverse, including reducing or increasing the binding of tau to microtubules, enhancing tau aggregation and affecting the ratio of the specific tau-isoforms (i.e. towards an increased ratio of four-repeat *versus* three-repeat tau) by affecting alternative splicing (reviewed in [70]). Interestingly, it appears that *MAPT* mutations almost exclusively lead to FTD with immunohistochemical evidence of both three- and four-repeat tau, while classic Pick's disease, which lacks the four-repeat isoform, has not yet been conclusively linked to *MAPT* or any other genetic defect [71]. The correlation between four-repeat tau and genetic variants in *MAPT* is further supported by genetic association studies showing almost unanimous support for a *MAPT* risk haplotype (H1) in samples with progressive supranuclear palsy or corticobasal degeneration, both characterized by the abundance of four-repeat tau, but no evidence of association with Pick's disease. Note, that this is the same haplotype that has also frequently been associated with PD (see above), possibly suggesting common and as yet uncharacterized tau-related pathogenic mechanisms between FTD and late-onset PD and/or the presence of substantial heterogeneity within the commonly defined late-onset PD samples.

While tau mutations have provided an important piece in the puzzle of FTD genetics, they probably account for less than 50% of the genetic variance in familial FTD [64]. However, the successful identification of additional contributing genetic factors largely depends on the development of precise phenotypic classification schemes, which

TABLE 39-3 Established genes causing other common neurodegenerative diseases

Gene	Protein	Location	Inheritance	Relevance to FTD pathogenesis
FTDP-17				
MAPT	Microtubule-associated protein tau	17q21	Dominant	Altered tau-production (4R:3R ratio ↑) and/or altered binding to microtubules
Amyotrophic lateral sclerosis				
SOD1	Superoxide dismutase 1	21q22	Dominant and recessive	Impaired oxidative stress response (?)
ALS2	Alsin	2q33	Recessive	Impaired neuroprotection (?)
Huntington's disease				
HD	Huntingtin	4p16	Dominant	Unknown
Familial prion diseases (fCJD, FFI, GSS)				
PRNP	Prion protein	20p13	Dominant and risk factor	Transformation of PrP^{c} into PrP^{sc}

fCJD, familial Creutzfeldt–Jakob disease; FFI, fatal familial insomnia; FTDP-17, Frontotemporal dementia with parkinsonism linked to chromosome 17; GSS, Gerstmann–Sträussler–Scheinker disease.

is particularly applicable to the FTD cluster of diseases. One relatively circumscribed phenotypic entity within the FTD-related syndromes is the co-occurrence of frontal lobe degeneration with motor neuron disease (i.e. amyotrophic lateral sclerosis, ALS). A recent full genome screen on a sample of familial ALS found significant evidence of linkage to chromosome 9q21, which was restricted to families showing both ALS and FTD [72]. Interestingly, this putative locus almost precisely overlaps with a late-onset AD linkage region [72a], again possibly suggesting a common genetic background for neurodegeneration in more than one disease entity. Finally, only relatively few studies have investigated the influence of *APOE* variation on the risk for FTD, yielding highly variable results. However, these studies were collectively based on only very small case-populations (none greater than 100 cases), leading to substantially reduced power in the face of only moderate effect sizes. On the other hand, a recent meta-analysis on all data published for *APOE* in FTD [73] detected a significant risk-effect associated with the ε2 allele, but no significant results with ε4, a finding similar to meta-analyses done on late-onset PD and opposite to those reported for AD (see above). While this observation may be purely incidental, it is similar to the findings of the H1-tau haplotype that has also been associated in some FTD syndromes and PD (see above).

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (also known as motor neuron disease or Lou Gehrig's disease) is characterized by a rapidly progressive degeneration of motor neurons in the brain and spinal cord, which ultimately lead to paralysis and premature death. The prevalence of ALS overall is low (≈5/100,000), but incidence increases with age, showing a peak between 55 and 75 years. Neuropathological features of ALS include intracellular accumulations and perikaryal inclusions of neurofilament, and Lewy-body-like cytoplasmic inclusions. While the precise pathogenic mechanism leading to neuronal death remains elusive, a number of biochemical events have been suggested to be probably involved: protein misfolding and aggregation, defective axonal transport, mitochondrial dysfunction and glutamate-related excitotoxicity [74]. Cognitive impairment and dementia co-occurs with ALS in at least 5% of cases and it was actually in a family displaying FTD with parkinsonism and amyotrophy that evidence of linkage to chromosome 17 was first described (see above).

Familial amyotrophic lateral sclerosis. Familial amyotrophic lateral sclerosis (FALS) is observed in ≈10% of all cases, but substantially more ALS cases are suspected to be influenced to some degree by genetic factors [75]. Mutations in two genes (*SOD1* and *ALS2*; Table 39-3) have been shown to cause FALS, apart from mutations in tau (*MAPT*) leading to FTD with parkinsonism and amyotrophy (see above). Following evidence in 1991 of genetic linkage to chromosome 21q [76], disease-causing mutations were identified in the gene encoding superoxide dismutase 1 (*SOD1*) [77], which catalyzes the conversion of superoxide radicals into hydrogen peroxide. More than 100 mutations in *SOD1* have been described worldwide since 1991 in over 200 pedigrees with FALS. These mutations account for ≈20% of FALS and for up to 10% of the 'sporadic' cases of ALS, i.e. those not showing an obvious Mendelian transmission [78]. Most of the known *SOD1* mutations are inherited in an autosomal dominant fashion, although one mutation (D90A) can act both dominantly and recessively [79]. The latter mode of inheritance was mainly observed in Scandinavian countries, a geographical region generally showing increased ALS incidence. Although the precise mechanisms of SOD1 dysfunction are still unknown, there is a growing consensus that it is most probably based on a toxic gain of function of mutant SOD1. Recently, mutations in a second FALS gene (*ALS2*, encoding alsin) were identified in independent families with a rare, juvenile-onset autosomal recessive form of ALS and primary lateral sclerosis, a syndrome restricted to upper motor neuron degeneration [80, 81]. Additional mutations in *ALS2* have also been described in families suffering from infantile-onset ascending hereditary spastic paralysis, suggesting considerable phenotypic variability of the *ALS2* mutations. The function of the encoded protein is unknown but it was recently suggested that it protects neurons from mutant SOD1 *in vitro* [82].

Several other FALS loci have been described by means of linkage analysis in individual families or larger FALS samples but none of the underlying gene defects has yet been uncovered. A recent full-genome screen in FALS has pinpointed significant linkage to the immediate vicinity of a putative AD locus on chromosome 9q21 in families with ALS and FTD [72], possibly indicating a common pathophysiological basis for neurodegeneration or dementia in these two disorders. Furthermore, various genetic associations with mostly nonfamilial ALS have been claimed but have met with only inconsistent replication to date. Specifically – as one of the few neurodegenerative conditions discussed in this chapter – *APOE*-ε4 has almost unanimously shown no evidence for association with risk or disease progression of ALS. Moreover, the genetic data supporting the postulated association between ALS and the neurofilament heavy-chain gene (*NFH*) on chromosome 22q12, a component of the neuronal inclusions observed histopathologically, remains scarce, despite the fact that it was initially described over a decade ago [83].

NEURODEGENERATIVE TRIPLET REPEAT DISORDERS

Triplet repeat disorders are characterized by 3-base-pair nucleotide repeats that lie in either coding or non-coding regions and give rise to a multitude of different

phenotypes that are inherited in an autosomal dominant, recessive or X-linked fashion. The nucleotide triplets are inherently unstable and the number of repeats tends to increase ('expand') across consecutive generations, especially following paternal transmission. This can lead to a decrease in onset age of the particular disease, a phenomenon described as 'anticipation'. The molecular basis of repeat instability is not well understood but the presence of pathogenetic triplet repeats gives rise to a variety of neurological and developmental disorders, such as Huntington's disease, spinocerebellar ataxias and fragile X-syndrome. Neurodegenerative triplet repeat disorders are all caused by coding poly-CAG expansions, which are translated into an elongated polyglutamine (polyQ) tract. Although most of these proteins do not share any homology aside from polyQ, several salient features are shared by the CAG-repeat disorders. For instance, they generally strike in mid-life and cause increasing neuronal dysfunction and eventual neuronal loss ≈10–20 years after onset of symptoms. Furthermore, all polyQ repeats confer a gain of function when they are pathologically expanded, and disease develops typically when the number of uninterrupted repeats exceeds 35–36 glutamines. Finally, despite the widespread expression of all currently known genes throughout the brain and other tissues, only a certain subset of neurons is vulnerable to dysfunction in each of the various diseases. In the remainder of this section we will discuss the genetics underlying Huntington's disease as an example of a neurodegenerative triplet repeat disorder leading to dementia.

Huntington's disease. Huntington's disease (HD) is caused by degeneration of neurons in the basal ganglia followed by cortical regions, leading clinically to involuntary movements (chorea), psychiatric symptoms and dementia. Its prevalence is similar to that of ALS but much less than that for most of the other dementing illnesses discussed above. Approximately 90% of HD cases are hereditary and are transmitted in an autosomal dominant fashion. The other 10% are considered to be '*de novo*', i.e. these cases originate from asymptomatic parents with normal repeat lengths that have expanded to symptomatic range (see below). The genetic locus underlying HD was originally mapped to chromosome 4q16 via conventional linkage analysis [84]. This was the first time that the genetic cause for an autosomal dominant disorder had been pinpointed solely via the genetic analyses of DNA variants. 10 years later the gene responsible for HD was identified as encoding a 350 kDa protein (huntingtin; gene: *HD*), the *HD* gene containing a poly-CAG repeat in exon 1 (Table 39-3) [85]. As is typical for CAG-repeat disorders, HD is symptomatic in the presence of 36 repeats or more, although lengths of 36–39 have been found to show only incomplete penetrance. The mean repeat length in HD patients is 40–45, although the variability is quite wide, ranging from 35 to 120 repeats [86]. The precise function of huntingtin remains elusive, but cloning experiments have demonstrated that it is highly conserved throughout evolution. Interestingly, CAG repeats in other mammalian and nonmammalian species are less frequent than in humans, suggesting that the polyQ segment is of little, if any, physiological importance [86]. One of the leading hypotheses regarding the mechanism underlying neurodegeneration in HD is that the pathologically expanded polyQ can cause mutant huntingtin to interact abnormally with other proteins. However, to date none of the many identified huntingtin-interacting proteins has shown a regional distribution correlating with neurodegeneration in the brains of HD patients, suggesting a more complex etiology [87].

It is well established that repeat length of the polyQ tract shows a strong inverse correlation with onset age. However, only ≈50% of the interindividual onset age variation can be explained by triplet number, suggesting the presence of other factors (genetic and nongenetic) as modifiers of onset age. A recently completed full genome screen performed to identify such onset age modifiers has revealed several suggestive linkage regions [88]. The most promising of these on chromosome 6q25 is close to the glutamate receptor 6 (*GRIK2*), which has been associated with a younger HD onset age in some studies, potentially supporting the notion of glutamate-induced excitotoxicity [89, 90]. However, this finding awaits further replication and functional characterization. Only few studies have investigated a potential onset-age effect of the *APOE* polymorphisms in HD but, as with ALS, the results thus far have been largely negative.

CREUTZFELDT–JAKOB DISEASE AND OTHER PRION DISEASES

Prion diseases include a rare and heterogenous spectrum of clinical and histopathological phenotypes, which are unique in the group of neurodegenerative diseases as they can be familial (e.g. familial Creutzfeldt–Jakob disease (fCJD), fatal familial insomnia (FFI), Gerstmann–Sträussler–Scheinker disease (GSS)), sporadic (e.g. CJD, sporadic fatal insomnia), or acquired by infection (e.g. kuru, iatrogenic CJD, variant CJD). While most forms are characterized by a rapidly progressing neurodegeneration with spongiosis and amyloid plaques consisting of prion protein (PrP) aggregates, this is not the case for all forms. Further, only a relatively small subset of cases with prion disease show a familial aggregation, but genetics has played a crucial role in elucidating the molecular mechanisms as well as facilitating the clinical classification of the various subtypes of disease [91]. In particular, mutations and polymorphisms in the gene encoding PrP (*PRNP*) on chromosome 20p13 at ≈5 Mb are major determinants of disease onset and phenotypic variation of familial prion diseases but also appear to affect risk for the sporadic forms (Table 39-3).

***PRNP* mutations cause neurodegeneration and influence disease progression.** The mechanisms by which

PRNP influences disease outcome are 2-fold. First, more than two dozen different amino-acid-changing mutations in the coding region of the gene have been identified as causing familial prion diseases, transmitted in an autosomal dominant fashion with nearly 100% penetrance. There is a remarkable heterogeneity in the sense that different mutations throughout the gene can give rise to a variety of different phenotypes associated with all three familial forms of prion disease, fCJD, FFI and GSS (reviewed in [91]). In addition to these point mutations, there are also rare cases of fCJD and GSS caused by variable numbers of octapeptide (i.e. 24 bp) repeats within the coding sequence of the *PRNP* gene. Second, clinical presentation and disease progression are further modified by a common polymorphism at codon 129, leading to a methionine (M) to valine (V) change. Most of the mutated *PRNP* missense alleles are on the same haplotype as the 129M allele, which includes virtually all forms of fCJD. In the rare cases where they co-occur with the 129V allele, they lead to a distinct clinical phenotype and, in the case of D178N–129M *versus* D178N–129V, even to a different disease entity: While the latter haplotype leads to typical fCJD, the former (i.e. M-associated) haplotype represents the only currently known genetic cause of FFI, which presents with a quite distinct clinical picture.

In addition to its effects on familial forms of prion disease, the M129V polymorphism was also found to increase the risk for sporadic forms of CJD (sCJD) [92, 93]. In particular, it was found that the homozygous state for either allele (i.e. M/M or V/V) was disproportionately more frequent in sCJD than the M/V genotype [92]. Furthermore, homozygosity at this polymorphism leads to a faster disease progression than heterozygosity in nearly all genetic as well as sporadic (including iatrogenic) forms of prion disease and, in most instances, the M/M genotype was associated with the most aggressive course of disease [94]. Interestingly, almost all individuals thus far known to be affected by the newly described 'variant' form of CJD (vCJD), which is characterized by a prion protein isotype resembling that found in bovine spongiform encephalopathies (BSE), have also been carriers of the M/M genotype. Furthermore, at least in Caucasian populations, there is evidence suggesting an overrepresentation of the M allele and M/M genotype in samples with AD as well (see above). This contrasts with observations made by other groups suggesting that the V allele predisposes to cognitive decline in the elderly [95], and Down's syndrome patients [96]. Only a few other genetic risk factors for the nonfamilial forms of CJD have been investigated and none of them has shown any noteworthy results to date. This includes *APOE*-ε4, which was found to both increase risk and/or accelerate disease progression in some studies. The majority of samples failed to replicate either of these effects, although more studies and meta-analyses on the available data are needed to more definitely exclude a prominent role of this gene in the etiology and progression of prion diseases.

CONCLUDING REMARKS

While displaying a diverse array of clinical and histopathological characteristics, the neurodegenerative disorders discussed in this chapter share a variety of epidemiologic and genetic aspects. First, with the exception of Huntington's disease, they all feature an etiological dichotomy with less frequent, relatively early-onset familial forms on the one hand, and more frequent multifactorial forms on the other. It is possible (and likely) that a substantial number of the hitherto nonfamilial cases of neurodegeneration will eventually turn out to originate from specific disease-causing mutations and that some, if not the majority, of the remaining seemingly 'sporadic' cases will prove to be governed by a variety of different, but *bona fide* genetic risk factors (such as *APOE*-ε4 in AD). Second, in some cases, the same mutations and polymorphisms have been linked and associated across clinically and neuropathologically diverse disease entities. For instance, based on recent meta-analyses the *APOE* polymorphisms may not only contribute to risk for AD but also for PD and FTD (albeit with different alleles). Along these lines, there have been reports of overlapping linkage regions for AD and PD (chromosome 10q), AD, PD and ALS-FTD (chromosome 9q), and AD, PD and LBD (chromosome 12p), possibly suggesting genetic commonalities. One possible explanation for such overlaps is that the samples were imperfectly ascertained and actually represent heterogenous diseases. Alternatively, these overlaps could point to one or several common genetic and mechanistic denominators for neuronal cell death. Finally, genetics has been one of the essential prerequisites for elucidating the molecular and biochemical pathways leading to neurodegeneration for almost all of the syndromes and disease entities discussed in this chapter. Likewise, a detailed understanding of the genetic basis of neurodegeneration will probably also be essential for the development of effective early-prediction and early-intervention/treatment strategies, with the prospect of largely decreasing the incidence of these devastating disorders in the future.

REFERENCES

1. Tanzi, R. E. A genetic dichotomy model for the inheritance of Alzheimer's disease and common age-related disorders. *J. Clin. Invest.* 104: 1175–1179, 1999.
2. Goate, A., Chartier-Harlin, M. C., Mullan, M. *et al.* Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349: 704–706, 1991.
3. Sherrington, R., Rogaev, E. I., Liang, Y. *et al.* Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375: 754–760, 1995.
4. Rogaev, E. I., Sherrington, R., Rogaeva, E. A. *et al.* Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature* 376: 775–778, 1995.

5. Levy-Lahad, E., Wasco, W., Poorkaj, P. *et al.* Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* 269: 973–977, 1995.
6. Human Genome Variation Society. Alzheimer Disease & Frontotemporal Dementia Mutation Database. Available on line at www.molgen.ua.ac.be/ADMutations/.
7. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T. and Selkoe, D. J. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* 398: 513–517, 1999.
8. Mayeux, R., Sano, M., Chen, J., Tatemichi, T. and Stern, Y. Risk of dementia in first-degree relatives of patients with Alzheimer's disease and related disorders. *Arch. Neurol.* 48: 269–273, 1991.
9. Strittmatter, W. J., Saunders, A. M., Schmechel, D. *et al.* Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl Acad. Sci. U.S.A.* 90: 1977–1981, 1993.
10. Schmechel, D. E., Saunders, A. M., Strittmatter, W. J. *et al.* Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. *Proc. Natl Acad. Sci. U.S.A.* 90: 9649–9653, 1993.
11. Farrer, L. A., Cupples, L. A., Haines, J. L. *et al.* Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. a meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *J.A.M.A.* 278: 1349–1356, 1997.
12. Poirier, J. Apolipoprotein E and Alzheimer's disease. A role in amyloid catabolism. *Ann. N.Y. Acad. Sci.* 924: 81–90, 2000.
13. Warwick Daw, E., Payami, H., Nemens, E. J. *et al.* The number of trait loci in late-onset Alzheimer disease. *Am. J. Hum. Genet.* 66: 196–204, 2000.
14. Bertram, L. and Tanzi, R. E. Alzheimer's disease: one disorder, too many genes? *Hum. Mol. Genet.* 13 Spec No 1: R135–R141, 2004.
15. Finckh, U., van Hadeln, K., Muller-Thomsen, T. *et al.* Association of late-onset Alzheimer disease with a genotype of *PLAU*, the gene encoding urokinase-type plasminogen activator on chromosome 10q22.2. *Neurogenetics* 4: 213–217, 2003.
16. Tanzi, R. E. and Bertram, L. New frontiers in Alzheimer's disease genetics. *Neuron* 32: 181–184, 2001.
17. Tanner, C. M., Ottman, R., Goldman, S. M. *et al.* Parkinson disease in twins: an etiologic study. *J.A.M.A.* 281: 341–346, 1999.
18. Maher, N. E., Golbe, L. I., Lazzarini, A. M. *et al.* Epidemiologic study of 203 sibling pairs with Parkinson's disease: the GenePD study. *Neurology* 58: 79–84, 2002.
19. De la Fuente-Fernandez, R. A note of caution on correlation between sibling pairs. *Neurology* 60: 1561, 2003.
20. Polymeropoulos, M. H., Lavedan, C., Leroy, E. *et al.* Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045–2047, 1997.
21. Vila, M. and Przedborski, S. Genetic clues to the pathogenesis of Parkinson's disease. *Nat. Med.* 10(Suppl): S58–S62, 2004.
22. Chartier-Harlin, M. C., Kachergus, J., Roumier, C. *et al.* Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* 364: 1167–1169, 2004.
23. Singleton, A. B., Farrer, M., Johnson, J. *et al.* alpha-Synuclein locus triplication causes Parkinson's disease. *Science* 302: 841, 2003.
24. Farrer, M., Kachergus, J., Forno, L. *et al.* Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. *Ann. Neurol.* 55: 174–179, 2004.
25. Ibanez, P., Bonnet, A. M., Debarges, B. *et al.* Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet* 364: 1169–1171, 2004.
26. Farrer, M., Maraganore, D. M., Lockhart, P. *et al.* alpha-Synuclein gene haplotypes are associated with Parkinson's disease. *Hum. Mol. Genet.* 10: 1847–1851, 2001.

26a. Zimprich, A. *et al.* Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 44: 601–607, 2004.

26b. Paisan-Ruiz, C. *et al.* Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 44: 595–600, 2004.

26c. Albrecht, M. LRRK2 mutations and Parkinsonism. *Lancet* 365: 1230, 2005.

27. Kitada, T., Asakawa, S., Hattori, N. *et al.* Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605–608, 1998.
28. Matsumine, H., Saito, M., Shimoda-Matsubayashi, S. *et al.* Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2–27. *Am. J. Hum. Genet.* 60: 588–596, 1997.
29. Petrucelli, L., O'Farrell, C., Lockhart, P. J. *et al.* Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. *Neuron* 36: 1007–1019, 2002.
30. Bonifati, V., Rizzu, P., van Baren, M. J. *et al.* Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299: 256–259, 2003.
31. Van Duijn, C. M., Dekker, M. C., Bonifati, V. *et al.* Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. *Am. J. Hum. Genet.* 69: 629–634, 2001.
32. Abou-Sleiman, P. M., Healy, D. G., Quinn, N., Lees, A. J. and Wood, N. W. The role of pathogenic DJ-1 mutations in Parkinson's disease. *Ann. Neurol.* 54: 283–286, 2003.
33. Valente, E. M., Abou-Sleiman, P. M., Caputo, V. *et al.* Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304: 1158–1160, 2004.
34. Valente, E. M., Bentivoglio, A. R., Dixon, P. H. *et al.* Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. *Am. J. Hum. Genet.* 68: 895–900, 2001.
35. Langston, J. W., Ballard, P., Tetrud, J. W. and Irwin, I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 219: 979–980, 1983.
36. Rohe, C. F., Montagna, P., Breedveld, G., Cortelli, P., Oostra, B. A. and Bonifati, V. Homozygous PINK1 C-terminus mutation causing early-onset parkinsonism. *Ann. Neurol.* 56: 427–431, 2004.
37. Hatano, Y., Li, Y., Sato, K. *et al.* Novel PINK1 mutations in early-onset parkinsonism. *Ann. Neurol.* 56: 424–427, 2004.
38. Leroy, E., Boyer, R., Auburger, G. *et al.* The ubiquitin pathway in Parkinson's disease. *Nature* 395: 451–452, 1998.
39. Le, W. D., Xu, P., Jankovic, J. *et al.* Mutations in NR4A2 associated with familial Parkinson disease. *Nat. Genet.* 33: 85–89, 2003.
40. Hering, R., Petrovic, S., Mietz, E. M. *et al.* Extended mutation analysis and association studies of Nurr1 (NR4A2) in Parkinson disease. *Neurology* 62: 1231–1232, 2004.

41. Maraganore, D. M., Lesnick, T. G., Elbaz, A. *et al.* UCHL1 is a Parkinson's disease susceptibility gene. *Ann. Neurol.* 55: 512–521, 2004.
42. Scott, W. K., Nance, M. A., Watts, R. L. *et al.* Complete genomic screen in Parkinson disease: evidence for multiple genes. *J.A.M.A.* 286: 2239–2244, 2001.
43. Martin, E. R., Scott, W. K., Nance, M. A. *et al.* Association of single-nucleotide polymorphisms of the tau gene with late-onset Parkinson disease. *J.A.M.A.* 286: 2245–2250, 2001.
44. Conrad, C., Andreadis, A., Trojanowski, J. Q. *et al.* Genetic evidence for the involvement of tau in progressive supranuclear palsy. *Ann. Neurol.* 41: 277–281, 1997.
45. Healy, D. G., Abou-Sleiman, P. M., Lees, A. J. *et al.* Tau gene and Parkinson's disease: a case-control study and meta-analysis. *J. Neurol. Neurosurg. Psychiatr.* 75: 962–965, 2004.
46. Huang, X., Chen, P. C. and Poole, C. APOE-epsilon2 allele associated with higher prevalence of sporadic Parkinson disease. *Neurology* 62: 2198–2202, 2004.
47. Blacker, D., Bertram, L., Saunders, A. J. *et al.* Results of a high-resolution genome screen of 437 Alzheimer's disease families. *Hum. Mol. Genet.* 12: 23–32, 2003.
48. DeStefano, A. L., Golbe, L. I., Mark, M. H. *et al.* Genome-wide scan for Parkinson's disease: the GenePD Study. *Neurology* 57: 1124–1126, 2001.
49. Bertram, L., Blacker, D., Mullin, K. *et al.* Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. *Science* 290: 2302–2303, 2000.
50. Li, Y. J., Scott, W. K., Hedges, D. J. *et al.* Age at onset in two common neurodegenerative diseases is genetically controlled. *Am. J. Hum. Genet.* 70: 985–993, 2002.
51. Pericak-Vance, M. A., Bass, M. P., Yamaoka, L. H. *et al.* Complete genomic screen in late-onset familial Alzheimer disease. Evidence for a new locus on chromosome 12. *J.A.M.A.* 278: 1237–1241, 1997.
52. Funayama, M., Hasegawa, K., Kowa, H., Saito, M., Tsuji, S. and Obata, F. *et al.* A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. *Ann. Neurol.* 51: 296–301, 2002.

52a. Funayama, M. *et al.* An LRRK2 mutation as a cause for the parkinsonism in the original PARK8 family. *Ann. Neurol.* 57: 918–21, 2005.

53. McKeith, I. G., Galasko, D., Kosaka, K. *et al.* Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. *Neurology* 47: 1113–1124, 1996.
54. Hamilton, R. L. Lewy bodies in Alzheimer's disease: a neuropathological review of 145 cases using alpha-synuclein immunohistochemistry. *Brain Pathol.* 10: 378–384, 2000.
55. Tsuang, D. W., DiGiacomo, L. and Bird, T. D. Familial occurrence of dementia with Lewy bodies. *Am. J. Geriatr. Psychiatr.* 12: 179–188, 2004.
56. Scott, W. K., Grubber, J. M., Conneally, P. M. *et al.* Fine mapping of the chromosome 12 late-onset Alzheimer disease locus: potential genetic and phenotypic heterogeneity. *Am. J. Hum. Genet.* 66: 922–932, 2000.
57. Blacker, D., Wilcox, M. A., Laird, N. M. *et al.* Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat. Genet.* 19: 357–360, 1998.
58. Sleegers, K., Roks, G., Theuns, J. *et al.* Familial clustering and genetic risk for dementia in a genetically isolated Dutch population. *Brain* 127: 1641–1649, 2004.
59. Singleton, A. B., Gibson, A. M., McKeith, I. G. *et al.* Alpha2-macroglobulin polymorphisms in Alzheimer's disease and dementia with Lewy bodies. *Neuroreport* 10: 1507–1510, 1999.
60. Bang, O. Y. , Kwak, Y. T., Joo, I. S. and Huh, K. Important link between dementia subtype and apolipoprotein E: a meta-analysis. *Yonsei Med. J.* 44: 401–413, 2003.
61. McKhann, G. M., Albert, M. S., Grossman, M. *et al.* Clinical and pathological diagnosis of frontotemporal dementia: report of the Work Group on Frontotemporal Dementia and Pick's Disease. *Arch. Neurol.* 58: 1803–1809, 2001.
62. Brun, A. Frontal lobe degeneration of non-Alzheimer type. I. Neuropathology. *Arch. Gerontol. Geriatr.* 6: 193–208, 1987.
63. Kertesz, A. Pick's complex and FTDP-17. *Mov. Disord.* 18(Suppl 6): S57–S62, 2003.
64. Bird, T., Knopman, D., VanSwieten, J. *et al.* Epidemiology and genetics of frontotemporal dementia/Pick's disease. *Ann. Neurol.* 54(Suppl 5): S29–S31, 2003.
65. Hutton, M., Lendon, C. L., Rizzu, P. *et al.* Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* 393: 702–705, 1998.
66. Wilhelmsen, K. C., Lynch, T., Pavlou, E., Higgins, M. and Nygaard, T. G. Localization of disinhibition-dementia-parkinsonism-amyotrophy complex to 17q21–22. *Am. J. Hum. Genet.* 55: 1159–1165, 1994.
67. Lynch, T., Sano, M., Marder, K. S. *et al.* Clinical characteristics of a family with chromosome 17-linked disinhibition–dementia–parkinsonism–amyotrophy complex. *Neurology* 44: 1878–1884, 1994.
68. Reed, L. A., Wszolek, Z. K. and Hutton, M. Phenotypic correlations in FTDP-17. *Neurobiol. Aging* 22: 89–107, 2001.
69. Rademakers, R., Dermaut, B., Peeters, K. *et al.* Tau (MAPT) mutation Arg406Trp presenting clinically with Alzheimer disease does not share a common founder in Western Europe. *Hum. Mutat.* 22: 409–411, 2003.
70. Ingelsson, M. and Hyman, B. T. Disordered proteins in dementia. *Ann. Med.* 34: 259–271, 2002.
71. Morris, H. R., Baker, M., Yasojima, K. *et al.* Analysis of tau haplotypes in Pick's disease. *Neurology* 59: 443–445, 2002.
72. Hosler, B. A., Siddique, T., Sapp, P. C. *et al.* Linkage of familial amyotrophic lateral sclerosis with frontotemporal dementia to chromosome 9q21–q22. *J.A.M.A.* 284: 1664–1669, 2000.

72a. Bertram, L. *et al.* Family-based association between Alzheimer's disease and variants in UBQLN1. *N. Engl. J. Med.* 352: 884–894, 2005.

73. Verpillat, P., Camuzat, A., Hannequin, D. *et al.* Apolipoprotein E gene in frontotemporal dementia: an association study and meta-analysis. *Eur. J. Hum. Genet.* 10: 399–405, 2002.
74. Cluskey, S. and Ramsden, D. B. Mechanisms of neurodegeneration in amyotrophic lateral sclerosis. *Mol. Pathol.* 54: 386–392, 2001.
75. Majoor-Krakauer, D., Willems, P. J. and Hofman, A. Genetic epidemiology of amyotrophic lateral sclerosis. *Clin. Genet.* 63: 83–101, 2003.
76. Siddique, T., Figlewicz, D. A., Pericak-Vance, M. A. *et al.* Linkage of a gene causing familial amyotrophic lateral sclerosis to chromosome 21 and evidence of genetic-locus heterogeneity. *N. Engl. J. Med.* 324: 1381–1384, 1991.
77. Rosen, D. R., Siddique, T., Patterson, D. *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362: 59–62, 1993.

78. Kato, S. *et al.* Amyotrophic lateral sclerosis. in D. Dickson (ed.), *Neurodegeneration – The Molecular Pathology of Dementia and Movement Disorders.* Basel: ISN Neuropathology Press, 2003, pp. 350–368.
79. Andersen, P. M., Nilsson, P., Ala-Hurula, V. *et al.* Amyotrophic lateral sclerosis associated with homozygosity for an Asp90Ala mutation in CuZn-superoxide dismutase. *Nat. Genet.* 10: 61–66, 1995.
80. Yang, Y., Hentati, A., Deng, H. X. *et al.* The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nat. Genet.* 29: 160–165, 2001.
81. Hadano, S., Hand, C. K., Osuga, H. *et al.* A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. *Nat. Genet.* 29: 166–173, 2001.
82. Kanekura, K., Hashimoto, Y., Niikura, T., Aiso, S., Matsuoka, M. and Nishimoto, I. Alsin, the product of ALS2 gene, suppresses SOD1 mutant neurotoxicity through RhoGEF domain by interacting with SOD1 mutants. *J. Biol. Chem.* 279: 19247–19256, 2004.
83. Figlewicz, D. A., Krizus, A., Martinoli, M. G. *et al.* Variants of the heavy neurofilament subunit are associated with the development of amyotrophic lateral sclerosis. *Hum. Mol. Genet.* 3: 1757–1761, 1994.
84. Gusella, J. F., Wexler, N. S., Conneally, P. M. *et al.* A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* 306: 234–238, 1983.
85. Huntington's Disease Collaborative Research Group. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72: 971–983, 1993.
86. Gusella, J. F. and MacDonald, M. E. Huntington's disease: CAG genetics expands neurobiology. *Curr. Opin. Neurobiol.* 5: 656–662, 1995.
87. Li, S. H. and Li, X. J. Huntingtin–protein interactions and the pathogenesis of Huntington's disease. *Trends Genet.* 20: 146–154, 2004.
88. Li, J. L., Hayden, M. R., Almqvist, E. W. *et al.* A genome scan for modifiers of age at onset in Huntington disease: the HD MAPS study. *Am. J. Hum. Genet.* 73: 682–687, 2003.
89. Rubinsztein, D. C., Leggo, J., Chiano, M. *et al.* Genotypes at the GluR6 kainate receptor locus are associated with variation in the age of onset of Huntington disease. *Proc. Natl Acad. Sci. U.S.A.* 94: 3872–3876, 1997.
90. MacDonald, M. E., Vonsattel, J. P., Shrinidhi, J. *et al.* Evidence for the GluR6 gene associated with younger onset age of Huntington's disease. *Neurology* 53: 1330–1332, 1999.
91. Gambetti, P., Kong, Q., Zou, W., Parchi, P. and Chen, S. G. Sporadic and familial CJD: classification and characterisation. *Br. Med. Bull.* 66: 213–239, 2003.
92. Palmer, M. S., Dryden, A. J., Hughes, J. T. and Collinge, J. Homozygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease. *Nature* 352: 340–342, 1991.
93. Collinge, J., Palmer, M. S. and Dryden, A. J. Genetic predisposition to iatrogenic Creutzfeldt–Jakob disease. *Lancet* 337: 1441–1442, 1991.
94. Pocchiari, M., Puopolo, M., Croes, E. A. *et al.* Predictors of survival in sporadic Creutzfeldt–Jakob disease and other human transmissible spongiform encephalopathies. *Brain* 127: 2348–2359, 2004.
95. Berr, C., Richard, F., Dufouil, C., Amant, C., Alperovitch, A. and Amouyel, P. Polymorphism of the prion protein is associated with cognitive impairment in the elderly: the EVA study. *Neurology* 51: 734–737, 1998.
96. Del Bo, R., Comi, G. P., Giorda, R. *et al.* The 129 codon polymorphism of the prion protein gene influences earlier cognitive performance in Down syndrome subjects. *J. Neurol.* 250: 688–692, 2003.
97. Bertram, L. and Tanzi, R. Genetics of Alzheimer's disease. In D. Dickson (ed.), *Neurodegeneration – The Molecular Pathology of Dementia and Movement Disorders.* Basel: ISN Neuropathology Press, 2003, pp. 40–46.

CHAPTER

40

Disorders of Amino Acid Metabolism

Marc Yudkoff

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INTRODUCTION

An aminoaciduria usually results from the congenital absence of an enzyme needed for metabolism of an amino acid. Aminoacidopathies typically involve an inherited deficiency of an enzyme that is important for the metabolism of a particular amino acid (Table 40-1). The concentration of that amino acid and its metabolites consequently rise sharply in blood, urine and body tissues, including the brain. When the enzymatic deficiency is nearly complete, the onset of disease tends to occur in infancy, even in the neonatal period. Partial enzyme deficiencies may not become apparent until later in life [1, 2].

Most untreated aminoacidopathies damage the brain. The pathophysiological factors that explain the encephalopathy remain unknown. Injury probably results from the accumulation of an amino acid or amino acid metabolite that is toxic to the nervous system, especially to the developing brain. Mental retardation and other manifestations of brain damage can result. Fortunately, these disorders often are amenable to therapy with a diet that is purposely low in the potentially offending amino acid. In some instances treatment is possible with a vitamin that serves as a cofactor for the defective biochemical reaction (Table 40-1). Antidotes to the neurotoxicity have been developed for a few syndromes. Outcome often depends upon early institution of treatment. The advent of mass screening of newborns for metabolic diseases has greatly improved the prognosis.

The major metabolic fate of amino acids is conversion into organic acids; absent an enzyme to oxidize an organic acid, an organic aciduria results. Three features characterize the metabolism of essentially all amino acids: (1) incorporation into protein; (2) conversion into messenger compounds such as hormones and neurotransmitters;

TABLE 40-1 Disorders of amino acid metabolism

Disorder	Biochemical derangement	Classical findings	Treatment
Branched-chain aminoaciduria (maple syrup urine disease)	Defective branched-chain amino acid breakdown (Fig. 40-1)	Coma, convulsions, vomiting, respiratory failure in neonate	Diet low in branched-chain amino acids Thiamine for B_1-responsive disorders (rare) Hepatic transplantation
Glutaric acidurias	Type I: Primary defect of glutarate oxidation Type II: Defect of electron transfer flavoprotein	Type I: Severe basal ganglia/cerebellar disease with macrocephaly. Onset 1–2 years Type II: Fulminant neurological syndrome of the neonate. Often with renal/hepatic cysts. Usually fatal	Diet low in lysine and tryptophan Supplementation with coenzyme Q, riboflavin, carnitine
Phenylketonuria (PKU)	Usually defect of phenylalanine hydroxylase. In care cases, defect of biopterin metabolism (Fig. 40-3; reaction 1)	Normal at birth. Mental retardation in untreated children. Avoidable with early institution of diet therapy. Prognosis less favorable in PKU secondary to defect of biopterin metabolism	Diet low in phenylalanine Carbidopa
Nonketotic hyperglycinemia	Defect of the glycine cleavage system (Fig. 40-4)	Intractable seizures in neonate. Usually fatal in first few weeks of life	Diet low in glycine NMDA receptor blockers (variable efficacy) Sodium benzoate to lower blood glycine
Homocystinuria	Usually a failure of cystathionine synthase (Fig. 40-2; reaction 6). Rarely associated with aberrant vitamin B_{12} metabolism (Fig. 40-2)	Thromboembolic diathesis, marfanoid habitus, ectopia lentis. Mental retardation is frequent.	Diet low in methionine Vitamin B_6 in pyridoxine-responsive syndromes Vitamin B_{12} in responsive syndromes Anticlotting agents
Urea cycle defects	Failure to convert ammonia to urea via urea cycle (Fig. 40-5).	Coma, convulsions, vomiting, respiratory failure in neonate. Often mistaken for sepsis of the newborn. Mental retardation, failure to thrive, lethargy, ataxia and coma in the older child. Associated with hyperammonemia and abnormalities of blood aminogram	Low protein diet Acylation therapy (sodium benzoate, sodium phenylacetate) Arginine therapy in selected syndromes Hepatic transplantation
Disorders of glutathione	Defective synthesis of glutathione, the major intracellular antioxidant	Spinocerebellar degeneration, mental retardation, cataracts, hemolysis. Severe acidosis in some cases	*N*-acetylcysteine (variable response)
Disorders of GABA	Vitamin B_6-dependent seizures Often an absence of succinic semialdehyde dehydrogenase	Hypotonia, ataxia, mental retardation in older child. Increased urine 4-OH-butyric acid.	Pyridoxine (B6-dependent disorder) Inhibitors of GABA transaminase
Canavan's disease	Absence of *N*-acetylaspartate acylase	Rapidly progressive demyelinating disease of infancy	Gene therapy (experimental)

For disorders of carbohydrate metabolism and the primary lactic acidoses, see Chapter 34.

and (3) oxidation, which involves the conversion of amino acid nitrogen into ammonia and of amino acid carbon into organic acids that enter the tricarboxylic acid cycle, where they become oxidized.

Generalized defects of protein synthesis have not yet been described, presumably because they would be lethal early in development. Disturbances in the synthesis of messenger compounds such as thyroxine or neurotransmitters may occur but they do not result in an aminoaciduria because relatively little amino acid is so disposed.

Many organic acidurias originate in the breakdown of the three branched-chain amino acids, leucine, isoleucine and valine (Fig. 40-1). Metabolism of the organic acids requires the presence of specific enzymes, congenital deficiencies of which give rise to the organic acidurias (Fig. 40-1). The clinical features of the organic acidurias are described in Table 40-2.

On rare occasions an organic aciduria occurs not because of an enzyme deficiency but from a failure to transport or activate a water-soluble vitamin that serves as a cofactor for the reaction in question. Thus, congenital deficiencies in the metabolism of vitamin B_{12} commonly give rise to methylmalonic aciduria (Fig. 40-1, Table 40-2). Similarly, deficiencies of biotin metabolism can cause a severe organic aciduria (Table 40-2). It is very important to be aware of the defects of vitamin metabolism because the administration of large doses of these cofactors may completely prevent brain damage.

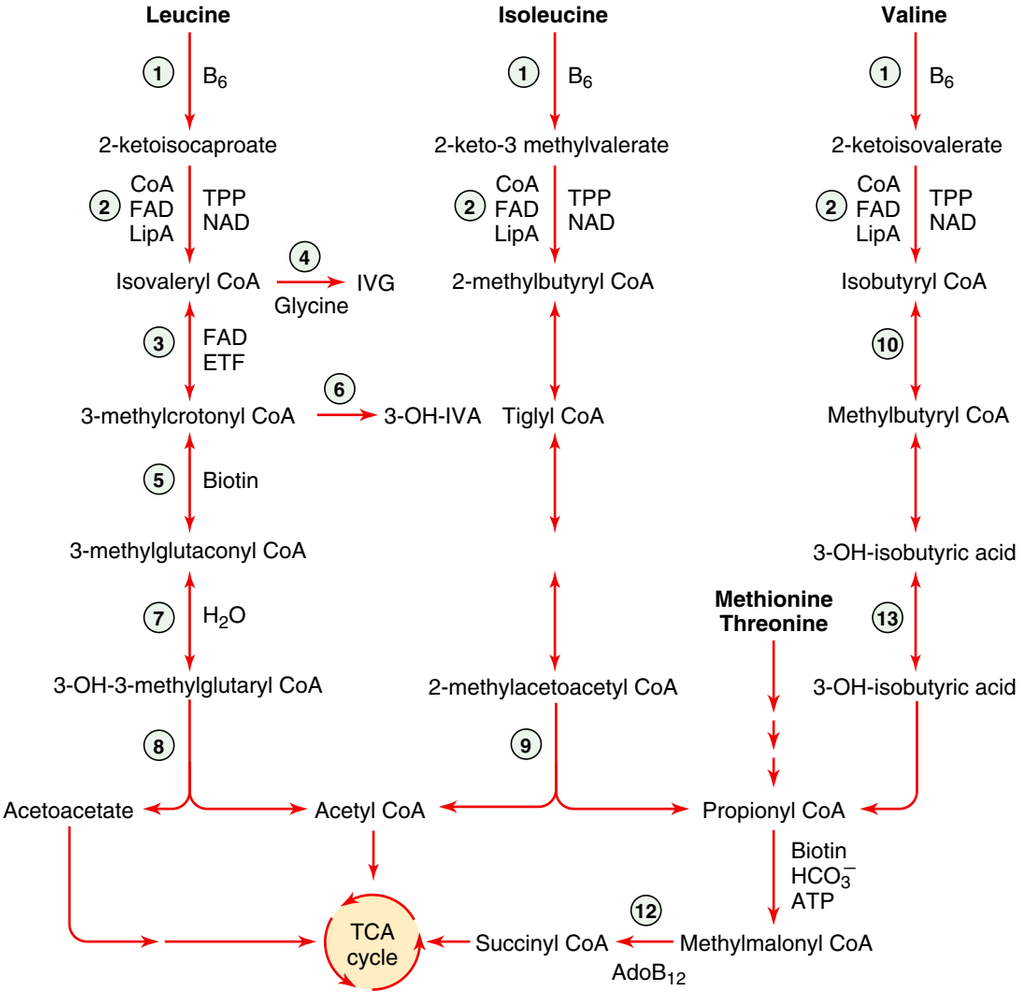

FIGURE 40-1 Major pathways of branched-chain amino acid metabolism. Maple syrup urine disease is caused by a congenital deficiency of reaction (2). Many of the primary organic acidurias, e.g. isovaleric acidemia and methylmalonic acidemia, are referable to inherited defects of enzymes involved in the oxidation of organic acids derived from the branched-chain amino acids. Enzymes: (1) Branched-chain amino acid transaminase; (2) Branched-chain amino acid decarboxylase; (3) Isovaleryl-CoA dehydrogenase; (4) Glycine-N-acylase; (5) 3-methylcrotonyl-CoA carboxylase; (6) 3-methylglutaconyl-CoA hydroxylase; (7) 2-methylglutaconyl-CoA hydratase; (8) 3-OH-3-methylglutaryl-CoA lyase; (9) 2-ketothiolase; (10) Isobutyryl-CoA dehydrogenase; (11) Propionyl-CoA carboxylase; (12) Methylmalonyl-CoA mutase; (13) 3-OH-isobutyryl-CoA deacylase. *$AdoB_{12}$*, adenosylcobalamin; *ETF*, electron transfer flavoprotein; *LipA*, lipoic acid; *T-PP*, thiamine pyrophosphate.

TABLE 40-2 The organic acidurias

Organic aciduria	Enzyme deficiency	Clinical findings	Treatment
Isovaleric acidemia	Isovaleryl-CoA dehydrogenase	Neonate: fulminant syndrome: coma, convulsions. Rancid odor of 'sweaty socks' Older child: developmental delay, mental retardation, recurrent vomiting. Odor of 'sweaty socks' Carnitine Glycine to promote acylation of isovaleryl-CoA	Low protein diet to restrict intake of leucine (precursor to isovaleric acidemia)
3-methylcrotonic aciduria	3-methylcrotonyl-CoA carboxylase (biotin dependent)	Infancy: vomiting, metabolic acidosis, hyperlactatemia, convulsions, coma Older child (2–5 years): recurrent vomiting, metabolic acidosis, hypoglycemia and progressive lethargy	Low protein diet to restrict intake of leucine
3-methylglutaconic aciduria	3-methylglutaconyl-CoA hydratase	Speech delay; developmental delay usually is mild	Low protein diet to restrict intake of leucine
3-hydroxy-3-methyl-glutaric aciduria	3-hydroxy-3-methyl-glutaryl-CoA lyase	Vomiting, lethargy, coma, convulsions, metabolic acidosis. Hypoglycemia without significant ketoaciduria	Low protein diet to restrict intake of leucine Avoidance of fasting to prevent hypoglycemia
2-methylacetoacetyl-CoA thiolase deficiency	2-methylacetoacetyl-CoA thiolase	Recurrent acidosis, ketosis, vomiting, often with hypoglycemia. Prompt clinical response to intravenous glucose. Mental retardation not usual	Avoid prolonged fasting. Glucose infusion during acute episode
3-OH-butyryl-CoA deacylase deficiency	3-OH-butyryl-CoA deacylase	Multiple congenital anomalies, tetralogy of Fallot, facial dysmorphism, brain dysgenesis	None
Propionic acidemia	Propionyl-CoA carboxylase	Fulminant syndrome in neonate: coma, convulsions. Hyperglycinemia and hyperammonemia common Milder, later-onset form: developmental retardation, failure to thrive, recurrent vomiting	Special diet low in isoleucine, valine, methionine, threonine. Carnitine supplementation. Liver transplantation may be beneficial.
Methylmalonic acidemia	Two forms: 1. Methylmalonyl-CoA mutase 2. Deficiency of metabolism of vitamin B_{12}, a cofactor for the reaction	Severe form: acidosis, hyperammonemia, hepatomegaly, hyperglycinemia, hypoglycemia, coma, convulsions, growth failure, psychomotor retardation. Basal ganglia damage ('metabolic stroke') is frequent, especially involving globus pallidus Defects of vitamin B_{12} metabolism may show homocystinemia	Special diet low in isoleucine, valine, methionine, threonine. Carnitine supplementation. Liver transplantation may be beneficial. Glucose and bicarbonate during acute episodes
Type I glutaric aciduria	Glutaryl-CoA dehydrogenase	Macrocephaly common. Initially normal. Develop hypotonia, opisthotonus, seizures, rigidity, dystonia, facial grimacing. Atrophy of caudate and putamen	Special diet low in lysine and tryptophan
Type II glutaric aciduria	Deficiency of electron transport flavoprotein or of ETF:ubiquinone reductase	Hepatomegaly, hypoglycemia without ketonuria, lipid storage myopathy with hypotonia, metabolic acidosis, rancid urine odor ('sweaty socks'), enlarged and cystic kidneys, hepatic cysts, facial dysmorphism	Typically fatal in infancy. Profound developmental delay in rare surviving infant
Biotinidase deficiency	Biotinidase	Eczema, seizures, deafness, lactic acidosis, hypotonia, lethargy, ataxia, neuropathy, immune deficiency, optic atrophy, alopecia	Biotin supplementation
Holocarboxylase synthetase deficiency	Holocarboxylase synthetase	Profound acidosis with ketonuria, lactic acidosis, tachypnea, lethargy, hypotonia, seizures, unusual urinary odors. Usually severe neonatal onset	Biotin supplementation

Untreated aminoacidurias can cause brain damage in many ways, often through impairing brain energy metabolism. Various biochemical changes occur in experimental models of these disorders. An adverse effect on brain energy metabolism has been observed in virtually all *in vitro* and animal models. *In vivo* evidence indicates that similar pathophysiological mechanisms probably occur in humans, particularly during metabolic decompensation. Thus, studies with ^{31}P-magnetic resonance spectroscopy in adults with phenylketonuria showed increases of brain levels of ADP in the basal state (see Ch. 58 for detailed discussion of spectroscopy). Loading with phenylalanine caused slowing of the electroencephalogram (EEG) and a further increase in the concentration of ADP [3]. Similarly, an elevation of brain lactate has been observed during metabolic decompensation in the brains of patients with maple syrup urine disease [4]. A variety of related factors probably are involved, including an uncoupling of oxidative metabolism, inhibition of the tricarboxylic acid cycle, impaired glucose homeostasis, alterations of the intracellular redox potential and excessive stimulation of brain *N*-methyl-D-aspartate (NMDA) receptors, thereby leading to consumption of ATP via activation of Na^+,K^+-ATPase (see pertinent topics of metabolism and neurotoxicity in Chs 5, 31, 32 and 42). These mechanisms are not necessarily mutually exclusive. For example, during hyperammonemia more than one mechanism probably is operative (see also Ch. 34) [5].

An imbalance of amino acids in the blood often alters the rate of transport of these compounds into the brain, thereby affecting levels of neurotransmitters and rates of protein synthesis. In almost all aminoacidopathies the blood concentrations of one or more amino acids

increase markedly. This factor may competitively inhibit the transport of other amino acids across the blood–brain barrier [6]. Amino acids are transferred into the CNS via specialized transporters that mediate the uptake of neutral compounds. Excessive plasma levels of one compound may inhibit the transport of others. This phenomenon has been studied most carefully with respect to phenylketonuria, in which there occurs diminished entry into brain of tyrosine and tryptophan, the respective precursors of dopamine and serotonin (see Chs 12 and 13). *In vivo* studies with magnetic resonance support this hypothesis. When adults with phenylketonuria receive dietary supplements of neutral amino acids, the blood concentrations of tyrosine and tryptophan increase but blood levels of phenylalanine are unchanged. In contrast, the brain concentration of phenylalanine declines toward the level observed in heterozygotes [7]. Some patients reported that neutral amino acid supplementation afforded relief from depression, perhaps reflecting a relative increase in brain levels of dopamine and/or tryptophan (see Catecholamines in Depression, Ch. 55).

Distortion of the plasma aminogram in individuals with an aminoaciduria also may lead to a relative failure of brain protein synthesis. Thus, in mice with a deficiency of phenylalanine hydroxylase, the blood concentration of phenylalanine is more than 20 times greater than the control value, leading to partial saturation of the transport system and a diminution in the brain level of neutral amino acids other than phenylalanine. Rates of protein synthesis were concomitantly reduced [8].

Treatment of aminoacidurias with a low-protein diet may influence brain chemistry. It should be emphasized that the treatment of the patient with an aminoaciduria may affect brain chemistry, perhaps in an adverse manner. Nearly all patients receive a low-protein diet. Indeed, undiagnosed patients sometimes avoid consumption of protein, which they feel intuitively can cause lethargy, headache, nausea and mental confusion. As dietary protein declines, the intake of carbohydrate frequently increases. The concomitant rise of endogenous insulin secretion favors an increase in the ratio of the concentration of blood tryptophan to that of other amino acids, thereby promoting the entry of tryptophan to the brain. The latter amino acid is precursor to brain serotonin, which tends to increase. This physiology is known to be operative in patients with urea cycle defects.

Imbalances of brain amino acids may hinder the synthesis of brain lipids, leading to a diminution in the rate of myelin formation. Decreases of lipids, proteolipids and cerebrosides (Ch. 3) have been noted in several of these syndromes, e.g. maple syrup urine disease, when intramyelinic edema is a prominent finding, particularly during the acute phase of metabolic decompensation [9]. Pathological changes in brain myelin are common, especially in infants who die early in life. The fundamental lesion may involve a failure of myelin protein synthesis as a consequence of the imbalanced brain amino acid content. It also is probable that disturbances of energy metabolism lead to a failure of myelin synthesis during development, when lipid formation is very active (see myelin formation in Ch. 4). Amino acid metabolites may directly inhibit the synthesis of crucial lipids such as arachidonic acid and docosahexaenoic acid (see detailed discussion of these lipids in Ch. 33) [10]. Similarly, excessive levels of phenylalanine appear to inhibit the synthesis of cholesterol [11].

In many aminoacidurias, there may occur deficits in neurotransmitters and receptors, particularly the *N*-methyl-D-aspartate receptor. Brain damage and dysfunction in the aminoacidurias may reflect injury to neurotransmitter receptors and transporters [12]. Thus, the presence of a glycine binding site on the NMDA glutamatergic receptor (see Ch. 15) prompted the hypothesis that the severe seizure disorder that typifies the congenital absence of the glycine cleavage system might be responsive to treatment with NMDA receptor antagonists [13]. Excessive stimulation of the NMDA receptor presumably contributes to the severe seizure disorder (see Ch. 37). Similarly, hyperammonemia appears to impair the high-affinity uptake of glutamate into both astrocytes and neurons. A compensatory decrease of NMDA receptors may then occur as an adaptation to increased external glutamate [14].

Brain edema, often associated with increased intracranial pressure, may accompany the acute phase of metabolic decompensation in the aminoacidurias. Brain swelling and increased intracranial pressure may occur during acute illness. As indicated above, intramyelinic edema may occur in maple syrup urine disease. Swelling of brain astrocytes occurs with acute hyperammonemia because water is drawn into glia secondary to accumulation of glutamine, which is preferentially formed in astrocytes from glutamate and ammonia (see Ch. 15). Increased brain blood flow during hyperammonemia accentuates swelling [15].

DISORDERS OF BRANCHED-CHAIN AMINO ACIDS: MAPLE SYRUP URINE DISEASE

Maple syrup urine disease involves a congenital failure to oxidize the three branched-chain amino acids. The first congenital defect of branched-chain amino acid (BCAA) catabolism to be described was maple syrup urine disease (MSUD), a deficiency of mitochondrial branched-chain ketoacid dehydrogenase (Fig. 40-1; reaction 2). The ketoacids are freely reaminated to the parent amino acids, the latter being readily measured in the blood

and urine. The characteristic odor of the branched-chain ketoacids imparts to the urine a smell that resembles maple syrup or burned sugar.

The decarboxylase is composed of four subunits: E1-a, E1-b, E2 and E3. A specific kinase and phosphatase, respectively, activate and deactivate the enzyme complex. Most MSUD patients have mutations involving the E1-a subunit that decarboxylates the ketoacid, although defects of the E1-b protein have been described [16]. The E1-a mutation usually causes faulty assembly of the heterotetrameric (a_2b_2) E1 protein. Mutations of either the E2 or E3 moieties are extremely rare. The E3 subunit is common to other decarboxylating systems, including pyruvate dehydrogenase and 2-oxo-glutarate dehydrogenase. Hence, mutations in this protein can cause lactic acidosis and deranged tricarboxylic acid cycle activity as well as an accumulation of the BCAAs.

Infants are protected during gestation because the placenta clears most potential toxins. The classical form of the disease therefore does not become clinically manifest until a few days after birth. An initial phase of alternating irritability and lethargy progresses over a period of days to frank coma and respiratory embarrassment. Irreversible brain damage is common in babies who survive, particularly those whose treatment is delayed until after the first week of life.

Survivors may suffer a metabolic relapse at any time. The most common cause of relapse is intercurrent infection, which favors endogenous protein catabolism. As a consequence, the patient's limited capacity to oxidize BCAAs is overwhelmed and these compounds, together with their cognate ketoacids, accumulate to a toxic level. Relapse also can occur in association with surgery, trauma and emotional upset.

Patients with partial enzymatic deficiencies may present later in life with intermittent ketoacidosis, prostration and recurrent ataxia. The plasma concentrations of BCAA are elevated during these episodes but they may be normal or near-normal during the periods when patients are metabolically compensated.

Rare patients respond to the administration of thiamine in large doses (10–30 mg/day). The clinical course is even more mild than that of patients with intermittent disease. Thiamine is a cofactor for the branched-chain ketoacid dehydrogenase, and the presumed mutation involves faulty binding of the apoprotein to this vitamin.

In many localities, newborn screening has become standard for this disorder, which in the general population has an approximate incidence of 1/250,000 live births. Carrier detection is possible, either by measurement of enzymatic activity in cultured fibroblasts or by study of restriction endonuclease fragments of DNA. Antenatal testing is also available.

Effective treatment of maple syrup urine disease involves the restriction of dietary branched-chain amino acids. Long-term treatment entails the dietary restriction of the BCAAs. This is accomplished by administration of a special formula from which these amino acids are removed. The outlook for intellectual development is favorable in youngsters in whom diagnosis is made early and who do not suffer recurrent, severe episodes of metabolic decompensation [17].

Gene therapy for this metabolic defect may become available within the next few years. *In vitro* studies have demonstrated the feasibility of retroviral-mediated gene transfer of both the E1-a and E2 subunits of the branched-chain decarboxylase complex [16, 18].

DISORDERS OF PHENYLALANINE METABOLISM: PHENYLKETONURIA

Phenylketonuria usually is caused by a congenital deficiency of phenylalanine hydroxylase. Phenylketonuria (PKU) is among the more common aminoacidurias (≈1:20,000 live births). The usual cause is a nearly complete deficiency of phenylalanine hydroxylase, which converts phenylalanine into tyrosine (Fig. 40-2; reaction 1).

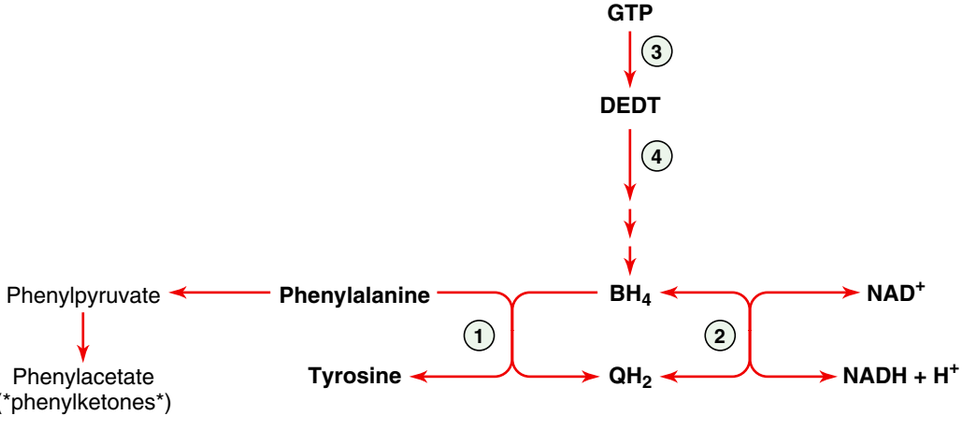

FIGURE 40-2 The phenylalanine hydroxylase (PAH) pathway. Phenylketonuria usually is caused by a congenital deficiency of PAH (reaction 1), but it also can result from defects in the metabolism of biopterin, which is a cofactor for the hydroxylase. Enzymes: (1) Phenylalanine hydroxylase; (2) Dihydropteridine reductase; (3) GTP cyclohydrolase; (4) 6-pyruvoyltetrahydrobiopterin synthase. *BH_4*, tetrahydrobiopterin; *DEDT*, D-*erythro*-dihydroneopterin triphosphate; *QH_2*, dihydrobiopterin.

In addition to 'classical' PKU, many youngsters have hyperphenylalaninemia caused by a partial deficiency of the enzyme. They do not suffer mental retardation but they may have more subtle neurological problems [19, 20].

The hydroxylase is a trimer (mol. wt ≈150,000) made up of identical subunits. It is located predominantly in the liver. The enzyme has been mapped to human chromosome 12q22–24.1, where the gene is made up of 13 exons extending over 90≈kb of genomic DNA.

Frank deletions of the gene are not common. A frequent mutation among northern Europeans (≈40%) is a G to A transition at the 5′ donor splice site in intron 12, resulting in absence of the C terminus. Another relatively common (≈20%) mutation in northern Europeans involves a C to T transition in exon 12, resulting in substitution of a tryptophan for an arginine residue [21]. Over 70 different mutations have been described to date in the American population [22].

Specific mutations have been associated with specific haplotypes, the latter determined by analysis of restriction fragment length polymorphisms. This approach has been utilized for prenatal diagnosis. The study of haplotypes also has revealed that the majority (≈75%) of northern European patients are compound PKU heterozygotes.

The outlook for patients who are treated at an early age is favorable. Affected babies are normal at birth but almost all will be impaired unless they receive dietary restriction by age 3 months. Mass screening has largely eliminated the untreated PKU phenotype of eczema, poor growth, irritability, musty odor (caused by phenylacetic acid) and tendency to self-mutilation. Progressive motor dysfunction has been described in children with long-term hyperphenylalaninemia.

The clinical utility of dietary restriction of phenylalanine (200–500 mg/day of phenylalanine) is clear. Well-controlled patients have normal intelligence, although there may be an increased risk of perceptual–learning disabilities, emotional problems and subtle motor difficulties [23]. Diet therapy must probably be maintained throughout adolescence and perhaps indefinitely. Performance may deteriorate after the diet is discontinued.

The genotypically normal offspring of an untreated mother may have microcephaly and irreversible brain injury as well as cardiac defects. Scrupulous monitoring of dietary phenylalanine intake in these women has improved outcome [24].

Rarely, phenylketonuria results from a defect in the metabolism of biopterin, a cofactor for the phenylalanine hydroxylase pathway. The electron donor for phenylalanine hydroxylase is tetrahydrobiopterin (BH4), which transfers electrons to molecular oxygen to form tyrosine and dihydrobiopterin (QH2; Fig. 40-2; reaction 2). BH_4 is regenerated from QH_2 in an NADH-dependent reaction that is catalyzed by dihydropteridine reductase (DHPR), which is widely distributed. In the brain, this enzyme and BH4 also are involved in hydroxylation of tyrosine and tryptophan (Chs 12 and 13). Human DHPR has been mapped to human chromosome 4p15.1–p16.1. The coding sequence shows little homology to other reductases, e.g. dihydrofolate reductase.

In rare instances, PKU is caused by defects in the metabolism of BH4, which is synthesized from GTP via sepiapterin (Fig. 40-2; reactions 3 and 4) [25]. Even careful phenylalanine restriction fails to avert progressive neurological deterioration because patients are unable to hydroxylate tyrosine or tryptophan, the synthesis of which also requires tetrahydrobiopterin. Thus, neurotransmitters are not produced in sufficient amount.

Patients sustain convulsions and neurological deterioration. The urine contains low levels of the metabolites of serotonin, norepinephrine and dopamine. The reductase also plays a role in the maintenance of tetrahydrofolate levels in brain, and some patients have had low folate levels in the serum and CNS. Treatment has been attempted with tryptophan and carbidopa to improve serotonin homeostasis and with folinic acid to replete diminished stores of reduced folic acid. This therapy is sometimes effective. Diagnosis involves assay of DHPR in skin fibroblasts or amniotic cells. Phenylalanine hydroxylase activity is normal.

Other causes of PKU secondary to defective tetrahydrobiopterin synthesis include GTP cyclohydrolase deficiency and 6-pyruvoyltetrahydrobiopterin synthase deficiency. Patients with either defect have psychomotor retardation, truncal hypotonia with limb hypertonia, seizures and a tendency to hyperthermia. The intravenous administration of BH4 may lower blood phenylalanine levels but this cofactor may not readily cross the blood–brain barrier. Treatment with synthetic pterin analogs or supplementation with tryptophan and carbidopa may prove more efficacious, particularly if treatment is started early in life.

DISORDERS OF GLYCINE METABOLISM: NONKETOTIC HYPERGLYCINEMIA

Nonketotic hyperglycinemia results from the congenital absence of the glycine cleavage system, which mediates the interconversion of glycine and serine. Glycine catabolism proceeds primarily via the glycine cleavage system, a mitochondrial system that interconverts glycine and serine (Fig. 40-3; reaction 1). Pyridoxal phosphate and tetrahydrofolate are cofactors. This reaction also provides precursor to the 'one-carbon pool' of folic acid intermediates that are pivotal to many synthetic reactions [26].

Nonketotic hyperglycinemia causes a severe seizure disorder and profound brain damage. Infants affected by deficiency of the glycine cleavage system become ill with seizures by the first or second day of life.

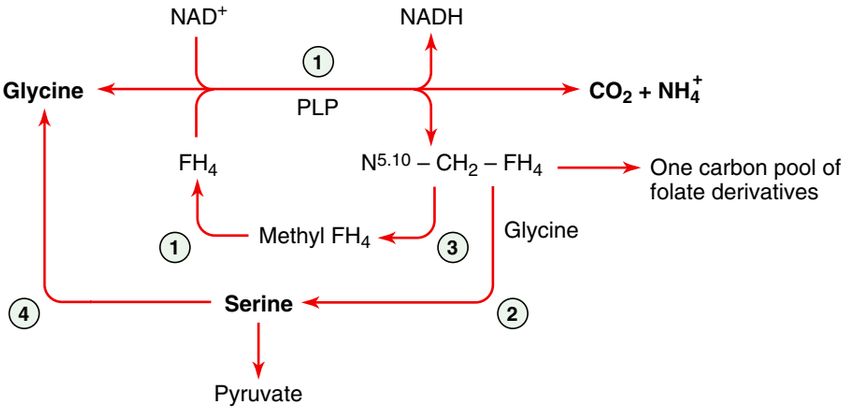

FIGURE 40-3 Glycine cleavage system and some related reactions. Glycine and serine are readily interchangeable. Enzymes: (1) Glycine cleavage system; (2) and (4) Serine hydroxymethyltransferase; (3) $N^{5,10}$-methylenetetrahydrolate reductase. *$N^{5,10}$-CH_2-FH_4*, $N^{5,10}$-methylenetetrahydrolate; *FH_4*, tetrahydrofolic acid.

Intrauterine seizures may occur. The electroencephalogram often displays a hypsarrhythmia or a burst-suppression pattern. Patients display myoclonic jerks, hiccuping and a profound hypotonia. The few patients who survive past the first week usually sustain profound mental retardation and neurological disability. Brain imaging shows atrophy and a loss of myelin. Rarely, patients present later in life with psychomotor retardation and growth failure. Others have had initial normal development followed by a progressive loss of developmental milestones. Some patients have manifested spinocerebellar degeneration and other symptoms of motor dysfunction [27].

In nonketotic hyperglycinemia, glycine is extremely high in the blood, often rising to >1 mmol/l (normal = 150–350 mmol/l). The concentration in the cerebrospinal fluid almost always exceeds 100 µmol/l (normal ≈10 µmol/l). The cerebrospinal fluid (CSF):blood ratio of glycine usually is five to ten times the control value (0.02), especially with the classical form of the disease. A transient form of nonketotic hyperglycinemia, probably reflecting delayed maturation of the glycine cleavage system, has been described in neonates with seizures but an otherwise normal neurological examination. The seizures ceased by 8 weeks of age and did not recur. Glycine concentrations of both the blood and the CSF were high. Urine organic acid analysis was normal. Prognosis is favorable in this situation.

Treatment for nonketotic hyperglycinemia is less effective than that available for other aminoacidurias. There is no specific therapy. Exchange transfusion and dialysis usually do not alter the progressive neurological deterioration. Sodium benzoate has been administered in the hope that glycine would react with it to form hippuric acid, but this approach is not helpful. It may be that a combination of benzoate and carnitine therapy is more effective [28]. Similarly, the restriction of dietary protein and the administration of pyridoxine or choline have not proved useful.

Glycine is a neurotransmitter, having a postsynaptic inhibitory activity in the spinal cord and in some central neurons (Ch. 16). Therapy with strychnine, which blocks the action of glycine at postsynaptic inhibitory receptors, has been unhelpful. Treatment with diazepam has been attempted because this drug displaces strychnine from its binding sites. The combination of benzoate and diazepam may be more effective, since high doses of the former reduce glycine levels in the CNS, thereby potentiating the ability of strychnine to block the glycine effect.

A few infants have been treated with antagonists of the NMDA receptor, an excitatory glutamatergic receptor for which glycine is a co-agonist (see Ch. 15) [29]. Ketamine and dextromethorphan have been used with inconclusive results. Some infants may have had an improvement of their irritability and electroencephalogram. One infant, treated with both benzoate and dextromethorphan, was seizure-free by 12 months of age and had only moderately delayed development. However, this favorable experience has not always been duplicated. Treatment with dextromethorphan at the recommended dosage (maximum 5 mg/kg/day) seems to be well-tolerated.

DISORDERS OF SULFUR AMINO ACID METABOLISM: HOMOCYSTINURIA

The transsulfuration pathway is the major route for the metabolism of the sulfur-containing amino acids. The transsulfuration pathway (Fig. 40-4) entails the transfer of the sulfur atom of methionine to serine to yield cysteine. The first step is activation of methionine, which reacts with ATP to form *S*-adenosylmethionine (Fig. 40-4; reaction 1). This compound is a key methyl donor and plays a prominent role in the synthesis of several

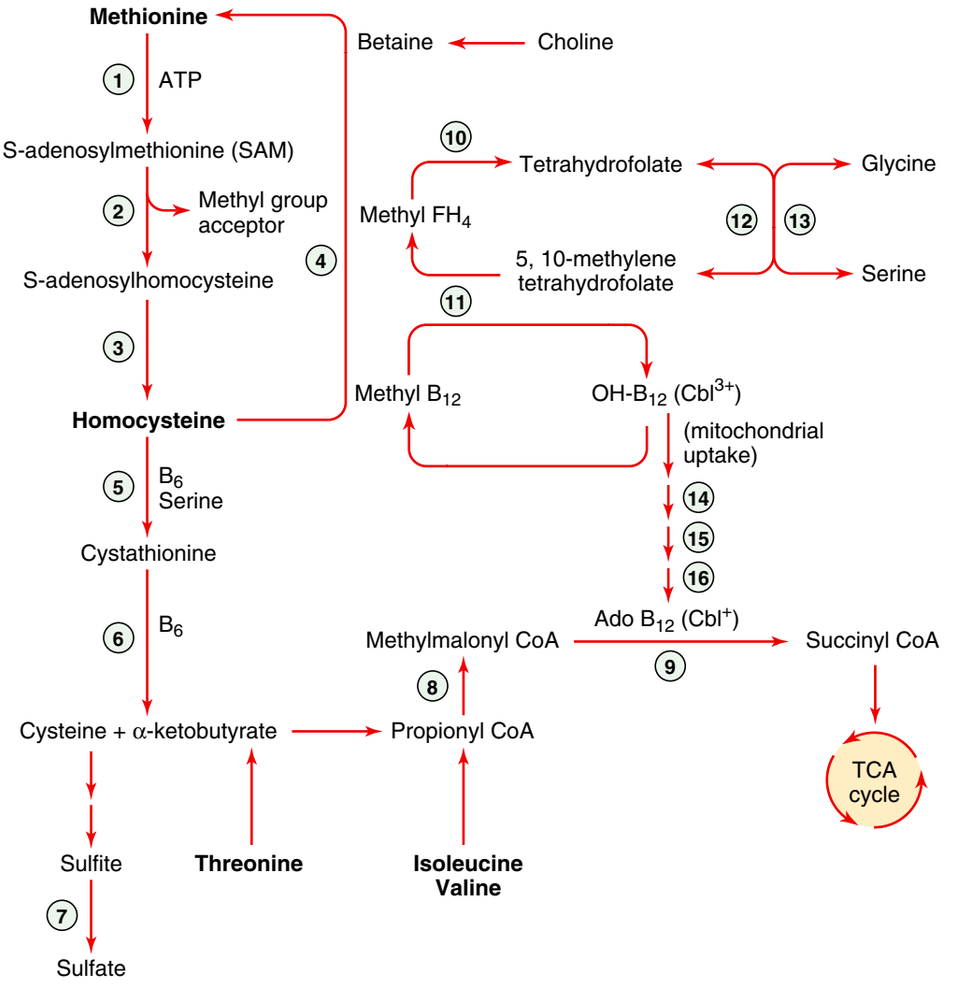

FIGURE 40-4 The transsulfuration pathway and related metabolic routes. Homocystinuria usually is caused by a congenital deficiency of cystathionine-β-synthase (reaction 5). Sometimes homocystinuria is caused by a failure of the remethylation of homocysteine. This may occur because of a failure to generate methylfolate or methylcobalamin. If there is a generalized failure of cobalamin activation or absorption, methylmalonic aciduria as well as homocystinuria may result because cobalamin derivatives are essential to both pathways. Enzymes: (1) Methionine-activating enzyme; (2) Generic depiction of methyl group transfer from *S*-adenosylmethionine; (3) *S*-adenosylhomocysteine hydrolase; (4) Homocysteine: methionine methyltransferase; (5) Cystathionine-β-synthase; (6) Cystathionase; (7) Sulfite oxidase; (8) Propionyl-CoA carboxylase; (9) Methylmalonyl-CoA mutase; (10) Homocysteine:methionine methyltransferase (essentially same as reaction 4, in which methyltetrahydrofolate is the methyl donor); (11) $N^{5,10}$-methylenetetrahydrolate reductase; (12) and (13) Glycine cleavage system; (14) and (15) Hydroxocobalamin reductases; (16) Cobalamin adenosyltransferase. *Ado-B_{12}*, adenosylcobalamin; *Methyl-B_{12}*, methylcobalamin; *OH-B_{12}*, hydroxocobalamin.

neurotransmitters and of creatine (Fig. 40-4; reaction 2). A portion of the carbon of spermidine and spermine is formed by decarboxylation of *S*-adenosylmethionine.

Transfer of a methyl group from *S*-adenosylmethionine yields *S*-adenosylhomocysteine, which potently inhibits several methyltransferases; this may partially explain the pathology of homocystinuria. Tissue levels of *S*-adenosylhomocysteine ordinarily are very low, since this metabolite is rapidly cleaved by a specific hydrolase to homocysteine and adenosine (Fig. 40-4; reaction 3).

About half of the homocysteine so generated is remethylated to methionine, with either betaine or 5-methyltetrahydrofolic acid (methyl-FH_4) serving as methyl donor. The enzyme mediating remethylation, 5-methyltetrahydrofolate-betaine methyltransferase (Fig. 40-4; reaction 4), utilizes methylcobalamin as a cofactor. The kinetics of the reaction favor remethylation. Faulty remethylation can occur secondary to: (1) dietary factors, e.g. vitamin B_{12} deficiency; (2) a congenital absence of the apoenzyme; (3) a congenital inability to convert folate or B_{12} to the methylated, metabolically active form (see below); or (4) the presence of a metabolic inhibitor, e.g. an antifolate agent that is used in an antineoplastic regimen.

The most common cause of homocystinuria is a congenital deficiency of cystathionine-β-synthase, a pyridoxine-dependent enzyme that condenses homocysteine and

serine to form cystathionine (Fig. 40-4; reaction 5). S-adenosylmethionine stimulates the forward reaction [30]. This enzyme has been mapped to human chromosome 21. The equilibrium favors cystathionine synthesis. Thus, homocysteine levels normally are very low, since both the remethylation pathway and the cystathionine synthetase route efficiently dispose of this amino acid.

Cleavage of cystathionine is accomplished by cystathionase, another pyridoxine-dependent enzyme that is coded on human chromosome 16 (Fig. 40-4; reaction 6). The enzyme functions almost entirely to produce cysteine, there being virtually no reversal of the reaction.

Homocystinuria is the result of the congenital absence of cystathionine synthase, a key enzyme of the transsulfuration pathway. A variety of mutations have been described, including the synthesis of an unstable enzyme, of a protein that loosely binds either pyridoxal phosphate, serine or homocysteine, or of an enzyme differing in size from the wild strain [31]. Cystathionine synthase is present in many organs, including the brain, and homocystinuric patients typically manifest deficient enzyme activity in these tissues. Blood homocysteine levels are elevated (50–200 μmol/l; normal <10 μmol/l) and the blood cysteine concentration tends to be low, reflecting the failure of cysteine synthesis. Increased remethylation of homocysteine that is not converted to cystathionine results in elevated blood methionine, often in excess of 200 μmol/l (normal = 20–40 μmol/l).

Homocystinuria can be treated in some cases by the administration of pyridoxine (vitamin B_6), which is a cofactor for the cystathionine synthase reaction. Some patients respond to the administration of pharmacological doses of pyridoxine (25–100 mg daily) with a reduction of plasma homocysteine and methionine. Pyridoxine responsiveness appears to be hereditary, with sibs tending to show a concordant pattern and a milder clinical syndrome. Pyridoxine sensitivity can be documented by enzyme assay in skin fibroblasts. The precise biochemical mechanism of the pyridoxine effect is not well understood but it may not reflect a mutation resulting in diminished affinity of the enzyme for cofactor, because even high concentrations of pyridoxal phosphate do not restore mutant enzyme activity to a control level.

About half of individuals who do not respond to pyridoxine will sustain *ectopia lentis* by age 5–10 years. Indeed, the diagnosis commonly is made by an ophthalmologist. The median IQ score for B_6 responsive and nonresponsive patients is 78 and 56 respectively. Some children present at 1–2 years with psychomotor retardation, convulsions (≈20% of cases) and psychiatric difficulties such as depression and personality disorders (≈50% of cases).

Patients with homocystinuria are at risk for cerebrovascular and cardiovascular disease and thromboses. The most striking feature is a thromboembolic diathesis that can occur in virtually any vessel, with thrombi common in the cerebral, cardiac and renal vasculature. Almost 25% of pyridoxine nonresponders sustain a major vascular insult during childhood. The comparable risk in untreated pyridoxine-responsive subjects is 25% by age 20 years. Vascular insults sometimes occur in association with dehydration secondary to vomiting and diarrhea. The stress of major surgery and anesthesia increase the risk of thrombosis by ≈5%. Homocystinuric patients who also have the relatively common Leiden mutation of clotting factor V are at sharply increased risk for developing a thrombosis [32].

Patients commonly manifest a marfanoid habitus with arachnodactyly, high-arched palate, tall stature and pes cavus. Bony abnormalities are common, with osteoporosis and scoliosis being frequent findings. The orthopedic features are more common and severe in patients who do not respond to pyridoxine treatment. Demyelination and spongy degeneration of the white matter have been reported. Frank infarctions are relatively common in virtually all parts of the brain. The arterial wall shows thickening of the intima and splitting of the smooth musculature of the media. The changes are not dissimilar to those of atherosclerosis.

The probable cause of the pathology is hyperhomocysteinemia rather than hypermethioninemia. The biochemical basis of homocysteine toxicity is the subject of intense scrutiny. Homocysteine increases the adhesiveness of platelets *in vitro*, perhaps by favoring the synthesis of selected thromboxanes. The administration of homocysteine to rats or baboons can cause endothelial injury. Homocysteine may diminish the mean survival time of peripheral blood platelets, possibly by a direct toxic effect on the vascular endothelium, which becomes denuded and thereby provides an atherogenic nidus. A direct effect of homocysteine on the blood clotting cascade also is possible. Thus, activation of factor V in cultured endothelial cells has been noted. This favors the conversion of prothrombin to thrombin. Homocysteine also promotes accumulation of copper in the vascular endothelium. This induces the oxidation of ceruloplasmin and the concomitant release of sufficient H_2O_2 to injure endothelial cells. Supplementation of the medium with catalase protects against such insult, thus confirming the role of oxidant injury.

High levels of homocysteine or one of its metabolites may directly affect brain function. The administration of homocysteine to rats induces grand mal convulsions, a phenomenon that is aggravated by either methionine or pyridoxine. Homocysteine-induced blockade of the γ-aminobutyric acid (GABA) receptor may be involved. In addition, brain can oxidize homocysteine to homocysteic acid, which has a glutamatergic activity.

A high intracerebral level of *S*-adenosylhomocysteine may inhibit methylation reactions involving *S*-adenosylmethionine. The metabolic repercussions would be extensive, including deficient methylation of proteins and of phosphatidylethanolamine as well as an inhibition of catechol-*O*-methyltransferase and histamine-*N*-methyltransferase.

Prognosis is more favorable in the pyridoxine-responsive patients. Patients who respond to large doses of vitamin B_6 (250–500 mg/day for several weeks) have the best prognosis. Efficacy of treatment usually is reflected in a reduction of blood homocystine and methionine to normal or near-normal levels. Since supplementation with pyridoxine can cause a deficiency of folic acid, the latter should be given (2–5 mg daily) at the same time. Any patient receiving pyridoxine should be monitored carefully for any signs of hepatotoxicity and for a peripheral neuropathy (see Ch. 36).

Management of the pyridoxine nonresponsive patient is difficult. Dietary restriction of methionine would seem logical, but this often is unpalatable, especially to an adult patient who has adapted to a diet that has not been purposefully restricted in protein.

A newer therapeutic approach is the administration of betaine (6–12 g daily), which lowers homocysteine levels by favoring remethylation [33]. A theoretical hazard of betaine treatment is increasing the blood methionine, sometimes to an extravagant degree (≈1 mmol/l). Experience to date indicates that betaine administration is safe, with no major side effects except for a 'fishy' odor to the urine. Other therapeutic approaches have included the administration of salicylate to ameliorate the thromboembolic diathesis. Patients also have been treated with dietary supplements of L-cystine, since the block of the transsulfuration pathway in theory could diminish the synthesis of this amino acid.

Homocystinuria can occur when homocysteine is not remethylated back to form methionine. This situation is termed 'remethylation deficiency homocystinuria'. In the form of *S*-adenosylmethionine, methionine is the major methyl donor for biosynthetic reactions in the brain. Most remethylation defects result from aberrant metabolism of methylfolate or methylcobalamin. Patients often present early in life with lethargy, poor feeding, psychomotor retardation and growth failure. Hematological abnormalities are common, including megaloblastosis, macrocytosis, thrombocytopenia and hypersegmentation of the leukocytes. Occasional patients present later, when seizures, dementia, hypotonia, mental retardation, spasticity or a myelopathy become evident.

Biochemical findings are variable. The blood cobalamin and folate levels often are normal. Patients often have homocysteinemia with hypomethioninemia, the latter finding discriminating this group from homocystinuria secondary to cystathionine-β-synthase deficiency. Urinary excretion of methylmalonic acid may be high, reflecting the fact that vitamin B_{12} serves as a cofactor for the methylmalonyl-CoA (coenzyme A) mutase reaction.

One form of remethylation deficit involves defective metabolism of folic acid, a key cofactor in the conversion of homocysteine to methionine. Methylenetetrahydrofolate reductase (Fig. 40-4; reaction 11) reduces $N^{5,10}$-methylenetetrahydrofolate to methyltetrahydrofolate in a reaction that is NADPH-dependent and inhibited by *S*-adenosylmethionine. In brain this enzyme also is important for the reduction of dihydropteridines (see Disorders of Phenylalanine Metabolism, above).

Patients typically present by 6–12 months with severe developmental retardation, convulsions, microcephaly and homocysteinemia (≈50 μmol/l) with hypomethioninemia (<20 μmol/l). A few individuals have had psychiatric disturbances. The blood concentration of vitamin B_{12} is normal, and, unlike individuals with defects of cobalamin metabolism, these patients manifest neither anemia nor methylmalonic aciduria. The blood folic acid level is usually low.

A thromboembolic diathesis is not unusual and strokes have been reported. Other pathological changes have included microgyri, demyelination, gliosis and brain atrophy. Lipid-laden macrophages have been described.

A relatively large number of agents have been utilized to treat this intractable disorder: folinic acid (5-formyltetrahydrofolic acid), folic acid, methyltetrahydrofolic acid, betaine, methionine, pyridoxine, cobalamin and carnitine. Betaine, which provides methyl groups to the betaine:homocysteine methyltransferase reaction, is a safe treatment that lowers blood homocysteine and increases methionine.

Methionine synthase deficiency (cobalamin-E disease) produces homocystinuria without methylmalonic aciduria. This enzyme mediates the transfer of a methyl group from methyltetrahydrofolate to homocysteine to yield methionine (Fig. 40-4; reaction 4). A cobalamin group bound to the enzyme is converted to methylcobalamin prior to formation of methionine.

In cobalamin-E (cblE) disease there is a failure of methyl-B_{12} to bind to methionine synthase. It is not known if this reflects a primary defect of methionine synthase or the absence of a separate enzyme activity. Patients manifest megaloblastic changes with a pancytopenia, homocystinuria and hypomethioninemia. There is no methylmalonic aciduria. Patients usually become clinically manifest during infancy with vomiting, developmental retardation and lethargy. They respond well to injections of hydroxocobalamin.

Cobalamin-c disease: remethylation of homocysteine to methionine also requires an 'activated' form of vitamin B_{12}. In the absence of normal B_{12} activation, homocystinuria results from a failure of normal vitamin B_{12} metabolism. Complementation analysis classifies defects in vitamin B_{12} metabolism into three groups: cblC (most common), cblD and cblF. Most individuals become ill in the first few months or weeks of life with hypotonia, lethargy and growth failure. Optic atrophy and retinal changes can occur. Methylmalonate excretion is excessive, but less than in methylmalonyl-CoA mutase deficiency, and without ketoaciduria or metabolic acidosis.

The fibroblasts do not convert cyanocobalamin or hydroxocobalamin to methylcobalamin or adenosylcobalamin, resulting in diminished activity of both N^5-methyltetrahydrofolate:homocysteine methyltransferase and methylmalonyl-CoA mutase. Supplementation with hydroxocobalamin rectifies the aberrant biochemistry. The precise nature of the underlying defect remains obscure. Diagnosis should be suspected in a child with homocystinuria, methylmalonic aciduria, megaloblastic anemia, hypomethioninemia and normal blood levels of folate and vitamin B_{12}. A definitive diagnosis requires demonstration of these abnormalities in fibroblasts. Prenatal diagnosis is possible.

Treatment involves the administration of large doses (as much as 1 mg) of intramuscular hydroxocobalamin. Administration of folate and betaine (see above) may be helpful, as is a reduction of protein intake.

Hereditary folate malabsorption presents with megaloblastic anemia, seizures and neurological deterioration. Levels of folate in both the blood and the cerebrospinal fluid have been very low. The anemia is correctable with injections of folate, or with the administration of large oral doses, but the concentration in the CSF is still low, suggesting that a distinct carrier system mediates folate uptake into the brain and that this system is the same as that facilitating intestinal transport.

THE UREA CYCLE DEFECTS

The urea cycle is essential for the detoxification of ammonia. The urea cycle (Fig. 40-5) converts ammonia to urea (10–20 g/day in the healthy adult). A urea cycle enzymopathy, whether associated with cirrhosis or an inherited metabolic defect, often causes a hyperammonemic encephalopathy and irreversible brain injury (see also Ch. 34).

The cycle 'begins' in hepatic mitochondria, where NH_3^-, HCO_3^- and ATP form carbamyl phosphate in a reaction catalyzed by carbamyl phosphate synthetase (CPS; Fig. 40-5; reaction 1). *N*-acetylglutamate (NAG), formed from glutamate and acetyl-CoA via *N*-acetylglutamate synthetase (Fig. 40-5; reaction 9), is an obligatory effector of CPS and an important regulator of ureagenesis. Various influences, including dietary protein, arginine and corticosteroids, augment the concentration of NAG.

Carbamyl phosphate condenses with ornithine to yield citrulline in the ornithine transcarbamylase (OTC) reaction. OTC is encoded on band p21.1 of the X chromosome, where the gene contains 8 exons and spans 85 kb of DNA. The activity of this enzyme is directly related to dietary protein. There may be 'tunneling' of ornithine transported from the cytosol to OTC, with the availability of intramitochondrial ornithine serving to regulate the reaction.

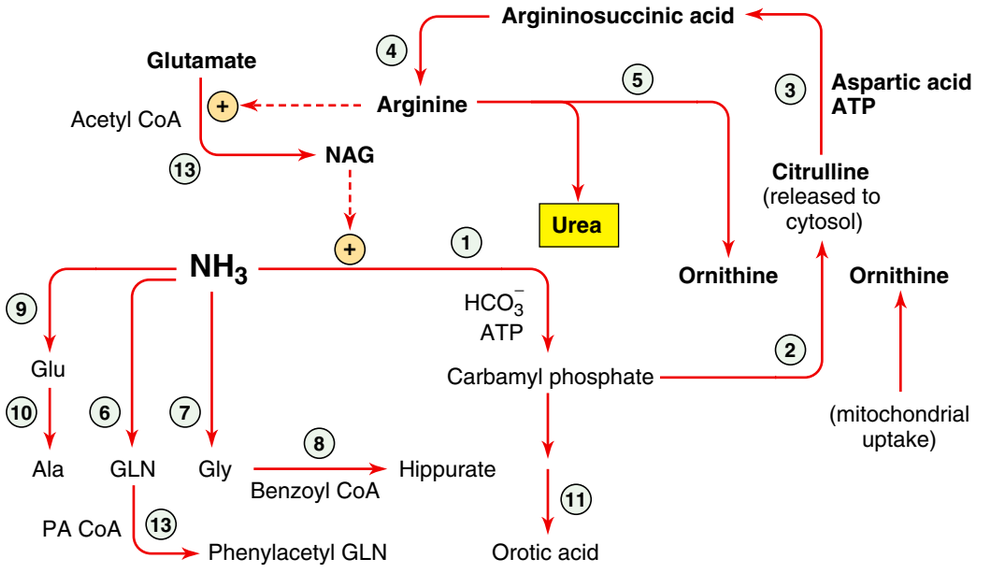

FIGURE 40-5 The urea cycle and related reactions of ammonia metabolism. Congenital hyperammonemia syndromes usually are caused by a deficiency of one of the enzymes of the urea cycle. Ammonia also can be metabolized to glutamate, alanine, glutamine and glycine. The administration of phenylacetate or of benzoate favors the formation of phenylacetylglutamine and hippurate, respectively, thereby providing an effective 'antidote' to ammonia toxicity. Enzymes: (1) Carbamyl phosphate synthetase; (2) Ornithine transcarbamylase; (3) Argininosuccinate synthetase; (4) Argininosuccinate lyase; (5) Arginase; (6) Glutamine synthetase; (7) Glycine cleavage system; (8) Glycine-*N*-acylase; (9) Glutamate dehydrogenase; (10) Alanine aminotransferase; (11) The cytosolic pathway of orotic acid synthesis (becomes prominent when there is a block at the level of reaction 2, thus resulting in increased orotic acid excretion); (12) N-acetylglutamate synthetase; (13) Phenylacetyl-CoA:glutamine transferase. *NAG*, *N*-acetylglutamate; *PA-CoA*, phenylacetyl-CoA. The + symbols denote that arginine and NAG are positive effectors for reactions 12 and 1 respectively.

In the hepatic cytosol, citrulline reacts with aspartate to form argininosuccinate, catalyzed by argininosuccinate synthetase (AS; Fig. 40-5; reaction 3). The mRNA for this enzyme is increased by starvation, corticosteroids or cyclic-AMP. Citrulline itself potently induces the mRNA.

Argininosuccinate lyase (AL) (Fig. 40-5; reaction 4) cleaves argininosuccinate to form fumarate, which is oxidized in the tricarboxylic acid cycle, and arginine, which is hydrolyzed to urea and ornithine via hepatic arginase. Both AL and arginase are induced by starvation, dibutyryl cyclic-AMP and corticosteroids.

Urea cycle defects cause a variety of clinical syndromes, including a metabolic crisis in the newborn infant. Severe urea cycle defects become manifest in infants with a severe syndrome of coma, convulsions and vomiting during the first few days of life. Clinical confusion with septicemia is common, and many infants are treated futilely with antibiotics. Hyperammonemia is usually severe, even in excess of 1 mmol/l (normal in term infants <100 μmol/l).

Diagnosis usually is made from the blood aminogram. The plasma concentrations of glutamine and alanine, the major nitrogen-carrying amino acids, are usually high and that of arginine is low. Patients with citrullinemia (deficiency of AS) or argininosuccinic aciduria (deficiency of AL) will manifest marked increases of the blood citrulline and argininosuccinate respectively.

Urinary orotic acid generally is very elevated in babies with OTC deficiency and normal or even low in the infant with CPS deficiency. Patients with OTC deficiency have orotic aciduria because carbamyl phosphate spills into the cytoplasm, where it enters the pathway of pyrimidine synthesis.

Diagnosis of CPS or OTC deficiency may not be apparent from the blood aminogram. Ornithine levels typically are normal. The presence of hyperammonemia, hyperglutaminemia, hyperalaninemia and orotic aciduria in a critically ill infant affords presumptive evidence for OTC deficiency. The presence of this blood aminogram without orotic aciduria suggests carbamyl phosphate synthetase deficiency.

Diagnosis of a urea cycle defect in the older child can be elusive. Patients may present with psychomotor retardation, growth failure, vomiting, behavioral abnormalities, perceptual difficulties, recurrent cerebellar ataxia and headache. It is therefore essential to monitor the blood ammonia in any patient with unexplained neurological symptoms, but hyperammonemia is inconstant with partial enzymatic defects. Measurement of blood amino acids and urinary orotic acid is indicated.

Hyperammonemia also occurs in some organic aciduria, particularly those that affect neonates. Therefore, the urine organic acids should be quantitated in all patients with significant hyperammonemia.

Except for patients with argininosuccinic aciduria, who may demonstrate varying degrees of hepatic fibrosis, there is little pathological change outside of the central nervous system.

Carbamyl phosphate synthetase deficiency. Carbamyl phosphate synthetase deficiency is rare. Neonates quickly develop lethargy, hypothermia, vomiting and irritability. The hyperammonemia typically is severe, even exceeding 1 mmol/l. Occasional patients with a partial enzyme deficiency have had a relapsing syndrome of lethargy and irritability upon exposure to protein. Brain damage can occur in both neonatal and late-onset groups.

***N*-acetylglutamate synthetase deficiency.** A deficiency of CPS also can arise because of the congenital absence of NAG synthetase, which catalyzes the formation of NAG from glutamate and acetyl-CoA. NAG is an obligatory effector of CPS. The few patients reported have had a malignant course of neonatal onset.

Ornithine transcarbamylase deficiency. This is the most common of the urea cycle defects. Presentation is variable, ranging from a fulminant, fatal disorder of neonates to a schizophrenic-like illness in an otherwise healthy adult. Males characteristically fare more poorly than do females with this X-linked disorder because of random inactivation (lyonization) of the X chromosome. If inactivation affects primarily the X chromosome bearing the mutant OTC gene, then a more favorable outcome can be anticipated. Conversely, the 'unfavorably lyonized' female has a more active disease.

The study of restriction fragment length polymorphisms is important to diagnosis. More than 80% of carriers can be detected, and antenatal diagnosis often is possible. Approximately one-third of the mothers of males and two-thirds of the mothers of females have been found to be noncarriers, reflecting the greater propensity for mutation in the male gamete.

Diagnosis of carriers (85–90%) can be made with measurement of urinary orotic acid following protein loading or administration of allopurinol. Loading studies with [^{15}N]-NH_4Cl as metabolic tracer indicate that symptomatic female carriers for OTC produce less [^{15}N]-urea than a control population. Asymptomatic heterozygotes form urea at a normal rate but overproduce [5-^{15}N]-glutamine. Thus, whole body nitrogen metabolism is abnormal even in this group [34].

Animal models for OTC deficiency include the 'sparse fur' (*spf*) mouse (15% control enzyme activity) and the 'sparse fur–abnormal skin and hair' (*spf-ash*) mouse (5% of control). Both kinds of mutant mouse manifest hyperammonemia, orotic aciduria, growth failure and sparse fur.

OTC deficiency must be suspected in any patient, male or female, with unexplained neurological symptoms. The absence of hyperammonemia should not rule out the diagnosis, especially with a history of protein intolerance, a suggestive family history or an untoward reaction

to infections. The blood amino acids and urinary orotic acid should be quantified in such individuals.

Citrullinemia. Neonates with AS deficiency usually die, and most survivors suffer major brain injury. Patients with a partial deficiency may have a milder course, and a few individuals with citrullinemia have been phenotypically normal. The diagnosis usually is apparent from the hyperammonemia and the extreme hypercitrullinemia. The activity of AS can be determined in both fibroblasts and chorionic villus samples, thus simplifying the problem of antenatal diagnosis.

Argininosuccinic aciduria. Patients manifest high levels of argininosuccinate in urine, blood and cerebrospinal fluid. Neonates have a stormy clinical course. Almost all have died or sustained severe brain injury. A peculiar finding is *trichorrhexis nodosa*, or dry brittle hair with nodular protrusions that are best visible with light microscopy. The precise cause is unknown.

Arginase deficiency. Most patients are thought to have psychomotor retardation during the first year of life, but the dominant presentation is a leukodystrophy with progressive spastic tetraplegia, especially in the lower extremities. Seizures and growth failure may occur, although some patients are of normal size. The motor dysfunction usually comes to clinical attention by age 2–3 years. Hyperammonemia is less severe than in neonatal-onset disorders. Plasma arginine is usually two to five times normal. Urine orotate is high, perhaps because arginine stimulates flux through the CPS reaction by favoring the synthesis of *N*-acetylglutamate.

Urea cycle defects sometimes result from the congenital absence of a transporter for an enzyme or amino acid involved in the urea cycle.

Hyperornithinemia, hyperammonemia, homocitrullinuria. Affected neonates commonly suffer growth failure and varying degrees of mental retardation. Sometimes symptoms are deferred until adulthood. Vomiting, lethargy and hypotonia are noted after protein ingestion. Recurrent hospitalizations for hyperammonemia are the rule. Some patients have manifested a bleeding diathesis and hepatomegaly. Electron microscopy of the liver has shown irregularities of mitochondrial shape.

The underlying biochemical defect is a failure of mitochondrial uptake of ornithine. This results in a failure of citrulline synthesis and a consequent hyperammonemia. Urinary orotic acid is high, presumably because of underutilization of carbamyl phosphate. In contrast, excretion of creatine is low, reflecting the inhibition of glycine transamidinase by excessive levels of ornithine.

Lysinuric protein intolerance. Infants manifest growth failure, hepatosplenomegaly, vomiting, hypotonia, recurrent lethargy, coma, abdominal pain and, in rare instances, psychosis. Rarefaction of the bones is common, and both fractures and vertebral compression have been reported. Most patients are not mentally retarded. Patients have died with interstitial pneumonia, which may respond to corticosteroid therapy.

The cause is defective transport of dibasic amino acids by the proximal tubule and intestine. The transport defect occurs at the basolateral rather than the luminal membrane. Hyperammonemia reflects a deficiency of intramitochondrial ornithine. An effective treatment is oral citrulline supplementation, which corrects the hyperammonemia by allowing replenishment of the mitochondrial pool of ornithine.

Successful management of urea cycle defects involves a low-protein diet to minimize ammonia production as well as medications that enable the excretion of ammonia nitrogen in forms other than urea. Protein restriction is the mainstay of therapy. In patients with severe disease, tolerance for dietary protein may be so limited that it is not possible to support growth.

Treatment with sodium benzoate and sodium phenylacetate represents an important therapeutic advance. Benzoyl-CoA reacts in the liver with glycine to form hippurate and phenylacetyl-CoA reacts with glutamine to yield phenylacetylglutamine, thereby allowing waste nitrogen elimination not as urea but as conjugates of benzoate and phenylacetate [35–37]. The clinical utility of phenylacetate is limited by its objectionable odor. Sodium phenylbutyrate, which is less malodorous and is converted to phenylacetate, has been used with success. Acylation therapy has greatly improved survival and morbidity. Thus, the outlook for female heterozygotes with OTC deficiency is favorable for those who are treated from an early age [37].

Patients who survive the neonatal period can be maintained with a low-protein diet and sodium benzoate. A useful therapeutic adjunct for citrullinemia and argininosuccinic aciduria is dietary arginine supplementation, which enhances the ability to eliminate nitrogen as either citrulline or argininosuccinate. Maintaining normal arginine levels also facilitates protein synthesis.

Liver transplantation has been employed, but the long-term utility is uncertain. Transplantation affords metabolic correction, although relatively minor deviations of amino acid concentration may persist postoperatively. The morbidity of organ transplantation restricts the utility of this approach.

Dialysis, including hemodialysis and peritoneal dialysis, relieves acute toxicity during fulminant hyperammonemia. Exchange transfusions also have been performed, but this technique has not been equally useful in removing ammonia.

The possibility of gene therapy for these disorders is a subject of intense scrutiny [38]. An adenoviral vector containing a cDNA for the OTC gene has been given to mice with a congenital deficiency of OTC. The result was

complete correction of hepatic OTC activity over a 2-month period. Transient correction of serum glutamine and urine orotic acid was reported. This experimental approach holds enormous promise for the management of this enzymopathy and other inborn errors of intermediary metabolism.

DISORDERS OF GLUTATHIONE METABOLISM

The tripeptide glutathione is the major intracellular antioxidant. The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is the major intracellular antioxidant. It is synthesized via these reactions (Fig. 40-6):

1. Glutamate + cysteine + ATP → γ-glutamylcysteine + ADP + P_i
2. γ-glutamylcysteine + glycine + ATP → glutathione + ADP + P_i

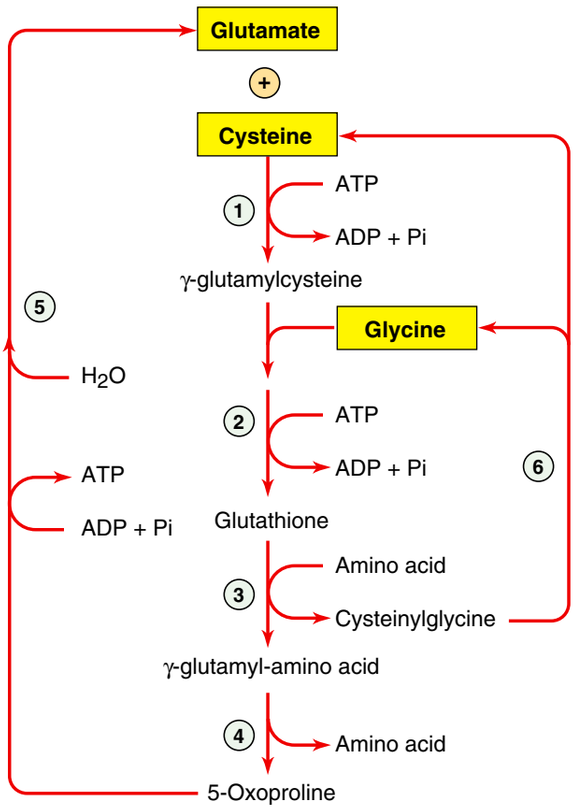

FIGURE 40-6 Metabolism of glutathione. Deficiency in reaction 2 leads to severe metabolic acidosis caused by excessive formation of 5-oxoproline from γ-glutamylcysteine in reaction 4. Deficiencies in reactions 1 and 3 also have neurological effects. Deficiencies in reaction 5 are known, but these patients have no significant neurological symptoms. Enzymes: (1) γ-glutamylcysteine synthetase; (2) Glutathione synthetase; (3) γ-glutamyltranspeptidase; (4) Cyclotransferase; (5) 5-oxoprolinase; (6) Peptidase.

Glutathione is subsequently metabolized in the γ-glutamyl cycle:

3. Glutathione + amino acid → γ-glutamyl-amino acid + cysteinylglycine
4. γ-glutamyl-amino acid → 5-oxoproline + amino acid
5. 5-oxoproline + ATP + $2H_2O$ → glutamate + ADP + P_i
6. Cysteinylglycine → cysteine + glycine.

The cycle is renewed after the cysteine formed in reaction 6 and the glutamate derived from reaction 6 are converted to γ-glutamylcysteine via γ-glutamylcysteine synthetase (reaction 1).

5-oxoprolinuria: glutathione synthetase deficiency. Patients have metabolic acidosis caused by excessive formation of 5-oxoproline (pyroglutamic acid; Fig. 40-6, reaction 2). This occurs because the diminution of intracellular glutathione relieves the feedback inhibition on the γ-glutamylcysteine synthetase pathway (reaction 1), thereby augmenting the concentration of γ-glutamylcysteine and the subsequent conversion of this dipeptide to cysteine and 5-oxoproline in the cyclotransferase pathway (reaction 4).

Clinical findings include mental retardation, severe metabolic acidosis, and evidence of a spastic quadriparesis and cerebellar disease. Some patients develop normally until late childhood, when a progressive loss of intellectual function became appreciated. Patients also may manifest a mild hemolysis. Pathological changes have included atrophy of the cerebellum and lesions in the cortex and thalamus. There is no specific therapy.

γ-glutamylcysteine synthetase deficiency. Patients with this rare disorder (Fig. 40-6, reaction 1) have spinocerebellar degeneration, peripheral neuropathy, myopathy and an aminoaciduria secondary to renal dysfunction. Psychosis and a hemolytic anemia have been noted.

γ-glutamyltranspeptidase deficiency. These patients display glutathionuria and varying degrees of mental retardation (Fig. 40-6, reaction 3). The enzyme is present in the brain, primarily in the capillaries. No specific treatment is available.

5-oxoprolinase deficiency. These patients excrete increased amounts of oxoproline and have a somewhat elevated plasma concentration (Fig. 40-6, reaction 5). They have not had significant neurological symptoms.

DISORDERS OF γ-AMINOBUTYRIC ACID METABOLISM

Congenital defects in the metabolism of γ-aminobutyric acid have been described. GABA is formed via the action of glutamate decarboxylase (see Ch. 16). Metabolism of this neurotransmitter is mediated first by uptake into

neurons and glia and subsequent transamination to succinic semialdehyde via γ-aminobutyric acid transaminase (GABA-T). The semialdehyde is oxidized to succinate via succinic semialdehyde dehydrogenase.

Pyridoxine dependency. Pyridoxine dependency is characterized by severe seizure activity of early onset, perhaps even *in utero*. Patients respond dramatically to parenteral administration of pyridoxine (10–100 mg). Speculation has centered on the possibility that the disease involves faulty binding of pyridoxine, a cofactor in the glutamate decarboxylase reaction, to the enzyme protein.

γ-aminobutyric acid transaminase deficiency. Patients with this rare disorder have severe psychomotor retardation and hyperreflexia. The concentrations of GABA and β-alanine are high in cerebrospinal fluid and blood. GABA-T activity is much diminished in blood lymphocytes and in the liver. A curious finding is increased stature, perhaps reflecting the ability of GABA to evoke release of growth hormone.

Succinic semialdehyde dehydrogenase deficiency. Patients have mental retardation, cerebellar disease, and hypotonia. They excrete large amounts of both succinic semialdehyde and 4-hydroxybutyric acid. There is no known therapy.

DISORDERS OF *N*-ACETYL ASPARTATE METABOLISM

Canavan's disease is the result of a deficiency of the enzyme that breaks down *N*-acetylaspartate, an important donor of acetyl groups for brain myelin synthesis. Infants seem normal at birth, but developmental delay is apparent by 3 months. Increased head circumference (>98th percentile) is common, and hydrocephalus sometimes is suspected. Neurological function deteriorates rapidly over a period of months. Optic atrophy leads to blindness. Infants manifest minimal interest in their environment. Spasticity is frequent and seizures may occur. Imaging of the brain shows demyelination and brain atrophy with enlargement of the ventricles and widening of the sulci. Pathological examination shows swelling of the astrocytes with elongation of the mitochondria. Vacuoles appear in the myelin sheets.

Urinary excretion of *N*-acetylaspartate is elevated and the cerebrospinal fluid concentration may be 50 times control values. The cause is a deficiency of aspartoacylase, which cleaves *N*-acetylaspartate to form aspartate and acetyl-CoA. The enzyme occurs primarily in the white matter, but *N*-acetylaspartate is most abundant in gray matter. The defect is expressed in skin fibroblasts.

N-acetylaspartate is among the most abundant amino acids in the brain, although its precise function remains elusive. Putative roles have included osmoregulation and the storage of acetyl groups that subsequently are utilized for myelin synthesis. The relationship of the enzyme defect to the clinical findings remains problematic. No specific therapy is yet available.

REFERENCES

1. Kahler, S. G. and Fahey, M. C. Metabolic disorders and mental retardation. *Am. J. Med. Genet.* 117C: 31–41, 2003.
2. Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. *The Metabolic and Molecular Basis of Inherited Disease.* New York: McGraw Hill, 2001.
3. Pietz, J., Rupp, A., Ebinger, F. *et al.* Cerebral energy metabolism in phenylketonuria: findings by quantitative in vivo ^{31}P MR spectroscopy, *Pediatr. Res.* 53:654–662, 2003.
4. Jan, W., Zimmerman, R. A., Wang, Z. J., Berry, G. T., Kaplan, P. B. and Kaye, E. M. MR diffusion imaging and MR spectroscopy of maple syrup urine disease during acute metabolic decompensation. *Neuroradiology* 45: 393–399, 2003.
5. Felipo, V. and Butterworth, R. Neurobiology of ammonia. *Prog. Neurobiol.* 67: 259–279, 2002.
6. Wagner, M., Coelho, D. M., Barschak, A. G. *et al.* Reduction of large neutral amino acid concentrations in plasma and CSF of patients with maple syrup urine disease. *J. Inher. Metab. Dis.* 23: 505–512, 2000.
7. Koch, R., Moseley, K. D., Yano, S., Nelson, M. Jr and Moats, R. A. Large neutral amino acid therapy and phenylketonuria: a promising approach to treatment. *Mol. Genet. Metab.* 79: 110–113, 2003.
8. Smith, C. B. and Kang, J. Cerebral protein synthesis in a genetic model of phenylketonuria. *Proc. Natl Acad. Sci. U.S.A.* 97: 11014–11019, 2000.
9. Morton, D. H., Strauss, K. A., Robinson, D. L., Puffenberger, E. G. and Kelley, R. I. Diagnosis and treatment of maple syrup disease: a study of 36 patients. *Pediatrics* 109: 999–1008, 2002.
10. Infant, J. P. and Huszagh, V. A. Impaired arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acid synthesis by phenylalanine metabolites as etiological factors in the neuropathology of phenylketonuria. *Mol. Genet. Metab.* 72: 185–198, 2001.
11. Shefer, S., Tint, G. S., Jean-Guillaume, D. *et al.* Is there a relationship between 3-hydroxy-3-methylglutaryl coenzyme a reductaseactivity and forebrain pathology in the PKU mouse? *J. Neurosci. Res.* 61: 549–563, 2000.
12. Hommes, F. A. Loss of neurotransmitter receptors by hyperphenylalaninemia in the HPH-5 mouse brain. *Acta Paediatr. Suppl.* 407: 120–121, 1994.
13. Deutsch, S. I., Rosse, R. B. and Mastropaolo, J. Current status of NMDA antagonist interventions in the treatment of nonketotic hyperglycinemia. *Clin. Neuropharmacol.* 21: 71–79, 1998.
14. Chan, H. and Butterworth, R. F. Cell-selective effects of ammonia on glutamate transporter and receptor function in the mammalian brain. *Neurochem. Int.* 43: 525–532, 2003.
15. Willard-Mack, C. L., Koehler, R. C., Hirata, T. *et al.* Inhibition of glutamine synthetase reduces ammonia-induced astrocyte swelling in rat. *Neuroscience* 71: 589–599, 1996.
16. Chuang, D. T., Davie, J. R., Wynn, R. M., Chuang, J. L., Koyatam H. and Cox, R. P. Molecular basis of maple syrup

urine disease and stable correction by retroviral gene transfer. *J. Nutr.* 125: 1766S–1772S, 1995.
17. Kaplan, P., Mazur, A., Field, M., Berlin, J.A., Berry, G.T., Heidenreich, R., Yudkoff, M. and Segal, S (1991). Intellectual outcome in children with maple syrup urine disease. *J. Pediatr.* 119: 46–50.
18. Mueller, G. M., McKenzie, L. R., Homanics, G. E., Watkins, S. C., Robbins, P. D. and Paul, H. S. Complementation of defective leucine decarboxylation in fibroblasts from a maple syrup urine disease patient by retrovirus-mediated gene transfer. *Gene Ther.* 2: 461–468, 1995.
19. Cedarbaum, S. Phenylketonuria: an update. *Curr. Opin. Pediatr.* 14: 702–706, 2002.
20. White, D. A., Nortz, M. J., Mandernach, T., Huntington, K. and Steiner, R. D. Deficits in memory strategy use related to prefrontal dysfunction during early development: evidence from children with phenylketonuria. *Neuropsychology* 15: 221–229, 2001.
21. Eisensmith, R. C. and Woo, S. L. Phenylketonuria and the phenylalanine hydroxylase gene. *Mol. Biol. Med.* 8: 3–10, 1991.
22. Guldberg, P., Levy, H. L., Hanley, W. B. *et al.* Phenylalanine hydroxylase gene mutations in the United States: report from the Maternal PKU Collaborative Study. *Am. J. Hum. Genet.* 59: 84–94, 1996.
23. Diamond, A. and Herzberg, C. Impaired sensitivity to visual contrast in children treated early and continuously for phenylketonuria. *Brain* 119: 523–538, 1996.
24. Levy, H. L. and Ghavami, M. Maternal phenylketonuria: a metabolic teratogen. *Teratology* 53: 176–184, 1996.
25. Kaufman, S. Hyperphenylalaninaemia caused by defects in biopterin metabolism. *J. Inherit. Met. Dis.* 8(Suppl. 1): 20–27, 1983.
26. Kikuchi, G. The glycine cleavage system: Composition, reactin mechanism and physiological significance. *Mol. Cell. Biochem.* 1: 169–175, 1973.
27. Steiner, R. D., Sweetser, D. A., Rohrbaugh, J. R., Dowton, S. B., Toone, J. R. and Applegarth, D. A. Nonketotic hyperglycinemia: atypical clinical and biochemical manifestations. *J. Pediatr.* 128: 243–246, 1996.
28. Van Hove, J. L., Kishnani, P., Muenzer, J. *et al.* Benzoate therapy and carnitine deficiency in non-ketotic hyperglycinemia. *Am. J. Med. Genet.* 59: 444–453, 1995.
29. Alemzadeh, R., Gammeltoft, K. and Matteson, K. Efficacy of low-dose dextromethorphan in the treatment of nonketotic hyperglycinemia. *Pediatrics.* 97: 924–926, 1996.
30. Kluijtmans, L. A., Boers, G. H., Stevens, E. M. *et al.* Defective cystathionine beta-synthase regulation by *S*-adenosylmethionine in a partially pyridoxine responsive homocystinuria patient. *J. Clin. Invest.* 98:285–289, 1996.
31. Kraus, J. P. Molecular basis of phenotype expression in homocystinuria. Komrower Lecture. *J. Inherit. Met. Dis.* 17: 383–390, 1994.
32. Mandel, H., Brenner, B., Berant, M. *et al.* Coexistence of hereditary homocystinuria and factor V Leiden–effect on thrombosis. *N. Engl J. Med.* 334: 763–768, 1996.
33. Dudman, N. P., Guo, X. W., Gordon, R. B., Dawson, P. A. and Wilcken, D. E. Human homocysteine catabolism: three major pathways and their relevance to development of arterial occlusive disease. *J. Nutr.* 126(4 Suppl): 295s–300s, 1996.
34. Yudkoff, M., Daikhin, Y., Nissim, I., Jawad, A., Wilson, J. and Batshaw, M. B. In vivo nitrogen metabolism in ornithine transcarbamylase deficiency. *J. Clin. Invest.* 98: 2167–2173, 1996.
35. Brusilow, S., Tinker, J. and Batshaw, M. L. Amino acid acylation: A mechanism of nitrogen excretion in inborn errors of urea synthesis. *Science* 207: 659, 1980.
36. Maestri, N. E., Hauser, E. R. Bartholomew, D. and Brusilow, S. W. Prospective treatment of urea cycle disorders. *J. Pediatr.* 119: 923, 1991.
37. Maestri, N. E., Brusilow, S. W., Clissold, D. B. and Bassett, S. S. Long-term treatment of girls with ornithine transcarbamylase deficiency. *N. Engl J. Med.* 335: 855–859, 1996.
38. Ye, X., Robinson, M. B., Batshaw, M. L., Furth, E. E., Smith, I. and Wilson, J. M. Prolonged metabolic correction in adult ornithine transcarbamylase-deficient mice with adenoviral vectors. *J. Biol. Chem.* 271: 3639–3646, 1996.

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CHAPTER

41

Lysosomal and Peroxisomal Diseases

Hugo W. Moser

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The past decade has witnessed a dramatic transition in studies of lysosomal and peroxisomal disorders, from a focus on gene products to the gene themselves. For most of these genetic disorders, the normal cDNAs and the genes responsible for the respective disorders have been isolated and characterized and, in many instances, specific disease-causing mutations have been identified. As expected, information at the genetic level demonstrates the highly complex genetic heterogeneity of these diseases, even when they appear homogeneous in clinical manifestations, analytical biochemistry and enzymatic defects. Detailed description of these disorders are found in the standard reference volume [1].

LYSOSOMAL DISEASES

The lysosome is the subcellular organelle responsible for physiological turnover of cellular constituents. Their combined incidence is estimated to be 1:8,000 [2]. Knowledge about the molecular and cellular defects has advanced a great deal in recent years. Platt and Walkley [3] have proposed a new classification based on the nature of the molecular defects (Table 41-1). This system will be used here.

Primary lysosomal hydrolase defects. Two-thirds of the lysosomal storage diseases involve defects in genes that code for acid hydrolases. Table 41-2 lists 29 defects that have been defined so far. They have an autosomal recessive mode of inheritance, except for Hunter's syndrome and Fabry's disease, where the mode is X-linked recessive. The defective genes have been identified and mutations have been defined for nearly all. The nervous system is involved in most. Many of the disorders show a wide range of clinical severity, which may range from death in early childhood to a moderate disability in adulthood.

Farber's disease is a rare disorder caused by deficiency of acid ceramidase and is associated with the accumulation of ceramide in subcutaneous tissues, joints, kidney, lung and neurons. Gangliosides also accumulate.

There are three allelic types of **Gaucher's disease**, all caused by deficiency of glucosylceramidase. The non-neuropathic type 1 is most common. The nervous system is not involved. Age of onset varies widely and it may not manifest until adulthood. Glucosylceramide is present in excess in the greatly enlarged liver and spleen and in the bone marrow, where it leads to anemia, thrombocytopenia and skeletal changes. In type II the nervous system is severely and progressively and this leads to death in early childhood. Type III is intermediate with nervous system abnormalities developing in adolescence. The accumulation of a toxic metabolite, glucosylsphingosine, may play

TABLE 41-1 Lysosomal storage diseases classified on basis of molecular defects

1.	Primary lysosomal hydrolase defect	$n = 29$
2.	Post-translational processing defect	$n = 1$
3.	Trafficking defect of lysosomal enzymes	$n = 3$
4.	Defect in lysosomal enzyme protection	$n = 1$
5.	Defect in soluble non-enzymatic proteins	$n = 4$
6.	Transmembrane (non-enzyme) protein defect	$n = 8$

Source: adapted from reference [3].

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TABLE 41-2 Primary lysosomal hydrolase defects

No. and name of disorder	Enzyme defect	Substrate
1. Farber's disease (lipogranulomatosis)	Acid ceramidase	Ceramide
2. Gaucher's disease	Glucosylceramidase	Glucosylceramide Galactosylceramide
3. Globoid cell leukodystrophy (Krabbe's disease)	Galactosylceramidase Galactosylsphingosine	Galactosylceramide
4. Metachromatic leukodystrophy	Sulfatide	Arylsulfatase A ('sulfatidase')
5. G_{M1}-gangliosidosis, Morquio B	G_{M1}-ganglioside galactose-rich fragments of glycoproteins	G_{M1} ganglioside Keratan sulfate
6. Tay–Sachs disease	G_{M2}-ganglioside	Hexosaminidase A
7. Sandhoff's disease	G_{M2}-ganglioside asialo-G_{M2}-ganglioside, globoside	Hexosaminidases A and B
8. Niemann–Pick A and B	Sphingomyelin	Acid sphingomyelinase
9. Fabry's disease	α-galactosidase (trihexoceramidase)	Trihexosylceramide (gal-gal-glc-cer digalactosylceramide)
10. Hurler's syndrome MPS1H Scheie's syndrome MPS1S	Alpha-iduronidase	Dermatan sulfate Heparan sulfate
11. Hunter's syndrome MPS 11	Iduronate sulfatase	Dermatan sulfate Heparan sulfate
12. Sanfilippo A MPS IIIA	Heparan-*N* sulfatase	Heparan sulfate
13. Sanfilippo B MPS IIIB	*N*-acetyl-glucosaminidase	Heparan sulfate
14. Sanfilippo C MPS IIIC	Acetyl CoA glucosamine N-acetyl transferase	Heparan sulfate
15. Sanfilippo D MPS IIID	*N*-acetyl-glucosamine 6-sulfatase	Heparan sulfate
16. Morquio A MPS IVA	*N*-acetyl-galactosamine 6-sulfatase	Keratan sulfate
17. Maroteaux–Lamy MPS VI	*N*-acetyl-galactosamine-4-sulfatase	Dermatan sulfate
18. Sly syndrome MPS VII	β-glucuronidase	Heparan sulfate Dermatan sulfate
19. Hyaluronidase deficiency	Hyaluronidase	Hyaluronic acid
20. Fucosidosis	Fucosidase	Fucoside glycolipids
21. α-mannosidosis	α-mannosidase	α-mannosides
22. β-mannosidosis	β-mannosidase	β-mannosides
23. Aspartylglucoaminuria	Aspartyl-glucosaminidase	Aspartyl-glucosamine
24. Schindler's disease	α-galactosidase-B	*N*-acetyl-galactosamide glycolipids
25. Sialidosis; mucolipidosis I	α-neuraminidase	Sialic acid
26. Pompe's disease	α-glucosidase	Glycogen
27. Wolman's disease Cholesterol ester storage disease	Acid lipase	Cholesterol ester
28. Infantile neuronal ceroid lipofuscinosis CNL1	Palmitoyl protein thioesterase	Saposins
29. Late infantile neuronal lipofuscinosis (*CNL2*)	Carboxypeptidase	Subunit C mitochondrial ATP synthase

Source: adapted from reference [3]

an important role in the pathogenesis of Gaucher's disease. The gene for glucosylceramidase has been cloned and characterized and many mutations responsible for the disease have been characterized. The most common N370S mutation has never been found in association with a neuropathic phenotype, either alone or in association with another mutation.

Globoid leukodystrophy (Krabbe's disease) is caused by a defect in galactocerebrosidase. 85–90% present with the infantile form before 6 months of age with general irritability, stiffness, arrest of mental and motor development, progressing to a decerebrate state with no voluntary movements. The main histopathological change is extensive demyelination and gliosis with the presence of characteristic globoid cells. The latter can be induced experimentally by injection of galactocerebroside. Biochemically there is accumulation not only of galactocerebroside but also of psychosine (galactosylsphingosine). Psychosine is a substrate for galactocerebrosidase. Even small accumulations of psychosine are toxic. The defective gene maps to 14q31. The 502T/del in which there is a 30 kb deletion is present in 40–50% of patients, is associated with the severe infantile form.

Metachromatic leukodystrophy is due to a defect in arylsulfatase A (ASA). There are three major forms: late infantile, juvenile and adult. The overall incidence is 1:40,000. In the late infantile and early juvenile forms, which comprise about 80% of patients, the initial symptoms involve the motor system, with falls, loss of ability to walk, flaccid paralysis, difficulty in swallowing, loss of speech, vision, seizures, decerebrate state and death 1–7 years after onset of symptoms. In the adolescent and

adult forms, which account for about 20% of cases, the initial symptoms are behavioral disturbances that may resemble a psychosis and dementia. The main biochemical abnormality is the accumulation of sulfatide in brain, peripheral nerve, kidney and gall bladder. In the brain there is also an increase in the concentration of lysosulfatide, the deacylated form of sulfatide, and the cytotoxicity of this compound is believed to contribute to the demyelinative process in MLD. The ASA gene has been mapped to chromosome 22 distal to q13. More than 100 variations have been found in patients with MLD. The most common is a G-to-A transition that eliminates a donor splice site at the start of intron 2. This causes a loss of all enzyme activity and is associated with the severe early onset disease.

G_{M1} gangliosidosis and Morquio's disease type B (Morquio B) are both caused by defects of acid β-galactosidase. These apparently clinically distinct disorders represent extremes of phenotypic variations of the same genetic defect. G_{M1} gangliosidosis is subdivided into infantile, juvenile and adult forms. Patients with the infantile form present at about 6 months with hypotonia and retarded psychomotor development and have facial and skeletal abnormalities that may have been recognized at birth. The neurological deficits are progressive and resemble those in Tay–Sachs disease (see below). Morquio B is a primary skeletal disorder without neurological manifestations except those caused by bone deformity. The main and characteristic biochemical abnormality is the accumulation of ganglioside G_{M1} and of keratan sulfate. More than 20 disease-causing mutations have been identified. Demonstration of diminished β-galactosidase activity with the G_{M1} ganglioside or artificial chromogenic or fluorogenic substrates is the recommended diagnostic procedure.

Tay–Sachs disease is due to hexosaminidase-A deficiency and is associated with the accumulation of G_{M2} ganglioside. It is panethnic but occurs most frequently in the Ashkenazi Jewish population, where, prior to the use of genetic counseling and prenatal screening, 1 in 4,000 births were affected. The infantile form is most frequent. The affected child may startle easily in the newborn period, appear listless, fail to follow objects visually at 3–6 months, and lose previously gained milestones. After age 1 year the child becomes increasingly immobile, with seizures and decerebrate posturing. Death occurs usually by 4–5 years. The cherry-red macular spot, due to the accumulation of gangliosides in retinal neurons except in the fovea, is a striking and characteristic finding. Juvenile variants and adult variants occur more rarely. Pathological studies show severe cortical atrophy and ballooned neurons that contain concentrically arranged electron dense lamellar structures known as membranous cytoplasmic bodies. Tay–Sachs disease is due to deficiency of the α subunit of HexA. At least 100 alterations in its nucleotide sequence are now known. Screening for virtually known mutations of the HexA subunit can be accomplished by mass screening of serum or leukocyte samples for HexA activity at pH 4.5 at 50° or 55°C. HexB is relatively stable at that temperature, whereas HexA is inactivated after 3 minutes at that temperature. Heterozygote screening of at-risk populations combined with prenatal diagnosis has greatly reduced the incidence of Tay–Sachs disease.

Sandhoff's disease is due to a deficiency of the HexB gene. Clinical features are similar to those of Tay–Sachs disease. Late infantile, juvenile and adults variants have also been described.

Niemann–Pick disease types A and B are due to a deficiency of acid sphingomyelinase. This leads to the accumulation of sphingomyelin in many organs and tissues. Type A patients present in the first few months of life with greatly enlarged liver and spleen, hypotonia, muscular weakness and feeding difficulties. The clinical features in type B patients are more variable but differ strikingly from type A patients in that neurological function is generally normal.

Fabry's disease is caused by α-galactosidase A deficiency. It is an X-linked recessive panethnic disorder with an estimated incidence of 1:40,000. It manifests in childhood and adolescence. Recurring episodes of excruciatingly severe burning pain in the fingers and toes, referred to as acroparesthesias, are the most distressing symptom. Other manifestations are angiokeratomas and characteristic opacities in the cornea and lens. Progressive renal failure and ischemic strokes occur in adulthood. The mean age at death in untreated males is 41 years. One variant, which has been recognized only recently, is late-onset cardiac disease without the other manifestations. Approximately 50% of heterozygous women have a milder corneal dystrophy, acroparesthesias in middle age or later. A variety of glycosphingolipids with a terminal α-glycosphingolipids with a terminal α-galactosyl moiety accumulate, particularly in the lysosomes of the endothelial cells in small blood vessels.

Clinically the **mucopolysaccharidoses (MPS)** can be subdivided into four major categories. Hurler's, Hunter's and Scheie's syndromes present with coarse facial features, hepatosplenomegaly, corneal clouding, skeletal abnormalities and psychomotor retardation. Scheie's syndrome is a milder variant and may be associated with survival to adulthood and normal intellect. Hunter's syndrome resembles Hurler's, except that the cornea is not involved. In patients with Sanfilippo's syndrome, the facial coarsening, hepatosplenomegaly and skeletal abnormalities are less severe, but psychomotor retardation and behavioral disturbances are severe. Conversely, patients with Maroteaux–Lamy syndrome have severe somatic manifestations, but normal intellect. Morquio's syndrome is associated with severe skeletal abnormalities, different in pattern from those in Hurler's syndrome. Intellectual function remains intact.

Patients with **fucosidosis** present with hypotonia, progressive mental retardation and motor deficits and also angiokeratomas in the skin, which resemble those in Fabry's disease. **Mannosidosis** patients show dysmorphic features, hepatosplenomegaly and mental retardation.

Patients with **sialidosis** show a striking syndrome characterized by action myoclonus, cerebellar ataxia and a macular cherry red spot similar to that in Tay–Sachs disease but with preserved intellect.

Pompe's disease is of historical significance because it was the first lysosomal storage disorder to be recognized (H. G. Hers, 1963). The most common infantile form presents at or soon after birth and is characterized by massive deposition of glycogen, especially in heart, muscle and liver. Severe hypotonia and weakness are associated with massive cardiomegaly, hepatomegaly and macroglossia. Cardiac or respiratory failures cause death, usually before the first year and invariably before the second year. In the rarer childhood and adult forms there is progressive weakness of muscles and respiratory insufficiency due to diaphragmatic involvement without cardiomegaly.

The neuronal ceroid lipofuscinoses (CLN), also referred to as Batten's disease, are a group of disorders characterized by the accumulation of autofluorescent lipopigments. Clinical hallmarks include blindness, seizures, cognitive and motor decline and early death. Age of onset varies from infancy to adulthood. Eight genetic forms have been identified [4]. Two involve lysosomal acid hydrolases. CLN1 codes for palmitoyl protein thioesterase 1. Clinically it presents most often in infancy and leads to loss of active movement and visual contact by 3 years of age. It is most common in Finland, where its incidence is 1:20,000. CLN2 codes for a lysosomal pepstatin-insensitive acid protease.

Other types of lysosomal disorder. These are listed in Table 41-3.

TABLE 41-3 Other types of lysosomal disease

Disease type	Disorder
Post-translational processing defect	Multiple sulfatase deficiency
Trafficking defects of lysosomal enzymes	Mucolipidosis type II (I-cell disease) Mucolipidosis type IIIA (pseudo-Hurler's) Mucolipidosis type IIIB
Defect in lysosomal enzyme protection	Galactosialidosis
Defects in soluble non-enzymatic lysosomal proteins	Niemann–Pick disease C type 2 G_{M2} activator protein deficiency Sphingolipid activator protein deficiency Neuronal; ceroid lipofuscinosis (CNL5)
Transmembrane (non-enzyme) protein defect	Danon's disease (LAMP-2 deficiency) Niemann–Pick disease C type 1 Cystinosis Infantile free sialic storage disease Salla's disease (free sialic acid storage) Juvenile ceroid lipofuscinosis (CNL3) Neuronal ceroid lipofuscinosis (CNL5) Neuronal ceroid lipofuscinosis (CNL6 and CNL8) Mucolipidosis type IV (mucolipin deficiency)

Source: adapted from reference [3]

Multiple sulfatase deficiency (MSD). The clinical presentation of MSD includes features of MLD and the MPS, with the features that resemble MLD, such as gait disturbances, psychomotor retardation and polyneuropathy predominating. MPS-like features, such as facial coarsening, hepatosplenomegaly and joint stiffness, are present to a milder and variable extent. A reduction in the activity of all sulfatases is the key diagnostic abnormality. Von Figura and associates have demonstrated that catalytic activity of all sulfatases requires a post-translational modification in which a cysteine residue (cysteine 69 in arylsulfatase A) is oxidized to an aldehyde. It is this modification that is defective in MSD [5].

The term mucolipidosis (ML) was coined because of the combined storage of lipids and mucopolysaccharides in the tissues and cells of these patients. Four subtypes have now been characterized. All involve the lysosome; three of them have entirely different genetic defects. **Mucolipidosis 1** (sialidosis, neuraminidase deficiency) presents clinically with myoclonus and seizures and gait disturbances that begin in the second decade. Cognitive function is usually intact. Eye examinations show a macular cherry red spot that resembles that seen in Tay–Sachs disease. **Mucolipidosis II** is also referred to as I cell disease, because of the striking inclusions in cultured skin fibroblasts. Patients present in infancy or early childhood with coarse facial features, large liver and spleen, a large tongue, gingival hyperplasia, an enlarged heart and kyphoscoliosis. The biochemical abnormalities are characteristic and their definition by Hickman and Neufeld [6] added greatly to the understanding of lysosomal biology. The levels of lysosomal enzymes are increased in serum and urine but deficient in cultured skin fibroblasts. The enzymes lack the mannose-6-phosphate signal that targets lysosomal enzymes to the lysosome; therefore they are excreted. The mannose-6 phosphate is added to the lysosomal enzymes by UDP-*N* acetylglucosamine: *N*-acetylglucosaminyl-1 phosphotransferase, and it is the deficiency of this enzyme that represents the basic defect in I cell disease and also in mucolipidosis III. **Mucolipidosis III**, also referred to as pseudo-Hurler's polydystrophy, has the same enzymatic defect basis as I cell disease, but clinical features are milder. Clinical onset is between 2 and 4 years of age; patients may live to adulthood and mental function may be normal. Joint and bone involvement are the principal source of disability. **Mucolipidosis IV** presents clinically during the first 2–3 years with psychomotor retardation, corneal clouding, retinal degeneration and strabismus. Patients may then remain relatively stable for the first two or three decades. The defective gene codes for mucolipin-1, a membrane protein that is involved in endosome processing and transport. It is of interest that patients with MLIV also have constitutive achlorhydria, suggesting a specific defect cellular trafficking in the gastric parietal cell. 80% of patients are of Ashkenazy Jewish origin, and a population screening program has been started in Israel as a prevention strategy.

Niemann–Pick disease type C (NPC) patients with the classical phenotype present with progressive neurological disease in late infancy to adolescence, manifested by clumsy gait, ataxia, school failure, behavior problems and a characteristic supranuclear downward gaze paralysis. There are varying degrees of hepatosplenomegaly. Infantile and adult-onset variants exist. Neuronal inclusions are present throughout the nervous system. Unesterified cholesterol, sphingolipids and glycolipids are stored in excess. Impaired processing of endocytosed free cholesterol is the fundamental defect. NPC can be caused by two different molecular defects: NPC1 and NPC2. 95 families have the NPC1 defect. The NPC1 gene maps to 18q11–12. It has homology to other proteins that are involved in cholesterol homeostasis. It is postulated that NPC1 regulates or mediates the retrograde transport of multiple lysosomal cargo in the late endosomal/lysosomal pathway. NPC1 and NPC2 function cooperatively [7]. The biochemical diagnosis of NPC depends on the demonstration of a specific defect of cholesterol processing in cultured skin fibroblasts. Cholesterol-depleted normal and NPC fibroblasts are cultured with low density lipoproteins for 24 hours, and the amount of free cholesterol is assessed by the degree of filipin-fluorescent staining.

Galactosialidosis is characterized by the simultaneous deficiencies of β-galactosidase and α-neuroaminidase. Clinical and pathological manifestations resemble those in G_{M1} gangliosidosis and like it show a range of severity. The underlying defect involves a protective protein, which stabilizes these two enzymes by a mechanism that is not understood. Curiously, the protective protein is itself a peptidase. The disorder is most common in Japan. The defective gene has been cloned and mutations have been identified.

G_{M2} activator deficiency was identified in 1977 during the analysis of a patient with the Tay–Sachs disease phenotype and massive storage of ganglioside G_{M2} and glycolipid GA2 in the brain in spite of the presence of hexosaminidases A and B. This led to the purification of an activator protein that itself is not catalytically active. It is a lysosomal glycoprotein of 17.6 kDa in the deglysosylated form. It 'lifts' the membrane-bound G_{M2} ganglioside a few angstroms out of the membrane and thus makes it accessible to the degradative enzymes. The gene is located on chromosome 5 and five different mutations have been identified.

PEROXISOMAL DISORDERS

Peroxisomes are single-membrane, typically spherical, organelles ranging in size from 0.1–1 μm in diameter and numbering from a few hundred to a few thousand in mammalian cells [8]. The matrix contains 50 or more enzymes that participate in various metabolic pathways; particularly those involved in the β-oxidation of straight and 2-methyl branched very-long-chain and long-chain fatty acids and in the synthesis of ether lipids. H_2O_2 produced by many of the peroxisomal oxidases is detoxified *in situ* by peroxisomal catalase. The peroxisomal membrane contains a unique set of peroxisomal membrane proteins (PMPs) including four ATP binding cassette (ABC) transporters and several proteins required for importing peroxisomal matrix proteins. Synthesized on free cytosolic ribosomes, peroxisomal matrix proteins are targeted post-translationally to the organelle by specific cytosolic receptors that recognize *cis*-acting sequences (peroxisomal targeting signals, PTSs) in the primary peptide sequence. Most peroxisome matrix proteins are targeted by peroxisome targeting sequence 1 (PTS1), a C-terminal serine–lysine–leucine–COOH (SKL) or conservative variant thereof. A few matrix proteins, particularly those with ether lipid and branched chain fatty acid metabolism, are targeted by PTS2, a degenerate sequence (–R/KX_5Q/HL–). Like matrix proteins, the peroxisomal membrane proteins (PMPs) are synthesized on free cytosolic ribosomes and targeted to the organelle by *cis*-acting targeting sequences, which are long and have only been partially characterized. The number and matrix content of peroxisomes is dynamic and varies with the metabolic state of the organism, and is mediated by activation of peroxisome proliferator receptor α. An increase in peroxisome number is achieved by maturation, enlargement and division of pre-existing peroxisomes but *de novo* synthesis is possible. The human peroxisomal disorders are subdivided into two major categories: the disorders of peroxisome biogenesis, in which the organelle fails to be formed normally, and defects that involve a single peroxisomal protein.

Disorders of peroxisome biogenesis.

Molecular defects. Proteins necessary for peroxisome biogenesis are termed peroxins and the genes encoding these proteins are referred to as PEX genes. More than 30 peroxins have been identified. 13 genetically determined human disorders have been shown to be associated with defects in a peroxin and are referred to as peroxisome biogenesis disorders (PBDs) [8]. They are listed in Table 41-4. Three peroxins (PEX19, PEX16 and PEX3) involve the formation or import of peroxisomal membrane proteins. When they are defective, the organelle is not formed and the phenotype has been severe in all patients reported so far. All the 10 other defects involve peroxisomal matrix proteins, and membranous structures that contain PMP and varying amounts of peroxisomal matrix proteins ('peroxisome ghosts') can be demonstrated by cytoimmunological techniques. Two of the defects involve PTS receptors: PEX5 involves the PTS1 receptor and PEX7 the PTS2 receptor. Defects that involve PEX7 account for 20% of human PBD. PEX5 and PEX7 are present mainly in the cytosol, where interaction with the proteins that contain the targeting sequence takes place. The other eight peroxins that are associated with human disease states are involved in the docking and import processes and in the

TABLE 41-4 Peroxisome biogenesis disorder

Peroxin	Function	Phenotype	Frequency (%)
19	Peroxisome membrane formation and import	ZS	<1
16	Peroxisome membrane import	ZS	<1
3	Peroxisome membrane import	ZS	<1
5	PTS1 receptor	ZS, NALD	2
7	PTS2 receptor	RCDP1, 2	20
14	PEX5 docking, recycling	ZS, NALD	<1
13	PEX5, PEX7 docking, recycling	ZS, NALD	<1
2	Matrix protein import after docking	ZS, IRD	2.5
10	Matrix protein import after docking	ZS, NALD, IRD	3
12	Matrix proteins after docking	ZS, NALD, IRD	4
1	Stabilizes PEX5 Late matrix import	ZS, NALD, IRD	48
6	Stabilizes *PEX5* Interacts with *PEX1*	ZS, NALD, IRD	8
26	Interacts with *PEX6*	ZS, NALD, IRD	4

See text and reference [9] for discussion.
IRD, infantile Refsum's disease; NALD, neonatal adrenoleukodystrophy; ZS, Zellweger's syndrome.

recycling of the receptor. PEX14 and PEX13 are required for docking, PEX2, PEX10 and PEX12 are thought to be involved with translocation into the matrix. PEX1, PEX6 and PEX26 are involved in the recycling of the receptors. PEX1 defects account for approximately 50% of human PBD.

Genotype–phenotype correlations. Most of the PBD were described and named before their relationship to the peroxisome was recognized. Re-evaluation of these clinical groupings has led to the conclusion that they fall into two major groups, the Zellweger spectrum and rhizomelic chondrodysplasia punctata (RCDP) [9, 10]. The Zellweger spectrum includes the classical Zellweger's syndrome, which was the first to be described, as well as neonatal adrenoleukodystrophy (NALD) and infantile Refsum's disease (IRD). They form a clinical continuum, with Zellweger's syndrome the most severe, IRD the least severe and NALD intermediate. Zellweger's syndrome patients present with characteristic dysmorphic features, hypotonia, severe psychomotor retardation, enlarged liver and spleen, impaired hearing and a characteristic defect in neuronal migration. Most patients also have seizures, cataracts, retinopathy renal cysts and chondrodysplasia punctata. Most patients die during the first 2 years. NALD and IRD patients are more mildly involved. Some survive to adulthood [11]. Patients with classical RCDP also have dysmorphic features different from those in Zellweger's syndrome, severe psychomotor delay, cataracts, impaired hearing, shortened limbs, chondrodysplasia punctata and coronal vertebral clefts. Clinically, the PBDs lead to malformations and dysfunction of many organs and tissues. Table 41-5 shows that the pattern of biochemical abnormalities of the Zellweger spectrum disorders is entirely

TABLE 41-5 Single peroxisomal protein deficiencies

Function	Disorder	Enzyme/protein defect	Phenotype
Fatty acid β-oxidation	X-ALD Acyl-CoA oxidase deficiency D-bifunctional protein deficiency Racemase deficiency	ALDP Acyl-CoA oxidase D-bifunctional Racemase	CNS childhood–adult adrenal insufficiency NALD-like Zellweger-spectrum-like Late onset neuropathy
Fatty acid α-oxidation	Refsum's disease	Phytanoyl CoA hydroxylase deficiency	Refsum's disease
Ether phospholipid biosynthesis	DHAPAT deficiency Alkyl-DHAP synthase deficiency	DHAPAT Alkyl-DHAP synthase	RCDP RCDP
Isoprenoid biosynthesis	Mevalonate kinase deficiency	Mevalonate kinase	1. Classic 2. Hyper-IgD–periodic fever
Hydrogen peroxide metabolism	Catalase deficiency	Catalase	Acatalasemia
Glyoxylate detoxification	Hyperoxaluria type I	Alanine-glyoxylate aminotransferase deficiency	Renal failure

ALDP, ; DHAP, dihydroxyacetone phosphate; DHAPAT, dihydroxyacetone phosphate acyltransferase; NALD, neonatal adrenoleukodystrophy; RCDP, rhizomelic chondrodysplasia punctata; X-ALD, X-linked adrenoleukodystrophy; ZS, Zellweger's syndrome.

distinct from RCDP. The clinical manifestations of 12 of the PBDs in which the gene defect has now been identified fall into the Zellweger spectrum. Only one, PEX7, is associated with the RCDP phenotype. In patients with *PEX7* defects the biochemical abnormalities involve phytanic acid and ether lipids. The metabolism of these substances involves enzymes that contain the PTS2 signal.

Table 41-4 shows that nine of the 12 Zellweger spectrum disorders show a considerable range of severity of clinical manifestations. Clinical and biochemical studies do not correlate with the type of gene defect. There is, however, considerable correlation between the severity of clinical manifestations and the degree to which a specific mutation compromises import function *in vitro.* For example in PEX1 deficiency, 75% of patients with the G843D allele, in which import is 15% retained, had the relatively mild IRD phenotype, compared to those with the C.2097insT, which abolishes import completely, where only 13% had the IRD phenotype. Such correlations also exist in patients with the PEX 7 defect [10].

Defects of single peroxisomal enzymes. **X-linked adrenoleukodystrophy (X-ALD)** is the most common peroxisomal disorder, with an estimated frequency of 1:17,000. *ABCD1*, the defective gene, maps to X-28 and codes for ALDP, a peroxisomal membrane protein that is a member of the ATP-binding cassette (ABC) transporter superfamily of proteins. More than 500 different mutations have been identified in X-ALD patients [12]. It is associated with the accumulation of saturated very-long-chain fatty acids (VLCFA) in the nervous system, adrenal cortex and Leydig cells in the testis, probably as a result of impaired capacity to oxidize these substances in the peroxisome. The pathogenesis of X-ALD and the function of ALDP is not yet understood. Most patients have primary adrenocortical insufficiency. There are two types of nervous system involvement. In the cerebral forms, which are most common in childhood, there is extensive demyelination associated with an intense inflammatory response. This manifests initially as a behavioral disturbance that resembles a hyperactivity-deficit disorder, but then progresses rapidly to severe neurological disability. In the adult form there is a noninflammatory distal axonopathy that affects the spinal cord most severely. It presents as slowly progressive paraparesis. The two different nervous system phenotypes often co-occur in members of the same family and do not correlate with the nature of the mutation. Search for modifier genes is in progress. Demonstration of abnormally high VLCFA in plasma is the most frequently used diagnostic technique.

Acyl-coenzyme (Co)A oxidase deficiency is a rare disorder that presents with progressive psychomotor and neurological deterioration and demyelination demonstrable by magnetic resonance imaging. Its clinical presentation and progression resembles that seen in NALD [13].

Bifunctional protein deficiency. The enzyme defect involves the D-bifunctional protein. This enzyme contains two catalytic sites, one with enoyl-CoA hydratase activity, the other with 3-hydroxyacyl-CoA activity [13]. Defects may involve both catalytic sites or each separately. The severity of clinical manifestations varies from that of a very severe disorder that resembles Zellweger's syndrome clinically and pathologically, to somewhat milder forms. Table 41-6 shows that biochemical abnormalities involve straight chain, branched chain fatty acids and bile acids. Bifunctional deficiency is often misdiagnosed as Zellweger's syndrome. Approximately 15% of patients initially thought to have a PBD have D-bifunctional enzyme deficiency. Differential diagnosis is achieved by the biochemical studies listed in Table 41-7 and by mutation analysis.

Refsum's disease. This disorder, first described nearly 60 years ago, was recently been shown due to a defect in the enzyme phytanoyl-CoA hydroxylase. Phytanic acid is a 3-methyl fatty acid that because of this methyl group cannot be oxidized directly. It is degraded by a peroxisomal α-oxidation to pristanic acid, a 2-methyl fatty acid which can be degraded by β-oxidation. The principal clinical features of Refsum's disease are progressive polyneuropathy, retinal degeneration, hearing loss, cardiomyopathy and ichthyosis, beginning in late childhood or later.

TABLE 41-6 Biochemical diagnosis of peroxisomal disorders

				Single protein defects					
	PBD Zellweger spectrum	**RCDP**	**X-ALD**	**Ox**	**Bif**	**Ref**	**Rac**	**DHAPAT**	**Alkyl PAT**
VLCFA	↑	N	↑	↑	↑	N	N	N	N
DHCA	↑	N	N	N	↑	N	↑	N	N
THCA	↑	↑	N	N	↑	↑	↑	N	N
Phytanic	↑	↑	N	N	↑	↑	N	N	N
Pristanic	↑	N	N	N	↑	↑	↑	N	N
Pipecolic	↑	N	N	N	N	N	nd	N	N
Ether lipids	↓	↓	N	N	N	N	N	N	N
Catalase	Cytosolic	N	N	N	N	N	N	N	N

Alkyl PAT, alkyl-dihydroxy phosphate synthase; Bif, bifunctional enzyme; DHAPAT, dihydroxyphosphate acyltransferase deficiency; DHCA, dihydroxycholestanoic acid; N, normal; nd, not determined; Ox, acyl-CoA oxidase; Rac, 2-methylacyl-CoA racemase; RCDP, rhizomelic chondrodysplasia punctata; Ref, Refsum's disease; THCA, trihydroxycholestanoic acid; VLCFA, very-long-chain fatty acid.

TABLE 41-7 Therapies of lysosomal and peroxisomal disorders that have shown success in clinical trials

Method	Disorder
Enzyme replacement	Gaucher's disease type I
	Fabry's disease
	Hurler's disease
	Pompe's disease
Hematopoietic cell transplantation	Hurler's syndrome
	Globoid leukodystrophy
	Metachromatic leukodystrophy
	X-linked adrenoleukodystrophy
Substrate reduction	Refsum's disease
	Cystinosis
	Adrenoleukodystrophy

Most, and possibly all of these changes result from the tissue accumulation of phytanic acid. Phytanic is of dietary origin exclusively, and dietary restriction of phytanic and plasmapheresis are of clinical benefit. The defective gene and pathogenic mutations have been identified. The clinical manifestations can also be mimicked by defects of PEX7.

Racemase deficiency. The biological role of 2-methylacyl-CoA racemase has only recently been clarified. This peroxisomal enzyme is essential for certain steps of the oxidation of phytol and bile acid derivatives, which are stereospecific. Biochemically there is accumulation of pristanic acid and C27 bile acid intermediates. Clinical symptoms may include adult-onset peripheral neuropathy, pigmentary degeneration of the retina and liver disease [13].

Alkyl-dihydroxy phosphate synthase and dihydroxyphosphate acyltransferase deficiencies. Both these disorders lead to severe deficiencies of ether phospholipids and lead to syndromes that are similar to RCDP, and point to the key physiological roles of ether phospholipids.

Mevalonate kinase deficiency. Mevalonate kinase and farnesyl-diphosphate synthase are localized in the peroxisome and are involved in the synthesis of isoprenoids. Mevalonate kinase deficiency causes severe developmental delay, dysmorphic features and early death. Mevalonate deficiency has also been observed in the hyperimmunoglobulinemia-and periodic fever syndrome.

Hyperoxaluria type 1 is due to functional efficiency of the liver specific peroxisomal enzyme alanine-glyoxylate aminotransferase. It leads to severe renal failure. The nervous system is not affected.

DIAGNOSIS OF LYSOSOMAL AND PEROXISOMAL DISORDERS

The diagnosis of lysosomal disorders is usually based on enzymatic assays in white blood cells or cultured skin fibroblasts [1]. Molecular studies required for identification of carriers. Initial diagnosis of peroxisomal disorders is based upon a panel of biochemical assays in blood or cultured skin fibroblasts. Table 41-6 shows that this scheme can be used to differentiate PBD disorders from those of single protein defects. Reference [13] provides an algorithm to accomplish this. Definition of the gene defects in the PBD and identification of heterozygote requires mutation analysis.

PATHOGENESIS OF LYSOSOMAL AND PEROXISOMAL DISORDERS

While the molecular defects in many of the lysosomal and peroxisomal disorders have now been defined, their pathogenesis is still poorly understood. Walkley has emphasized that many children with lysosomal storage disease exhibit little or no evidence of disease at birth. The primary gene defects do not, in themselves, necessarily impede an orderly and normal development of many organ systems, including brain. This suggests that the primary defect leads to a series of downstream or cascade events [14]. Some of these events have been defined. Some are specific to particular diseases, others apply generally. Animal models are now available for most of the lysosomal [15] and peroxisomal disorders [8], and their study will enhance greatly our understanding of pathogenesis.

The earlier hypothesis that storage in tertiary lysosomes itself leads to mechanical disruption of cytoplasm appears insufficient, in light of the realization that tertiary lysosomes cannot be viewed as end-organelles but are part of a continuum consisting of the entire endosomal–lysosomal system. The metabolic defects may lead not only to accumulation of the compounds that make up the bulk of the storage material, such as galactocerebroside in globoid leukodystrophy and sulfatide in metachromatic leukodystrophy, but also to lesser accumulation in terms of total quantity of other compounds that play a key role in pathogenesis, such as psychosine in globoid leukodystrophy. Extralysosomal accumulation of G_{M2} ganglioside in the perikaryon, axon hillocks and dendrites appears to be a major cause of nerve system damage in Tay–Sachs and Sandhoff's diseases. The accumulation in axons and dendrites leads to the formation of meganeurites and, intriguingly, to ectopic dendritogenesis. The ectopic dendrites become functional in the overall dendritic arbor and contribute to the seizure disorder. Other pathogenetic cascades lead to apoptosis that involves neurons in infantile ceroid lipofuscinosis and Farber's disease, and oligodendrocytes in globoid leukodystrophy. Macrophage infiltration and release of inflammatory cytokines contribute to oligodendrocyte damage in metachromatic leukodystrophy. In X-ALD the accumulation of saturated VLCFAs increases membrane viscosity, decreases the stability of biological membranes and impairs their function. This may the cause of the distal axonopathy in adrenomyeloneuropathy. Mitochondrial damage also appears to contribute. The inflammatory demyelination in the

childhood cerebral X-ALD may involve autoimmune response to gangliosides or proteolipids containing saturated VLCFAs that involves $CD8^+$ cytotoxic cells and CD1-mediated lipid antigen presentation. Studies in the mouse model of Zellweger's syndrome suggest that the characteristic defect in neuronal migration is secondary to glutamate receptor dysfunction [8].

THERAPY OF LYSOSOMAL AND PEROXISOMAL DISORDERS

New therapeutic approaches are now available. Table 41-7 lists the therapies that have been shown to be beneficial in clinical trials. The development of intravenously administered enzyme replacement therapy is an exciting new development. Successful therapy of type 1 Gaucher's disease with macrophage-targeted glucocerebrosidase [16] is now well documented. Key to its development was the recognition that lysosomal enzymes are targeted by mannose 6 phosphate [6]. It was only after glucocerebrosidase was modified to uncover the signaling mannose residues that efficient endocytosis was achieved. Results of enzyme replacement therapy for Fabry's disease [17], Hurler's syndrome [18] and Pompe's disease [19] are highly encouraging. While these therapies benefit the hematological, skeletal, hepatic, corneal and connective tissue abnormalities, a serious limitation is that intravenously administered enzyme does not cross the blood–brain barrier and thus has little or no effect on neurological manifestations. Methods to circumvent the blood–brain barrier for lysosomal enzyme delivery are under study.

Hematopoietic cell transplantation has been shown to provide some degree of neurological benefit in lysosomal disorders, specifically in Hurler's disease [20], globoid leukodystrophy [21] and metachromatic leukodystrophy [21]. Studies in experimental animals and postmortem studies on individuals who had received sex-mismatched bone marrow transplants suggest that 1–10% of microglia are bone-marrow-derived. The degree of neurological benefit is variable. The benefit usually consists of stabilization rather than the reversal of neurological deficits, so that the best chance for clinically meaningful favorable effect occurs when it is performed when neurological disability is still mild. It is not clear to what extent the benefit results from the bone-marrow-derived cells themselves and how much is the result of secretion of enzyme from donor cells and uptake by host glial cells and neurons. Direct evidence of active enzyme in neurons following bone marrow transplant has been demonstrated in the feline model of α-mannosidosis. Hematopoietic cell transplantation is beneficial in the childhood cerebral form of X-ALD when performed in the early stage of the illness [22]. It is not clear to what extent this relates to immunomodulation or anti-inflammatory effects, versus reduction of brain VLCFA levels or other metabolic effects attributable to donor-derived microglia.

Gene therapy and stem cell therapy are under intensive investigation in animal models. Other promising new approaches are the inhibition of substrate synthesis by pharmacological agents [23] and chemical chaperone therapy designed to increase the stability of mutant enzymes that have retained some degree of catalytic activity. Results in a mouse model of G_{M1} gangliosidosis are encouraging [24]. Last but not least, therapy of Refsum's disease by dietary restriction of phytanic acid intake and cysteamine therapy of cystinosis are well established forms of therapy.

REFERENCES

1. Scriver, C.R., Beaudet, A. L., Sly, W. S. and Valle, D. *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. New York: McGraw-Hill, 2001.
2. Meikle, P. J., Hopwood, J. J., Clague, A. E. and Carey, W. F. Prevalence of lysosomal storage disorders. *J.A.M.A.* 281: 249–254, 1999.
3. Platt, F. M. and Walkley, S. U. *Lysosomal Disorders of the Brain*. New York: Oxford University Press, 2004.
4. Haltia, M. The neuronal ceroid-lipofuscinoses. *J. Neuropathol. Exp. Neurol.* 62: 1–13, 2003.
5. Dierks, T., Schmidt, B., Borissenko, L. V. *et al.* Multiple sulfatase deficiency is caused by mutations in the gene encoding the human Cα-formylglycine generating enzyme. *Cell* 113: 435–444, 2003.
6. Hickman, S. and Neufeld, E. F. A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes. *Biochem. Biophys. Res. Commun.* 49: 992–999, 1972.
7. Sleat, D. E., Wiseman, J. A., El-Banna, M. *et al.* Genetic evidence for nonredundant functional cooperativity between NPC1 and NPC2 in lipid transport. *Proc. Natl Acad. Sci. U.S.A.* 101: 5886–5891, 2004.
8. Weller, S., Gould, S. J. and Valle, D. Peroxisome biogenesis disorders. *Annu. Rev. Genomics Hum. Genet.* 4: 165–211, 2003.
9. Gould, S. J., Raymond, G. V. and Valle, D. The peroxisome biogenesis disorders. In C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (eds), *The Metabolic and Molecular Bases of Inherited Diseases.* New York: McGraw Hill, 2001, pp. 3181–3217.
10. Braverman, N., Chen, L., Lin, P. *et al.* Mutation analysis of PEX7 in 60 probands with rhizomelic chondrodysplasia punctata and functional correlations of genotype with phenotype. *Hum. Mutat.* 20: 284–297, 2002.
11. Barth, P. G., Majoie, C. B., Gootjes, J. *et al.* Neuroimaging of peroxisome biogenesis disorders (Zellweger spectrum) with prolonged survival. *Neurology* 62: 439–444, 2004.
12. Peroxisomal Diseases Laboratory, Kennedy Krieger Institute, Baltimore/Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam. X-linked adrenoleukodystrophy database. Available on line at www. x-ald. nl/.
13. Wanders, R. J. A., Barth, P. G. and Heymans, H. S. A. Single peroxisomal enzyme deficiencies. In C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle (eds), *The Metabolic and*

Molecular Bases of Inherited Disease. New York: McGraw Hill, 2001, pp. 3219–3256.
14. Walkley, S. U. Pathogenic cascades in brain dysfunction. In F. M. Platt and S. U. Walkley (eds), *Lysosomal Disorders of the Brain*. New York: Oxford University Press, 2004, pp. 290–324.
15. Hopwood, J. J., Crawley, A. C. and Taylor, R. M. Spontaneous and engineered mammalian storage disease models. In F. M. Platt and S. U. Walkley (eds), *Lysosomal Disorders of the Brain*. New York: Oxford University Press, 2004, pp. 257–289.
16. Barton, N. W., Brady, R. O., Dambrosia, J. M. *et al.* Replacement therapy for inherited enzyme deficiency–macrophage-targeted glucocerebrosidase for Gaucher's disease. *N. Engl. J. Med.* 324: 1464–1470, 1991.
17. Eng, C. M., Guffon, N., Wilcox, W. R. *et al.* Safety and efficacy of recombinant human alpha-galactosidase A replacement therapy in Fabry's disease. *N. Engl. J. Med.* 345: 9–16, 2001.
18. Kakkis, E. D., Muenzer, J., Tiller, G. E. *et al.* Enzyme-replacement therapy in mucopolysaccharidosis I. *N. Engl. J. Med.* 344: 182–188, 2001.
19. Winkel, L. P., Van den Hout, J. M., Kamphoven, J. H. *et al.* Enzyme replacement therapy in late-onset Pompe's disease: a three-year follow-up. *Ann Neurol* 55: 495–502, 2004.
20. Staba, S. L., Escolar, M. L., Poe, M. *et al.* Cord-blood transplants from unrelated donors in patients with Hurler's syndrome. *N. Engl. J. Med.* 350: 1960–1969, 2004.
21. Krivit, W., Peters, C. and Shapiro, E. G. Bone marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux–Lamy, and Sly syndromes, and Gaucher disease type III. *Curr. Opin. Neurol.* 12: 167–176, 1999.
22. Peters, C., Charnas, L. R., Tan, Y. *et al.* Cerebral X-linked adrenoleukodystrophy: The international hematopoietic cell transplantation experience from 1982 to 1999. *Blood* 104: 881–888, 2004.
23. Platt, F. M. and Butters, T. D. Inhibition of substrate synthesis: a pharmacological approach for glycosphingolipid storage disease therapy. In F. M. Platt and S. U. Walkley (eds), *Lysosomal Disorders of the Brain*. New York: Oxford University Press, 2004, pp. 381–408.
24. Matsuda, J., Suzuki, O., Oshima, A. *et al.* Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. *Proc. Natl Acad. Sci. U.S.A.* 100: 15912–15917, 2003.

CHAPTER

42

Diseases of Carbohydrate, Fatty Acid and Mitochondrial Metabolism

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Darryl C. De Vivo

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Defects of energy metabolism cause profound disturbances in the function of muscle and brain. Such defects may present as a myopathy, encephalopathy or encephalomyopathy. Clinical features are best appreciated by understanding the preferred oxidizable substrates for brain and muscle.

Muscle in the resting state predominantly utilizes fatty acids. The immediate source of energy for muscle contraction is ATP, which is rapidly replenished at the expense of creatine phosphate by the phosphorylation of ADP by creatine kinase. During exercise of moderate intensity, the fuel choice depends on the duration of work. Initially, glycogen is the main fuel source; after 5 or 10 minutes, blood glucose becomes the more important fuel. As work continues, fatty acid utilization increases and, after approximately 4 hours, lipids are the primary source of energy. During high-intensity exercise, at near-maximal power, additional ATP is generated by the anaerobic breakdown of glycogen and by glycolysis. Intense exercise is performed in essentially anaerobic conditions, whereas mild or moderate exercise is accompanied by increased blood flow to exercising muscles, facilitating substrate delivery and favoring aerobic metabolism. This adaptation is known as the 'second-wind' phenomenon [1].

Brain utilizes glucose predominantly, with regional variations of the metabolic rate depending on the mental or motor task being performed. As with muscle, the immediate intracellular energy source is ATP, buttressed by the creatine phosphate stores. Glycogen provides very little energy reserve because brain concentrations of glycogen are extremely low, approximately only one-tenth the amount found in muscle per gram wet weight. Therefore, brain is exquisitely sensitive to fluctuations in the blood glucose concentration. Movement of glucose across the blood–brain barrier is facilitated by a carrier protein, the glucose transporter (GLUT-1) [2]. The facilitated transport of glucose ensures adequate brain glucose concentrations to meet the needs of cerebral metabolism under normal conditions. During starvation, the brain uses little, if any, fatty acids. However, fatty acids of varying chain lengths may be taken up by brain, as the efficiency of transport across the blood–brain barrier is much greater for short- or medium-chain fatty acids than for

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long-chain fatty acids. Ketone bodies represent the preferred cerebral fuel source during starvation when glucose supply is limited [3] (see Ch. 31). Defective fatty acid oxidation, therefore, may affect muscle directly by blocking oxidation of this substrate and brain indirectly by limiting hepatic ketogenesis. Elevated circulating free fatty acids may also have a direct toxic effect on brain, but the precise mechanisms for this effect are poorly understood (see Chs 35 and 38).

Energy metabolism has been studied extensively in skeletal muscle, and several metabolic disorders have been documented [1, 4]. Comparatively less is known about metabolic defects in cerebral energy metabolism. This may be because muscle tissue is more accessible for biochemical analysis and because certain cerebral enzyme defects are lethal.

DISEASES OF CARBOHYDRATE AND FATTY ACID METABOLISM IN MUSCLE

One class of glycogen or lipid metabolic disorders in muscle is manifest as acute, recurrent, reversible dysfunction. These disorders occur with exercise intolerance and myoglobinuria, with or without cramps. Among the glycogenoses, this is characteristic of deficiencies in phosphorylase, phosphofructokinase (PFK), aldolase, phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), β-enolase and lactate dehydrogenase (LDH). Among the disorders of lipid metabolism, this is characteristic of deficiencies in very-long-chain acyl-CoA dehydrogenase (VLCAD), trifunctional protein (TP), carnitine palmitoyltransferase II (CPT II) and short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD). Figures 42-1 and 42-2 schematically illustrate the pathways of glycogen and fatty acid metabolism.

Phosphorylase deficiency (McArdle's disease, glycogenosis type V) is an autosomal recessive myopathy caused by a genetic defect of the muscle isoenzyme of glycogen phosphorylase (Fig. 42-1). Intolerance of strenuous exercise is present from childhood, but usually onset is in adolescence, with cramps after exercise [1, 5]. Myoglobinuria occurs in about one-half of patients. If they avoid intense exercise, most patients can live normal lives; however, about one-third of them develop some degree of fixed weakness, usually as a late-onset manifestation of the disease. In a few patients, weakness rather than exercise-related cramps and myoglobinuria characterizes the clinical picture.

In patients with myoglobinuria, renal insufficiency is a possible life-threatening complication. Physical examination between episodes of myoglobinuria may be completely normal or show some degree of weakness and, occasionally, wasting of some muscle groups.

Even between episodes, most patients have increased serum creatine kinase (CK); forearm ischemic exercise causes no rise of venous lactate concentration. This is a useful but nonspecific test in McArdle's disease. The electromyogram (EMG) at rest shows nonspecific myopathic features in about one-half of patients.

Muscle biopsy demonstrates subsarcolemmal blebs that contain periodic-acid–Schiff (PAS)-positive material, a marker for glycogen [1]. The histochemical stain for phosphorylase is negative, except in regenerating fibers. Biochemical documentation of the enzyme defect requires muscle biopsy because the defect is not expressed in more easily accessible tissues, such as leukocytes, erythrocytes and cultured fibroblasts. The gene encoding muscle phosphorylase has been assigned to chromosome 11, and more than 30 distinct mutations have been identified in patients [5]. By far the most common among these in Caucasian patients is a nonsense mutation in codon 49 (*mut-49*). This allows diagnosis of 90% of patients through molecular analysis of genomic DNA isolated from blood, thus making muscle biopsy unnecessary in most cases [5].

Phosphofructokinase deficiency (Tarui's disease, glycogenosis type VII) is an autosomal recessive myopathy caused by a genetic defect of the muscle (M) subunit of the rate-limiting enzyme of glycolysis, PFK (Fig. 42-1). Presenting symptoms are cramps after intense exercise, followed by myoglobinuria in some patients. A few patients may have mild jaundice, reflecting excessive hemolysis, or typical symptoms and signs of gout. In patients with typical presentation, fixed weakness appears to be less common than in phosphorylase deficiency. However, in PFK deficiency, as in phosphorylase deficiency, a few patients have only weakness, without cramps or myoglobinuria. In addition to renal insufficiency due to myoglobinuria, other possible complications include renal colic due to urate stones and gouty arthritis [1].

Physical examination may show slight jaundice. Neurological examination is normal. Serum CK is variably increased in most patients. Forearm ischemic exercise causes no rise of venous lactate concentration. Serum bilirubin is elevated in most patients and the number of reticulocytes is increased. Serum uric acid is also increased in most patients. The EMG is usually normal. Muscle biopsy shows focal, mostly subsarcolemmal, accumulation of glycogen. In some patients, a small portion of the glycogen is abnormal. By histochemical analysis, it is shown to be diastase-resistant; by electron microscopy, it appears finely granular and filamentous in structure. The enzyme defect can be demonstrated by a specific histochemical reaction for PFK. Although a partial defect of PFK activity is manifest in erythrocytes from patients, firm diagnosis usually requires biochemical studies of muscle. The gene encoding PFK-M is on chromosome 1, and about 15 mutations have been identified in patients of different ethnic origins. Recognition of a specific mutation in genomic DNA from blood cells can eliminate the need for a muscle biopsy in suspected PFK-deficient patients [1].

Muscle aldolase deficiency. The first patient with muscle aldolase deficiency was identified in 1996: this young boy suffered from a hemolytic trait but also

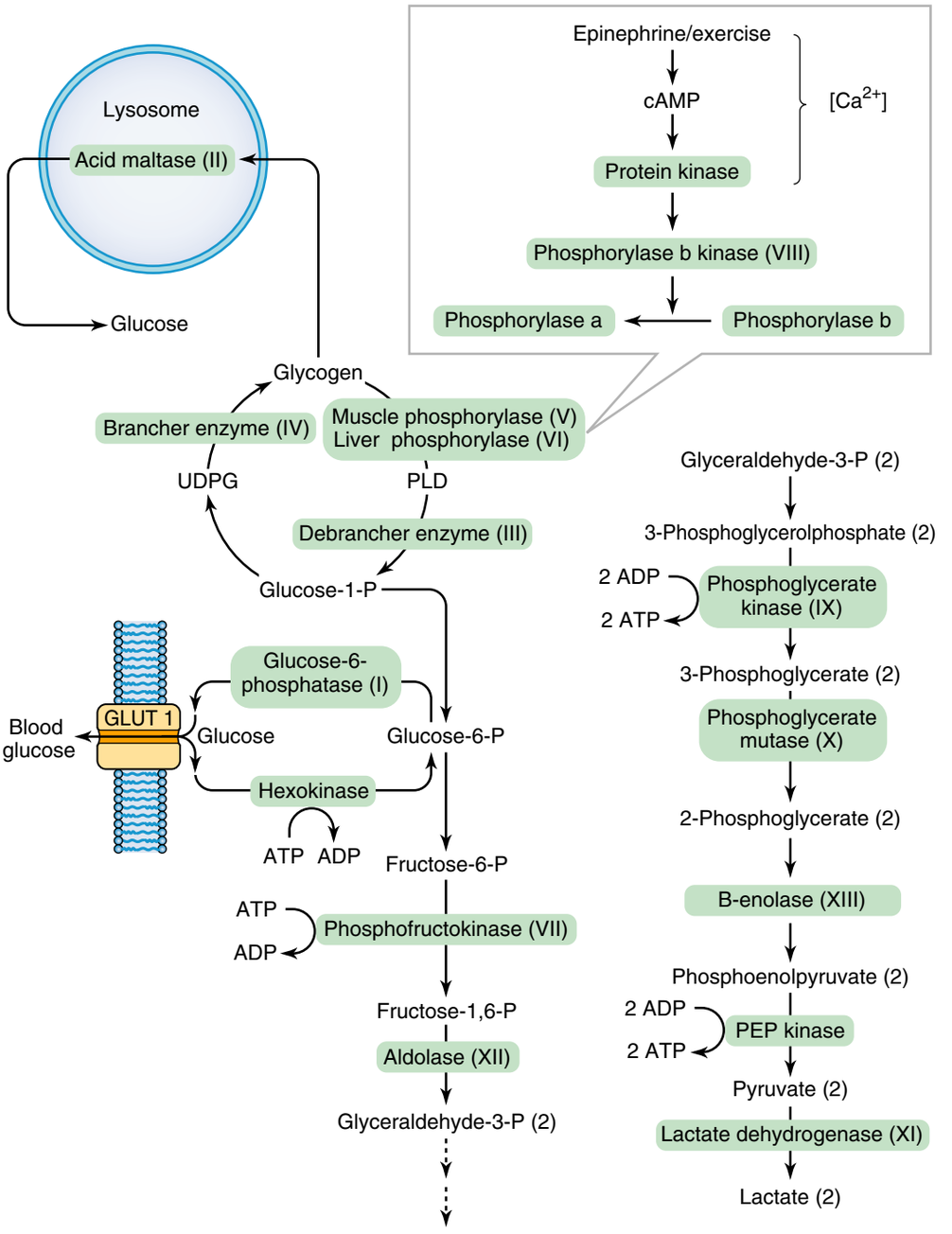

FIGURE 42-1 Schematic representation of glycogen metabolism and glycolysis. *Roman numerals* indicate the sites of identified enzyme defects: *I*, glucose-6-phosphatase; *II*, acid maltase; *III*, debrancher enzyme; *IV*, brancher enzyme; *V*, muscle phosphorylase; *VI*, liver phosphorylase; *VII*, phosphofructokinase; *VIII*, phosphorylase kinase; *IX*, phosphoglycerate kinase; *X*, phosphoglycerate mutase; *XI*, lactate dehydrogenase; *XII*, aldolase; *XIII*, β-enolase. *GLUT 1*, glucose transporter; *P*, phosphate; *PEP*, phosphoenolpyruvate; *PLD*, phosphorylase-limit dextrin; *UDPG*, uridine diphosphate glucose.

complained of exercise intolerance and experienced several episodes of myoglobinuria during febrile illnesses [1].

Phosphoglycerate kinase deficiency is an X-linked recessive disease (type IX, Fig. 42-1). The most common clinical presentation includes hemolytic anemia with or without CNS involvement (see below). Thus far, ten patients have been described with a purely myopathic syndrome, characterized by exercise-induced cramps and myoglobinuria. Between episodes of myoglobinuria, physical and neurological examinations were normal. Forearm ischemic exercise caused contracture and no rise of venous lactate concentration.

Because the enzyme defect is expressed in all tissues except sperm, diagnosis can be made by biochemical

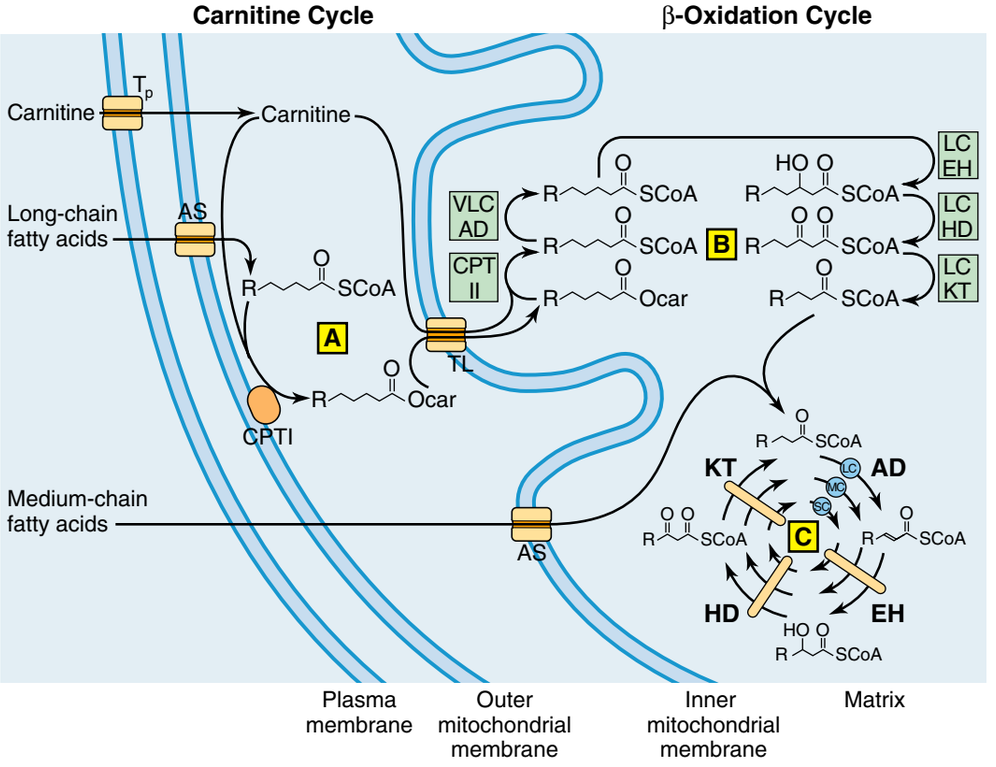

FIGURE 42-2 Schematic representation of fatty acid oxidation. This metabolic pathway is divided into the carnitine cycle (**A**), the inner mitochondrial membrane system (**B**), and the mitochondrial matrix system (**C**). The carnitine cycle includes the plasma membrane transporter (*Tp*), carnitine palmitoyltransferase I (*CPT I*), the carnitine–acylcarnitine translocase system (*Tl*) and carnitine palmitoyltransferase II (*CPT II*). The inner mitochondrial membrane system includes the very-long-chain acyl-CoA dehydrogenase (*VLCAD*) and the trifunctional protein with three catalytically active sites. Long-chain acylcarnitines enter the mitochondrial matrix by the action of CPT II to yield long-chain acyl-CoAs. These thioesters undergo one or more cycles of chain shortening catalyzed by the membrane-bound system. Chain-shortened acyl-CoAs are degraded further by the matrix β-oxidation system. Medium-chain fatty acids enter the mitochondrial matrix directly and are activated to the medium-chain acyl-CoAs before degradation by the matrix β-oxidation system. *AD*, acyl-CoA dehydrogenase; *AS*, acyl-CoA synthetase; *CoA*, coenzyme A; *CPT*, carnitine palmitoyltransferase; *EH*, 2-enoyl-CoA hydratase; *HD*, 3-hydroxyacyl-CoA dehydrogenase; *KT*, 3-ketoacyl-CoA thiolase; *LC*, long chain; *MC*, medium chain; *SC*, short chain; *TL*, carnitine-acylcarnitine translocase; T_p, carnitine transporter; *VLC*, very long chain. (With permission from reference [6].)

studies of muscle, erythrocytes, leukocytes and cultured fibroblasts. Two distinct mutations have been identified in patients with myopathy [1].

Phosphoglycerate mutase deficiency is an autosomal recessive myopathy caused by a genetic defect of the muscle subunit of the enzyme PGM (type X, Fig. 42-1). 12 patients with this enzyme deficiency have been identified thus far.

The clinical picture includes cramps and recurrent myoglobinuria following intense exercise. Aside from episodes of myoglobinuria, none of the patients was weak. Forearm ischemic exercise caused a 1.5–2.0-fold increase in venous lactate concentration, an abnormally low but not absent response. Muscle biopsy showed normal or only moderately increased glycogen concentration. Because other accessible tissues, such as erythrocytes, leukocytes and cultured fibroblasts, express a different isoenzyme, the diagnosis of PGM-M subunit deficiency must be established by biochemical studies of muscle. Four different mutations have been identified in the PGM-M gene, which is located on chromosome 7 [1].

Beta-enolase deficiency (type XIII, Fig. 42-1) has been identified in a 47-year-old man with late-onset but rapidly progressive exercise intolerance and exercise-triggered myalgia. He did not complain of cramps, never experienced pigmenturia, but had chronically elevated serum CK. The patient was a compound heterozygote for two mutations in the *ENO3* gene, which is located on chromosome 17 [1].

Lactate dehydrogenase deficiency is an autosomal recessive myopathy caused by a genetic defect of the muscle subunit, which is encoded by a gene on chromosome 11 (type XI, Fig. 42-1). Thus far, several Japanese families and two Caucasian patients with this disease have been described. The clinical picture is characterized by cramps and myoglobinuria after intense exercise.

Forearm ischemic exercise showed a subnormal rise of lactate concentration, contrasting with an increased rise

of pyruvate. The diagnosis can be established by electrophoretic studies of LDH in serum, erythrocytes and leukocytes, showing lack of subunit-M-containing isoenzymes. Nevertheless, it should be confirmed by biochemical studies of muscle or by molecular analysis of genomic DNA as five different mutations have been documented in patients [1].

Carnitine palmitoyltransferase deficiency is an autosomal recessive myopathy caused by a genetic defect of the mitochondrial enzyme CPT (Fig. 42-2). The disease is prevalent in men (male:female ratio, 5.5:1) and appears to be the most common cause of recurrent myoglobinuria in adults [4].

Clinical manifestations are limited to attacks of myoglobinuria not preceded by contractures and usually precipitated by prolonged exercise of several hours duration, prolonged fasting or a combination of the two conditions. Less common precipitating factors include intercurrent infection, emotional stress and cold exposure, but some episodes of myoglobinuria occur without any apparent cause. Most patients have two or more attacks, probably because the lack of muscle cramps deprives them of a warning signal of impending myoglobinuria.

For unknown reasons, some women seem to have milder symptoms, such as myalgia after prolonged exercise, without pigmenturia. This has been observed in sisters of men with recurrent myoglobinuria. The only serious complication is renal failure following myoglobinuria.

Physical and neurological examinations are completely normal. Prolonged fasting at rest, which should be conducted under close medical observation, causes a sharp rise of serum CK in about one-half of patients. Also, in about one-half of patients, ketone bodies fail to increase normally after prolonged fasting. Forearm ischemic exercise causes a normal increase of venous lactate concentration. Aside from episodes of myoglobinuria, the serum CK concentration and EMG are normal. A muscle biopsy specimen may appear completely normal or show variable, but usually moderate, accumulation of lipid droplets. Most patients with CPT deficiency benefit from a high-carbohydrate, low-fat diet and the therapeutic response may serve as an indirect diagnostic clue. Because the enzyme defect appears to be generalized, tissues other than muscle, such as mixed leukocytes or isolated lymphocytes or platelets, can be used to demonstrate CPT deficiency; but the diagnosis should be confirmed in muscle.

The myopathic form of CPT deficiency is due to a defect of CPT II. The gene for CPT II has been localized to chromosome 1, and several mutations have been identified in patients [4]. As in the case of McArdle's disease (see above), one mutation, a serine-to-leucine substitution at codon 113, is far more common than the others in Caucasians and can be screened for in genomic DNA from blood cells, thus potentially avoiding muscle biopsy.

Very-long-chain acyl-CoA dehydrogenase and trifunctional protein are the two inner membrane-bound enzymes of fatty acid β-oxidation. Genetic defects of either can mimic the clinical presentation of CPT II deficiency by causing recurrent myoglobinuria in otherwise apparently healthy young adults. As in CPT II deficiency, precipitating factors include prolonged exercise, prolonged fasting, cold exposure, intercurrent illnesses or emotional stress [4].

Short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency has been described in three patients. It is associated with additional defects of β-oxidation, which have been associated with limb weakness and attacks of myoglobinuria, and it is potentially fatal.

A second class of disorders of glycogen and fatty acid metabolism causes progressive weakness. These disorders are associated with acid maltase, debrancher enzyme, brancher enzyme and triosephosphate isomerase deficiencies among the glycogenoses. These are also associated with carnitine deficiency, some defects of β-oxidation and other biochemically undefined lipid-storage myopathies among the disorders of lipid metabolism. Figures 42-1 and 42-2 schematically illustrate the pathways of glycogen and fatty acid metabolism.

Acid maltase deficiency (AMD; glycogenosis type II) is an autosomal recessive disease caused by a genetic defect of the lysosomal enzyme acid maltase, an α-1,4- and α-1,6-glucosidase capable of digesting glycogen completely to glucose (Fig. 42-1). Two major clinical syndromes are caused by AMD. The first is Pompe's disease, which is a severe, generalized and invariably fatal disease of infancy; the second is a less severe neuromuscular disorder beginning in childhood or in adult life (see Ch. 41).

Infantile, generalized cardiomegalic AMD, or Pompe's disease, usually becomes manifest in the first weeks or months of life, with failure to thrive, poor suck, generalized hypotonia and weakness, also termed floppy infant syndrome. Macroglossia is common, as is hepatomegaly, which, however, is rarely severe. There is massive cardiomegaly, with congestive heart failure. Weak respiratory muscles make these infants susceptible to pulmonary infection; death usually occurs before the age of 1 year and invariably before the age of 2 years [6].

The childhood- and adult-onset forms of AMD cause signs and symptoms that are limited to the musculature, with progressive weakness of truncal muscles and of proximal, more than distal, limb muscles, usually sparing facial and extraocular muscles. In the childhood form, onset is in infancy or childhood and progression tends to be rapid. In the adult form, onset is usually in the third or fourth decade but occasionally even later and the course is slower [7].

The clinical picture in male children can closely resemble Duchenne-type muscular dystrophy; in adults, it mimics limb-girdle dystrophy or polymyositis. The early and severe involvement of respiratory muscles in most patients with AMD is a distinctive clinical clue. Respiratory failure and pulmonary infection are the most common causes of death.

Serum CK is consistently increased in all forms of AMD. Forearm ischemic exercise causes a normal rise of venous lactate concentration in patients with childhood or adult AMD. The electrocardiogram (ECG) is altered in Pompe's disease, with a short P-R interval, giant QRS complexes and left ventricular or biventricular hypertrophy, but is usually normal in the later-onset forms. The EMG shows myopathic features and fibrillation potentials, bizarre high-frequency discharges and myotonic discharges.

Muscle biopsy shows vacuolar myopathy of very severe degree affecting all fibers in Pompe's disease but of varying degree and distribution in childhood and adult AMD. In adult AMD, biopsy specimens from unaffected muscles may appear normal by light microscopy. The vacuoles contain PAS-positive material, a marker for glycogen. Electron microscopy shows abundant glycogen, both within membranous sacs, presumably lysosomes, and free in the cytoplasm.

The enzyme defect is expressed in all tissues, and the diagnosis can be made by biochemical analysis of urine, lymphocytes (mixed leukocytes do not give reliable results) or cultured skin fibroblasts. Fibroblasts cultured from amniotic fluid can be used for prenatal diagnosis of Pompe's disease. The gene encoding acid maltase is on chromosome 17, and numerous mutations have been identified in patients with both forms of AMD, confirming that infantile and late-onset AMD are allelic disorders. Predictably, more severe mutations are associated with Pompe's disease; however, many patients are compound heterozygotes, and there is no strict genotype/phenotype correlation [6].

Debrancher enzyme deficiency (glycogenosis type III, Cori–Forbes disease) is an autosomal recessive disease (Fig. 42-1). In its more common presentation, debrancher enzyme deficiency causes liver dysfunction in childhood, with hepatomegaly, growth retardation, fasting hypoglycemia and seizures [1]. Myopathy has been described in about 20 patients [1]. In most, onset of weakness was in the third or fourth decade. Wasting of distal leg muscles and intrinsic hand muscles is common, and the association of late-onset weakness and distal wasting often suggests the diagnosis of motor neuron disease or peripheral neuropathy. The course is slowly progressive. In a smaller number of patients, onset of weakness is in childhood, with diffuse weakness and wasting. The association of hepatomegaly and growth retardation facilitates the diagnosis.

There is no glycemic response to glucagon or epinephrine (Fig. 42-1), whereas a galactose load causes a normal glycemic response. Forearm ischemic exercise produces a blunted venous lactate rise or no response. Serum CK activity is variably, often markedly, increased. The ECG shows left ventricular or biventricular hypertrophy in most patients, and the EMG may show myopathic features alone or associated with fibrillations, positive sharp waves and myotonic discharges. This 'mixed' EMG pattern in patients with weakness and distal wasting often reinforces the erroneous diagnosis of motor neuron disease. Motor nerve conduction velocities are moderately decreased in one-fourth of patients, suggesting a polyneuropathy.

Muscle biopsy shows severe vacuolar myopathy with glycogen storage. On electron microscopy, the vacuoles correspond to pools of glycogen free in the cytoplasm.

In most patients, the enzyme defect is generalized, and it has been demonstrated in erythrocytes, leukocytes and cultured fibroblasts. In patients with myopathy, the diagnosis is securely established by measurement of debrancher enzyme activity in muscle biopsy specimens or by studies of iodine adsorption spectra of glycogen isolated from muscle; there is a shift in the spectrum toward lower wavelengths, indicating that the polysaccharide has abnormally short peripheral branches. The gene for the debrancher enzyme has been assigned to chromosome 1, and more than 15 mutations have been identified in patients [1].

Branching enzyme deficiency (glycogenosis type IV; Andersen's disease) is an autosomal recessive disease of infancy or early childhood, typically causing liver dysfunction with hepatosplenomegaly, progressive cirrhosis and chronic hepatic failure (Fig. 42-1). Death usually occurs in childhood. Myopathy has been reported in numerous patients, either alone or in association with hepatopathy or cardiopathy. Some infants had severe hypotonia, wasting, contractures and hyporeflexia, suggesting the diagnosis of spinal muscular atrophy [1].

There are no diagnostic laboratory tests. A muscle biopsy specimen may be normal or show focal accumulations of abnormal glycogen, which is intensely PAS-positive and partially resistant to diastase digestion. With the electron microscope, the abnormal glycogen is found to have a finely granular and filamentous structure. The gene that encodes the branching enzyme has been cloned and assigned to chromosome 3 and several mutations have been identified in patients. [1].

Carnitine deficiency is a clinically useful term describing a diversity of biochemical disorders affecting fatty acid oxidation. Carnitine deficiency may be tissue-specific or generalized.

Tissue-specific carnitine deficiency has previously been termed myopathic carnitine deficiency because patients have generalized limb weakness, starting in childhood. Limb, trunk and facial musculature may be involved. The course is slowly progressive, but weakness may fluctuate in severity. Laboratory investigations show normal or near-normal serum carnitine concentrations and variably increased serum CK values. The EMG shows myopathic features with or without spontaneous activity at rest. Muscle biopsy reveals severe triglyceride storage, best seen with the oil red O stain in frozen sections. This condition is transmitted as an autosomal recessive trait. Originally, it was thought that the primary biochemical defect involved the active transport of carnitine from blood into muscle. However, no such defect has ever been documented [4]. Rather, an increasing number of patients have a tissue-specific defect involving the short-chain isoform of

acyl-CoA dehydrogenase (SCAD). As such, the muscle carnitine deficiency is secondary to a primary enzyme defect.

Generalized carnitine deficiency, in its primary form and inherited as an autosomal recessive trait, is due to a defect of the specific high-affinity, low-concentration, carrier-mediated carnitine-uptake mechanism. The defect has been documented in cultured fibroblasts and muscle cultures, but the same uptake system is probably shared by heart and kidney, thus explaining the cardiomyopathy and the excessive 'leakage' of carnitine into the urine. Oral L-carnitine supplementation results in dramatic improvement in cardiac function [4, 8].

Systemic carnitine deficiency was first described in 1975 and was thought to represent a defect in the *de novo* biosynthesis of carnitine [4]. However, no such defect has been documented. Patients with systemic carnitine deficiency have a generalized decrease in the tissue and plasma concentrations of carnitine and an excessive urinary excretion of carnitine. Many of the patients originally reported to have systemic carnitine deficiency have been reinvestigated and found to have a primary enzyme defect, such as medium-chain acyl-CoA dehydrogenase deficiency (MCAD). This deficiency is the prototype of a defect in β-oxidation that produces secondary carnitine deficiency. β-oxidation defects are also associated with dicarboxylic aciduria. This finding is particularly prominent during a metabolic crisis and may be rather inconspicuous between attacks. The differential diagnosis of systemic carnitine deficiency and dicarboxylic aciduria includes other defects of β-oxidation, such as deficiencies of the long-chain isoform of acyl-CoA dehydrogenase (LCAD), SCAD, electron transfer flavoprotein (ETF) and ETF oxidoreductase [4], the long- and short-chain isoforms of 3-hydroxyacyl CoA dehydrogenase (LCHAD, SCHAD), β-ketothiolase and the newly described TP enzyme that includes the catalytic activities of enoyl hydratase, LCHAD and β-ketothiolase. Cardiac involvement is particularly prominent in conditions that involve the metabolism of long-chain fatty acids. Other genetically determined biochemical defects involving organic acid metabolism and respiratory chain function may produce secondary carnitine deficiency. Carnitine deficiency also may result from acquired diseases, such as chronic renal failure treated by hemodialysis, renal Fanconi's syndrome, chronic hepatic disease with cirrhosis and cachexia, kwashiorkor and total parenteral nutrition in premature infants [4]. The mechanisms of carnitine depletion in these diverse conditions include excessive renal loss and excessive accumulation of acyl-CoA thioesters. These potentially toxic compounds are esterified to acylcarnitines and excreted in the urine, resulting in an excessive loss of carnitine.

The genetically determined defect of membrane carnitine transport is the only known condition that fulfills the criteria for primary carnitine deficiency [4, 9]. This condition, like the other conditions involving the carnitine cycle, is not associated with dicarboxylic aciduria. It is transmitted as an autosomal recessive trait and produces a life-threatening cardiomyopathy in infancy or early childhood, which is effectively treated with carnitine supplementation. The untreated patient also manifests systemic features of hypotonia, failure to thrive and alterations of consciousness, including coma. Carnitine concentrations are extremely low in plasma and body tissues, and the excretion of carnitine in the urine is extremely high. The excessive urinary carnitine losses are caused by a defect in renal tubular uptake of filtered carnitine, resulting from the primary defect of the plasma membrane carnitine transporter. This condition can be documented by carnitine-uptake studies in cultured skin fibroblasts from patients. Uptake studies in parents give intermediate values, consistent with a heterozygous state.

A few patients have been described with a defect involving the carnitine–acylcarnitine translocase system, which facilitates the movement of long-chain acylcarnitine esters across the inner membrane of the mitochondrion (Fig. 42-2). These patients have extremely low carnitine concentrations and minimal dicarboxylic aciduria [4].

Carnitine concentrations are normal to high in patients with a primary defect of CPT I. Patients with CPT II have normal carnitine concentrations. Two clinical syndromes have emerged in relationship to CPT II. The more common syndrome, as discussed previously, involves recurrent myoglobinuria provoked by fasting or intercurrent infection and later may be associated with fixed limb weakness. The less common syndrome involves infants and produces hypoketotic hypoglycemic coma with a Reye-like clinical signature. All cases thought to be recurrent Reye's syndrome should be investigated for defects involving fatty acid oxidation. Low serum carnitine concentrations and increased urinary dicarboxylic acids implicate a biochemical defect of β-oxidation. Low serum carnitine concentrations and normal urinary dicarboxylic acids implicate a defect of the membrane carnitine transporter or the mitochondrial inner membrane carnitine–acylcarnitine translocase system. Normal to high serum carnitine concentrations and no dicarboxylic aciduria suggests a defect of CPT I or CPT II.

Oral administration of L-carnitine is life-saving in patients with the genetically determined defect of the plasma membrane carnitine transporter [4, 8, 9]. It also is recommended as a supplement in all patients who have documented carnitine deficiency, even though clear evidence of benefit is lacking. Medium-chain triglyceride supplementation has proved beneficial in CPT I deficiency and should be beneficial also in the other defects of the carnitine cycle. Medium-chain fatty acids cross the plasma membrane and the mitochondrial membranes directly and are esterified to the thioesters in the mitochondrial matrix (Fig. 42-2). A ketonemic response to medium-chain triglycerides documents the biological integrity of β-oxidation and implicates a biochemical defect of the carnitine cycle or of β-oxidation involving the metabolism of the longer-chain fatty acids.

The impairment of energy production from carbohydrate or fatty acids, which is the common consequence of these defects, should result in similar, exercise-related signs and symptoms. Except for debrancher deficiency, this is the case [1]. Of the nine glycolytic enzyme defects described above, eight affect glycogen breakdown or glycolysis: phosphorylase, debrancher, PFK, aldolase, PGK, PGAM, β-enolase, LDH deficiencies. Patients with phosphorylase, PFK, aldolase, PGK, β-enolase, PGAM or LDH deficiency have exercise intolerance manifested by premature fatigue, cramps and, often, myoglobinuria. As predicted by the crucial role of glycogen as a fuel source, these patients are more prone to experience cramps and myoglobinuria when they engage in isometric exercise, such as lifting weights, or in intense dynamic exercise, such as walking uphill. Energy for these types of exercise derives mainly from anaerobic or aerobic glycolysis. The block of glycogen utilization leads to a shortage of pyruvate and, therefore, of acetyl CoA (Fig. 42-3), the pivotal substrate of the Krebs cycle, and to a decreased mitochondrial energy output. Moderate exercise typically causes premature fatigue and myalgia, but these symptoms usually resolve after brief rest or slowing of pace; thereafter, patients find that they can resume or continue exercise without problems. This second-wind phenomenon seems to be due to early mobilization of fatty acids and to increased blood flow to exercising muscles.

Conversely, patients with fatty-acid oxidation defects experience myalgia and myoglobinuria after prolonged, though not necessarily high-intensity, exercise. Fasting exacerbates these complaints. Thus, myoglobinuria occurs in CPT deficiency under metabolic conditions that favor oxidation of fatty acids in normal muscle [4]. This observation suggests that impaired cellular energetics are the common cause of myoglobinuria in diverse metabolic myopathies. However, biochemical proof of energy depletion is still necessary. No abnormal decrease of ATP concentration has yet been measured in muscle of patients with McArdle's disease during fatigue, which is defined as failure to maintain the required or expected force, or during ischemic exercise-induced contracture. The possibility cannot be excluded, however, that contracture as well as necrosis may involve only a relatively small percentage of fibers. Measurements of ATP and

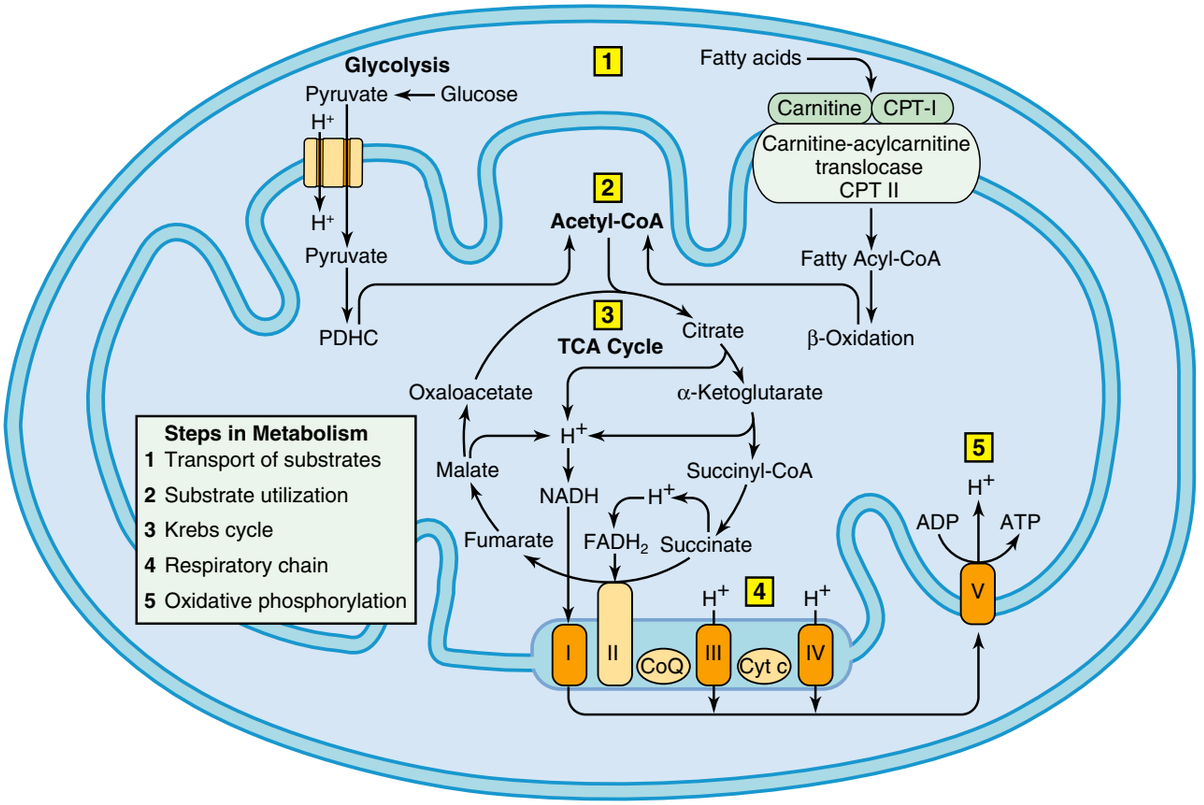

FIGURE 42-3 Schematic representation of mitochondrial metabolism. Respiratory chain complexes or components encoded exclusively by the nuclear genome are *light orange*. Complexes containing some subunits encoded by the nuclear genome and others encoded by mitochondrial DNA are *dark orange*. *CPT*, carnitine palmitoyltransferase; *PDHC*, pyruvate dehydrogenase complex; *CoA*, coenzyme A; *TCA*, tricarboxylic acid; *CoQ*, coenzyme Q; *Cyt c*, cytochrome c. (Modified from reference [12], with permission of McGraw-Hill, New York).

phosphocreatine in whole muscle might fail to detect loss of high-energy phosphate compounds in selected fibers. Additionally, ATP deficiency may affect a specific subcellular compartment.

The cause of weakness is also poorly understood. Chronic impairment of energy provision is unlikely because two of the three glycogenoses causing weakness involve a glycogen-synthesizing enzyme, branching enzyme deficiency, and a lysosomal glycogenolytic enzyme, acid maltase deficiency (see Ch. 41), neither of which is directly involved in energy production [1].

A more likely explanation is that weakness may be due to a net loss of muscle fibers because regeneration cannot keep pace with the rate of degeneration. With fewer functioning fibers, the muscle cannot exert full force. EMG reinforces this interpretation: motor unit potentials are of smaller amplitude and briefer duration than normal due to loss of muscle fibers from a motor unit. Fibrillations are attributed to areas of focal necrosis of muscle fiber, isolating areas of the cell from the neuromuscular junction in a form of 'microdenervation'. Muscle fiber degeneration may be due to excessive storage of glycogen, as in acid maltase and debrancher enzyme deficiencies, or lipid droplets, as in carnitine deficiency. In agreement with this hypothesis is the observation that in at least two of the glycogenoses causing weakness, infantile acid maltase deficiency and debrancher enzyme deficiency, glycogen storage is much more severe than in the glycogenoses causing cramps and myoglobinuria. Similarly, lipid storage is much more severe in carnitine deficiency than in CPT deficiency [4, 8, 9].

An additional cause of weakness may be involvement of the anterior horn cells of the spinal cord, which is very conspicuous in infantile acid maltase deficiency. All three glycogenoses causing weakness are in fact due to generalized enzyme defects, but histological signs of denervation are not evident.

DISEASES OF CARBOHYDRATE AND FATTY ACID METABOLISM IN BRAIN

The concentration of glycogen in brain is small: approximately 0.1 g per 100 g fresh tissue, compared with 1.0 g per 100 g in muscle and 6–10 g per 100 g in liver. The functional significance of glycogen in the brain is not completely understood, but it is generally assumed that it represents available energy to be tapped during glucose depletion; however, the limited glycogen reserve renders the brain vulnerable to injury within minutes of onset of hypoglycemia or hypoxia.

The role of fatty acids as oxidizable fuels for brain metabolism is negligible, but ketone bodies, derived from fatty acid oxidation, can be utilized, particularly in the neonatal period. Diseases of carbohydrate and fatty acid metabolism may affect the brain directly or indirectly [1, 10].

Defective transport of glucose across the blood–brain barrier is caused by deficiency in the glucose transporter protein. Glucose crosses the blood–brain barrier by a mechanism of facilitated diffusion (see Chs 5 and 32). This stereospecific system has a relatively high K_m for glucose, approximately 6 mmol/l. Normally, transport of glucose across the blood–brain barrier is not rate-limiting for cerebral metabolism. Two patients were reported with a defect involving the GLUT-1 carrier protein [2]. The clinical presentation was infantile-onset seizures and developmental delay. One patient had deceleration of head growth with resulting microcephaly. The metabolic signature of this condition is a persistent hypoglycorrhachia with low–normal or low CSF lactate values. The patients responded to a ketogenic diet that was implemented to provide ketone bodies as an alternative fuel source for cerebral metabolism [2, 3]. The GLUT-1 protein also is present in erythrocyte membranes. Decreased binding of cytochalasin B, a ligand that selectively binds to glucose transporters, was documented in both cases, and decreased uptake of 3-*O*-methylglucose by freshly isolated erythrocytes was documented in one case. The molecular and genetic basis for this condition involves mutation in the GLUT-1 gene on chromosome 1 [11]. These patients may be misdiagnosed as examples of cerebral palsy, suspected hypoglycemia or sudden infant death syndrome.

One class of carbohydrate and fatty acid metabolism disorders is caused by defects in enzymes that function in the brain. **Acid maltase deficiency** is characterized by large amounts of glycogen in the perikaryon of glial cells in both gray and white matter, whereas cortical neurons contain much smaller quantities of glycogen. In the spinal cord, the neurons of the anterior horn appear ballooned and contain glycogen, as shown by the abundant PAS-positive material that is digested by diastase. Schwann cells of both anterior and posterior spinal roots and of peripheral nerves also contain excessive glycogen. By electron microscopy, the most striking feature is the presence of glycogen granules within membrane-bound vacuoles. These glycogen-laden vacuoles are particularly abundant in anterior horn cells, in neurons of brainstem motor nuclei and in Schwann cells, whereas they are scarce in cortical neurons. Glycogen is increased in postmortem brain, and acid maltase activity is undetectable. The severe involvement of spinal and brainstem motor neurons and the massive accumulation of glycogen in muscle contribute to the profound hypotonia, weakness and hyporeflexia seen in Pompe's disease [6].

Debrancher enzyme deficiency appears to be generalized. Accordingly, although neither pathology nor debrancher enzyme activity has been reported, increased glycogen concentration has been observed in the brain of a patient. Thus, in debrancher enzyme deficiency, the nervous system seems to be involved biochemically, although clinical signs of brain dysfunction are limited to hypoglycemic seizures in childhood [1].

Branching enzyme deficiency has been described in multiple tissues, reflecting the fact that the enzyme is expressed as a single molecular form. Branching enzyme deficiency causes the accumulation of an abnormal glucose polymer resembling amylopectin and called polyglucosan. A form of polyglucosan body disease (adult polyglucosan body disease, APBD) was described in patients with a characteristic neurological syndrome consisting of progressive upper and lower motoneuron involvement, sensory loss, neurogenic bladder, and, in one-half of the patients, dementia without myoclonus or epilepsy. Onset is in the fifth or sixth decade, and the course varies between 3 and 30 years. Polyglucosan bodies are disseminated throughout the CNS and PNS in processes of neurons and astrocytes but not in perikarya. Other tissues are also affected, including liver, heart and skeletal and smooth muscle. In Ashkenazy Jewish patients with this disorder, branching enzyme activity is decreased in leukocytes, peripheral nerve and, presumably, brain, but is normal in muscle. A 'mild' mutation in the branching enzyme gene was found in seven patients from five Ashkenazy Jewish families with APBD. The mild nature of the mutation may explain the late onset of this variant, but the preferential brain involvement remains unclear [1].

Phosphoglycerate kinase deficiency, as it is most commonly manifest, includes nonspherocytic hemolytic anemia and CNS dysfunction. Neurological problems vary in severity. All patients show some degree of mental retardation, with delayed language acquisition and behavioral abnormalities, and some have hemiplegia or seizures. The enzyme defect has been directly proved in the brain, and the severe brain involvement can be explained by impairment of the glycolytic pathway. The lack of symptoms of brain dysfunction in some patients with PGK deficiency, such as the patients with recurrent myoglobinuria described above, are probably attributable to the presence of sufficient residual enzyme activity to prevent severe energy shortage.

Lafora's disease manifest an accumulation of polyglucosan, in the CNS and PNS as well as in other tissues [1]. Lafora's disease is transmitted as an autosomal recessive trait and is characterized by epilepsy, myoclonus and dementia. Other neurological manifestations include ataxia, dysarthria, spasticity and rigidity. Onset is in adolescence, and death occurs in most patients before 25 years of age.

The pathological hallmark of the disease is the presence in the brain of Lafora bodies: round, basophilic, PAS-positive intracellular inclusions varying in size from small, 'dust-like' bodies less than 3 nm in diameter to large bodies up to 30 nm in diameter. Lafora bodies are typically seen in neuronal perikarya and processes, not in glial cells, and are more abundant in cerebral cortex, substantia nigra, thalamus, globus pallidus and dentate nucleus. Ultrastructural studies have shown that Lafora bodies consist of two components: amorphous electron-dense granules and irregular branched filaments. Linkage analysis in nine families localized the gene responsible for Lafora's disease to chromosome 6q, and the gene product, a tyrosine phosphatase, was called 'laforin'. It has been suggested that laforin may play a role in the cascade of reactions controlling glycogen synthesis and degradation, and that mutations in laforin (documented in patients) may alter the ratio of glycogen synthetase:branching enzyme activities in favor of the former. Indirect support for this hypothesis comes from the observation that polyglucosan accumulates in muscle of patients with PFK deficiency, in whom the increased concentration of glucose-6-phosphate resulting from the enzyme defect may keep an excessive amount of glycogen synthetase in the active form [1].

Another class of carbohydrate and fatty acid metabolism disorders is caused by systemic metabolic defects that affect the brain. **Glucose-6-phosphatase deficiency (glycogenosis type I, Von Gierke's disease)** results in hypoglycemia and excessive intracellular accumulation of glucose-6-phosphate (Fig. 42-1). Hypoglycemia may produce lethargy, coma, seizures and brain damage in gluconeogenic and glycogen synthetase deficiencies [12]. As a result, there is formation of lactic acid, uric acid and lipids. A second form of the disease (type Ib) has been described. The defect in this form involves the glucose-6-phosphate translocation system that is important in facilitating the movement of the substrate into the microsomal compartment for enzymatic conversion to glucose by glucose-6-phosphatase. The clinical features of types Ia and Ib are similar, but normal enzyme activity is present in type Ib. Hepatomegaly, bleeding diathesis and neutropenia are present. The neurological signs result from the chronic hypoglycemia. Recent studies indicate that lactate may be used by the brain as an alternative cerebral metabolic fuel when hypoglycemia is associated with lactic acidosis. Nocturnal intragastric feeding and frequent daytime meals ameliorate most of the clinical and metabolic abnormalities of this condition.

Fructose-1,6-bisphosphatase deficiency, first described by Baker and Winegrad in 1970, has now been reported in approximately 30 cases. It is more common in women and is inherited as an autosomal recessive disorder. Initial manifestations are not strikingly dissimilar from those of glucose-6-phosphatase deficiency. Neonatal hypoglycemia is a common presenting feature, associated with profound metabolic acidosis, irritability or coma, apneic spells, dyspnea, tachycardia, hypotonia and moderate hepatomegaly. Lactate, alanine, uric acid and ketone bodies are elevated in the blood and urine [11]. The enzyme is deficient in liver, kidney, jejunum and leukocytes. Muscle fructose-1,6-bisphosphatase activity is normal.

Fructose-1,6-bisphosphatase is an important rate-limiting step in gluconeogenesis. This gluconeogenic step antagonizes the opposite reaction that forms fructose-1, 6-bisphosphate from fructose-6-phosphate and ATP

(see Ch. 31). A futile cycle exists between these two enzymes, one forming fructose-1,6-bisphosphate and the other disposing of this substrate. Small amounts of fructose-2, 6-bisphosphate also are formed by the PFK reaction. This metabolite stimulates the PFK reaction and inhibits the fructose-1,6-bisphosphatase reaction. This finding nicely explains the subtle interplay between the key rate-limiting step in glycolysis, which is PFK-dependent, and the rate-limiting step in gluconeogenesis catalyzed by fructose-1,6-bisphosphatase.

Phosphoenolpyruvate carboxykinase (PEPCK) deficiency is distinctly rare and even more devastating clinically than deficiencies of glucose-6-phosphatase or fructose-1,6-bisphosphatase. PEPCK activity is almost equally distributed between a cytosolic form and a mitochondrial form. These two forms have similar molecular weights but differ by their kinetic and immunochemical properties. The cytosolic activity is responsive to fasting and various hormonal stimuli. Hypoglycemia is severe and intractable in the absence of PEPCK [12]. A young child with cytosolic PEPCK deficiency had severe cerebral atrophy, optic atrophy and fatty infiltration of liver and kidney.

Pyruvate carboxylase deficiency has been documented in 36 cases [10, 12]. This enzyme is mitochondrial in location and catalyzes the conversion of pyruvate to oxaloacetate in a biotin-dependent manner (Chs 35 and 39). The first report of pyruvate carboxylase deficiency involved an infant with subacute necrotizing encephalomyelopathy, or Leigh's syndrome. Subsequent reports have failed to confirm this causal relationship between pyruvate carboxylase deficiency and the neuropathological features of Leigh's syndrome. Leigh's syndrome has now been assigned to several other biochemical defects, including pyruvate dehydrogenase deficiency, cytochrome-oxidase deficiency, biotinidase deficiency and defects involving complex I and complex V of the respiratory chain.

Most patients with pyruvate-carboxylase deficiency present with failure to thrive, developmental delay, recurrent seizures and metabolic acidosis. Lactate, pyruvate, alanine, β-hydroxybutyrate and acetoacetate concentrations are elevated in blood and urine. Hypoglycemia is not a consistent finding despite the fact that pyruvate carboxylase is the first rate-limiting step in gluconeogenesis.

Sixteen patients had an associated hyperammonemia, citrullinemia and hyperlysinemia. This presentation is the most malignant, with death in early infancy. This French phenotype is commonly associated with the absence of any immunological cross-reacting material (CRM) corresponding to the pyruvate carboxylase apoenzyme protein.

The North American phenotype is associated with the presence of CRM. Possibly as a result, the clinical presentation is less devastating in early infancy, although the outcome is almost invariably fatal in later infancy or early childhood. These patients do not have the associated abnormalities of ammonia metabolism, and the serum aspartic acid concentrations are not as severely depleted. Only one patient has been described with the North American phenotype and a benign clinical syndrome. She has had recurrent episodes of metabolic acidosis requiring hospitalization. Otherwise, her growth and neurological development have been normal.

Prenatal and postnatal diagnoses can be made by enzyme assay of cultured amniocytes, fibroblasts or white blood cells. Treatment remains symptomatic. Sodium bicarbonate is necessary to correct the acidosis. Aspartic acid supplementation will improve the systemic condition but has no effect on the neurological disturbances. Biotin supplementation is of no value.

Biotin-dependent syndromes are manifest in infants, who may present with developmental delay and may demonstrate laboratory abnormalities resulting from deficiencies of the four biotin-dependent carboxylases (see Ch. 39). Three of the carboxylases, located in the mitochondria, are involved in organic acid metabolism. Multiple carboxylase deficiency, when present in the newborn period, is the result of a deficiency of holocarboxylase synthetase, the enzyme that catalyzes the binding of biotin to the apocarboxylase. These infants often die shortly after birth. Older infants gradually develop neurological signs, with developmental delay and seizures associated with alopecia, rash and immunodeficiency. There is a deficiency of biotinidase, the enzyme responsible for the breakdown of biocytin, the lysyl derivative of biotin, to free biotin. Biotinidase deficiency can be recognized at birth by measuring the serum activity. Biotinidase deficiency occurs in 1 in 41,000 live births, and it is eminently treatable by the oral administration of biotin.

Glycogen synthetase deficiency has been described in three families. It caused stunted growth and severe fasting hypoglycemia with ketonuria. Mental retardation was reported in the three children who survived past infancy. The liver was virtually devoid of glycogen and showed fatty degeneration in all cases. In two patients, the brain showed diffuse, nonspecific changes in the white matter, seen as the presence of reactive astrocytes and increased microglia, which were considered secondary to prolonged hypoglycemia or anoxia. Biochemical studies showed that glycogen synthetase activity was markedly decreased in liver but normal in muscle, erythrocytes and leukocytes, suggesting the existence of multiple tissue-specific isoenzymes under separate genetic control. It is not known whether brain glycogen synthetase is different from that in liver.

In liver phosphorylase deficiency (glycogenosis type VI, Hers' disease; Fig. 42-1) and in two genetic forms of phosphorylase kinase deficiency, one of which is X-linked recessive, the other of which is autosomal recessive, hypoglycemia is either absent or mild. Symptoms of brain dysfunction do not usually occur (type VIII, Fig. 42-1) [1].

Fatty acid oxidation defects often produce recurrent disturbances of brain function [4, 8]. Drowsiness, stupor and coma occur during acute metabolic crises and mimic the Reye's syndrome phenotype. The neurological symptoms have been attributed to hypoglycemia, hypoketonemia and the deleterious effects of potentially toxic

organic acids. Hypoglycemia is caused by a continuing demand for glucose by brain and other organs, resulting from the primary biochemical defect of fatty-acid oxidation (Fig. 42-2). Avoidance of catabolic circumstances that require the utilization of fatty acids is the basic principle of treatment. L-carnitine supplementation is recommended for all conditions associated with generalized carnitine deficiency. Some patients may benefit from medium-chain triglyceride supplementation, as discussed previously. Certain forms of ETF-oxidoreductase deficiency respond to riboflavin supplementation. The riboflavin-responsive multiple acyl CoA dehydrogenase deficiency represents the milder form of glutaric aciduria type II.

DISEASES OF MITOCHONDRIAL METABOLISM

Mitochondrial dysfunction produces syndromes involving mainly muscle and the central nervous system. Although some energy can be obtained quickly from glucose or glycogen through anaerobic glycolysis, most of the energy derives from oxidation of carbohydrates and fatty acids in the mitochondria. The common metabolic product of sugars and fats is acetyl-CoA, which enters the Krebs cycle. Oxidation of one molecule of acetyl-CoA results in the reduction of three molecules of NAD and one of FAD. These reducing equivalents flow down a chain of carriers (Fig. 42-3) through a series of oxidation-reduction events. The final hydrogen acceptor is molecular oxygen, and the product is water. The released energy 'charges' the inner mitochondrial membrane, converting the mitochondrion into a veritable biological battery. This oxidation process is coupled to ATP synthesis from ADP and inorganic phosphate (P_i), catalyzed by mitochondrial ATPase [10, 13, 14]. Considering the enormous amount of information collected since 1960 on mitochondrial structure and function, it is surprising that diseases of terminal mitochondrial metabolism, i.e. the Krebs cycle and respiratory chain, have attracted the attention of clinical investigators only recently [10, 13, 14].

Initial clues that some diseases might be due to mitochondrial dysfunction came from electron-microscopic studies of muscle biopsies showing fibers with increased numbers of structurally normal or abnormal mitochondria. These fibers have a 'ragged red' appearance in the modified Gomori trichrome stain. Because the diagnosis was based on mitochondrial changes in muscle biopsies, these disorders were initially labeled mitochondrial myopathies. It soon became apparent, however, that many mitochondrial diseases with ragged red fibers were not confined to skeletal muscle but were multisystem disorders. In these patients, the clinical picture is often dominated by signs and symptoms of muscle and brain dysfunction, probably due to the great dependence of these tissues on oxidative metabolism. This group of disorders, often called mitochondrial encephalomyopathies, includes four more common syndromes (Table 42-1) [10, 13, 14].

The first, **Kearns–Sayre syndrome**, is characterized by childhood onset of progressive external ophthalmoplegia and pigmentary degeneration of the retina. Heart block, cerebellar syndrome or high CSF protein may also appear. Almost all cases are sporadic. The second syndrome, **myoclonus epilepsy with ragged red fibers (MERRF)**, is characterized by myoclonus, ataxia, weakness and generalized seizures. The third syndrome, **mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS)**, affects young children, who show stunted growth, episodic vomiting and headaches, seizures and recurrent cerebral insults resembling strokes and causing hemiparesis, hemianopsia or cortical blindness. The fourth syndrome, **neuropathy, ataxia, retinitis pigmentosa/maternally inherited Leigh's syndrome (NARP/MILS)** comes in two flavors: NARP is usually seen in young adults and is characterized by retinitis pigmentosa, dementia, seizures, ataxia, proximal weakness and sensory neuropathy, whereas MILS affects infants and children (often in the same family) and is a devastating encephalomyopathy with the neuroradiological and neuropathological symmetrical CNS lesions of Leigh's syndrome. Unlike Kearns–Sayre syndrome, MERRF, MELAS and NARP/MILS are transmitted by non-mendelian maternal inheritance. All four conditions are due to mutations in mitochondrial DNA (mtDNA); Kearns–Sayre syndrome is associated with single, large-scale mtDNA deletions; MERRF is typically due to a point mutation (A8334G) in the $tRNA^{Lys}$; MELAS is most commonly due to the A3243G mutation in the $tRNA^{Leu(UUR)}$, whereas NARP/MILS is caused by mutations in the ATPase-6 gene. The different severity of NARP and MILS is due to different mutation loads, much higher in MILS than in NARP [10, 13, 14].

Mitochondrial DNA is inherited maternally. What makes mitochondrial diseases particularly interesting from a genetic point of view is that the mitochondrion has its own DNA (mtDNA) and its own transcription and translation processes. The mtDNA encodes only 13 polypeptides; nuclear DNA (nDNA) controls the synthesis of 90–95% of all mitochondrial proteins. All known mitochondrially encoded polypeptides are located in the inner mitochondrial membrane as subunits of the respiratory chain complexes (Fig. 42-3), including seven subunits of complex I; the apoprotein of cytochrome *b*; the three larger subunits of cytochrome *c* oxidase, also termed complex IV; and two subunits of ATPase, also termed complex V.

In the formation of the zygote, all mitochondria are contributed by the ovum. Therefore, mtDNA is transmitted by maternal inheritance in a vertical, nonmendelian fashion. Strictly maternal transmission of mtDNA has been documented in humans by studies of restriction fragment length polymorphisms (RFLPs) in DNA from platelets. As exemplified by the disorders outlined above,

TABLE 42-1 Clinical features of mitochondrial diseases associated with mtDNA mutations

		Δ-mtDNA		*tRNA*		*ATPase6*	
Tissue	**Symptom/sign**	**KSS**	**Pearson**	**MERRF**	**MELAS**	**NARP**	**MILS**
CNS	Seizures	–	–	+	+	–	+
	Ataxia	+	–	+	+	+	±
	Myoclonus	–	–	+	±	–	–
	Psychomotor retardation	–	–	–	–	–	+
	Psychomotor regression	+	–	±	+	–	–
	Hemiparesis/hemianopia	–	–	–	+	–	–
	Cortical blindness	–	–	–	+	–	–
	Migraine-like headaches	–	–	–	+	–	–
	Dystonia	–	–	–	+	–	+
PNS	Peripheral neuropathy	±	–	±	±	+	–
Muscle	Weakness	+	–	+	+	+	+
	Ophthalmoplegia	+	±	–	–	–	–
	Ptosis	+	–	–	–	–	–
Eye	Pigmentary retinopathy	+	–	–	–	+	±
	Optic atrophy	–	–	–	–	±	±
	Cataracts	–	–	–	–	–	–
Blood	Sideroblastic anemia	±	+	–	–	–	–
Endocrine	Diabetes mellitus	±	–	–	±	–	–
	Short stature	+	–	+	+	–	–
	Hypoparathyroidism	±	–	–	–	–	–
Heart	Conduction block	+	–	–	±	–	–
	Cardiomyopathy	±	–	–	±	–	±
Gastrointestinal	Exocrine pancreas dysfunction	±	+	–	–	–	–
	Intestinal pseudo-obstruction	–	–	–	–	–	–
ENT	Sensorineural hearing loss	–	–	+	+	±	–
Kidney	Fanconi's syndrome	±	±	–	±	–	–
Lab	Lactic acidosis	+	+	+	+	–	±
	Muscle bx: RRF	+	±	+	+	–	–
Inheritance	Maternal	–	+	+	±	+	+
	Sporadic	+	–	–	±	–	–

Boxes highlight typical features of different syndromes, except for maternally inherited Leigh's syndrome (MILS), which is defined on the basis of neuroradiologic or neuropathologic criteria. Pearson's syndrome is a hematological disorder of infancy characterized by sideroblastic anemia and exocrine pancreas dysfunction. –, absent; +, present; Δ-mtDNA, deleted mtDNA; KSS, Kearns–Sayre syndrome; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonus epilepsy with ragged-red fibers; MILS, maternally inherited Leigh's syndrome; NARP, neuropathy, ataxia and retinitis pigmentosa.

diseases caused by mutations of mtDNA are also transmitted by maternal inheritance: an affected mother ought to pass the disease to all of her children, were it not for the 'threshold effect', which is described later, but only her daughters can transmit the trait to subsequent generations [10, 13, 14]. Characteristics that distinguish maternal from mendelian inheritance include the following:

1. The number of affected individuals in subsequent generations should be higher than in autosomal dominant disease, again were it not for the 'threshold effect' (see below).
2. Inheritance is maternal, as in X-linked diseases, but children of both sexes are affected.
3. Because there are hundreds or thousands of copies of mtDNA in each cell, the phenotypic expression of a mitochondrially encoded gene depends on the relative proportions of mutant and wild-type mtDNAs within a cell; this is termed the 'threshold effect'.
4. Because mitochondria replicate more often than do nuclei, the relative proportion of mutant and wild-type mtDNAs may change within a cell cycle.
5. At the time of cell division, the proportion of mutant and wild-type mtDNAs in the two daughter cells can shift, thus giving them different genotypes and, possibly, different phenotypes, a phenomenon called mitotic segregation.

As described above, maternal inheritance has been documented in diseases due to point mutations of mtDNA, while most diseases due to mtDNA deletions or duplications are sporadic.

The genetic classification of mitochondrial diseases divides them into three groups. **Defects of mtDNA** include point mutations and deletions or duplications. From a biochemical point of view, these disorders will be associated with dysfunction of the respiratory chain

because all 13 subunits encoded by mtDNA are subunits of respiratory chain complexes. Diseases due to point mutations are transmitted by maternal inheritance, and the number has rapidly increased since the first pathogenic mtDNA mutations were described 16 years ago. The main syndromes include MERRF; MELAS; NARP/MILS (Table 42-1); Leber's hereditary optic neuropathy, a disorder causing blindness in young adult men. Diseases due to deletions or duplications are usually sporadic, for reasons that are not completely clear. They include, besides Kearns–Sayre syndrome (Table 42-1), isolated progressive external ophthalmoplegia and Pearson's syndrome, a usually fatal infantile disorder dominated by sideroblastic anemia and exocrine pancreas dysfunction.

Defects of nuclear DNA also cause mitochondrial diseases. As mentioned above, the vast majority of mitochondrial proteins are encoded by nDNA, synthesized in the cytoplasm and 'imported' into the mitochondria through a complex series of steps. Diseases can be due to mutations in genes encoding respiratory chain subunits, ancillary proteins controlling the proper assembly of the respiratory chain complexes, proteins controlling the importation machinery, or proteins controlling the lipid composition of the inner membrane. All these disorders will be transmitted by mendelian inheritance. From a biochemical point of view, all areas of mitochondrial metabolism can be affected (see below).

Defects of communication between nDNA and mtDNA can also cause mitochondrial diseases. The nDNA controls many functions of the mtDNA, including its replication. It is, therefore, predictable that mutations of nuclear genes controlling these functions could cause alterations in the mtDNA. Two groups of human diseases are due to faulty intergenomic communication [10, 13, 14]. The first is associated with multiple mtDNA deletions and is characterized clinically by ptosis, progressive external ophthalmoplegia (PEO), weakness of limb and respiratory muscles, peripheral neuropathy, parkinsonism and – often – psychiatric disorders. Transmission is more commonly autosomal dominant than recessive. Mutations in four genes have been associated with autosomal dominant PEO syndromes (adenine nucleotide transporter 1, *ANT1*, and *Twinkle*, a helicase), with autosomal dominant or recessive PEO syndromes (polymerase gamma, *POLG*), and with a multisystem disorder called mitochondrial neurogastrointestinal encephalomyopathy, or MNGIE (thymidine phosphorylase, *TP*) [10, 13, 14]. The second group of disorders is associated with mtDNA depletion in one or more tissues, more commonly in muscle (myopathic form) or in liver and brain (hepatocerebral form). Depending on the tissues affected and the severity of the mtDNA decrease, the clinical picture can be a rapidly fatal congenital myopathy, a slightly more benign myopathy of childhood, a fatal hepatopathy or a multisystem disorder. Transmission is autosomal recessive, and two genes have been associated with mtDNA depletion syndromes, deoxyguanosine kinase (*dGK*) and thymidine kinase 2 (*TK2*). Mutations in *dGK* tend to cause the hepatocerebral phenotype and mutations in *TK2* are generally associated with the myopathic variant. However, there are many patients with mtDNA depletion but without mutations in either gene: clearly, more genes are implicated and have to be identified.

The biochemical classification of mitochondrial DNA is based on the five major steps of mitochondrial metabolism. These steps are illustrated in Figure 42-3 and divide mitochondrial diseases into five groups: defects of mitochondrial transport, defects of substrate utilization, defects of the Krebs cycle, defects of the respiratory chain and defects of oxidation–phosphorylation coupling.

All disorders except those in group 5 are due to defects of nDNA and are transmitted by Mendelian inheritance. Disorders of the respiratory chain can be due to defects of nDNA or mtDNA. Usually, mutations of nDNA cause isolated, severe defects of individual respiratory complexes, whereas mutations in mtDNA or defects of intergenomic communication cause variably severe, multiple deficiencies of respiratory chain complexes. The description that follows is based on the biochemical classification.

Defects of mitochondrial transport interfere with the movement of molecules across the inner mitochondrial membrane, which is tightly regulated by specific translocation systems. The carnitine cycle is shown in Figure 42-2 and is responsible for the translocation of acyl-CoA thioesters from the cytosol into the mitochondrial matrix. The carnitine cycle involves four elements: the plasma membrane carnitine transporter system, CPT I, the carnitine–acyl carnitine translocase system in the inner mitochondrial membrane and CPT II. Genetic defects have been described for each of these four steps, as discussed previously [4, 8, 9].

Defects of substrate utilization. Pyruvate dehydrogenase (PDH) deficiency can cause alterations of pyruvate metabolism, as can defects of pyruvate carboxylase, as discussed earlier. Over 200 patients have been described with a disturbance of the PDH complex (PDHC) [15, 16]. The clinical picture includes several phenotypes ranging from a severe, devastating metabolic disease in the neonatal period to a benign, recurrent syndrome in older children. There is considerable overlap clinically and biochemically with other disorders (see below).

The PDHC catalyzes the irreversible conversion of pyruvate to acetyl-CoA (Fig. 42-3) and is dependent on thiamine and lipoic acid as cofactors (see Ch. 35). The complex has five enzymes: three subserving a catalytic function and two subserving a regulatory role. The catalytic components include PDH, E1; dihydrolipoyl transacetylase, E2; and dihydrolipoyl dehydrogenase, E3. The two regulatory enzymes include PDH-specific kinase and phospho-PDH-specific phosphatase. The multienzyme complex contains nine protein subunits, including

protein X. Protein X anchors the E3 component to the E2 core of the complex. The E1α subunit is encoded by a gene on the short arm of the X chromosome and a gene on chromosome 4. The E1β subunit is encoded by a gene on chromosome 3, the E2 component is encoded by a gene on chromosome 11 and the E3 component is encoded by a gene on chromosome 7. Biochemical defects have been documented for the E1α subunit, E2, E3, protein X and the phospho-PDH-specific phosphatase. The great majority of cases involve a mutation defect of the E1α subunit [15, 16]. Both sexes are equally represented despite the location of the E1α-subunit gene on the X chromosome.

The most devastating phenotype of PDH deficiency presents in the newborn period. The majority of patients are male and critically ill with a severe metabolic acidosis. There is an elevated blood or CSF lactate concentration and associated elevations of pyruvate and alanine. These patients have seizures, failure to thrive, optic atrophy, microcephaly and dysmorphic features. Multiple brain abnormalities have been described, including dysmyelination of the cortex, cystic degeneration of the basal ganglia, ectopic olivary nuclei, hydrocephalus and partial or complete agenesis of the corpus callosum. A less devastating phenotype presents in early infancy. These patients demonstrate the histopathological features of Leigh's syndrome. Other patients affected in infancy survive with a chronic neurodegenerative syndrome manifested by mental retardation, microcephaly, recurrent seizures, spasticity, ataxia and dystonia.

Mutations involving the E1α subunit behave clinically like an X-linked dominant condition. These mutations usually are lethal in boys during early infancy. The clinical spectrum in the heterozygous girl is more varied, ranging from a devastating condition in early infancy to a mild chronic encephalopathy with mental retardation. The least symptomatic woman may give birth to affected male and female progeny and pose a significant problem in clinical diagnosis and genetic counseling.

Treatment is largely symptomatic, and the prognosis ranges from dismal to guarded. Thiamine, lipoic acid, ketogenic diet and physostigmine have been tried in different concentrations and doses with equivocal results. Some patients with periodic ataxia resulting from PDHC deficiency may respond to acetazolamide.

Glutaric aciduria type II, which is a defect of β-oxidation, may affect muscle exclusively or in conjunction with other tissues. Glutaric aciduria type II, also termed multiple acyl-CoA dehydrogenase deficiency (Fig. 42-2), usually causes respiratory distress, hypoglycemia, hyperammonemia, systemic carnitine deficiency, nonketotic metabolic acidosis in the neonatal period and death within the first week. A few patients with onset in childhood or adult life showed lipid-storage myopathy, with weakness or premature fatigue [4]. Short-chain acyl-CoA deficiency (Fig. 42-2) was described in one woman with proximal limb weakness and exercise intolerance. Muscle biopsy showed marked accumulation of lipid droplets. Although no other tissues were studied, the defect appeared to be confined to skeletal muscle, suggesting the existence of tissue-specific isozymes [4].

Defects of the Krebs cycle. Fumarase deficiency was reported in children with mitochondrial encephalomyopathy. Usually, there is developmental delay since early infancy, microcephaly, hypotonia and cerebral atrophy, with death in infancy or early childhood. The laboratory hallmark of the disease is the excretion of large amounts of fumaric acid and, to a lesser extent, succinic acid in the urine. The enzyme defect has been found in muscle, liver and cultured skin fibroblasts [16].

Abnormalities of the respiratory chain. These are increasingly identified as the hallmark of 'mitochondrial diseases' or 'mitochondrial encephalomyopathies' [13]. They can be identified on the basis of polarographic studies showing differential impairment in the ability of isolated intact mitochondria to use different substrates. For example, defective respiration with NAD-dependent substrates, such as pyruvate and malate, but normal respiration with FAD-dependent substrates, such as succinate, suggests an isolated defect of complex I (Fig. 42-3). However, defective respiration with both types of substrates in the presence of normal cytochrome c oxidase activity, also termed complex IV, localizes the lesions to complex III (Fig. 42-3). Because frozen muscle is much more commonly available than fresh tissue, electron transport is usually measured through discrete portions of the respiratory chain. Thus, isolated defects of NADH–cytochrome c reductase, or NADH-coenzyme Q (CoQ) reductase suggest a problem within complex I, while a simultaneous defect of NADH and succinate–cytochrome c reductase activities points to a biochemical error in complex III (Fig. 42-3). Isolated defects of complex III can be confirmed by measuring reduced CoQ-cytochrome c reductase activity.

Defects of complex I. Known or presumed nuclear defects of complex I usually affect infants or children. Some have fatal infantile multisystem disorders, characterized by severe congenital lactic acidosis, psychomotor delay, diffuse hypotonia and weakness, cardiopathy, and cardiorespiratory failure. Some have the neuroradiological and neuropathological features of Leigh's syndrome. Interestingly, a handful of mutations in nuclear genes encoding complex I subunits have been identified in these children, making prenatal diagnosis possible for some families [7].

Complex I deficiency due to mtDNA mutations (seven subunits of complex I are encoded by mtDNA) can be divided into encephalomyopathies and myopathies. The most important encephalomyopathy is Leber's hereditary optic neuropathy, characterized by acute or subacute loss of vision due to severe bilateral optic atrophy, with onset usually between 18 and 30 years and marked predominance in men. Three mutations (in *ND1*, *ND4* and *ND6*)

are considered 'primary', i.e. pathogenic in and by themselves. Increased number of mutations in *ND5* have been associated with clinical phenotypes resembling MELAS, Leigh's syndrome, MERRF or overlapping syndromes. As expected, all these disorders are inherited maternally [14].

Myopathies have been described in several sporadic cases with lactic acidosis, cytochrome oxidase (COX)-positive RRF in muscle, and isolated complex I deficiency, and have been attributed to various pathogenic mutations in ND genes. The sporadic nature of these myopathies suggests that the ND mutations are de novo, arising spontaneously in myogenic stem cells after germ-layer differentiation (somatic mutations) [14].

Defects of complex II. These have not been fully characterized in the few reported patients, and the diagnosis has often been based solely on a decrease of succinate–cytochrome *c* reductase activity (Fig. 42-3). However, partial complex II deficiency was documented in muscle and cultured fibroblasts from two sisters with clinical and neuroradiological evidence of Leigh's syndrome, and molecular genetic analysis showed that both patients were homozygous for a point mutation in the flavoprotein subunit of the complex [17]. This was the first documentation of a molecular defect in the nuclear genome associated with a respiratory chain disorder.

Coenzyme Q10 (CoQ10) deficiency. This mitochondrial encephalomyopathy has three main clinical presentations. A predominantly myopathic form is characterized by the triad of exercise intolerance, recurrent myoglobinuria, and CNS involvement. A more frequent ataxic form is dominated by ataxia and cerebellar atrophy, variously associated with weakness, developmental delay, seizures, pyramidal signs, and peripheral neuropathy, often simulating spinocerebellar atrophy. A third presentation with fatal infantile encephalomyopathy and renal involvement, has been described in two families. The biochemical defect (or defects) presumably involve different steps in the biosynthesis of CoQ10, but are still unknown, as are the molecular defects. Diagnosis, however, is important because all patients – and especially those with the myopathic and infantile forms – benefit from CoQ10 supplementation [13, 14].

Defects of complex III. Like defects of complex I, these can be due to nDNA mutations or to mtDNA mutations. The only nuclear defect described thus far does not affect a complex III subunit, but an ancillary protein needed for proper assembly, BCS1L. Mutations in *BCS1L* can cause a Leigh's-syndrome-like disorder or a fatal infantile disease called GRACILE (growth retardation, aminoaciduria, cholestasis, iron overload, lactacidosis, and early death).

Mutations in the only mtDNA-encoded subunit of complex III (cytochrome *b*) can cause multisystem disorders or – more commonly – isolated myopathies, manifested by exercise intolerance with or without exercise-related myoglobinuria. Patients with myopathy are almost invariably sporadic, suggesting that the cytochrome *b* mutations are somatic. Muscle biopsies in these patients show COX-positive RRF and isolated complex III deficiency [18].

Defects of complex IV. These disorders, also termed COX deficiency, have clinical phenotypes that fall into two main groups: one in which myopathy is the predominant or exclusive manifestation and another in which brain dysfunction predominates (Fig. 42-3). In the first group, the most common disorder is fatal infantile myopathy, causing generalized weakness, respiratory insufficiency and death before age 1 year. There is lactic acidosis and renal dysfunction, with glycosuria, phosphaturia and aminoaciduria, also termed DeToni–Fanconi–Debre syndrome. The association of myopathy and cardiopathy in the same patient and myopathy and liver disease in the same family has also been described [14].

In patients with pure myopathy, COX deficiency is confined to skeletal muscle, sparing heart, liver and brain. The amount of immunologically reactive enzyme protein is markedly decreased in muscle by enzyme-linked immunosorbent assay (ELISA) and by immunocytochemistry of frozen sections. Benign infantile mitochondrial myopathy, in contrast, has been described in a few children with severe myopathy and lactic acidosis at birth, who then improve spontaneously and are virtually normal by age 2 years. This condition is due to a reversible COX deficiency. The enzyme activity is markedly decreased, <19% of normal, in muscle biopsies taken soon after birth but returns to normal in the first year of life. Immunocytochemistry and immunotitration show normal amounts of enzyme protein in all muscle biopsies. This finding differs from the virtual lack of CRM in patients with fatal infantile myopathy and may represent a useful prognostic test. The selective involvement of one or more tissues and the reversibility of the muscle defect in the benign form suggest the existence of tissue-specific and developmentally regulated COX isoenzymes in humans [13]. While the molecular bases of both the 'fatal' and the 'benign' myopathies described above remain uncertain, but presumably involve nuclear genes, some patients with exercise intolerance and myoglobinuria have somatic mutations in mtDNA-encoded genes [14].

Subacute necrotizing encephalomyelopathy, also termed Leigh's syndrome, typifies the second group of disorders of complex IV, dominated by involvement of the CNS. Leigh's syndrome usually starts in infancy or childhood and is characterized by psychomotor retardation, brainstem abnormalities and apnea [10, 13, 14]. The pathological hallmark consists of focal, symmetrical necrotic lesions from thalamus to pons, involving the inferior olives and the posterior columns of the spinal cord. Microscopically, these spongy brain lesions show demyelination, vascular proliferation and astrocytosis. In these patients, COX deficiency is generalized, including cultured fibroblasts in most, but not all, cases. This may provide a useful tool for prenatal diagnosis in at least some families. Immunological studies show CRM in all tissues.

Great strides have been made in our understanding of the molecular bases of COX-deficient Leigh's syndrome [13, 14]. As is the case for complex III deficiency, thus far no mutations have been found in genes encoding COX subunits, but several COX-assembly genes have been involved. Most mutations causing typical Leigh's syndrome are in the *SURF1* gene. Mutations in other genes often affect one other tissue besides the brain, such as the heart (*SCO2* and *COX15*), the liver (*SCO1*), or the kidney (*COX10*). Even though there is no effective therapy for these disorders, at least we can now offer prenatal diagnosis to many affected families.

Defects of complex V. The most important disorder is NARP/MILS, an encephalomyopathy already described above, which can present in the same family as Leigh's syndrome in infants or as milder multisystem disorder in young adults (NARP). In fact NARP/MILS is a good example of the importance of the mutation load. When the common mutation (T8993G in the ATPase-6 gene of mtDNA) is present in great abundance (>90%), the phenotype is MILS; when the same mutation is less abundant (about 70%), the phenotype is NARP. Another interesting aspect of the NARP/MILS phenotype is that it can also be caused by a distinct mutation in the same nucleotide (T8993C). Both clinical and biochemical expressions are milder with the T-to-C than with the T-to-G mutation. As expected, ATP production by mitochondria isolated from cultured fibroblasts harboring almost homoplasmic levels of the T89993G mutation was markedly decreased. A few other mutations in the ATPase 6 gene have been associated with Leigh's syndrome or with a similar but milder condition called familial bilateral striatal necrosis.

Mutations in one nuclear gene (*ATP12*), encoding an ATPase assembly protein, have been associated with complex V deficiency in an infant with congenital lactic acidosis and a rapidly fatal disorder affecting brain, liver, heart, and muscle [19].

Defects of oxidation–phosphorylation coupling. The best known example of such a defect is Luft's disease, or nonthyroidal hypermetabolism. Only two patients with this condition have been reported. Family history was noncontributory in both cases. Symptoms started in childhood or early adolescence with fever, heat intolerance, profuse perspiration, resting tachypnea and dyspnea, polydipsia, polyphagia and mild weakness. The basal metabolic rate was markedly increased in both patients, but all tests of thyroid function were normal. Muscle biopsies showed ragged red fibers and proliferation of capillaries. Other tissues were morphologically normal. Studies of oxidative phosphorylation in isolated muscle mitochondria from both patients showed maximal respiratory rate even in the absence of ADP, an indication that respiratory control was lost. Respiration proceeded at a high rate independently of phosphorylation, and energy was lost as heat, causing hypermetabolism and hyperthermia [13, 14].

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REFERENCES

1. DiMauro, S., Hays, A. P. and Tsujino, S. Nonlysosomal glycogenoses. In A. G. Engel and C. Franzini-Armstrong (eds), *Myology*, 3rd edn. New York: McGraw-Hill, 2004, pp. 1535–1558.
2. De Vivo, D. C., Trifiletti, R. R., Jacobson, R. I., Ronen, G. M., Behmand, R. A. and Harik, S. I. Defective glucose transport across the blood–brain barrier as a cause of persistent hypoglycorrhachia, seizures, and developmental delay. *N. Engl. J. Med.* 325: 703–709, 1991.
3. De Vivo, D. C. The effects of ketone bodies on glucose utilization. In J. V. Passonneau, R. A. Hawkins, W. D. Lust and F. A. Welsh (eds), *Cerebral Metabolism and Neural Function.* Baltimore: Williams & Wilkins, 1980, pp. 243–254.
4. DiDonato, S., Taroni, F. Disorders of lipid metabolism. In R. N. Rosenberg, S. B. Prusiner, S. DiMauro, R. L. Barchi and E. J. Nestler (eds), *The Molecular and Genetic Basis of Neurological Disease.* Boston: Butterworth-Heinemann, 2003, pp. 591–601.
5. DiMauro, S., Andreu, A. L., Bruno, C. and Hadjigeorgiou, G. M. Myophosphorylase deficiency (glycogenosis type V; McArdle disease) *Curr. Mol. Med.* 2: 189–196, 2002.
6. Engel, A. G., Hirsschhorn, R. and Huie, M. L. Acid maltase deficiency. In A. G. Engel and C. Franzini-Armstrong (eds), *Myology*, 3rd edn. New York: McGraw-Hill, 2004, pp. 1559–1586.
7. Smeitink J. and van den Heuvel, L. Human complex I in health and disease. *Am. J. Hum. Genet.* 11: 144–149, 1999.
8. Tein, I. Carnitine transport: pathophysiology and metabolism of known molecular defects. *J. Inher. Metab. Dis.* 26: 147–169, 2003
9. Treem, W. R., Stanley, C. A., Finegold, D. N., Hale, D. E. and Coates, P. W. Primary carnitine deficiency due to a failure of carnitine transport in kidney, muscle, and fibroblasts *N. Engl. J. Med.* 319: 1331–1336, 1988.
10. De Vivo, D. C. and DiMauro, S. Mitochondrial diseases. In K. S. Swaiman and S. Ashwal (eds), *Pediatric Neurology.* St Louis: C. V. Mosby, 1999, pp. 494–509.
11. De Vivo, D. C., Wang, D. and Pascual, J. Disorders of glucose transport. In R. N. Rosenberg, S. B. Prusiner, S. DiMauro, R. L. Barchi and E. J. Nestler (eds), *The Molecular and Genetic Basis of Neurological Disease.* Boston: Butterworth-Heinemann, 2003, pp. 625–634.
12. Haymond, M. W., Sunehag, A. L. Hypoglycemia. In C. D. Rudolph, A. M. Rudolph, M. K. Hostetter, G. Lister and N. J. Siegel (eds), *Pediatrics*, 21st edn. New York, McGraw-Hill, 2003, pp. 2106–2111.
13. DiMauro, S. and Schon, E. A. Mitochondrial respiratory-chain diseases. *N. Engl. J. Med.* 348: 2656–2668, 2003.

14. DiMauro, S. and Bonilla, E. Mitochondrial encephalomyopathies. In: A. G. Engel and C. Franzini-Armstrong (eds), *Myology*, 3rd edn. New York: McGraw-Hill, 2004, pp. 1623–1662.
15. Lissens, W., De Meirleir, L., Seneca, S. *et al.* Mutations in the X-linked pyruvate dehydrogenase (E1) alpha subunit gene (PDHA1) in patients with a pyruvate dehydrogenase complex deficiency. *Hum. Mut.* 15: 209–219, 2000.
16. De Meirleir,L. Defects of pyruvate metabolism and the Krebs cycle. *J. Child Neurol.* 17(Suppl. 3): S26–S33, 2002.
17. Bourgeron, T., Rustin, Chretien, D. *et al.* Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency *Nat. Genet.* 11: 44–149, 1995.
18. Andreu, A. L., Hanna, M. G., Reichmann, H. *et al.* Exercise intolerance due to mutations in the cytochrome b gene of mitochondrial DNA. *N. Engl. J. Med.* 341: 1037–1044, 1999
19. De Meirleir, L., Seneca, S., Lissens, W. *et al.* Respiratory chain complex V deficiency due to a mutation in the assembly gene *ATP12. J. Med. Genet.* 41: 120–124, 2004.

CHAPTER

43

Disorders of Muscle Excitability

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ORGANIZATION OF THE NEUROMUSCULAR JUNCTION

Nerve and muscle communicate through neuromuscular junctions. The transmission of the nerve impulse to muscle to generate movement and force takes place at the neuromuscular junction (NMJ), a specialized type of synapse where the nerve action potential triggers a muscle action potential through the intervention of a chemical relay: the release of acetylcholine (ACh). Each motor neuron in the brainstem and spinal cord innervates a larger or smaller group of muscle cells (fibers) – all together constituting the motor unit – whose number depends on the precision of movement and strength of force to which the muscle is accustomed (Fig. 43-1). The entire pre- and postsynaptic apparatus, including the intercellular synaptic space, is contained in a differentiated area of exceeding complexity that only measures about 40 µm in its largest dimension (Fig. 43-2). The neural Schwann cells delimit the surface of the NMJ by contributing a protective enveloping process devoid of myelin and containing specialized membrane proteins such as neural cell adhesion molecule. Entering slightly into the muscle, the nerve endings form boutons that are separated from a highly invaginated region of raised muscle plasma membrane by a synaptic space of 50 nm. The basal lamina of both nerve and muscle, mainly composed of type IV collagen, continues uninterrupted across the synaptic cleft but differentiates at the NMJ by harboring specialized proteins such as acetylcholinesterase (AChE), laminin, agrin

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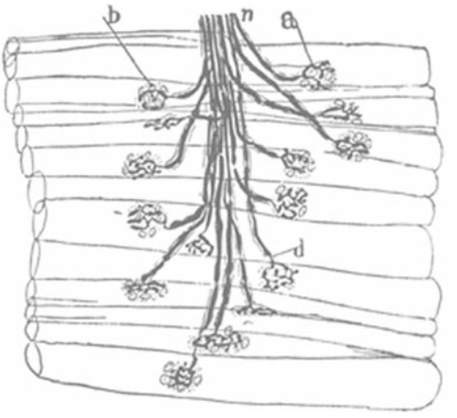

FIGURE 43-1 Motor endplates from rabbit intercostal muscle stained with Löwit's gold chloride method. *a*, terminal axonal arborization; *b*, nucleus; *d*, region where the myelin sheath ends; *n*, neural branch. (From Ramón y Cajal, S. *Textura del sistema nervioso del hombre y los vertebrados.* Madrid: N. Moya, 1899.)

and neuregulins. Presynaptic terminal mitochondria provide ATP for the energetically demanding vesicular ACh turnover process and a fraction of the synaptic vesicles in the terminal are associated with regions of increased membrane density called active or release zones (Fig. 43-3). These zones contain high densities of voltage-gated Ca^{2+} channels of the P/Q type as well as calcium-activated K^+ channels, and are precisely located across from the infoldings of the postsynaptic membrane. Following nerve stimulation, vesicles at these sites fuse with the plasma membrane and release their content of ACh directly over the postsynaptic receptor molecules on the folds below. A significant fraction of synaptic vesicles, however, is directly associated with actin (see Ch. 8) away from the active zones and not available for immediate release. The postsynaptic muscle membrane is also highly specialized. Nicotinic ACh receptors (AChR) cluster in the infoldings and directly associate with 43 kDa rapsyn (receptor-aggregating protein at the synapse). Utrophin – a large protein similar to dystrophin – and α-dystrobrevin are thought to link the AChR complexes to actin filaments and to a much larger network of proteins, many of which are coming to attention as the molecular cause of an increasing number of human myopathies is being determined.

Acetylcholine acts as a chemical relay between the electrical potentials of nerve and muscle. ACh is synthesized in the nerve terminal by choline acetyltransferase (ChAT) from choline and acetyl-CoA – a product of glycolysis and pyruvate oxidation – and, in most regards, this process does not differ from that carried out in central nervous system synapses (see Ch. 11). ACh is released after the arrival of the nerve action potential to the resting terminal, whose potential is maintained by the Na^+,K^+-ATPase. Depolarization leads to activation of Ca^{2+}

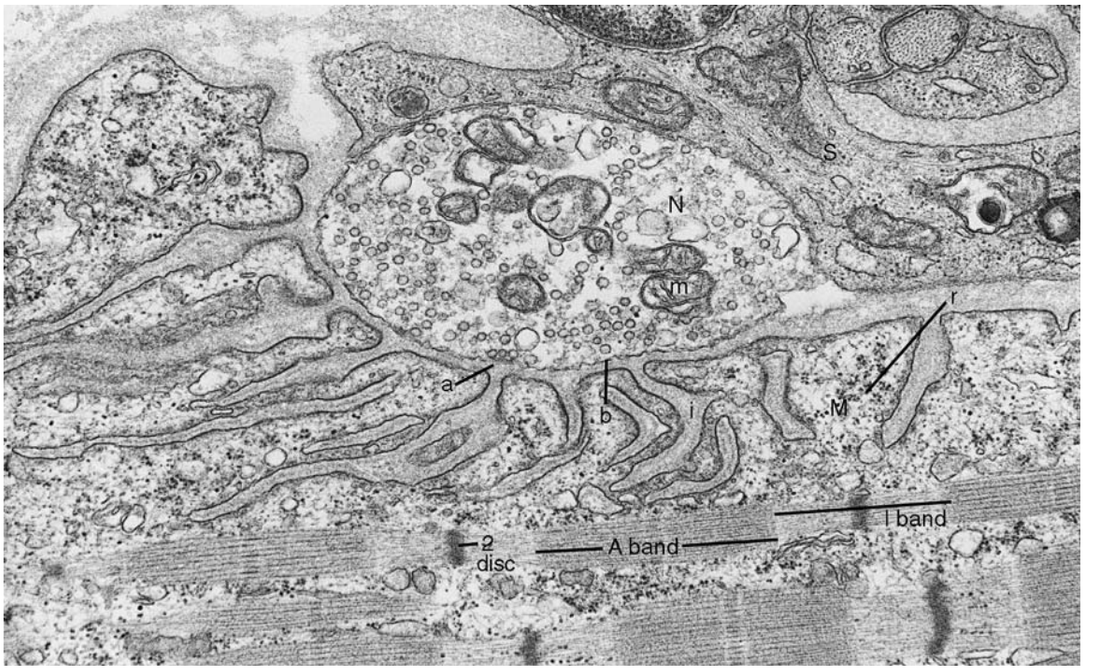

FIGURE 43-2 Photomicrograph of the human neuromuscular junction. In normal muscle, Ach receptors are associated with the terminal expansions of the junctional folds and the architecture of the postjunctional membrane follows closely the distribution of active zones in the presynaptic membrane. *b*, basal lamina; *I*, infoldings; *m*, mitochondria; *M*, myocyte; *N*, nerve terminal; *r*, ribosomes; *s*, synaptic space; *S*, Schwann cell. Courtesy of A. Engel.

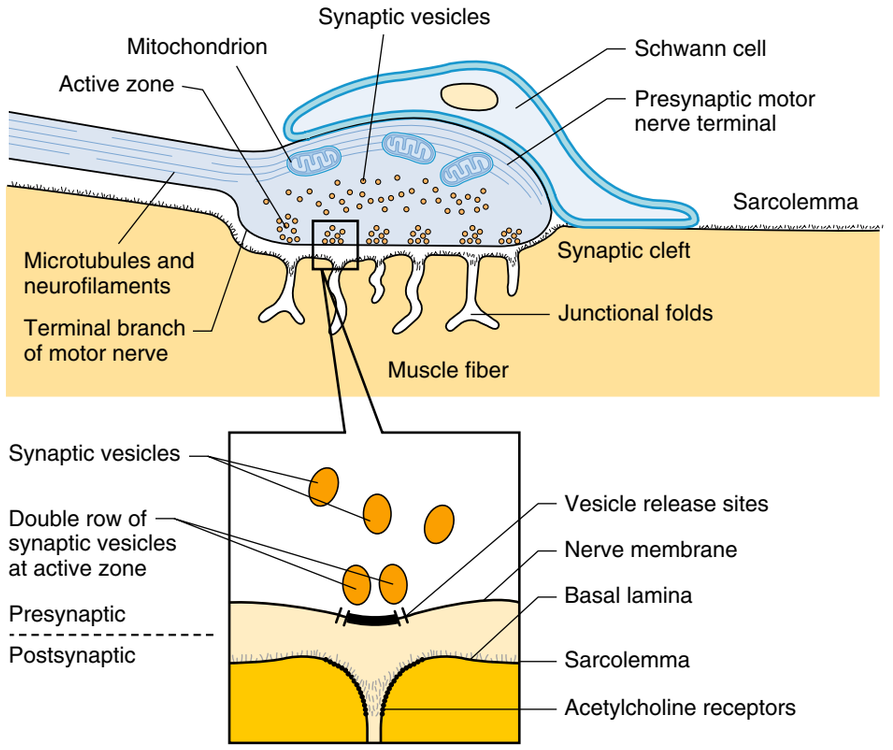

FIGURE 43-3 Schematic representation of the key synaptic elements of the neuromuscular junction.

channels and to a surge of Ca^{2+} entry (see Ch. 22) reaching an impressive concentration of 0.1 mmol/l for a few milliseconds. Later, Ca^{2+} is buffered in the terminal and extruded by the Na^+/Ca^{2+} exchanger, as the opening of voltage-gated K^+ channels also helps restore membrane potential. While still in the cytosol, Ca^{2+} interacts with the sensing vesicular protein synaptotagmin, priming other vesicular proteins for docking against and fusion with the plasma membrane. ACh then is freed to diffuse across the junctional cleft and interact with AChR [1] until it encounters AChE and is hydrolyzed into choline and acetate, later to be recovered by the terminal. AChE exists in a peculiar trimeric conformation composed of three groups of four active globular heads linked by a collagen strand named ColQ. An enzyme of great therapeutic significance, AChE is reversibly blocked by anticholinesterases of medical use, and irreversibly phosphorylated and disabled by organophosphate insecticides, among many other modulators. Following fusion and diffusion of ACh, the vesicular membrane is retrieved by endocytosis of clathrin-coated vesicles and refilled with ACh. While being refilled, vesicles are tethered to actin filaments as mentioned above via synapsin, a vesicular protein that loosens its attachment after Ca^{2+}-dependent phosphorylation by calmodulin kinase. Only then is a vesicle ready for localization near the active zone pending release.

The fidelity of signal transmission relies on the orchestration of innumerable stochastic molecular events. Neuromuscular transmission relies on the generation of a junctional muscle membrane depolarization large enough to sustain the formation of a propagated action potential; the difference between the magnitude of the first parameter and the threshold of the action potential is called the safety factor of neuromuscular transmission. Inexorably, impulse transmission must proceed across multiple conformational changes in the molecules that underlie NMJ excitability. An inherently stochastic (as opposed to a deterministic all-or-nothing) process, normal transmission necessitates a large safety factor in order to operate with fidelity. Among the processes that are nondeterministic, i.e. governed by probability distribution laws, are the opening of voltage-gated Ca^{2+} channels, the number of ACh vesicles released each time, the diffusion of ACh in the cleft, the binding of two ACh molecules to the AChR, the onset of desensitization and closing of the AChR, and the encounter of the neurotransmitter with AChE, to name but a few events for which the probability laws are known. The origin of this stochastic behavior is intrinsic to the molecules that change conformation but is accentuated by the limited dimensions of the NMJ, which includes a relatively small number of molecules. In contrast, an enzymatic reaction taking place, for example,

in solution carried out by an ensemble of many more molecules behaves, as a whole, predictably. The limitation to the size of the NMJ may be due to the large energetic expense associated with its maintenance, particularly when it is subject to frequent use (think, for example, of a flying hummingbird) and, thus, energy may be safely spared by reducing synaptic size as long as the safety factor of transmission remains large. In line with the importance of neuromuscular transmission for the survival of the organism, all key molecular elements of the NMJ are encoded by highly conserved genes whose absence or severe dysfunction is incompatible with life. Instead, diseases of the NMJ tend to shift the probability distribution laws for the different conformations of its key molecules, and may thus be viewed as processes that decrease the safety factor of transmission. This is the central pathophysiological phenomenon of neuromuscular diseases.

EXCITATION AND CONTRACTION OF THE MUSCLE FIBER

The excitable apparatus of muscle is composed of membranous compartments. The muscle cell is surrounded by a plasma membrane that, together with the various connective tissue elements and collagen fibrils, forms the sarcolemma [2]. The interior of the resting cell is maintained at an electrical potential about 80 mV more negative than the exterior by the combined action of pumps and channels in the plasma membrane. Unlike membranes of nerves, muscle membranes have a high conductance (G) to chloride ions in the resting state; G_{Cl^-} accounts for about 70% of the total membrane conductance. Potassium ion conductance accounts for most of the remainder, and the membrane potential is normally close to the Nernst potential for these two ions (see Ch. 6). Asymmetrical concentration gradients for sodium and potassium ions are maintained at the cost of energy by the membrane Na^+,K^+-ATPase. During the generation of a muscle action potential, a rapid and stereotyped membrane depolarization is produced by an increase in sodium ion conductance mediated by voltage-dependent Na^+ channels. The conductance increase is self-limiting and membrane repolarization is assisted by the delayed opening of a potassium ion conductance pathway. Action potentials originating at the NMJ spread in a nondecremental fashion over the entire surface of the muscle. Action potentials traveling on the sarcolemma penetrate the interior of the muscle cell along transverse (T) tubules that are continuous with the outer membrane (Fig. 43-4). These tubules are seen as openings on the surface of the

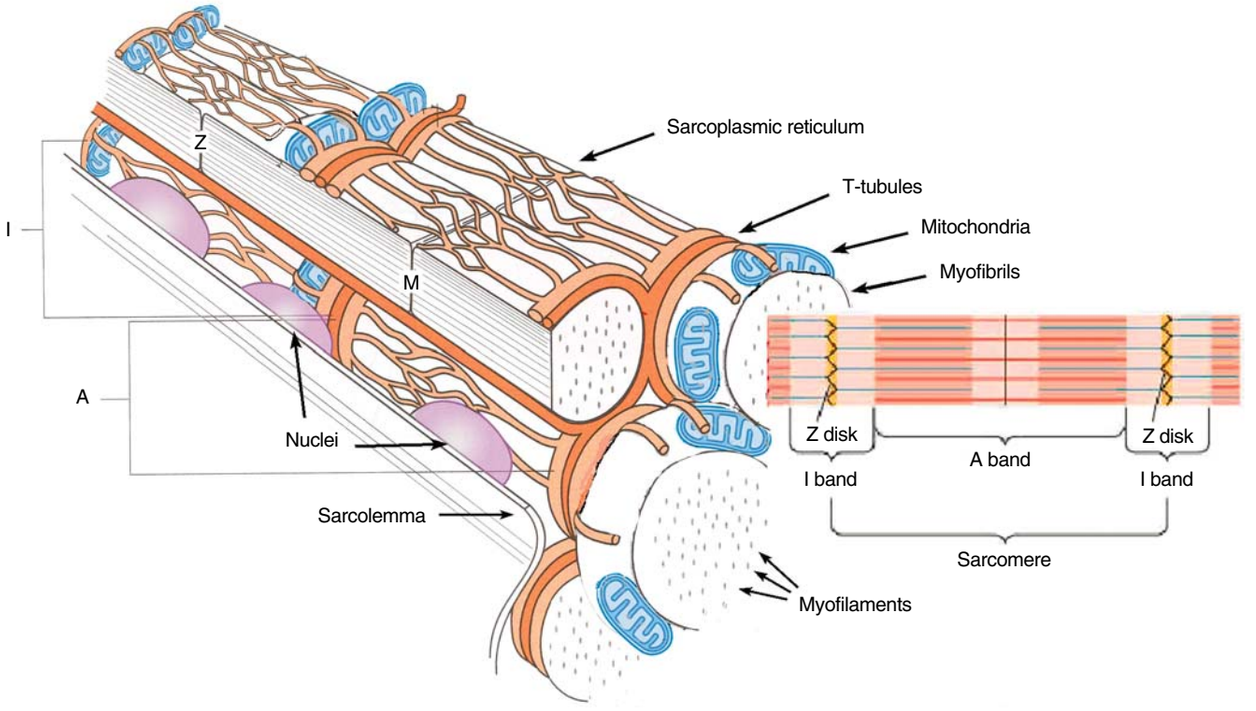

FIGURE 43-4 Cellular and molecular organization of the skeletal myocyte.

muscle cell and the depolarization of this membrane system spreads activity to the center of the fiber. As this T-tubular network courses inward, close associations are formed with specialized terminal elements of the sarcoplasmic reticulum (SR). At the electron microscope level, the structure formed by a single tubule interposed between two terminal SR elements is called a triad. The SR stores Ca^{2+} in relaxed muscle and releases it into the sarcoplasm upon depolarization of the cell membrane and the T-tubular system.

Myofibrils are designed and positioned to produce movement and force. The physiological unit of muscle, the myocyte or fiber, contains repeating structures known as sarcomeres that are separated from each other by dark lines called Z disks. Within each sarcomere, the A and I bands are seen; the A band, lying between two I bands, occupies the center of each sarcomere and is highly birefringent. Within the A band is a central, lighter zone, the H band, and in the center of the H band is the darker M band. The Z disk is at the center of the I band (Fig. 43-4). The difference in birefringence between the A and I bands produces the characteristic striated appearance of voluntary muscle when seen through the light microscope. The repeating optical characteristics of the A and I bands in each sarcomere reflect the regular arrangement of two sets of filaments. The thin filaments have a diameter of ≈180 Å, appear to be attached to the Z bands and are found in the I band and part of the A band. The thick filaments have a diameter of ≈150 Å, occupy the A band and are connected crosswise by material in the M band. In cross section, the thick filaments are arranged in a hexagonal lattice and the thin filaments occupy the centers of the triangles formed by the thick filaments. With the identification of two sets of discontinuous filaments in the sarcomere came the recognition that (1) the two kinds of filaments become cross-linked only on excitation and (2) contraction of muscle does not depend on shortening the length of the filaments but rather on the relative motion of the two sets of filaments, termed the sliding-filament mechanism [3]. Thus, the length of the muscle depends on the length of the sarcomeres and, in turn, variation in sarcomere length is based on variation in the degree of overlap between the thin and thick filaments.

In addition to actin and myosin, other proteins are found in the two sets of filaments. Tropomyosin and a complex of three subunits collectively called troponin are present in the thin filaments and play an important role in the regulation of muscle contraction. Although the proteins constituting the M and the Z bands have not been fully characterized, they include α-actinin and desmin as well as the enzyme creatine kinase, together with other proteins. A continuous elastic network of proteins, such as connectin, surround the actin and myosin filaments, providing muscle with a parallel passive elastic element. Actin forms the backbone of the thin filaments [4]. The thin filaments of muscle are linear polymers of slightly elongated, bilobar actin subunits, each about 4×6 nm, arranged in a helical fashion, with the longer dimension roughly at right angles to the filament axis. Each monomer has a molecular weight of about 42 kDa and contains a single nucleotide binding site. Hydrolysis of ATP to ADP takes place during actin polymerization but is not involved in muscle contraction. A wide variety of proteins interact with actin in both muscle and nonmotile cells. They may affect the polymerization–depolymerization of actin and are involved in the attachment of actin to other cellular structures, including the Z disks in muscle as well as membranes in both muscle and nonmuscle cells.

Myosin, the chief constituent of thick filaments, is a multisubunit protein. It is an asymmetrical molecule of ≈500 kDa with an overall length of ≈150 nm. Its width varies between about 2 and 10 nm. In contrast to actin, myosin consists of several peptide subunits. Each myosin molecule contains two heavy chains of ≈200 kDa; these extend through the length of the molecule. Over most of their length, the two chains are intertwined to form a double α-helical rod; at one end they separate, each forming an elongated globular portion. The two globular portions contain the sites responsible for ATP hydrolysis and interaction with actin. In addition to the two heavy chains, each myosin molecule contains four light chains of ≈20 kDa. These light chains modulate myosin activity and some of them can be phosphorylated by kinases. Myosin molecules form end-to-end aggregates involving the rod-like segments, which then grow into the thick filaments. The polarity of the myosin molecules is reversed on either side of the central portion of the filament. The globular ends of the molecules form projections, termed cross-bridges, on the aggregates that interact with actin. Conformational changes within this region, driven by ATP hydrolysis, provide the force that propels the movement of actin fibrils with respect to the myosin filament (Fig. 43-5). The ATPase activity of myosin itself is stimulated by Ca^{2+} and is low in Mg^{2+}-containing media. The hydrolysis of ATP releases sufficient free energy to induce conformational changes that are converted into cellular movement and, ultimately, mechanical work [4].

Tropomyosin and troponin are proteins located in the thin filaments, and together with Ca^{2+}, they regulate the interaction of actin and myosin (Fig. 43-3) [5]. Tropomyosin is an α-helical protein consisting of two polypeptide chains; its structure is similar to that of the rod portion of myosin. Troponin is a complex of three proteins. If the tropomyosin–troponin complex is present, actin cannot stimulate the ATPase activity of myosin unless the concentration of free Ca^{2+} increases substantially, while a system consisting solely of purified actin and myosin does not exhibit any Ca^{2+} dependence. Thus, the actin–myosin interaction is controlled by Ca^{2+} in the presence of the regulatory troponin–tropomyosin complex [6].

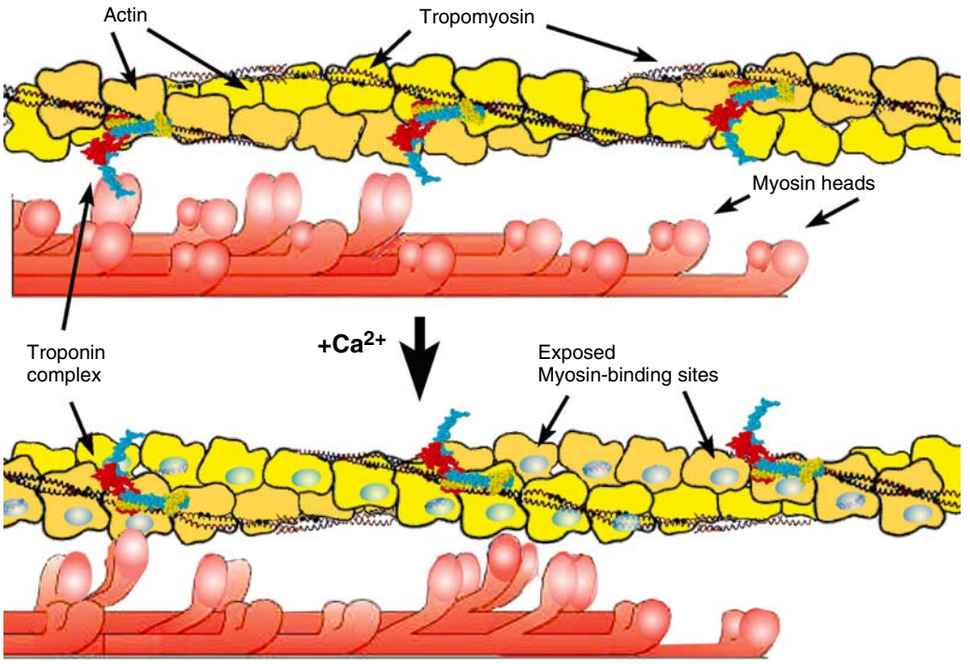

FIGURE 43-5 Myofibrillar apparatus and muscular contraction. Troponin is essential in Ca^{2+} regulation of contraction and is represented as a small, boomerang-shaped trimer. It consists of three subunits (TnT (*yellow*), TnC (*red*) and TnI (*cyan*)) and, together with tropomyosin (drawn as a helicoidal dimeric string), is located in apposition to the actin filament (represented as an *orange-yellow* helical chain with its myosin binding sites in *blue*). Myosin filament heads, represented in the lower part of each panel, possesses ATPase activity. Redrawn from Takeda, S. *et al.* Structure of the core domain of human cardiac troponin in the Ca^{2+}-saturated form, *Nature* 424: 35–41 and Brown J. H. *et al.* Deciphering the design of the tropomyosin molecule, *PNAS* 98: 8496–8501, 2001.

Calcium couples muscle membrane excitation to filament contraction. Important work has focused on the proteins present in the T-tubule/SR junction. One protein, an integral component of the T-tubular membrane, is a form of L-type, dihydropyridine-sensitive, voltage-dependent calcium channel. Another, the ryanodine receptor (RyR), is a large protein associated with the SR membrane in the triad that may couple the conformational changes in the Ca^{2+} channel protein induced by T-tubular depolarization to the Ca^{2+} release from the SR (Fig. 43-6).

The first molecule, the Ca^{2+} channel, is required for coupling at the triad. Skeletal muscle contains higher concentrations of this L-type Ca^{2+} channel that can be accounted for on the basis of measured voltage-dependent Ca^{2+} influx because much of the Ca^{2+} channel protein in the T-tubular membrane does not actively gate calcium ion movement but, rather, acts as a voltage transducer that links depolarization of the T-tubular membrane to Ca^{2+} release through a receptor protein in the SR membrane. The ryanodine receptor mediates sarcoplasmic reticulum Ca^{2+} release. The bar-like structures that connect the terminal elements of the SR with the T-tubular membrane in the triad are formed by a large protein that is the principal pathway for Ca^{2+} release from the SR. This protein, which binds the

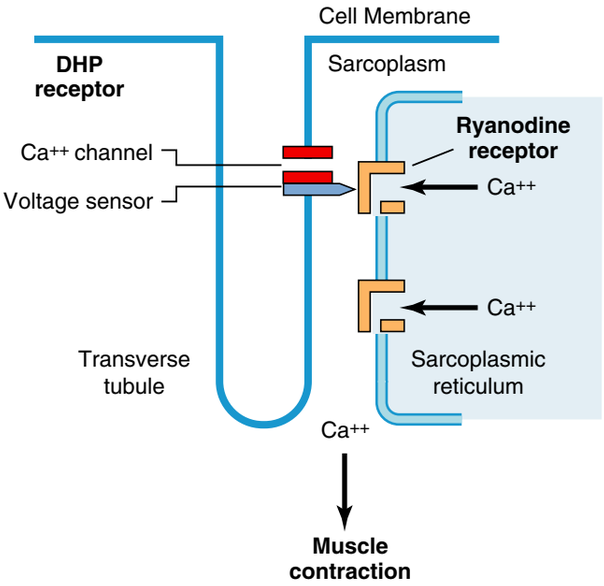

FIGURE 43-6 Molecular physiology of muscle excitation–contraction coupling.

plant alkaloid ryanodine with high affinity, is a huge homotetramer of 565 kDa polypeptide subunits [7].

The purified complex, when incorporated into planar bilayers, exhibits Ca^{2+} channel activity that is modulated by Ca^{2+} itself, Mg^{2+}, and ATP among many other regulators. The manner in which activation of the ryanodine receptor complex is coupled to events at the T-tubular membrane probably involves direct mechanical linkage through a conformational change in the dihydropyridine receptor protein [8]. After diffusion towards the myofibrils, Ca^{2+}reuptake in the sarcoplasmic reticulum allows the relaxation of muscle and the maintenance of a low resting intracellular Ca^{2+} concentration by means of an ATP-dependent Ca^{2+} pump, located in the SR membrane. The free energy of ATP hydrolysis is utilized here for the concentrative uptake of Ca^{2+} into the SR vesicle through a phosphorylated enzyme intermediate (see Ch. 5).

Other SR proteins assist in Ca^{2+} uptake and storage. Phospholamban is prominent in cardiac muscle and slowly contracting muscle, where phosphorylation participates in the control of Ca^{2+}-ATPase and Ca^{2+}-uptake activity. Another protein, calsequestrin, containing numerous low-affinity Ca^{2+}-binding sites, is present in the lumen of the SR and also participates in Ca^{2+} storage. Fast-twitch muscle contains a soluble Ca^{2+}-binding protein, parvalbumin, which is structurally related to troponin-C. Parvalbumin may also regulate the Ca^{2+} concentration in the initial stages of relaxation to facilitate rapid contraction.

The disorders of muscle excitability are best classified according to their cause [9] and are listed in Table 43-1.

TABLE 43-1 Disorders of muscle excitability

Inheritable diseases	
Disorders of the neuromuscular junction: congenital myasthenic syndromes	
Choline acetyltransferase deficiency	MIM 254210
Acetylcholine receptor deficiency	MIM 254210
Rapsyn deficiency	MIM 601592
Slow channel syndrome	MIM 601462
Fast channel syndrome	MIM 254210
Acetylcholinesterase deficiency	MIM 603034
Disorders of the muscle membrane	
Hyperkalemic periodic paralysis	MIM 170500
Paramyotonia congenita	MIM 168300
Hypokalemic periodic paralysis	MIM 170400
Andersen syndrome	MIM 170390
Myotonic dystrophy (Steinert's disease)	MIM 160900
Myotonia congenita (Thomsen's disease and Becker's myotonia)	MIM 160800 and 255700
Malignant hyperthermia	MIM 145600
Brody's disease	MIM 601003
Immunological diseases	
Myasthenia gravis	
Lambert–Eaton syndrome	
Neuromyotonia (Isaac's syndrome)	
Intoxications and metabolic diseases	
Botulism	
Animal envenomations	
Electrolyte imbalances	

Mutations associated with inheritable diseases are numbered in MIM notation as described in: McKusick, V. A. *Mendelian Inheritance in Man. A Catalog of Human Genes and Genetic Disorders*,12th edn. Baltimore: Johns Hopkins University Press, 1998. An updated Internet version is found at: *Online Mendelian Inheritance in Man, OMIM™*. McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2000,: www.ncbi.nlm.nih.gov/omim/.

GENETIC DISORDERS OF THE NEUROMUSCULAR JUNCTION

Congenital myasthenic syndromes impair the operation of the acetylcholine receptor. These disorders result in impaired neuromuscular transmission due to mutations of either presynaptic, synaptic basal lamina-associated or postsynaptic proteins and cause fatigable weakness in specific muscle distribution patterns [10]. Traditionally termed 'congenital myasthenia', they should be differentiated from acquired, immune-mediated myasthenia gravis, and it is recognized that additional types of congenital myasthenic syndrome are likely to be discovered. In fact, in a patient with typical myasthenic symptoms, mutations of *SCN4A* – the gene that encodes the $Na_V1.4$ skeletal muscle sodium channel – leading to enhanced channel inactivation have been found.

Choline acetyltransferase deficiency. The distinguishing clinical feature is sudden episodes of severe respiratory difficulty and oropharyngeal (bulbar) weakness leading to apnea (cessation of respiration) precipitated by infections, fever or excitement, or occurring even spontaneously. In some patients, the disease presents at birth with hypotonia (diminished muscle resistance to mobilization) and severe bulbar and respiratory weakness, requiring ventilatory support that gradually improves through life, but is followed by episodes of apnea attacks and bulbar paralysis later. Other patients are normal at birth and may develop myasthenic symptoms and apneic attacks after infancy or childhood. Muscle action potentials decline abnormally when neuronal impulse flow is increased and recovers slowly, indicating an underlying defect in resynthesis or vesicular packaging of ACh. Several recessive mutations have been identified, all of which decrease the abundance or the efficiency of ChAT. Although still largely unexplained, the selective neuromuscular *versus* CNS involvement in ChAT deficiency may be due to the enzyme rate-limiting characteristics for ACh synthesis at the NMJ.

Acetylcholine receptor deficiency. The degree of disease severity may vary from mild to very severe. In general, patients harboring low-expressor or even homozygous null mutations in the AChR ε subunit may experience

mild symptoms, presumably owing to the compensatory replacement of this peptide by the analogous γ subunit. Conversely, patients with low-expressor mutations in the other non-interchangeable subunits are severely affected, and no patients with null mutations in both alleles of a non-ε subunit have been observed. AChR deficiency results from mutations that cause premature termination of the translational chain by frameshift, by altering splice sites, or by generating stop codons; from point mutations in the promoter region; from chromosomal microdeletion; and even from missense mutations, some of which affect the signal peptide or residues essential for assembly of the AChR. Patients respond moderately well to anti-AChE drugs.

Rapsyn deficiency. These patients manifest AChR deficiency with decreased rapsyn as well as secondary AChR abundance and resulting impaired postsynaptic morphologic development. None of the known rapsyn mutations hinders rapsyn self-association, but all diminish clustering of AChR with this protein. In addition to weakness, some patients manifest striking facial malformations. Patients are treated with anti-AChE drugs and with 3,4-diaminopyridine (3,4-DAP), a blocker of open K^+ channels. 3,4-DAP decreases presynaptic membrane resting conductance, causing hyperpolarization and therefore resetting more Ca^{2+} channels into their closed, non-inactivated state from which they can readily open, increasing Ca^{2+} influx into the terminal and facilitating a compensatory increase in ACh release.

Slow-channel syndrome. Abnormally long-lived openings of mutant AChR channels result in prolonged endplate currents and potentials, which in turn elicit one or more repetitive muscle action potentials of lower amplitude that decrement. The morphologic consequences stem from prolonged activation of the AChR channel that causes cationic overload of the postsynaptic region – the endplate myopathy – with Ca^{2+} accumulation, destruction of the junctional folds, nuclear apoptosis, and vacuolar degeneration of the terminal. Some slow-channel mutations in the transmembrane domain of the AChR render the channel leaky by stabilization of the open state, which is populated even in the absence of ACh. Curiously, some slow-channel mutants can be opened by choline even at the concentrations that are normally present in serum. Quinidine, an open-channel blocker of the AchR, is used for therapy.

Fast-channel syndrome. The clinical features resemble those of autoimmune myasthenia gravis (see below) with variable severity. Conversely to what is found in slow-channel syndrome, the open state of the AChR is destabilized, manifesting as fast dissociation of ACh from the receptor and/or excessively reduced open times. One mutation has also caused multiple congenital joint contractures owing to fetal hypomotility *in utero*. In most cases, the mutant allele causing the kinetic abnormality is accompanied by a null mutation in the second allele, so that the kinetic mutation dominates the phenotype, but homozygous fast-channel mutations also exist. Therapy includes anti-AChE agents and 3,4-DAP.

Acetylcholinesterase deficiency. Inhibition of the AChE results in prolonged exposure of AChR to ACh, leading to prolonged endplate potentials, desensitization of AChR and depolarization block. Endplate myopathy with loss of AChR may result. In most patients, the disease presents in the neonatal period and is highly disabling, but exceptions with mild neonatal onset and severe adult impairment exist. Some patients manifest an excessively slow pupillary response to light. An array of mutations may cause (1) reduced attachment of AChE to ColQ, (2) truncation of the collagen domain rendering it insertion-incompetent, or (3) hindrance of the triple-helical assembly of the collagen domain.

HEREDITARY DISEASES OF MUSCLE MEMBRANES

Mutations of the sodium channel cause hyperkalemic periodic paralysis and paramyotonia congenital. An important group of inherited muscle diseases called the periodic paralyses is characterized by intermittent episodes of skeletal muscle weakness or paralysis that occur in individuals who otherwise appear normal or are just mildly weak between attacks [11]. The periods of paralysis are often associated with changes in serum K+ concentration; while the serum K+ concentration may increase or diminish, the direction of change is usually consistent for a particular family and forms one basis for classifying these diseases as either hyperkalemic or hypokalemic. A variant of periodic paralysis, in which spells of weakness are less frequent and in which a form of muscle hyperexcitability is often seen, is called paramyotonia congenita.

Recordings from muscle fibers isolated from patients during an attack of periodic paralysis have shown that the paralytic episodes are associated with acute depolarization of the sarcolemma. In all forms of periodic paralysis, this depolarization is due to an increase in membrane conductance to Na^+. In the case of hyperkalemic periodic paralysis and paramyotonia congenita, this abnormal conductance can be blocked by tetrodotoxin, a small polar molecule that is highly specific for the voltage-dependent Na^+ channel. Foods with a high K^+ content may trigger an attack, while carbohydrate-rich substances are abortive of the paralytic episode. Single-ion channel recordings in hyperkalemic periodic paralysis have revealed that some of the muscle membrane Na^+ channels show abnormal inactivation kinetics, intermittently entering a mode in which they fail to inactivate. These channels produce a persistent, non-inactivating Na^+ current that in turn produces membrane depolarization. Because normal Na^+ channels enter an inactivated state after depolarization

(see Ch. 6), the net result of long-term depolarization is loss of sarcolemmal excitability and paralysis.

Both hyperkalemic periodic paralysis and paramyotonia are caused by mutation of the adult skeletal muscle Na^+ channel SkM1 gene *SCN4A* on chromosome 17. The hypokalemic form of periodic paralysis, however, is not linked to this Na^+ channel gene (see below). Numerous mutations alter the coding region of the *SCN4A* gene in families with hyperkalemic periodic paralysis or paramyotonia congenita. Although these mutations are distributed through a wide span of the channel coding region, a number of them are clustered in a cytoplasmic linker region known from biophysical studies to control inactivation. Others are located near the cytoplasmic ends of transmembrane domains S5 and S6, and these residues may in fact constitute the binding site for the closing inactivation gate. Mutations in these regions may destabilize this closed conformation, leading to abnormalities in channel inactivation.

Mutants associated with the paramyotonia congenita phenotype show, on the other hand, a marked slowing in the major component of fast inactivation. In some cases, the voltage dependence of the inactivation rate constant, known to biophysicists as τ_h, is markedly reduced as well, and the mutations appear to uncouple the inactivation process from the voltage-dependent channel conformational changes associated with inactivation. In some families with hyperkalemic periodic paralysis, the mutations cause a small, persistent inward Na^+ current in the myocyte that is the result of a shift in channel modal gating. Normal skeletal muscle Na^+ channels can shift between a fast and a slow inactivation gating mode and, usually, the channels are found in the fast inactivation mode. Channels with hyperkalemic periodic paralysis mutations, however, spend a greater percentage of the time in the slow inactivation gating mode, and late openings associated with this slow gating mode contribute to the persistent inward current seen in cells harboring these mutations. Under voltage-clamp conditions at the single-channel level, SkM1 channels with paramyotonia congenita mutations show multiple late openings and prolonged openings after depolarization. These late openings account for the slow inactivation of the Na^+ current observable in the cell. Hyperkalemic periodic paralysis mutations also show multiple late openings at the single-channel level, but these abnormal events are temporally clustered, consistent with an underlying shift in modal gating.

Single-channel conductance is not altered by any of these mutations, but all sodium ion channel mutations in periodic paralysis produce dominant-negative effects. Although some mutant channels only intermittently exhibit abnormal inactivation, this small population of abnormally inactivating channels can modify the behavior of the remaining mutant and normal channels present in the membrane. Unlike the ClC-1 chloride channel mutations in myotonia congenita (see below), which produce dominant-negative effects within a single channel multimer, these Na^+ channel mutations produce dominant-negative effects that reflect the relationship of normal channel inactivation to membrane potential. In either case, the persistent inward current carried by a small population of noninactivating channels, or the prolonged inward current resulting from mutant channels with slowed inactivation rates, results in a slight but long-lasting membrane depolarization. Since the relationship between voltage and inactivation in normal channels is very steep near the resting potential, this slight depolarization can produce inactivation of normal channels. If depolarization is sufficient, too few channels will remain in the noninactivated state to satisfy the requirements for a regenerative action potential and the muscle will become paralyzed.

Hypokalemic periodic paralysis is due to calcium channel mutations. Hypokalemic periodic paralysis, in which serum potassium drops during a paralytic episode, is the most common of the inherited periodic paralyses. Although paralysis is associated with membrane depolarization and increased resting Na^+ conductance, this conductance is not sensitive to tetrodotoxin. This disease is linked to a region of chromosome 1 that encodes the α subunit of the skeletal muscle, dihydropyridine-sensitive Ca^{2+} channel, designated CACNL1A3 [12]. Although this channel in skeletal muscle contains at least five subunits, the channel-forming α subunit has a structure that bears strong sequence homology to the voltage-gated Na^+ channel.

Several mutations have been identified in the Ca^{2+} channel gene in families with hypokalemic periodic paralysis and some of these are in locations where mutations in the homologous Na^+ channel also occur. The pathophysiological mechanism linking these L-type muscle Ca^{2+} channel mutations to the depolarization seen during episodes of weakness in hypokalemic periodic paralysis is less clear. This dihydropyridine-sensitive Ca^{2+} channel is present in the T-tubular membrane at the triadic junction with the terminal elements of the SR. There, it is thought to provide the coupling between T-tubular depolarization and activation of the SR ryanodine-sensitive Ca^{2+} release channel with which it interacts, instead of conducting ionic currents. Perhaps another uncharacterized form of the sarcolemmal Ca^{2+} channel, in which CACNL1A3 is complexed with different accessory subunits, is responsible for the abnormal Na^+ currents seen in this disorder.

Abnormal potassium channels in Andersen's syndrome cause more than periodic paralysis. Andersen's syndrome includes periodic paralysis, prolongation of the electrocardiographic QT interval causing susceptibility to cardiac ventricular arrhythmias, and characteristic physical features including low-set ears, a small jaw and malformation of the digits; it is inherited in an autosomal dominant fashion. The disease is unique due to the combination of both a skeletal and a cardiac muscle phenotype and may be caused by mutations in *KCNJ2*, a gene that encodes the inward rectifier K^+ channel Kir2.1, which is expressed in both cardiac and skeletal muscles. Kir2.1 is an important contributor to the cardiac inward rectifier

K^+ current, I_{K1}, which provides substantial current during the repolarization phase of the cardiac action potential [13]. All KCNJ2 mutations cause a dominant negative effect on channel function and a reduction in I_{K1} prolongs the terminal repolarization phase, rendering the myocardium prone to repetitive ectopic action potentials. Attacks of paralysis can be associated with hypo-, hyper- or normokalemia and, although serum potassium levels during attacks differed among kindreds, they are consistent within an individual kindred. Mutations in Kir2.1 may sufficiently reduce the muscle resting K^+ conductance such that the membrane depolarizes, leading to inactivation of Na^+ channels making them unavailable for initiation and propagation of action potentials.

Ribonuclear inclusions are responsible for the multiple manifestations of myotonic dystrophy. Myotonic dystrophy (DM; Steinert's disease) is a multisystemic disorder that is now recognized as one of the most common forms of muscular dystrophy in adults. In addition to hereditary muscular dystrophy and myotonia (involuntary persistence of muscle contraction), DM causes a constellation of seemingly unrelated clinical features including cardiac conduction defects, cataracts, and endocrine and immunological abnormalities. Two clinical and genetic types of DM exist [14]. The genetic features of DM type 1 (DM1) include variable penetrance, anticipation (a tendency for the disease to worsen in subsequent generations), and a maternal transmission bias for congenital forms despite the location of the causative gene on chromosome 19. The cause of DM1 is a $(CTG)_n$ repeat in the 3-UTR of a protein kinase (DMPK) gene. Type 2 DM, in contrast, predominantly causes pelvic girdle weakness, and is often referred to as proximal myotonic myopathy, ascribed to the genetic locus encoding zinc finger protein 9 on chromosome 3. DM2 is caused by a $(CCTG)_n$ expansion. Nevertheless, all individuals affected by DM1 and DM2 experience weakness, pain and myotonia, and cardiac involvement may lead to conduction defects, arrhythmias and sudden death. Endocrine abnormalities in both DM1 and DM2 result in hyperinsulinemia, hyperglycemia and insulin insensitivity, with type 2 diabetes occurring in each disorder. Testicular failure is also common, with associated hypotestosteronism, elevated follicle-stimulating hormone (FSH) levels and oligospermia. Other serological abnormalities in both disorders include reduced levels of immunoglobulins G and M. The brain is also affected as assessed by magnetic resonance imaging (MRI), but mental retardation is only a feature of DM1.

The unusual multisystemic clinical parallels between DM1 and DM2 suggest a similar pathogenic mechanism. The discovery that DM2 mapped to chromosome 3 and not to the DM1 region of chromosome 19 makes it unlikely that specific gene expression defects cause the common clinical features of the disease. The discovery that a CCTG repeat expansion located on chromosome 3 which is expressed at the RNA (but not at the protein level) causes DM2, and the observation that both CUG and CCUG repeat-containing foci accumulate in affected muscle nuclei suggests that a gain-of-function RNA mechanism underlies the clinical features common to both diseases. This is, in fact, but one of an emerging class of disorders [15]. RNA-binding proteins, including CUG-binding protein (CUG-BP) and *muscleblind* isoforms bind to – being sequestered – or are dysregulated by the repeat-containing RNA transcripts resulting in specific trans-alterations in pre-mRNA splicing. Specific changes in pre-mRNA splicing have been associated with several genes, including the insulin receptor, the chloride channel ClC-1 and cardiac troponin T, and are probably correlated with insulin resistance, myotonia and cardiac abnormalities. For example, CUG-BP, which is elevated in DM1 skeletal muscle, binds to the ClC-1 pre-mRNA, causing an aberrant pattern of ClC-1 splicing. Thus, altered splicing regulation of ClC-1 decreases its abundance in the muscle plasma membrane causing hyperexcitability and leading to the DM feature of myotonia. Further, mutant RNA transcripts bind and sequester transcription factors, leading to the depletion of as much as 90% of several of them, resulting in secondary depletion of proteins such as the chloride channel ClC-1.

Congenital myotonia is caused by mutant Cl^- channels. In two diseases, dominant myotonia congenita (Thomsen's disease) and recessive myotonia congenita (Becker's myotonia), myotonia is the major presenting symptom and often the only abnormality, although the muscles may be overdeveloped (conferring individuals a herculean appearance) in Thomsen's disease. Patients afflicted with these diseases have difficulty relaxing their muscles normally: doorknobs and handshakes are difficult to release, clumsiness is a problem and falls often occur. In both Thomsen's and Becker's myotonia, multiple mutations have been found in chromosome 7 and the sarcolemma exhibits a severe reduction in membrane Cl^- conductance [16]. This locus encodes the ClC-1 skeletal muscle Cl^- channel family, whose members control anion flux in a number of tissues and are closely related in structure, forming dimers of two ClC subunits. The fact that mutations in the gene encoding ClC-1 can produce either dominant or recessive effects is surprising. Mutants that introduce frameshifts of stop codons early in the coding sequence produce a nonfunctional protein product. With one defective gene copy, wild-type channels encoded by the second allele should produce a net Cl^- conductance about 50% of normal. When both gene copies carry the mutation, expression of the functional channels will be very low or absent and, as a recessive disorder, the myotonia can be severe. Point mutations can also lead to the alteration of a single amino acid in the primary structure of an otherwise full-length channel monomer, and channels formed from this protein may not function normally.

The possibility also exists that channels containing even a single mutant subunit may fail to function even though the other subunits are encoded by a normal copy of the gene. Such a dominant-negative effect, which has been demonstrated for a number of myotonia congenita mutations, leads to a dominant transmission of the disease phenotype. Since mixed channels containing different numbers of mutant subunits may have different levels of residual activity, the resulting membrane Cl^- conductance may be more variable and the disease phenotype less severe than in the recessive form of the disease.

Malignant hyperthermia caused by mutant ryanodine receptor calcium release channels. A rare complication of general inhalation anesthesia is a syndrome characterized by muscle stiffness and fever due to a state of elevated skeletal muscle metabolism that occurs in genetically predisposed individuals. If untreated, this syndrome, called malignant hyperthermia (MH), can be rapidly fatal. Although the inheritance pattern of the disease is difficult to trace, it is likely to be transmitted from generation to generation as an autosomal dominant trait. A similar disease occurs in a strain of pigs, and this experimental animal model has proven to be very useful in studying the physiology of the disease. Measurements on isolated muscle from affected pigs or individuals show that the defect is at the level of excitation-contraction coupling. Specifically, the muscles release Ca^{2+} when exposed to caffeine at concentrations much lower than those required for Ca^{2+} release from normal muscle. Once released, this Ca^{2+} produces persistent activation of tropomyosin and sustained contraction, which associates with hypermetabolism. Analysis of the ryanodine receptor (RyR) gene sequence in affected individuals has uncovered several mutations in the coding region of the protein that cosegregates with the MH phenotype [17]. All of the RyR mutations result in amino acid substitutions in the myoplasmic portion of the protein, with the exception of the mutation in the C-terminus, which resides in the luminal/transmembrane region. Functional analysis shows that MH and central core disease – a related myopathy – mutations produce RyR abnormalities that alter the channel kinetics for calcium inactivation and make the channel hyper- and hyposensitive to activating and inactivating ligands, respectively. The deciding factors in determining whether a particular RyR mutation results in MH alone or MH and central core disease are the sensitivity of the RyR mutant proteins to agonists, the degree of abnormal channel-gating caused by the mutation, the extent of decrease in the size of the releasable calcium store and increase in resting concentration of calcium, and the level of compensatory calcium homeostasis achieved by the muscle.

Calcium channel mutations may also cause malignant hyperthermia. At least five loci other than the RyR have been associated with MH, implicating great genetic heterogeneity. One such gene, *CACNA1*, encoding the main subunit of the voltage-gated dihydropyridine receptor, has a confirmed role in MH, so that this type of MH is allelic to hypokalemic periodic paralysis. In contrast to the mutations specific for hypokalemic periodic paralysis, the mutations so far described for MH are situated in the myoplasmic loop connecting repeats III and IV, the function of which is unknown but may serve as a link to RyR.

Brody's disease is an unusual disorder of the sarcoplasmic reticulum calcium ATPase. Brody's disease, a dominant or recessive disorder, is characterized by exercise-induced delay in skeletal muscle relaxation, which is most pronounced in the legs, arms and eyelids. The muscles stiffen during sustained strenuous muscular activities, leading to painless or only mildly painful cramping. Early studies provided evidence for reduced ATP-dependent Ca^{2+} uptake and Ca^{2+}-ATPase activities in the sarcoplasmic reticulum (SERCA) of Brody's disease patients. Several mutations in *ATP2A1*, the gene encoding SERCA1, have been found in families afflicted by the recessive form of the disease, and all have been predicted to truncate SERCA1 [18]. These mutant enzymes would be inactive and degraded *in situ*. The disease may also cosegregate in an autosomal dominant fashion with an apparently balanced constitutional chromosome translocation (2;7)(p11.2;p12.1), providing evidence for another causative locus.

IMMUNE DISEASES OF MUSCLE EXCITABILITY

Myasthenia gravis is caused by antibodies that promote premature AChR degradation. Myasthenia gravis (MG), a frequent disorder of neuromuscular transmission, is an acquired autoimmune disease affecting the AChR on the postsynaptic membrane. Clinically, the disorder is characterized by weakness and abnormal fatigability. Most patients have circulating antibodies against the AChR in their serum and many of them are directed against the main immunogenic region of the receptor, located extracellularly [19]. Patients with MG typically show fluctuating symptoms; weakness and fatigability may be worse in the evenings and usually become more severe with exercise. Weakness may involve only the extraocular muscles, producing diplopia (double vision), or may be so extensive as to cause quadriparesis (weakness of all limbs) and respiratory compromise. Although spontaneous remissions can occur, the untreated disease is often progressive and can eventually lead to death from respiratory failure. The classic electrophysiological observation in MG is a decremental response in the extracellularly recorded compound muscle action potential with

repeated nerve stimulation (Fig. 43-7). The decrease in action potential amplitude is due to a reduction in the number of AChR molecules present in the postsynaptic membrane combined with a pathological alteration in the architecture of the postsynaptic membrane. The density of receptors in the myasthenic postsynaptic membrane may be as low as 20% of normal. In addition, the architecture of the postsynaptic membrane is lost. The distance between pre- and postsynaptic membrane is often increased, and the postsynaptic membrane is highly simplified. The destruction seen at the postsynaptic membrane is mediated by antibodies directed against the AChR. The major effects of anti-AChR antibodies seem to be twofold. First, antibodies cross-link receptor proteins and increase their rates of endocytosis and lysosomal degradation. In the absence of an increased rate of receptor synthesis, this results in a net decrease in receptor density in the postsynaptic membrane. Second, these antibodies target the postsynaptic membrane for complement fixation and activation of the lytic phase of the complement reaction cascade. Although the mechanism of chronic neuromuscular damage in this disease has been defined, the nature of the initial triggering event is unclear. One hypothesis involves the role of muscle-like cells that exist in the thymus gland. These cells express AChRs on their surface membranes. An initial inflammatory response in the thymus may trigger the generation of cross-reacting antibodies that subsequently target AChRs on muscle. This hypothesis would help to explain the beneficial role of the removal of the thymus (thymectomy) as well as that of immunosuppressive steroids in patients with MG. AChE inhibitors and removal of antibodies by plasma filtration are also of therapeutic value. Careful reduction of endplate AChE activity by administration of inhibitors can prolong the action of released ACh sufficiently to increase the amplitude of an abnormally low endplate potential above that required for successful neuromuscular transmission. Excessive inhibition, however, produces long-term postsynaptic depolarization, receptor inactivation and transmission block.

Antibodies against the muscle-specific receptor kinase mimic myasthenia gravis. Another salient antigenic target of the NMJ is the muscle-specific receptor kinase, MuSK. During development, MuSK participates in the orchestration of agrin-dependent clustering of AChR and of other components that make up the postsynaptic architecture of the NMJ. It is unclear how the reaction of this cytoplasmic target with antibodies causes the symptoms of myasthenia in adults. One possibility is that these antibodies bind complement and cause loss of AChR-containing postsynaptic membrane, similar to the action of AChR antibodies. Another possible explanation is that the antibodies do penetrate the postsynaptic cytoplasm and alter the function of MuSK at the NMJ. Exposure of myocytes to plasma containing MuSK antibodies can inhibit agrin-dependent AChR clustering substantially and, in the absence of added agrin, they can cause a modest clustering of AChR, suggesting that they are able to cross-link and activate MuSK on the cell surface. Therefore, it is possible that the antibodies lead to reduced clustering of AChRs and reduced stability of the adult postsynaptic membrane architecture [20].

Antibodies cause calcium channel dysfunction in Lambert–Eaton syndrome. Some patients with cancer, especially small-cell carcinoma of the lung, develop a syndrome of weakness associated with autonomic dysfunction as part

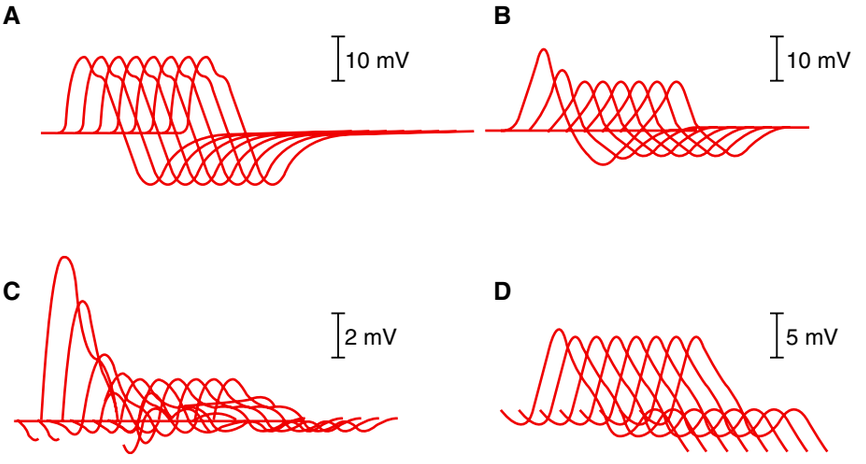

FIGURE 43-7 Block of the acetylcholine receptor in myasthenia gravis. Compound muscle action potentials recorded from the abductor digiti minimi of human subjects during repetitive stimulation of the ulnar nerve at three stimuli per second. (**A**) A recording from a normal individual shows no change in the amplitude of the muscle electrical response during the stimulation interval. (**B**) In a patient with myasthenia gravis, the same stimuli produce a 40% decrement in response amplitude over the first five stimuli, with a slow partial recovery during subsequent stimulation. Similar studies from a patient with severe myasthenia gravis before (**C**) and 2 minutes after (**D**) intravenous administration of 10 mg edrophonium, a short-acting acetylcholinesterase inhibitor. Note the change in amplitude scale between the two recordings and the reversal of the abnormal decrement by edrophonium.

of the Lambert–Eaton myasthenic syndrome (LEMS). Complaints often begin with progressive proximal muscle weakness and fatigue; unlike myasthenia gravis, bulbar involvement is usually mild and respiratory compromise is unusual.

These patients demonstrate a remarkable reduction in the amplitude of the compound muscle action potential produced by single supramaximal stimulus to the motor nerve of a resting muscle. Repeated stimulation of the same nerve, however, results in progressive improvement in response amplitude, often returning to nearly normal levels. These clinical findings are indicative of a defect in presynaptic neurotransmitter release. When analyzed at the cellular level, LEMS is characterized by a reduction of ACh release, often to 10% or less of normal values. Repetitive stimulation causes a progressive increase in ACh release, consistent with the improvement seen in the compound muscle action potential. LEMS, like MG, is an autoimmune disease. The autoantibodies in LEMS are heterogeneous and target a number of presynaptic proteins. Antibodies are most commonly observed against P/Q-type voltage-gated Ca^{2+} channels, but antibodies against L- and N-type channels are also present [21]. Antibodies against the synaptic vesicle protein synaptotagmin have also been identified. The S5–6 loop in the channel α subunit is a particularly active target for LEMS autoantibodies. The large intramembranous particles that form the synaptic active zones are disrupted in the disease. The number of these arrays is reduced, with many particles found instead in irregular aggregates. In small-cell lung carcinomatous cells, voltage-gated Ca^{2+} channels are expressed on their surface, and Ca^{2+} currents in these cells are inhibited by LEMS serum. It seems likely that an immune response against the voltage-gated Ca^{2+} channel population in these tumor cells causes the pathology in LEMS by cross-reaction with similar epitopes present in the presynaptic channels of autonomic and NMJ nerve terminals.

Potassium channel antibodies in Isaac syndrome cause neuromyotonia. Dysfunction of voltage gated K^+ channels is a potential cause of acquired neuronal hyperexcitability disorders, as patients with different point mutations in the potassium channel Kv1.1 can manifest varying combinations of episodic ataxia, neuromyotonia and seizures. Antibodies directed against presynaptic voltage gated K^+ channels have been found in acquired neuromyotonia, also called Isaac's disease, a disorder associated with peripheral nerve hyperactivity that results in muscle twitching (myokymia) and cramps, increased sweating and other autonomic nervous system abnormalities [20]. Approximately 20% of patients with neuromyotonia have an associated thymoma and, occasionally, patients may also experience central nervous system disturbances including insomnia, hallucinations and confusion. Application of the antibodies to the NMJ mimics the electrophysiological effect of 3,4-DAP exposure, with enhanced excitation of muscle owing to pronounced ACh release from hyperpolarized nerve terminals.

TOXINS AND METABOLITES THAT ALTER MUSCULAR EXCITATION

Bacterial botulinum toxin blocks presynaptic acetylcholine release. Botulism is caused by intoxication with an exotoxin with metalloprotease activity synthesized and excreted by bacterial strains from the genus *Clostridium* (*botulinum*, *barati* and *butirycum*). The effect of botulinum toxin on the neuromuscular junction is one of the most potent processes known in biology and recovery from intoxication may persist for months. A dose of 1 ng toxin per kilogram of body weight is lethal to mice. In infancy, the toxin gains access to the neuromuscular junction after bacterial colonization of the gut by *C. botulinum*, whereas food-borne toxin ingestion and wound contamination give rise to more rapid forms of the intoxication. In all its forms, botulism causes a severe and persistent blockade of endplate potentials, rendering the postsynaptic membrane unexcitable after depolarization of the nerve terminal. The neurotoxin greatly reduces the frequency, but not the amplitude, of evoked miniature endplate potentials, indicating that the number of vesicles capable of undergoing fusion and release of ACh is diminished, without affecting the ACh quantum, which indicates an unaltered neurotransmitter synthesis, uptake and storage. In fact, the only structural abnormality of the synapse is an increase in the number of synaptic vesicles near the presynaptic membrane. A structurally related toxin synthesized by *C. tetani* produces tetanus, a process that causes rigid paralysis – often resulting in death from diaphragmatic spasm – due to retrograde transport of this toxin beyond the neuromuscular junction and transcytosis into inhibitory spinal cord interneurons. Another member of this infamous genus, *C. perfringens,* is associated with gas gangrene, an often lethal complication of wound infection in the preantibiotic era.

Seven serotypes (A–G) of botulinum toxin exist. All share a degree of structural homology – different amino acid sequences can be part of each serotype – but differ in potency and duration of action, a phenomenon that has allowed the beneficial use of type A toxin in conditions associated with persistent muscular contraction such as dystonia or spasticity and with excessive autonomic cholinergic activity. At high doses, however, some of the specificity of action is lost and type A toxin may be transported retrogradely, acting on the central nervous system. Botulinum toxins, synthesized as single polypeptides of ≈150 kDa (comprising 1295 amino acids in the case of type A toxin), are encoded in the genome of a bacteriophage virus that infects the bacterium lysogenically [22]. Prior to acquiring its active conformation, the peptide is cleaved into a light (LC) and a heavy (HC) chain that

weigh about 50 and 100 kDa respectively and remain linked via a disulfide bond. The LC domain is a potent zinc endopeptidase designed against specific elements inside the presynaptic nerve terminal, while the HC region contains regions for binding of the toxin to the membrane of the cholinergic motor neuron and for translocation of the LC domain into the terminal. The molecular targets of LC are several synaptic members of the SNARE (soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) attachment protein receptor) family (see Ch. 10), the facilitators of all membrane fusion events in eukaryotic cells.

Upon exposure of the neuromuscular junction to toxin and binding to the neural membrane, a chain of events comprising internalization into synaptic vesicles, translocation into the cytoplasm and inhibition of acetylcholine release unfolds inexorably. Once internalized, the toxin cannot be neutralized by antiserum. The first step of intoxication, involving contact between the carbohydrate-binding, lectin-like HC domain and the terminal membrane, is highly specific. It requires the presence of gangliosides, in particular GD1b, GT1b and GQ1b, together with a protein receptor – possibly synaptotagmin (see Ch. 10) – all embedded and crowded in lipidic rafts that help anchor HC with high affinity. Thus, multivalent binding of HC is accomplished by several near-simultaneous molecular recognition events in a restricted two-dimensional plane taking place between multiple carbohydrate-binding sites on HC and selected sialic-acid-containing gangliosides and glycoproteins. Consequently, the toxin coats the terminal in a punctate distribution and different toxin types bind independently of each other. The presence of the glycosylated portion of synaptotagmin I and II in the synaptic space, however, is contingent upon the fusion of synaptic vesicles with the plasma membrane during neurotransmission and exposure of the luminal aspect of the vesicle to the extracellular medium.

Upon binding, the toxins are internalized via endocytosis and reach vesicular or endosomal compartments that undergo acidification as part of their normal recycling process. Targeting to the appropriate compartment is attributed to the protein receptor present in the endocytic vesicle. The reduction in pH is believed to facilitate a conformational change in the toxin, increasing the exposure of hydrophobic residues on its surface and facilitating its penetration through the membrane to reach the cytosol. Two histidine residues (H551 and H560) lie on a suitably exposed location on botulinum toxin type A, the 'translocation belt' portion of HC, and may therefore be susceptible to titration by pH. The HC domain undergoes conformational changes at low pH *in vitro* and forms somewhat cation-selective channels in artificial membranes of ≈15 Å in diameter as judged from their unitary conductance of 110 pS.

It is thought that LC also experiments a structural change inside the vesicle or the endosome that allows it to translocate via – or next to – the small transmembrane HC channel in an unfolded conformation. This is the least understood event of the intoxication process, as it is very difficult to envision the ≈55×55×62 Å folded, intact LC domain crossing the membrane. Nevertheless, protein-translocating channels are part of the repertoire used by other toxins such as diphtheria and cholera and by the mitochondrial protein import system, the latter with a pore diameter of 13 Å. After translocation and refolding of LC, the toxin is preactivated by proteolysis of a surface-exposed loop accomplishable by a variety of cellular proteases that separate the peptidic continuity after the first 419–449 residues of LC, giving rise to the LC and HC chains proper, and which remain bound by the disulfide bond between C430 and C454 and by noncovalent interactions. Disulfide reduction then releases active LC into the cytosol.

The ultimate localization of LC depends on toxin type, with, for example, type A residing near the plasma membrane and type E remaining in the cytosol, a phenomenon that may explain the differential stability of the toxin isoforms and the persistence of their action. Nevertheless, the LC fragment is always effectively sequestered from the degradative cycle of the terminal. Tyrosines in both LC and HC may be phosphorylated by Src (see Ch. 24), enhancing the stability and proteolytic activity of LC.

The target of LC proteolysis is SNARE proteins, including SNAP25, synaptobrevin and syntaxin, all of which share repeats of a motif that is recognized by LC. The SNARE proteins exist in a complex stabilized by a bundle of tightly packed α-helices, each containing the SNARE motif (distinct from the recognition motif above), and which is disassembled following transmitter release. Binding of LC occurs only after individual SNARE members dissociate and results in their proteolysis at the level of the SNARE motif, preventing reassembly and thus causing impairment of synaptic vesicular release. Residues H222, E223 and H226 form part of the LC active site and are typical of zinc proteases (which display the sequence HEXXH), with H222 and H226 and E261 coordinating zinc and E223 coordinating a water molecule. Additionally, after cleavage, the complex LC-SNAP25 may not dissociate, reentering the otherwise intact SNARE complex and continuing to interfere with neurotransmission.

The long-term effects of botulism are reversible as both nerve and muscle remain alive. The muscle undergoes a transient atrophy with loss of acetylcholinesterase and dispersion of ACh receptors from the endplate over the course of several weeks. During recovery, the release of trophic factors from paralyzed muscle fibers causes enlargement of the endplate, and nerve sprouts develop. The number of endplates per muscle fiber also increases and some muscle fibers may be innervated by more than one motor axon. After axonal sprouting and reformation of functional neuromuscular junctions, the muscle eventually regains its normal size and then redundant sprouts degenerate. The muscle atrophy induced by botulism in animals and humans is therefore largely reversible.

Snake, scorpion, spider, fish and snail peptide venoms act on multiple molecular targets at the neuromuscular junction. β-neurotoxins from the venoms of snakes and scorpions are endowed with phospholipase A_2 (PLA_2) activity. PLA_2 subtypes are essential in phospholipid turnover and in the production of inflammatory mediators (see Ch. 3). PLA_2-directed toxic potency measured as mouse LD_{50} ranges from 1 mg/kg for taipoxin (present in the venom of the Australian snake *Oxyuranus scutellatus*) to 750 mg/kg for pseudexin B (produced by *Pseudechis porphyriacus*), although this wide difference is partly due to toxin pharmacokinetics. There are over 50 neuromuscular toxins with PLA_2 activity and, although their effects differ to some extent, all tend to cause death from respiratory failure due to paralysis of respiratory muscles. This final event may be preceded by hyperexcitability (as in the intoxication with β-bungarotoxins) or by flaccid paralysis of the limbs (after intoxication with crotoxin) that evolves over at least 1 hour, regardless of toxin dose. Muscle use accelerates paralysis and, in the end, the synaptic membrane appears to have experienced massive fusion of ACh vesicles with inhibition of their recycling. ACh release from the nerve terminal, however, undergoes three phases: (1) a short initial phase displaying either decreased or unchanged ACh release, (2) a longer phase (10–30 min) of stimulation of evoked release and (3) complete and irreversible inhibition (30–120 min). In addition, some toxins with PLA_2 activity also inhibit voltage-gated K^+ channels at the synaptic terminal. In the late stage of intoxication, severe alterations of plasma and of synaptic organelle – including mitochondrial – membrane permeability become apparent.

Four types of PLA_2 toxin structure exist. Class I comprises single-chain toxins of ≈14 kDa containing seven disulfide bridges. This class includes agkistrodotoxin from *Agkistrodon* snakes, ammodytoxin from *Vipera ammodytes ammodytes*, caudotoxin from *Bitis caudalis*, notexin from *Notechis scutatus scutatus*, OS toxin from *Oxyuranus scutellatus scutellatus* and pseudexin from *Pseudechis porphyriacus*. Class II includes toxins with two noncovalently linked homologous subunits such as crotoxin and related rattlesnake neurotoxins produced by the genus *Crotalus*. Class III includes heterodimers composed of unrelated subunits, such as the most studied of PLA_2 toxins, β-bungarotoxin. This toxin is manufactured by *Bungarus multicinctus* (together with the ACh-receptor-specific α-bungarotoxin). β-bungarotoxin is similar to dendrotoxin, a neurotoxin that binds to the voltage-gated potassium channels of presynaptic membranes. Class IV toxins comprises noncovalently associated oligomers of homologous subunits and includes taipoxin and paradoxin both of which are heterotrimers. Textilotoxin, produced by *Pseudonaja textilis textilis,* is the most complex of these neurotoxins including five homologous subunits, all of them endowed with PLA_2 activity.

Binding of PLA_2 toxin to the nerve membrane, although selective, is less well understood than that of clostridial toxins. A limited number of high-affinity binding sites exist along with unsaturable low-affinity binding sites. While lipids of the presynaptic membrane may be involved in low-affinity interactions, proteins or glycoproteins are thought to constitute the high-affinity sites. For example, taipoxin may bind the neuronal pentraxin, facilitating its access to the endoplasmic reticulum, where the toxin would be retained via its binding to other specific proteins. But even less well understood is how phospholipid hydrolysis in the endoplasmic reticulum (located in the cellular body) would lead to inhibition of ACh release at the nerve terminal. The structure of the simpler, class I toxin notexin, however, sheds some light into the PLA_2 catalytic mechanism. The toxin is folded into three α-helices with two β-strands and it is both stabilized and activated by Ca^{2+}. The divalent metal binding site consists of the consensus sequence $YGC^{Y}/_{F}CXGG$ and may be occupied, instead of Ca^{2+}, by Sr^{2+}, Ba^{2+} and Zn^{2+}, all of which inhibit PLA_2 activity potently. The toxin also contains a hydrophobic channel that accommodates and secures the fatty acid chains of the phospholipid molecule and places the ester bond to be cleaved into the active site. The activity of the toxin is much higher against membrane-inserted than isolated phospholipids because of the higher efficiency of interfacial catalysis, which depends on the absorption of the enzyme on to the lipid–water interface, facilitating the seamless diffusion of the phospholipid molecule from the membrane to the active site channel.

Elapidae and Hydrophiidae snake α-neurotoxins, in contradistinction, block postsynaptic AChR, inhibiting neuromuscular transmission in a manner analogous to (+)-tubocurare (see Ch. 11). Some 100 α-neurotoxins have been isolated, the most used being α-bungarotoxin, synthesized by the Asian krait *Bungarus multicinctus*. The LD_{50} for these toxins is ≈100 μg/kg in mice. Exposure of humans to low doses of toxin that induce partial blockade of junctional receptors can produce weakness and fatigability that resemble acquired myasthenia gravis. Higher doses can lead to complete neuromuscular block, paralysis, respiratory failure and death.

The α-toxins responsible for the postsynaptic curarimimetic activity are proteins of 7–8 kDa. Structurally, they fall into two groups: long toxins, which comprise 71–74 amino acids and five internal disulfide bonds, and short toxins, with 60–62 amino acids and four internal disulfide bonds. All the α-toxins have about the same equilibrium dissociation constant for the AChR, but they differ markedly in their binding kinetics. The short toxins bind to and dissociate from the AChR five to nine times faster than the long toxins, explaining the near irreversibility of long toxin binding. The stability of the binding of the long α-neurotoxins to the AChR, especially that of α-bungarotoxin, has made them valuable tools for the purification and characterization of the receptor. α-bungarotoxin binds to a site on each α subunit of the AChR that sterically overlaps and interferes with ACh binding at the

interfaces between the two α and the γ and δ subunits. All α-toxins exhibit a high degree of homology and are shaped as concave disks with a small projection at one end. Their elliptical dimensions are approximately 3.8×2.8×1.5 nm, and for the most part, the structure is only a single polypeptide chain thick. The reactive site of the protein is on the concave surface and involves the regions encompassed by residues 32–45 and 49–56 as well as isolated residues from other regions of the molecule.

In contrast with toxins that block neuromuscular transmission, latrotoxins (LTX) cause excessive ACh release and excitation. These toxins are produced by black or brown widow spiders from the genus *Latrodectus* and are directed predominantly against vertebrates (α-LTX), although five homologous insect-specific latrotoxins and one crustacean-specific latrotoxin also coexist in the venom of *Latrodectus mactans tredecimguttans*. This family of toxins may have arisen through a process of gene duplication in the spider. Envenomation by spiders of the genus *Latrodectus* cause lactrodectism, a generalized poisoning syndrome that develops within an hour of being bitten, with pain first localized at regional lymph nodes. Rapidly, generalized muscle cramps and rigidity develop together with hypertension and transient tachycardia followed by bradycardia, profuse sweating and oliguria. A similar envenomation with prominent autonomic nervous system dysfunction is caused by stonefish stings and all these symptoms can be ascribed to hyperexcitability of various nerve terminals. Trachynilysin, a toxin of ≈150 kDa, has been isolated from the venom of the stonefish *Synanceia trachynis* and similar-sized stonustoxin and verructoxin are synthesized by *S. horrida* and *S. verrucosa* respectively. The poisoned neuromuscular junction terminals exhibits enlarged and swollen synaptic boutons depleted of ACh vesicles and containing swollen mitochondria. Exocytosis of synaptic vesicles takes place at active zones and is followed by unaltered vesicular recycling.

Two types of high-affinity LTX receptor have been distinguished in the neuronal membrane on the basis of their Ca^{2+} dependence, with affinity in the nanomolar range. Binding of LTX to the plasmalemma of neuronal cells causes a large influx of Ca^{2+}. After a delay of a few dozens of seconds, LTX induces the formation of long-lasting channels, which are nonselective for cations and display a large conductance of tens of picosiemens, leading to influx of Ca^{2+} and Na^{+} and consequent membrane depolarization. Ion influxes through LTX channels are such that they can well account for the massive neurotransmitter release. However, LTX is capable of inducing neurotransmitter release even in the absence of extracellular Ca^{2+}, provided that the terminal is supplemented with millimolar concentrations of other divalent cations. Unlike the Ca^{2+}-dependent LTX-induced neurotransmitter release, which is mediated via SNARE proteins, LTX may cause Ca^{2+}-independent phospholipase C stimulation, leading to exocytosis. In addition, neurexin Ia and a 120 kDa integral membrane protein associated with the G protein α-subunit (see Ch. 19), syntaxin and synaptotagmin both bind LTX. If these interactions were indeed functional, binding of LTX could activate Ca^{2+} channel/syntaxin complexes coupled to neurexin Ia and to the 120 kDa LTX receptor via a G protein.

LTX consists of 1,381 residues distributed in four different regions: part I is a poorly conserved signal sequence that is removed during toxin maturation, whereas part II is highly conserved and contains two putative membrane-inserting segments, which may account for the channel-forming activity of these neurotoxins; part III is less conserved and includes many ankyrin repeats, suggestive of an adaptor role linking membrane proteins to the cytoskeleton (see Ch. 8). Part IV is of variable size and is not conserved, being removed during maturation and activation of latrotoxin.

Other fish toxins are yet to be characterized, but it appears that all fish venoms act both pre- and postjunctionally to cause depolarization and that the venoms possess cytolytic activity. In contrast, venoms produced by the marine predatory snails of the genus *Conus* are more diverse, the total number of peptides in the venom of a single Conus species ranging from 50 to 200. About 50,000 different *Conus* peptide toxins are estimated to exist, owing to a synthetic strategy that amounts to a combinatorial library scheme [23]. The most prominent venoms belong to the conotoxin type, a class of peptides containing multiple disulfide bonds usually targeted against ion channels. α-conotoxins, produced by the fish-, snail- and worm-hunting marine snails *C. striatus*, *C. magus* and *C. purpurascens*, are competitive antagonists of the AChR with nanomolar affinity. In particular, subtypes α-conotoxin GI and MI inhibit all AChR types from vertebrate muscle without affecting neuronal receptors. *C. purpurascens* also secretes ψ-conotoxin PIIIE, an unrelated noncompetitive AChR antagonist structurally similar to the μ-conotoxins (the potent Na^{+} channel blockers), that probably acts as AChR channel blocker. α-conotoxins bind preferentially to the α:δ subunit interface of the AChR versus the α:γ interface (see Ch. 11) with an affinity difference of 10,000-fold between the two sites in some cases. AChR residues γK34, γS111 and γF172 at the interface with the α subunit confer lower affinity than δS36, δY113 and δI178 at the equivalent α subunit interface.

The overall structure of α-conotoxin GI is triangular, with both the positively charged amino group of the first residue (E1) and the guanidino group of R9 located at two of the corners and a central hydrophobic scaffold held together by the disulfide bonds. Not unexpectedly, conotoxin ψ-conotoxin PIIIE is very differently shaped, as a flat disc with five exposed positive charges that may penetrate the AChR channel pore when its intrinsic electrostatic potential becomes less positive as a result of opening [24].

Electrolyte imbalances alter the voltage sensitivity of muscle ion channels. While the effect of changing ionic concentrations across cellular membranes on membrane

potential and excitability is dictated by the Nernst equation (see Ch. 6), plasmatic electrolyte disturbances affect excitable tissues differently depending on the rate of exchange of the extracellular solution and the mobility of ions across the often narrow intercellular spaces [9]. Hypercalcemia causes divalent metal ionic screening of membrane surface charges and is associated with weakness and fatigability due to muscle dysfunction caused by offsetting of the extracellular potential, to which the voltage sensors of all ion channels are exposed and which can eventually lead to chronic myopathy. Effects on the brain include altered consciousness ranging from apathy to agitation and seizures. Common causes of hypercalcemia are hyperparathyroidism, metastatic disease and vitamin D intoxication. Hypocalcemia, a feature of hypoparathyroidism, malabsorption, vitamin D deficiency and – rarely – thyroid and parathyroid surgery, initially causes numbness of mouth, hands and feet, followed by tetany or spasms in the same distribution. The encephalopathy associated with hypocalcemia is more dramatic, with hallucinations, psychosis and seizures. Effects remote from the cerebral cortex include parkinsonism and chorea (see Ch. 45) and even spinal cord dysfunction. The effects of magnesium electrolyte imbalance resemble those of calcium, except that hypomagnesemia may escape analytical detection because magnesium is predominantly an intracellular ion. Renal tubular acidosis may cause hypomagnesemia, while renal failure commonly causes hypermagnesemia.

Alterations in potassium concentration are worse tolerated because of the fundamental role of potassium in setting resting membrane potential in almost all excitable cells of the organism. Hyperkalemia usually causes cardiac arrhythmia before nerve and muscle dysfunction. The latter abnormalities are manifested as weakness preceded by burning sensation (paresthesia) and are sometimes accompanied by mental changes. Hypokalemia, on the other hand, causes primarily neuromuscular disturbance, with fatigability, weakness of large (proximal) muscles and, ultimately, lysis of the muscle membrane (rhabdomyolysis with release of myoglobin to the plasma). When accompanied by alkalosis, hypokalemia causes tetany.

The neuromuscular junction and muscle are more resistant to changes in sodium concentration, to which they are minimally permeable at rest. In fact, the consequences of sodium disturbance relate instead to the role of this ion in maintaining the osmotic equilibrium between the brain and plasma and range from depression of consciousness, coma and seizures caused by hyponatremia, to brain shrinkage and tearing of superficial blood vessels due to excessive serum osmolarity due to hypernatremia.

REFERENCES

1. Karlin, A. and Akabas, M. H. Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* 15: 1231–1244, 1995.
2. Franzini-Armstrong, C. Studies of the triad. I. Structure of the junction of frog twitch fibers. *J. Cell Biol.* 47: 488–499, 1979.
3. Huxley, H. E. The mechanism of muscle contraction. *Science* 164: 1356–1366, 1969.
4. Pollard, T. D. and Cooper, J. A. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55: 987–1035, 1986.
5. Hibberd, M. G. and Trentham, D. R. Relationships between chemical and mechanical events during muscular contraction. *Annu. Rev. Biophys. Biophys. Chem.* 15: 119–161, 1986.
6. Zot, A. S. and Potter, J. D. Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. *Annu. Rev. Biophys. Biophys. Chem.* 16: 535–560, 1987.
7. Campbell, K. P., Knudson, C. M., Imagawa, T. *et al.* Identification and characterization of the high affinity [^{3}H]ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. *J. Biol. Chem.* 262: 6460–6463, 1987.
8. Wagenknecht, T., Grassucci, R., Frank, I. *et al.* Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. *Nature* 338: 167–170, 1989.
9. Jones, H. R. Jr, De Vivo, D. C. and Darras, B. T. (eds) *Neuromuscular Disorders of Infancy, Childhood, and Adolescence – A Clinician's Approach.* Butterworth-Heinemann. Philadelphia, 2003.
10. Engel, A. G., Ohno, K. and Sine, S. M. Congenital myasthenic syndromes: a diverse array of molecular targets. *J. Neurocytol.* 32: 1017–1037, 2003.
11. Lehmann-Horn, F. and Jurkat-Rott, K. Voltage-gated ion channels and hereditary disease. *Physiol Rev.* 79: 1317–1372, 1999.
12. Ptacek, L., Tawil, R., Griggs, R. *et al.* Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. *Cell* 77: 863–898, 1994.
13. Plaster, N. M., Tawil, R., Tristani-Firouzi, M. *et al.* Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. *Cell* 105: 511–519, 2001.
14. Ranum, L. P. and Day, J. W. Myotonic dystrophy: RNA pathogenesis comes into focus. *Am. J. Hum. Genet.* 74: 793–804, 2004.
15. Waxman, S. G. Transcriptional channelopathies: an emerging class of disorders. *Nat. Rev. Neurosci.* 2: 652–659, 2001.
16. Steinmeyer, K., Klocke, R., Ortland, C. *et al.* Inactivation of muscle chloride channel by transposon insertion in myotonic mice. *Nature* 354: 304–306, 1991.
17. MacLennan, D. H., Duff, C., Zorzato, F. *et al.* Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia.*Nature* 343: 559–561, 1990.
18. Odermatt, A., Taschner, P. E., Khanna, V. K. *et al.* Mutations in the gene-encoding SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+} ATPase, are associated with Brody disease. *Nat. Genet.*14: 191–194, 1996.
19. Lindstrom, J. Immunobiology of myasthenia gravis, experimental autoimmune myasthenia gravis, and Lambert–Eaton syndrome. *Annu. Rev. Immunol.* 3: 109–131, 1985.
20. Vincent, A., McConville, J., Farrugia, M. E. *et al.* Antibodies in myasthenia gravis and related disorders. *Ann. N.Y. Acad. Sci.* 998: 324–335, 2003.
21. Lang, B. and Newsom-Davis, J. Immunopathology of the Lambert–Eaton myasthenic syndrome. *Springer Semin. Immunopathol.* 17: 3–15, 1995.

22. Schiavo, G., Matteoli, M. and Montecucco, C. Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* 80:717–766, 2000.
23. Terlau, H. and Olivera, B. M. *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol. Rev*. 84: 41–68, 2004.
24. Pascual, J. M. and Karlin, A. State-dependent accessibility and electrostatic potential in the channel of the acetylcholine receptor. *J. Gen. Physiol.* 111: 717–739, 1998.

CHAPTER

44

Motor Neuron Diseases

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AMYOTROPHIC LATERAL SCLEROSIS IS THE MOST COMMON ADULT ONSET MOTOR NEURON DISEASE

The motor neuron diseases (MND), including amyotrophic lateral sclerosis (ALS), are chronic, progressive illnesses characterized by: severely disabling clinical features involving motor systems (weakness, muscle atrophy and, in ALS, spasticity); relatively selective involvement of lower motor neurons and, in classical ALS, upper motor neurons; disease-related abnormalities associated with, in some instances, the presence of intracellular protein aggregates (inclusions); alterations in axonal transport; and death of motor neurons. Some forms of the disease are inherited as autosomal dominants, others as recessives. In some instances, the presence of specific gene products confer risk. The majority of cases appear to be sporadic. Although symptomatic treatments are available, there are, at present, no effective mechanism-based therapies [1–4]. The MND represent challenges for science and medicine, not because of their prevalence but because of the tragedy of these illnesses for affected individuals, their families and caregivers. Recent research, particularly studies utilizing animal models, has provided new insights into the mechanisms of these disorders, identified potential new targets for therapy and allowed design and testing of treatments.

Before discovery of mutant genes linked to MND, scientists created and studied surrogate models of dysfunction/death of motor neurons [4, 5], including: axotomy models (axonal transection and avulsion); models of neurofilamentous abnormalities of axons induced by exposure to toxins; and spontaneously occurring animal models, such as hereditary canine spinal muscular atrophy (HCSMA), a genetic disease occurring in Brittany spaniels. Although useful for testing hypotheses about pathogenic mechanisms, these models have variable direct relevance to human MND. However, because investigations of these models set the stage and established experimental strategies for later work, we briefly describe studies

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of several of these disorders as examples of the types of investigations that lead to investigations of the new models, many of which were developed following the exciting discoveries of the roles of mutant genes in MND.

The identification of causative mutations in specific genes in cases of human MND, including familial ALS (fALS) and spinal muscular atrophy (SMA) [6–8], has provided new opportunities, using transgenic and gene targeting approaches, to investigate the molecular participants in disease processes [1, 3]. In autosomal dominant fALS, the mutant proteins often acquire toxic properties that directly or indirectly impact on the functions and viability of neurons [3, 4, 7, 9, 10], and introduction of mutant genes into mice reproduces some features of these diseases [4, 11–13]. In contrast, autosomal recessive diseases, such as SMA, which usually lack the functional protein encoded by the mutant gene (*SMN* – 'survival motor neuron' – in SMA), can often be modeled by gene targeting strategies [1, 4]. Studies of some MND in animals, including progressive motor neuronopathy (PMN) [14], have led to discovery of mutant genes/proteins whose roles in neurons illuminate potential disease mechanisms in humans [14].

In models of MND, therapeutic manipulations, manipulation of expression of selected genes in specific cell populations [15, 16], creation of chimeric animals to test whether abnormalities are cell autonomous [17], administration of trophic factors to prevent trophic cell death [18–20] and testing of a variety of drug therapies [21–24] have been used to try to ameliorate phenotypes and thus provide insights into disease mechanisms and potential treatment strategies [1, 3, 4, 15, 25, 26]. Results of these studies are being used to design novel therapies to be tested in clinical trials in humans.

In this review, we focus on ALS, particularly its genetic variants, and relevant model systems with the belief that understanding these inherited illnesses will help to clarify the mechanisms of the more common sporadic forms of MND. SMA, a major cause of MND in infancy and childhood, is beyond the scope of this chapter and is the subject of several recent reviews [4, 8, 27].

Motor neuron disease is characterized clinically by weakness, muscle atrophy and spasticity. This illness, often termed Lou Gehrig's disease in the United States, is the most common adult-onset form of MND with a prevalence of approximately 2–3 per 100,000 people [1–3, 10, 25, 28]. Each year in the United States, in excess of 5,000 people are diagnosed with ALS. In parts of the United Kingdom, 1 in ≈500 deaths are attributed to some form of MND. The principal clinical signs of ALS include progressive limb weakness, which may be symmetrical or asymmetrical; atrophy of appendicular, bulbar and respiratory muscles; and spasticity [1, 2, 26, 28]. The paralysis/muscle atrophy and spasticity are the result of degeneration of motor neurons in the spinal cord/brain stem and motor cortex respectively. The onset of this illness is typically in the fifth or sixth decade of life; affected individuals usually die within 2–5 years of appearance of symptoms. Both sporadic (sALS) and familial (fALS) forms of illness exist; familial cases make up approximately 5–10% of the total. While the causes of the majority of cases of ALS are yet to be identified, the shared features of the clinical presentations and pathologies occurring in both sporadic and familial cases suggest the existence of some common disease mechanisms.

The pathological processes impact particularly on the spinal and corticospinal motor neurons.

These processes appear to evolve through a series of stages influencing size, shape, content, metabolism and physiology of these cells [28–30]. Years ago, it was observed that proximal axonal segments were swollen with maloriented arrays of neurofilaments (NF) [31, 32]. Similar abnormalities were documented in experimental models [5, 33–37]. Investigations, described below, tested the hypothesis that this type of pathology was the result of defects in axonal transport [5, 38–43] (see Ch. 28 for discussion of axonal transport.). Moreover, it was hypothesized that impairments in transport could also be associated with 'a dying back' phenomenon (i.e. degeneration proceeding retrogradely from distal nerve terminals to proximal cell body), which was hypothesized to be related to impaired delivery of essential constituents to the most distant axons and terminal [5]. In MND, it was hypothesized that these abnormalities become more extensive over time with clinical signs becoming increasingly evident. Moreover, as a part of the dying-back process and discrimination of neurons from targets, retrogradely transported trophic support to neuronal cell bodies is compromised, which, in turn, impacts on the viability of these cells [18, 20, 44]. In ALS, motor neurons show a variety of abnormalities, including chromatolysis (enlargement of cell bodies with dispersal/margination of Nissl substance) and protein aggregates and inclusions, which are often ubiquitinated [28]. Later, neurons may become atrophic. There is wallerian degeneration of motor axons (see Ch. 30). In the final stages, motor neurons exhibit several features of apoptosis (see Ch. 35), which are discussed below [4, 29, 30, 45–47]. Ultimately, the numbers of motor neurons in brainstem nuclei and spinal cord are reduced and there is also a loss of large pyramidal neurons in motor cortex associated with secondary degeneration of the corticospinal tracts [28].

Excitotoxicity (see Chs 15 and 32) has been suggested to be a mechanism by which motor neurons are damaged in ALS [25, 48, 49]. About 60–70% of sporadic ALS patients have a 30–95% reduction in the levels of the astroglial glutamate transporter EAAT2 (excitatory amino acid transporter 2), also termed GLT-1, in motor cortex and spinal cord [25, 48, 49]. Reduction in level of activity of this major glutamate transporter leads to increased extracellular concentrations of glutamate at synapses and evidence of excitotoxicity exists in some patients with ALS.

The mechanisms of cell death are the subject of very active research [29, 30, 45–47, 50–53]. Three variants of cell death have been described: apoptosis or programmed

cell death (PCD); necrosis associated with cytoplasmic swelling and loss of membrane integrity; and autophagy (an intracellular catabolic process that occurs by lysosomal degradation of damaged or expendable organelles). The morphological distinctions between these different forms of cell death are blurred and it has been suggested that the death process may involve a continuum with varying overlapping contributions, particularly of apoptosis and necrosis. In ALS, current evidence indicates that apoptosis plays a role in the degeneration of motor neurons, albeit, perhaps, in a nonclassical form [4, 29, 30, 45–47, 50, 54–56].

Thus, in ALS, motor neuron degeneration evolves over time showing the following: chromatolysis; appearance of inclusions and aggregates; somatodendritic atrophy; and accumulations of vacuoles in mitochondria in dendrites and cell bodies. In late stages, DNA fragmentation is evident and cytochrome *c* and cleaved caspase-3 appear in cytoplasm. The increase in caspase-3 activity and the activation of DNA fragmentation factor endonuclease (DFF-45) leads to the appearance of internucleosomal fragmentation of genomic DNA in cytoplasm, an increase in levels of Bax and Bak and a decrease in levels of Bcl-2 in mitochondrial membrane-enriched fractions derived from selectively vulnerable motor regions [29, 30, 45, 50, 55, 57]. Some investigations support the idea of inappropriate re-emergence of a PCD mechanism involving p53 activation and cytosol-to-mitochondria redistributions of cell death proteins. In this scenario, it has been suggested that death of motor neurons in ALS appears to be linked to a p53-driven, intrinsic mitochondrial caspase-3-dependent apoptotic pathway, possibly involving a Bax channel model [30, 57]. In studies of mouse models of ALS [47, 54, 58–63], there is no clear consensus on the type or mechanism of cell death [30] and some of these issues are discussed below.

Some cases of amyotrophic lateral sclerosis are familial. Approximately 10% of cases of ALS are familial and, in the majority of these cases, the disease is inherited as an autosomal dominant [1, 3, 25]. Several of the genes that cause or confer risk in ALS are reviewed below because the information serves as important background for subsequent discussion of genetically engineered models. In ALS1, mutations in the superoxide dismutase 1 (SOD1) gene occur in ≈5–10% of autosomal dominant cases of fALS. Mutations in dynactin $p150^{glued}$ have been recently linked to autosomal dominant fALS [7] and may, as an allelic variant, serve as a risk factor [64]. In ALS2, autosomal recessive deletion mutations have been identified in *ALS2*, which encodes alsin, a protein that regulates GTPases [65, 66]. In ALS4, a rare autosomal dominant form of juvenile ALS, mutations have been identified in the gene (*SETX*) that encodes senataxin [67], which contains a DNA/RNA helicase domain with homology to other proteins known to have roles in the processing of RNA [68]. Finally, following an observation that deletion of the hypoxia response element in the promoter of the vascular endothelial growth factor (VEGF) gene causes degeneration of motor neurons in mice [69], it has been reported that individuals homozygous for certain haplotypes in the VEGF promoter have an increased risk for ALS [15]. Thus, VEGF, a cytokine involved in angiogenesis but with many other functions, may play a role as a susceptibility gene for ALS [70].

Selected aspects of the genetics of MND are reviewed in greater detail below with the belief that understanding of the biology of some of the proteins encoded by these genes and subsequent generation of mouse models are of value in clarifying the molecular mechanisms of selective dysfunction and death of motor neurons in MND, and, eventually, for identifying targets for new treatments.

Several genes have been shown to cause amyotrophic lateral sclerosis.

Amyotrophic lateral sclerosis 1 is caused by expression of mutant *SOD1*. Approximately 15–20% of patients with autosomal dominant fALS (i.e. ≈2% of all ALS cases) have mutations in the gene (chromosome 21) that encodes cytosolic Cu/Zn SOD1, a 153 amino acid enzyme that, as a homodimer, catalyzes the conversion of O_2^- to O_2 and H_2O_2 [3, 6]. To date, investigators have identified approximately 100 mutations (see on line at: alsod.org), all of which lead to autosomal dominant fALS [3]; the exception is homozygous D90A SOD1, which is inherited as recessive. These mutations are scattered throughout the structure of SOD1 and are not preferentially localized near the active site or the dimer interface. While some fALS SOD1 mutants show reduced enzymatic activities, many mutant proteins retain activity [9, 71]. It is thought that the mutant enzyme causes selective neuronal degeneration through a gain of toxic property [11, 71], consistent with the dominant pattern of inheritance. Supporting this concept are the following observations: $SOD1^{-/-}$ mice do not develop disease [72]; the levels of enzyme activity do not correlate with disease [9]; and, in transgenic mice, increasing wild-type SOD1 activity does not ameliorate the disorder [3, 73]. The presence of aggregates containing mutant SOD1 in affected neurons has generated several hypotheses regarding disease pathogenesis including: the sequestration of essential molecules by mutant proteins in aggregates; damaging mutant peptides are mislocated (i.e. to mitochondria) and cause problems at these sites; malfolded proteins in aggregates are toxic and impact on molecular motor, axonal transport, proteosomal degradation and glutamate transport; and mutant SOD1 participates in aberrant copper chemistry. Some of the potential mechanisms are discussed below.

Amyotrophic lateral sclerosis 2 is linked to mutant alsin. In several families with autosomal recessive juvenile ALS, mutations have been identified in *ALS2 (*chromosome 2), encoding alsin [65, 66]. This illness, which was originally described in a Tunisian kindred, is characterized by spasticity (involvement of upper motor neurons) and weakness/amyotrophy (involvement of lower

motor neurons) [65]. The functions of alsin are not well understood, but the protein has several sequence motifs that have homologies to GTPase regulatory proteins important in cell signaling and in protein trafficking. Alsin appears to be a guanine–-nucleotide exchange factor (GEF) for at least the RAB5 family of GTPases. The *ALS2* mutations are believed to be unstable [74] and it has been hypothesized that loss of functional activities of the GEF domains of alsin could impact on signal transduction pathways, regulation of the cytoskeleton and/or intracellular trafficking [65, 66]. However, at present the mechanisms whereby mutations in this gene cause MND are unknown.

Amyotrophic lateral sclerosis 4 is linked to mutations in a helicase gene. This rare autosomal dominant form of juvenile ALS has been linked to mutations in *SETX* (chromosome 9), encoding senataxin [67], the mammalian ortholog of a yeast RNA helicase [68]. This disease is manifest by distal weakness (beginning at approximately 25 years of age), slow progression and a normal lifespan [75]. There is some evidence of partial denervation of muscle and some affected individuals have signs of involvement of upper motor neurons [75]. Sensation is normal. At autopsy, the number of spinal motor neurons is reduced and swelling of axons occur among a variety of populations of neurons [75]. The corticospinal tract shows evidence of degeneration. Sensory neurons exhibit some mild abnormalities. Senataxin has a helicase domain homologous to that of other proteins known to have roles in the processing of RNA [67, 76–78]. Significantly, recessive mutations in *SETX*, which are believed to result in a truncated protein (loss of function), have also been reported in ataxia–oculomotor apraxia type 2. Interestingly, infantile spinal muscular atrophy with respiratory distress type1, manifest as weakness and difficulty with respiration at age 1–6 months, results from mutations in another helicase gene, which encoded the immunoglobulin M-binding protein (IGH MBP2) on chromosome 11 [79, 80].

Mutant dynactin p150^{glued} causes a form of familial amyotrophic lateral sclerosis. A family with a slowly progressive autosomal dominant lower motor neuron disease (lacking sensory signs) has recently shown linkage to a G59S mutation in the p150^{glued} subunit of dynactin (DCTN1) [7], a motor protein that, along with dynein, plays a role in retrograde axonal transport [81–83]. This inherited disease begins in early adult life with vocal cord paralysis (associated with breathing difficulties), facial weakness and atrophy of muscles in the hands [7]. Subsequently, weakness and atrophy appear in the distal lower extremities. The dynactin complex, which includes p150 and dynamitin, provides a linker between cargos, microtubules and dynein. The mutation in the index family occurs in a motif in the p150^{glued} subunit that binds to microtubules; modeling studies suggest that this mutation impacts on protein structure to create steric hindrance and distortion of the folding of the microtubule binding domain [7]. Consistent with this concept is the observation that the mutant protein binds less well to micro tubules [7]. More recently, heterozygous missense mutations have been described in several familial cases of ALS and one apparently sporadic case; these observations have been interpreted to suggest that variants in the p150 subunit can confer risk in ALS [64].

Taking advantage of the discovery of this fALS-linked mutation in a motor protein, members of our group (Drs Laird, Ackerly, Price, Wong) have recently created a mutant p150^{glued} model of fALS with a robust MND phenotype.

NON-TRANSGENIC, INDUCED MODELS OF MOTOR NEURON DISEASE

Interruption of the communication between the motor neuron cell body and axon by transection, crush or avulsion induces motor neuron injury.

Peripheral nerve axonal injury. These highly reproducible models of neuronal injury, which interrupt anterograde and retrograde transport in axons, are associated with distal wallerian degeneration and retrograde responses of neurons [5, 84–86]. Following axotomy, the proximal and distal stumps become enlarged, with accumulations of membranous elements [86]; shortly thereafter the distal stump begins to undergo wallerian degeneration [86]. The cell bodies of axotomized neurons may show: chromatolysis [84]; alterations in levels of specific mRNAs [87–89]; changes in the synthesis and transport of specific proteins; aberrant distributions of cytoskeletal proteins (i.e. phosphorylated neurofilaments appear in perikarya) [88–91]; and synaptic disconnection [85]. Moreover, by choosing certain experimental parameters (i.e. age of animal, motor or sensory neurons, proximal or distal locations of lesion, nature of the lesion (crush, transection or avulsion)), investigators can study responses associated with regeneration (distal lesions) [86, 88, 91–93] and degeneration (proximal avulsions) [18–20, 94]. Over the years, the axotomy model has provided important information concerning the influence of axonal injury on the expression of genes encoding cytoskeletal proteins, including NF proteins, peripherin, tubulin, etc. [88, 89, 91, 92, 95]. For example, following unilateral crush of the sciatic nerve, sensory neurons exhibit reduced levels of NF gene expression [88]; the amounts of NF proteins entering axons are decreased and a wave of reduced axonal caliber moves down axons at the rate of transport of NF proteins [91]. Following regeneration, the synthesis of NF proteins returns to normal and axonal caliber is restored [88, 91]. These experiments provided the first insights into the role of NF as a determinant of axon caliber [91, 95]. Following axonal injury,

the expression of other genes may be increased (i.e. the levels of β-tubulin and peripherin mRNAs increase following axotomy [96]and levels of p75 NGF-R immunoreactivity transiently reappear in motor neurons [97]. Levels of neurotransmitter-related proteins, including choline acetyltransferase, often decrease after axotomy of motor neurons; when reinnervation occurs, these transmitter-related markers usually return to normal. During outgrowth of regenerating axons, fast transport delivers membranous constituents required for new growth cones and the axolemma [93, 98]. When regeneration is complete, levels of these mRNA return to control values. Fast transport delivers many of the proteins essential for resuming normal functions at the neuromuscular junction.

Proximal axotomies (including rhizotomies) of facial and spinal motor neurons, particularly in young animals, causes death of neurons [18–20, 44, 94]. These models have proved to be very useful for examining the influences of trophic factors on the survival of axotomized motor neurons [18–20, 44, 94]. For example, brain-derived neurotrophic factor (BDNF), a survival factor for motor neurons, is expressed in the local environment and in muscle targets of motor neurons; expression in muscle is upregulated by denervation [18]. (Neurotrophic factors are discussed in Ch. 27.) Significantly, motor neurons express the gene encoding $p145^{trkB}$, a receptor involved in BDNF signal transduction [97] and it is believed that BDNF and phosphorylated TrkB are carried by retrograde axonal transport to motor neurons from skeletal muscles [97, 99]. In the facial nerve axotomy model, human recombinant BDNF, placed in proximity to the proximal stump, reduces cell death as compared to the vehicle-treated group [18, 94, 100]. NT-4/5 has a similar effects on axotomized facial motor neurons [20]. Glial derived neurotrophic factor (GDNF), a member of the TGFβ superfamily, binds to specific receptors and is also a potent trophic factor for motor neurons *in vitro* and *in vivo* [19, 101]. The administration of neurotrophins may have beneficial effects in other models of MND. If delivery problems and biological toxicities can be overcome, trophic approaches may offer potential for treatment and certain types of MND [102, 103].

β,β′-iminodipropionitrile-induced neurofilamentous axonal pathology. Administration of β,β′-iminodipropionitrile (IDPN) produces a pathology in proximal axons [38, 104] similar to that described in ALS [31]. IDPN selectively impairs slow transport, particularly of the three NF proteins [38]; the transport of tubulin and actin are also somewhat slowed, but fast anterograde and retrograde transport appear to be relatively normal. Exposure to IDPN impairs the transport of NF proteins beyond the proximal internodes and the toxin appears to dissociate NF from microtubules [39, 40]. In this model, a relatively selective defect in the transport of NF leads to formation of massive filament-filled proximal axonal swellings and atrophy of distal axons [104]. Secondary to the axonal abnormalities, changes occur in Schwann cells and myelin sheaths [105, 106]. Studies of several toxic neurofilamentous axonopathies, including those induced by IDPN, acrylamide and aluminum, with their highly reproducible pathology, were among the first to be investigated with radiolabeling methods developed in the 1970s. This research demonstrated, for the first time, the roles of impaired axonal transport in generating cytoskeletal axonal abnormalities resembling those identified in ALS [5, 37–43]. Moreover, other investigations were the first to demonstrate that retrograde axonal transport was also important in the disease (i.e. it provided a pathway by which tetanus toxin enters the nervous system [107]). Years after these studies were published, interest in the roles of axonal transport in normal neuronal functions, in the perturbations seen in experimental models (including axotomy, dying back neuropathies and neurodegenerative diseases) and in experimental therapeutics has undergone an extraordinary renaissance [1, 5, 10, 108–112].

Hereditary canine spinal muscular atrophy. This disease of Brittany spaniels, discovered by Dr Linda Cork, manifests as weakness and atrophy of skeletal muscles, with sparing of eye movements and sphincters [34, 113, 114]. Three HCSMA phenotypes have been identified: pups with accelerated disease, produced by mating affected to affected dogs, are tetraplegic by 3–4 months of age; intermediately affected dogs become weak at approximately 6 months of age and are paralyzed at 2–3 years of age; and chronically affected dogs become mildly weak later in life and show very slow rates of progression. Neurofilamentous swellings are abundant in the anterior horns involving proximal axons of motor neurons. Moreover, there are reductions in the size of these cells and, possibly, in the content of transmitter markers [34, 36]. Axonal transport of NF proteins and, to a lesser extent, tubulin are impaired. In ventral roots, axonal diameters are smaller than controls and evidence of axonal degeneration is not conspicuous. The clinical phenotypes, selective involvement of motor neurons and cytoskeletal pathology that occur in HCSMA resembles the abnormalities described in cases of ALS. To date, the genetic basis for the canine disorder has not been defined but it would not be surprising if the mutant gene product impacted on the motor axonal transport.

SELECTED GENETIC MODELS OF RELEVANCE TO AMYOTROPHIC LATERAL SCLEROSIS

Information concerning the functions of some of the genes/proteins implicated in causation of and risk for these illnesses and has been of great value for the generation of mouse models, the study of which has enhanced our understanding of the pathogenesis of ALS and its variants.

Transgenic mice expressing wild type or mutant neurofilament genes develop motor neuron disease and neurofibrillary pathology. Neurofilaments are assembled as obligate heteropolymers from three subunits, including NF-L (68 kDa), NF-M (95 kDa) and NF-H (115 kDa) [115–117]. As described above, NFs are an important determinant of axonal caliber [88, 91, 117, 118]. To determine whether increased NF content or the expression of NF transgene (without or with) mutations can cause disease, investigators have generated a variety of lines of *NF* mice. Approximately twofold overexpression of wild-type mouse NF-L is not associated with an overt phenotype [119] but greater elevations of NF protein leads to accumulations of NF in cell bodies, and accumulations of NF in distended axons and there is evidence of denervation [120]. Animals die within 3–4 weeks of age. Doubling NF-H content by overexpression of wild-type human NF-H results in an similar pathology; however, the onset of signs is later (4–5 months) and the disease progresses more slowly. In this model, axonal transport is impaired [121].

Significantly, mutations in NF genes can cause MND. Mutant *NF-L* mice with a single amino acid substitution at a conserved residue develop clinical signs between 3–4 weeks and 3 months, depending on levels of a mutant NF-L subunit [122]. The mice develop clinical signs, NF axonal swellings, evidence of motor neuron dysfunction (including altered axonal transport) and death, denervation, and muscle atrophy [122]. Thus, perturbations in the biology of NF can cause clinical disease that resembles those features occurring in cases of ALS.

Familial-amyotrophic-lateral-sclerosis-linked mutant *SOD1* mice reproduce many of the clinical and pathological features of amyotrophic lateral sclerosis. SOD1 is normally present in neurons (and other cells) [123]. To attempt to model an inherited form of fALS, investigators have introduced mutant *SOD1* into mice. These mice develop progressive weakness and muscle atrophy as well as cellular abnormalities which closely resemble the features of ALS [11, 12, 124]. For example, the *G37R SOD1* mice, which accumulate three to 12 times the endogenous levels of SOD1 in the spinal cord, develop an MND phenotype [1, 4]. In some lines of mice, high-molecular-weight complexes of mutant SOD1 accumulate in neural tissues [125, 126]; these complexes are rarely found outside the nervous system other than in skeletal muscle [127], suggesting that factors in neural tissues promote the formation or lead to failure of clearance of these complexes. Motor neurons also exhibit SOD1 inclusions, ubiquitin and phosphorylated NF-H immunoreactivities [12, 128, 129]. Aggregates are present in neurons, in some mutant lines of mice, and in glial cells [11, 12, 73, 126, 129]. SOD1, transported with the slow anterograde component [130], accumulates in irregular, swollen, intraparenchymal portions of motor axons and is often associated with vacuolization of mitochondria and disorganized bundles of filaments. Approximately 2–3 months before the appearance of clinical signs, axonal transport appears to be abnormal [131]. The presence of wallerian degeneration correlates with development of weakness [11, 12]. In mutant mice, cleaved products caspase 1 and 3 accumulate in spinal cord (early and late, respectively), followed by evidence of cleavage of caspase 9 [58], suggesting caspase activation. However, more recent studies demonstrated that caspase-1 and caspase-3 activation is not crucial for motor neuron degeneration in mutant SOD1 mice [132]. Investigations have defined a motor-neuron-specific, cell-autonomous death pathway mediated by Fas signaling and involving p38 [54]. Interestingly, embryonic murine motor neurons expressing mutant SOD1 display increased susceptibility to the activation of this pathway (but not to deprivation of trophic factors or to excitotoxic manipulations) [54]. Eventually, in mutant *SOD1* mice, motor neurons degenerate and the anterior horns show reduced number of neurons as well as evidence of local proliferation of glial cells.

Following the discovery that the *SOD1* mutations cause disease independent of levels of dismutase activity [9, 71], several hypotheses were proposed to explain the ways in which mutations lead to abnormalities of motor neurons: mutant SOD1 damages cells by carrying out aberrant copper chemistries; the protein becomes mislocalized, perhaps leading to mitochondrial damage; the mutant protein is improperly folded and shows an increased propensity to aggregate; the aggregates of mutant SOD1 cause toxicity, perhaps because high-molecular-weight complexes sequester molecules critical for viability, deplete chaperones essential for proper protein folding and impair proteosomal degradation processes; the mutant protein, by unknown mechanism alters (reduces) glutamate transport leading to excitotoxicity; and the presence of the mutant enzyme is associated with impaired axonal transport, perhaps by interfering with molecular motors. These hypothetical mechanisms are not mutually exclusive. However, the mechanism whereby the mutant SOD1 gains an adverse property remains to be defined [3, 11] (see below).

Lines of mice with mutant genes encoding motor proteins develop an amyotrophic-lateral-sclerosis-like phenotype. For many years, axonal transport has been suggested to play roles in the pathogenesis of human diseases, including ALS, and in various animal models [5, 38, 41, 107]. As described above, these concepts have been validated in experimental investigations of models of traumatic, toxic and neurodegenerative diseases [37, 38, 41, 107, 110]. In recent studies of mutant SOD1 mice, disruption of transport has been shown to antedate the onset of clinical signs [131]; a similar scenario has long been postulated in ALS [5]. Although the mechanisms leading to impaired transport are not entirely clear, the most obvious consequences of impaired transport are the failure of critical proteins synthesized in the cell body and

destined for the synapses to reach their target(s) at terminals, and/or altered retrograde transport of signals, originating in the periphery, to reach cell bodies [18, 81–83, 99, 103, 133]. Extrapolating from investigations of many models, including those related to mutant motor protein, either or both of these abnormalities could lead to clinical signs and to dysfunction and death of motor neurons.

Compelling evidence in support of roles of impaired transport leading to disease comes from recent studies of the impact of mutations on the properties of motor proteins essential for normal axonal transport. These proteins include: the kinesins and the dyneins, members of two superfamilies of molecular motors, which are responsible for the anterograde and retrograde transport of cargos along microtubules within axons; and dynactin, a large multi-subunit complex, involved in dynein-mediated retrograde transport [81–83, 133]. Selected investigations relevant to these issues are reviewed below.

Mutant dynactin mice. The dynein–dynactin complex is a critical component of fast retrograde transport of vesicles and organelles [81–83]. Dynein is responsible for the minus end movement along microtubules and dynactin has been postulated to enhance the processing and efficiency of the motor with $p150^{glued}$ interacting with dynein and tubulin [134]. The overexpression of the dynactin subunit dynamitin (dynamitin; GenBank number NM 006400) disrupts the dynactin complex (presumably by causing dissociation of dynactin at the junction of the $p150^{glued}$ and Arp1 filament). The *in vivo* result is a mouse model displaying a late-onset MND phenotype, including weakness, trembling, abnormal posture and abnormal gait [134]. These mice exhibit denervation and atrophy of muscles and degeneration of motor neurons.

Mutant dynein mice. Dynein, comprised of heavy, intermediate, light intermediate and light chains [83], is responsible, in conjunction with dynactin complex, for the minus end movement of cargos [81–83]. A late-onset, progressive MND in mice, identified following an *N*-ethyl-*N*-nitrosourea-mediated mutagenesis screen, is linked to mutations in the cytoplasmic dynein heavy chain 1(Dnchc1; GenBank number NM030238) [135]. Heterozygous missense mutations are associated with impaired transport and degeneration of motor neurons.

Mutant *Tbce* mice. Progressive motor neuropathy (PMN), an autosomal recessive murine disease, manifests as weakness beginning within a few weeks of birth [14, 136]. These mice are homozygous for a Trp 524 Gly substitution of *Tbce* (tubulin-specific chaperone E), localized to mouse chromosome 13 [14]. *Tbce* mRNA is present in neurons in the spinal cord. Degenerative changes are conspicuous in motor axons, and ultrastructural studies of peripheral nerves of *PMN* mice disclose reduced numbers of microtubules in these axons. Mutations of the highly conserved Trp524 residue, which appears to influence protein stability, are believed to impact on the biology of tubulin in axons. Transgenic complementation restores the *PMN* mouse line to a normal phenotype [14]. *Tbce* is an ortholog of human *TBCE* and mutations in the human gene are associated with two multiorgan system degeneration syndromes, which are distinct from PMN [14].

A variety of experimental therapeutic strategies have been tested in mutant *SOD1* mice. Lines of mutant *SOD1* mice have been used for pharmacological and 'genetic' therapeutic trials [10, 21, 22, 25, 137–139]. Selected examples of these approaches are briefly described below.

Results obtained during several drug trials have been less than encouraging (vitamin E, selenium, riluzole, gabapentin and D-penicillamine, a copper chelator). Treatment with creatine appear to have modest benefits [138]. Although oral administration of creatine to G93A *SOD1* mice results in a dose-dependent improvement in motor tasks and extended survival, a creatine trial in humans showed no beneficial effects compared to placebo [140]. Minocycline, a second generation tetracycline antimicrobial and anti-inflammatory, which acts as a neuroprotectant by upstream inhibition of the activities of caspase-1, caspase-3, inducible NOS matrix metalloproteinases and p38 mitogen-activated protein kinase (MAPK) and, by reducing cytochrome *c* release, also had a modest effect (i.e. delayed disease onset and prolonged survival) [23, 63]. Because some evidence suggests that several pathogenic pathways converge in ALS, investigators have also turned to combination treatments. Mutant mice received minocycline (which inhibits microglial activation and the pathways described above), riluzole (a glutamate antagonist used to treat patients with ALS) and nimodipine (a voltage-gated calcium channel blocker), beginning in the late preclinical stage. This dietary regimen delays onset, slows progression of weakness and increases survival [22]. Inhibition of inhibitors of intracellular-1β-converting enzyme (ICE) also slows progress of disease in mutant *SOD1* mice; in these studies, a mouse line with a dominant negative mutation of ICE was crossed to mutant *SOD1* mice. The outcome of the mating results in a modest increase in survival [63].

The reasons that outcome studies in mutant mice may not necessarily translate to benefits for humans with ALS are discussed in a recent editorial [21].

As indicated in the discussion of axotomy models, the beneficial influences of trophic factors on motor neurons provide potential therapeutic opportunities. Although previous human trials have been disappointing (for review see [111, 112]), adenoassociated viral (AAv) delivery of insulin-like growth factor (IGF)-1 to muscle prolongs survival in mutant *SOD1* mice [111]. Thus, following intramuscular injection, the retrograde transport of AAV-IGF-1 appears to provide trophic influences to motor neurons.

To test whether increasing or decreasing SOD1 has an impact on disease in the mutant *SOD1* mice, these animals were mated with wild-type *SOD1* or $SOD1^{-/-}$ mice.

Neither elevations nor reductions of SOD1 influence the clinical course or the character of pathology [73].

The molecular mechanisms whereby mutant SOD1 causes selective motor neuron death have not yet been defined. It has been hypothesized that the toxic property of mutant SOD1 may be related to mutation-induced conformational changes in SOD1 that result in aberrant oxidative activities [141]. In this scenario, cell dysfunction and death could be initiated by aberrant oxidative chemistries catalyzed by the copper bound in the active site of mutant SOD1 [141]. Copper within the active site of SOD1 is essential for dismutase activity [16]. However, copper is extremely toxic and there is virtually no free intracellular copper [142, 143]. Instead, copper is delivered to specific proteins by copper chaperones [142, 143]. The copper chaperone for SOD1, termed CCS in mammals and Lys 7 in yeast (where it was discovered), is present within nerve cells, including neurons and astrocytes [144]. CCS binds to SOD1 [142] and delivers copper to the dismutase, where it is bound by specific histidine residues. To determine the role of CCS in copper homeostasis in the nervous system of mammals, mice were generated with targeted disruption of *CCS* alleles ($CCS^{-/-}$ mice). Although $CCS^{-/-}$ mice are viable and possess normal levels of SOD1, the levels of SOD1 activity are markedly reduced as compared to values of control littermates. Metabolic labeling with copper-64 demonstrates that the reduction of SOD1 activity in $CCS^{-/-}$ mice is the result of impaired copper incorporation into SOD1; this effect is specific because no abnormalities are observed in copper uptake, distributions, or incorporation into other cuproenzymes. Consistent with this loss of SOD1 activity, $CCS^{-/-}$ mice exhibit increased sensitivity to paraquat and reduced fertility in females, phenotypes that are characteristic of $SOD1^{-/-}$ mice. Moreover, in $CCS^{-/-}$ mice, as in $SOD1^{-/-}$ mice, motor neurons are more vulnerable to axotomy.

The hypothesis that the toxicity of mutant SOD1 involves aberrant copper chemistry bound to SOD1 has been tested by two approaches: in one set of experiments, mutant *SOD1* mice were crossed to $CCS^{-/-}$ mice (thus preventing delivery of copper to SOD1) [16]; in a second set of experiments, mutant *SOD1* mice also carried mutations in the histidine residues (H46R/H48Q) that disrupt the copper binding site [126]. The $CCS^{-/-}$ mutant *SOD1* mice possess virtually no copper-loaded SOD1 or SOD1 activity [16]. Significantly, these mice exhibit no differences in the onset or progression of disease as compared to mutant *SOD1* mice [16]. Similarly, the mutant *SOD1* mice with the mutated copper binding sites develop pathological features of disease including (fibrillar inclusions) identical to those occurring in mutant *SOD1* mice with the normal histidine residues at these sites [126]. Thus, copper bound within the active site of the enzyme is not essential for mutant SOD1-mediated toxicity. Although proponents of aberrant copper chemistry hypothesis suggest that copper bound elsewhere on SOD1 could be involved in such reactions, the results of these studies are more consistent with the idea that the effects of improper folding/aggregation of mutant SOD1 is a critical feature of disease [126].

To test the role of NF in mutant *SOD1* mice, the latter animals were crossbred to several lines of mice that have altered distributions of NF. The progeny of mutant *SOD1* mice crossed to mice expressing an NF-H-β-galactosidase fusion protein (NF-H-lacZ) [118], which crosslinks NF and prevents their export to axons, show no effect on disease progression. In contrast, mating with $NF\text{-}L^{-/-}$ mice increases the lifespan of mutant *SOD1* mice [145]. However, crosses with mice overexpressing either wild type NF-H or wild type NF-L are associated with slowing of disease progression, increased lifespan and relative sparing of motor neurons [139, 146]. It has been suggested that the common properties shared by these various lines of transgenic mice are the reduced content of assembled NF in the axonal compartment and an increase in NF proteins in cell bodies. Given the varied results, it is uncertain as to the ways by which the distributions of NF influence the mutant *SOD1* phenotype. Some of the observed effects may be related to strains of mice used in the experiments.

Vascular endothelial growth factor (VEGF) (*VEGF*; GenBank number NM 003376) influences the growth and permeability of blood vessels during development and during the response to altered metabolic demands [147]. Importantly, VEGF stimulates survival of motor neurons under certain conditions of stress. Mice homozygous for a targeted disruption of the hypoxia response element (HRE) within the VEGF promoter ($VEGF^{\delta/\delta}$) develop an adult onset progressive MND with clinical and pathological features reminiscent of ALS [15, 69]. $VEGF^{\delta/\delta}$ mice show reduced lower basal levels of VEGF and are unable to regulate VEGF levels in response to tissue hypoxia. It has been hypothesized that reduced perfusion of neurons results in chronic ischemia and/or the loss of VEGF-mediated neuroprotection, and that the impaired response may lead to the degeneration of nerve cells [75, 148]. An inability to regulate levels of VEGF has been reported in mutant *SOD1* mice, and the progeny of crosses of $VEGF^{\delta/\delta}$ mice and mutant *SOD1* mice do not survive as well as mice with mutant *SOD1* alone. Finally, it has been reported that *VEGF* may act as a modifier gene for cases of human ALS [15].

It is not known why mutations in SOD1, an ubiquitously expressed protein, cause selective dysfunction and death of motor neurons. Mutations in *SOD1* do not have obvious impacts on many other cells in CNS or cells in other organs other than possibly skeletal muscle. The question of whether the toxicity of mutant SOD1 is cell autonomous has been tested by selectively expressing mutant *SOD1* in different cell types. Neither restricted expression of mutant *SOD1* to neurons or to astrocytes appears to be sufficient to produce disease [17]. Complex experiments in chimeric mice (with different mixtures of wild-type or mutant SOD1-expressing cells) disclose that motor neuron toxicity can be influenced by expression of wild-type or mutant SOD1 in glial cells. For example,

in the chimeric mice, normal motor neurons develop abnormalities interpreted to be related to the presence of mutant SOD1 in non-neuronal cells. Moreover, when motor neurons accumulate mutant SOD1, the presence of a higher proportion of wild-type SOD1 in non-neuronal cells reduces the incidence of motor neuron death and prolong survival in the chimeric mice. These observations suggest that disease may be result of different populations of cells acting in concert.

It has been hypothesized that individuals with ALS have problems with excessive glutamate at synapses and excitotoxicity related to reduced levels of the glutamate transporter GLT1 (EAAT2) [25, 48, 49]. Using a screen of more than 1000 FDA-approved compounds, investigators discovered that β-lactam antibiotics can stimulate expression of GLT1, via increased transcription of the *GLT1* gene. When animals were treated with the β-lactam ceftriaxone, the levels of GLT1 in the brain increased, as did the biochemical and functional activity parameters of this transporter [24]. Significantly, this antibiotic, which is neuroprotective *in vitro* for models of ischemia and neuronal degeneration, delayed the pathology and increased survival in mutant *SOD1* mice [24].

Mouse models offer opportunities for discovering disease mechanisms and for testing novel treatment strategies. Riluzole is the major drug approved for therapy in human ALS [3, 25]. However, the benefits are modest. Investigators are examining other ways to ameliorate excitotoxicity, possibly by influencing the levels or activity of GLT-1 [24]. Trials with growth factors were truncated because of evidence of complications [25]. Trials of antiaggregation strategies, novel ways to deliver trophic factors, inhibitors of apoptosis, treatment with gene therapy and the use of engineered cells or stem cells are some of the therapeutic approaches for the future [1, 3, 17, 18, 21, 25, 30, 57, 63, 70, 111, 126, 147, 149, 150].

The identification of genes mutated or deleted in the inherited forms of neurodegenerative diseases, including MND, has allowed investigators to create genetically engineered models of these illnesses [1, 3, 17, 18, 21, 30, 57, 63, 70, 111, 126, 147, 149, 150]. Investigations of these mouse models have greatly increased our understanding of the molecular mechanisms of neurodegenerative diseases critical for identifying potential therapeutic targets. These models are of great value for testing a variety of novel therapeutic approaches. Investigations of the pathogeneses of the neurodegenerative diseases, have made spectacular progress over the past few years. We anticipate that knowledge of disease mechanisms will lead to novel treatments for these devastating illnesses.

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REFERENCES

1. Wong, P. C., Cai, H., Borchelt, D. R. and Price, D. L. Genetically engineered mouse models of neurodegenerative diseases. *Nat. Neurosci.* 5, 633–639, 2002.
2. Rowland, L. P. and Shneider, N. A. Amyotrophic lateral sclerosis. *N. Engl. J. Med.* 344, 1688–1700, 2001.
3. Bruijn, L. I., Miller, T. M. and Cleveland, D. W. Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annu. Rev. Neurosci.* 27: 723–749, 2004.
4. Price, D. L., Wong, P. C., Borchelt, D. R., Koliatsos, V. E. and Rothstein, J. D. In C. M. Clark and J. Q. Trojanoswki (eds), *Neurodegenerative Dementias.* New York: McGraw-Hill, 2000, pp. 373–385.
5. Griffin, J. W. and Price, D. L. In J. Andrews and R. Johnson (eds), *Amyotrophic Lateral Sclerosis: Recent Research Trends.* New York: Academic Press, 1976, pp. 33–67.
6. Rosen, D. R., Siddique, T., Patterson, D. *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362: 59–62, 1993.
7. Puls, I., Jonnakuty, C., LaMonte, B. H. *et al.* Mutant dynactin in motor neuron disease. *Nat. Genet.* 33: 455–456, 2003.
8. Anderson, K. and Talbot, K. Spinal muscular atrophies reveal motor neuron vulnerability to defects in ribonucleoprotein handling. *Curr. Opin. Neurol.* 16: 595–599, 2003.
9. Borchelt, D. R., Lee, M. K., Slunt, H. H. *et al.* Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc. Natl Acad. Sci. U.S.A.* 91: 8292–8296, 1994.
10. Julien, J.-P. Amyotrophic lateral sclerosis: unfolding the toxicity of the misfolded. *Cell* 104: 581–591, 2001.
11. Wong, P. C., Pardo, C. A., Borchelt, D. R. *et al.* An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 14: 1105–1116, 1995.
12. Bruijn, L. I., Becher, M. W., Lee, M. K. *et al.* ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18: 327–338, 1997.
13. Dal Canto, M. C. and Gurney, M. E. Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. *Am. J. Pathol.* 145: 1271–1280, 1994.
14. Martin, N., Jaubert, J., Gounon, P. *et al.* A missense mutation in Tbce causes progressive motor neuronopathy in mice. *Nat. Genet.* 32: 443–447, 2002.
15. Lambrechts, D., Storkebaum, E., Morimoto, M. *et al.* VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat. Genet.* 34: 383–394, 2003.
16. Subramaniam, J. R., Lyons, W. E., Liu, J. *et al.* Mutant SOD1 causes motor neuron disease independent of copper chaperone- mediated copper loading. *Nat. Neurosci.* 5: 301–307, 2002.

17. Clement, A. M., Nguyen, M. D., Roberts, E. A. *et al.* Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 302: 113–117, 2003.
18. Koliatsos, V. E., Clatterbuck, R. E., Winslow, J. W., Cayouette, M. H. and Price, D. L. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons *in vivo*. *Neuron* 10: 359–367, 1993.
19. Henderson, C. E., Phillips, H. S., Pollock, R. A. *et al.* GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* 266: 1062–1064, 1994.
20. Koliatsos, V. E., Cayouette, M. H., Berkemeier, L. R., Clatterbuck, R. E., Price, D. L. and Rosenthal, A. Neurotrophin 4/5 is a trophic factor for mammalian facial motor neurons. *Proc. Natl Acad. Sci. U.S.A.* 91: 3304–3308, 1994.
21. Rothstein, J. D. Of mice and men: reconciling preclinical ALS mouse studies and human clinical trials. *Ann. Neurol.* 53: 423–426, 2003.
22. Kriz, J., Gowing, G. and Julien, J. P. Efficient three-drug cocktail for disease induced by mutant superoxide dismutase. *Ann. Neurol.* 53: 429–436, 2003.
23. Zhu, S., Stavrovskaya, I. G., Drozda, M. *et al.* Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* 417: 74–78, 2002.
24. Rothstein, J. D., Patel, S., Regan, M. R. *et al.* Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* 433: 73–77, 2005.
25. Cleveland, D. W. and Rothstein, J. D. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat. Rev. Neurosci.* 2: 806–819, 2001.
26. Wong, P. C., Rothstein, J. D. and Price, D. L. The genetic and molecular mechanisms of motor neuron disease. *Curr. Opin. Neurobiol.* 8: 791–799, 1998.
27. Le, T. T., Pham, L. T., Butchbach, M. E. *et al.* SMNΔ7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum. Mol. Genet.* 14: 845–857, 2005.
28. Ince, P. G. In R. H. Brown Jr, V. Meininger and M. Swash (eds), *Amyotrophic Lateral Sclerosis*. London: Martin Dunitz, 2000, pp. 83–112.
29. Martin, L. J. and Liu, Z. Injury-induced spinal motor neuron apoptosis is preceded by DNA single- strand breaks and is p53- and Bax-dependent. *J. Neurobiol.* 50: 181–197, 2002.
30. Martin, L. J., Liu, Z., Troncoso, J. C. and Price, D. L. In M. Holcik, E. LaCasse, R. Korneluk and A. MacKenzie (eds), *Apoptosis in Health and Disease*. Cambridge: Cambridge University Press, 2005, pp. 242–315.
31. Carpenter, S. Proximal axonal enlargement in motor neuron disease. *Neurology* 18: 841–851, 1968.
32. Delisle, M. B. and Carpenter, S. Neurofibrillary axonal swellings and amyotrophic lateral sclerosis. *J. Neurol. Sci.* 63: 241–250, 1984.
33. Clark, A. W., Parhad, I. M., Griffin, J. W. and Price, D. L. Neurofilamentous axonal swellings as a normal finding in the spinal anterior horn of man and other primates. *J. Neuropathol. Exp. Neurol.* 43: 253–262, 1984.
34. Cork, L. C., Griffin, J. W., Munnell, J. F., Lorenz, M. D., Adams, R. J. and Price, D. L. Hereditary canine spinal muscular atrophy. *J. Neuropathol. Exp. Neurol.* 38, 209–221, 1979.
35. Cork, L. C., Griffin, J. W., Adams, R. J. and Price, D. L. Motor neuron disease: spinal muscular atrophy and amyotrophic lateral sclerosis. Animal model: hereditary canine spinal muscular atrophy. *Am. J. Pathol.* 100: 599–602, 1980.
36. Cork, L. C., Griffin, J. W., Choy, C., Padula, C. A. and Price, D. L. Pathology of motor neurons in accelerated Hereditary Canine Spinal Muscular Atrophy. *Lab. Invest.* 46: 89–99, 1982.
37. Troncoso, J. C., Price, D. L., Griffin, J. W. and Parhad, I. M. Neurofibrillary axonal pathology in aluminum intoxication. *Ann. Neurol.* 12: 278–283, 1982.
38. Griffin, J. W., Hoffman, P. N., Clark, A. W., Carroll, P. T. and Price, D. L. Slow axonal transport of neurofilament proteins: impairment by β,β'-iminodipropionitrile administration. *Science* 202: 633–635, 1978.
39. Griffin, J. W., Fahnestock, K. E., Price, D. L. and Hoffman, P. N. Microtubule-neurofilament segregation produced by β,β′- iminodipropionitrile: evidence for the association of fast axonal transport with microtubules. *J. Neurosci.* 3: 557–566, 1983.
40. Griffin, J. W., Fahnestock, K. E., Price, D. L. and Cork, L. C. Cytoskeletal disorganization induced by local application of β,β′-iminodipropionitrile and 2,5-hexanedione. *Ann. Neurol.* 14: 55–61, 1983.
41. Gold, B. G., Griffin, J. W. and Price, D. L. Slow axonal transport in acrylamide neuropathy: different abnormalities produced by single-dose and continuous administration. *J. Neurosci.* 5: 1755–1768, 1985.
42. Troncoso, J. C., Hoffman, P. N., Griffin, J. W., Hess-Kozlow, K. M. and Price, D. L. Aluminum intoxication: a disorder of neurofilament transport in motor neurons. *Brain Res.* 342: 172–175, 1985.
43. Griffin, J. W., Price, D. L. and Drachman, D. B. Impaired axonal regeneration in acrylamide intoxication. *J. Neurobiol.* 8: 355–370, 1977.
44. Clatterbuck, R. E., Price, D. L. and Koliatsos, V. E. Further characterization of the effects of brain-derived neurotrophic factor and ciliary neurotrophic factor on axotomized neonatal and adult mammalian motor neurons. *J. Comp Neurol.* 342: 45–56, 1994.
45. Martin, L. J. Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism. *J. Neuropathol. Exp. Neurol.* 58: 459–471, 1999.
46. Sathasivam, S., Ince, P. G. and Shaw, P. J. Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. *Neuropathol. Appl. Neurobiol.* 27: 257–274, 2001.
47. Guegan, C., Vila, M., Rosoklija, G., Hays, A. P. and Przedborski, S. Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. *J. Neurosci.* 21: 6569–6576, 2001.
48. Rothstein, J. D., Tsai, G., Kuncl, R. W. *et al.* Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. *Ann. Neurol.* 28: 18–25, 1990.
49. Rothstein, J. D., Van Kammen, M., Levey, A. I., Martin, L. J. and Kuncl, R. W. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann. Neurol.* 38: 73–84, 1995.
50. Waldmeier, P. C. Prospects for antiapoptotic drug therapy of neurodegenerative diseases. *Prog. Neuropsychopharmacol. Biol. Psychiatr.* 27: 303–321, 2003.

51. Yuan, J. and Yankner, B. A. Apoptosis in the nervous system. *Nature* 407: 802–809, 2000.
52. Yuan, J., Lipinski, M. and Degterev, A. Diversity in the mechanisms of neuronal cell death. *Neuron* 40: 401–413, 2003.
53. Mattson, M. Apoptosis in neurodegenerative disorders. *Nature* 1: 120–129, 2001.
54. Raoul, C., Estevez, A. G., Nishimune, H. *et al.* Motoneuron death triggered by a specific pathway downstream of Fas. potentiation by ALS-linked SOD1 mutations. *Neuron* 35: 1067–1083, 2002.
55. Martin, L. J., Price, A. C., Kaiser, A., Shaikh, A. Y. and Liu, Z. Mechanisms for neuronal degeneration in amyotrophic lateral sclerosis and in models of motor neuron death. *Int. J. Mol. Med.* 5: 3–13, 2000.
56. Nijhawan, D., Honarpour, N. and Wang, X. Apoptosis in neural development and disease. *Annu. Rev. Neurosci.* 23: 73–87, 2000.
57. Martin, L. J. and Liu, Z. Opportunities for neuroprotection in ALS using cell dealth mechanism rationales. *Drug Discov. Today* 1: 135–143, 2004.
58. Li, M., Ona, V. O., Guegan, C. *et al.* Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* 288: 335–339, 2000.
59. Kuntz, C., Kinoshita, Y., Beal, M. F., Donehower, L. A. and Morrison, R. S. Absence of p53: no effect in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Exp. Neurol.* 165: 184–190, 2000.
60. Prudlo, J., Koenig, J., Graser, J. *et al.* Motor neuron cell death in a mouse model of FALS is not mediated by the p53 cell survival regulator. *Brain Res.* 879: 183–187, 2000.
61. Bendotti, C., Calvaresi, N., Chiveri, L. *et al.* Early vacuolization and mitochondrial damage in motor neurons of FALS mice are not associated with apoptosis or with changes in cytochrome oxidase histochemical reactivity. *J. Neurol. Sci.* 191: 25–33, 2001.
62. Kostic, V., Jackson-Lewis, V., de Bilbao, F., Dubois-Dauphin, M. and Przedborski, S. Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* 277: 559–562, 1997.
63. Friedlander, R. M., Brown, R. H., Gagliardini, V., Wang, J. and Yuan, J. Inhibition of ICE slows ALS in mice. *Nature* 388: 31, 1997.
64. Munch, C., Sedlmeier, R., Meyer, T. *et al.* Point mutations of the p150 subunit of dynactin (DCTN1) gene in ALS. *Neurology* 63: 724–726, 2004.
65. Hadano, S., Hand, C. K., Osuga, H. *et al.* A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. *Nat. Genet.* 29: 166–173, 2001.
66. Yang, Y., Hentati, A., Deng, H.-X. *et al.* The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nat. Genet.* 29: 160–165, 2001.
67. Chen, Y. Z., Bennett, C. L., Huynh, H. M. *et al.* DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am. J. Hum. Genet.* 74: 1128–1135, 2004.
68. Moreira, M. C., Klur, S., Watanabe, M. *et al.* Senataxin, the ortholog of a yeast RNA helicase, is mutant in ataxia-ocular apraxia 2. *Nat. Genet.* 36: 225–227, 2004.
69. Oosthuyse, B., Moons, L., Storkebaum, E. *et al.* Deletion of the hypoxia-response element in the vascular endothelial growth factor promotor causes motor neuron degeneration. *Nat. Genet.* 28: 131–138, 2001.
70. Cleveland, J. L. A new piece of the ALS puzzle. *Nat. Genet.* 34: 357–358, 2003.
71. Bowling, A. C., Barkowski, E. E., McKenna-Yasek, D. *et al.* Superoxide dismutase concentration and activity in familial amyotrophic lateral sclerosis. *J. Neurochem.* 64: 2366–2369, 1995.
72. Reaume, A. G., Elliott, J. L., Hoffman, E. K. *et al.* Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.* 13: 43–47, 1996.
73. Bruijn, L. I., Houseweart, M. K., Kato, S. *et al.* Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science* 281: 1851–1854, 1998.
74. Yamanaka, K., Vande, V. C., Eymard-Pierre, E., Bertini, E., Boespflug-Tanguy, O. and Cleveland, D. W. Unstable mutants in the peripheral endosomal membrane component ALS2 cause early-onset motor neuron disease. *Proc. Natl Acad. Sci. U.S.A.* 100: 16041–16046, 2003.
75. Rabin, B. A., Griffin, J. W., Crain, B. J., Scavina, M., Chance, P. F. and Cornblath, D. R. Autosomal dominant juvenile amyotrophic lateral sclerosis. *Brain* 122: 1539–1550, 1999.
76. Seeburg, P. H. RNA helicase participates in the editing game. *Neuron* 25: 261–263, 2000.
77. Tanner, N. K. and Linder, P. DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Mol. Cell* 8: 251–262, 2001.
78. Caruthers, J. M. and McKay, D. B. Helicase structure and mechanism. *Curr. Opin. Struct. Biol.* 12: 123–133, 2002.
79. Grohmann, K., Varon, R., Stolz, P. *et al.* Infantile spinal muscular atrophy with respiratory distress type 1 (SMARD1). *Ann. Neurol.* 54: 719–724, 2003.
80. Grohmann, K., Schuelke, M., Diers, A. *et al.* Mutations in the gene encoding immunoglobulin mu-binding protein 2 cause spinal muscular atrophy with respiratory distress type 1. *Nat. Genet.* 29: 75–77, 2001.
81. Goldstein, L. S. B. and Yang, Z. Microtubule-based transport systems in neurons: the roles of kinesins and dyneins. *Annu. Rev. Neurosci.* 23: 39–71, 2000.
82. Schroer, T. A. Dynactin. *Annu. Rev. Cell Dev. Biol.* 20: 759–779, 2004.
83. Vallee, R. B., Williams, J. C., Varma, D. and Barnhart, L. E. Dynein: an ancient motor protein involved in multiple modes of transport. *J. Neurobiol.* 58: 189–200, 2004.
84. Price, D. L. and Porter, K. R. The response of ventral horn neurons to axonal transection. *J. Cell Biol.* 53: 24–37, 1972.
85. Price, D. L. The response of amphibian glial cells to axonal transection. *J. Neuropathol. Exp. Neurol.* 31: 267–277, 1972.
86. Griffin, J. W., Price, D. L., Engel, W. K. and Drachman, D. B. The pathogenesis of reactive axonal swellings: role of axonal transport. *J. Neuropathol. Exp. Neurol.* 36: 214–227, 1977.
87. Koo, E. H., Hoffman, P. N. and Price, D. L. Levels of neurotransmitter and cytoskeletal mRNAs during nerve regeneration in sympathetic ganglia. *Brain Res.* 449: 361–363, 1988.

88. Hoffman, P. N., Cleveland, D. W., Griffin, J. W., Landes, P. W., Cowan, N. J. and Price, D. L. Neurofilament gene expression: a major determinant of axonal caliber. *Proc. Natl Acad. Sci. U.S.A.* 84: 3472–3476, 1987.
89. Muma, N. A., Hoffman, P. N., Slunt, H. H., Applegate, M. D., Lieberburg, I. and Price, D. L. Alterations in levels of mRNA coding for neurofilament protein subunits during regeneration. *Exp. Neurol.* 107: 230–235, 1990.
90. Rosenfeld, J., Dorman, M. E., Griffin, J. W. *et al.* Distribution of neurofilament antigens after axonal injury. *J. Neuropathol. Exp. Neurol.* 46: 269–282, 1987.
91. Hoffman, P. N., Thompson, G. W., Griffin, J. W. and Price, D. L. Changes in neurofilament transport coincide temporally with alterations in the caliber of axons in regenerating motor fibers. *J. Cell Biol.* 101: 1332–1340, 1985.
92. Hoffman, P. N., Griffin, J. W., Gold, B. G. and Price, D. L. Slowing of neurofilament transport and the radial growth of developing nerve fibers. *J. Neurosci.* 5: 2920–2929, 1985.
93. Griffin, J. W., Price, D. L., Drachman, D. B. and Morris, J. Incorporation of axonally transported glycoproteins into axolemma during nerve regeneration. *J. Cell Biol.* 88, 205–214, 1981.
94. Yan, Q., Elliott, J. and Snider, W. D. Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* 360: 753–755, 1992.
95. Hoffman, P. N., Griffin, J. W. and Price, D. L. Control of axonal caliber by neurofilament transport. *J. Cell Biol.* 99: 705–714, 1984.
96. Tierney, M. C., Fisher, R. H., Lewis, A. J. *et al.* The NINCDS-ADRDA Work Group criteria for the clinical diagnosis of probable Alzheimer's disease: a clinicopathologic study of 57 cases. *Neurology* 38: 359–364, 1988.
97. Koliatsos, V. E., Crawford, T. O. and Price, D. L. Axotomy induces nerve growth factor receptor immunoreactivity in spinal motor neurons. *Brain Res.* 549: 297–304, 1991.
98. Griffin, J. W., Drachman, D. B. and Price, D. L. Fast axonal transport in motor nerve regeneration. *J. Neurobiol.* 7: 355–370, 1976.
99. Ehlers, M. D., Kaplan, D. R., Price, D. L. and Koliatsos, V. E. NGF-stimulated retrograde transport of trkA in the mammalian nervous system. *J. Cell Biol.* 130: 149–156, 1995.
100. Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H. and Barde, Y. A. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature* 360: 757–759, 1992.
101. Oppenheim, R. W., Houenou, L. J., Johnson, J. E. *et al.* Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* 373: 344–346, 1995.
102. Elliott, J. L. and Snider, W. D. Motor neuron growth factors. *Neurology* 47, S47–S53, 1996.
103. Yuen, E. C. and Mobley, W. C. Therapeutic potential of neurotrophic factors for neurological disorders. *Ann. Neurol.* 40: 346–354, 1996.
104. Clark, A. W., Griffin, J. W. and Price, D. L. The axonal pathology in chronic IDPN intoxication. *J. Neuropathol. Exp. Neurol.* 39: 42–55, 1980.
105. Griffin, J. W. and Price, D. L. Demyelination in experimental IDPN and hexacarbon neuropathies: evidence for an axonal influence. *Lab. Invest.* 45: 130–141, 1981.
106. Griffin, J. W. and Price, D. L. Schwann cell and glial responses in β,β′-iminodipropionitrile intoxication. I. Schwann cell and oligodendrocyte ingrowths. *J. Neurocytol.* 10: 995–1007, 1981.
107. Price, D. L., Griffin, J., Young, A., Peck, K. and Stocks, A. Tetanus toxin: direct evidence for retrograde intraaxonal transport. *Science* 188: 945–947, 1975.
108. Goldstein, L. S. Do disorders of movement cause movement disorders and dementia? *Neuron* 40: 415–425, 2003.
109. Morfini, G., Pigino, G. and Brady, S. T. Polyglutamine expansion diseases: failing to deliver. *Trends Mol. Med.* 11: 64–70, 2005.
110. Raff, M. C., Whitmore, A. V. and Finn, J. T. Axonal self-destruction and neurodegeneration. *Science* 296: 868–871, 2002.
111. Kaspar, B. K., Llado, J., Sherkat, N., Rothstein, J. D. and Gage, F. H. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science* 301: 839–842, 2003.
112. Boillee, S. and Cleveland, D. W. Gene therapy for ALS delivers. *Trends Neurosci.* 27: 235–238, 2004.
113. Lorenz, M. D., Cork, L. C., Griffin, J. W., Adams, R. J. and Price, D. L. Hereditary spinal muscular atrophy in Brittany spaniels: clinical manifestations. *J. Am. Vet. Med. Assoc.* 175: 833–839, 1979.
114. Sack, G. H. Jr, Cork, L. C., Morris, J. M., Griffin, J. W. and Price, D. L. Autosomal dominant inheritance of Hereditary Canine Spinal Muscular Atrophy. *Ann. Neurol.* 15: 369–373, 1984.
115. Lee, M. K., Xu, Z., Wong, P. C. and Cleveland, D. W. Neurofilaments are obligate heteropolymers *in vivo*. *J. Cell Biol.* 122: 1337–1350, 1993.
116. Ching, G. Y. and Liem, R. K. Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *J. Cell Biol.* 122: 1323–1335, 1993.
117. Lee, M. K. and Cleveland, D. W. Neurofilament function and dysfunction: involvement in axonal growth and neuronal disease. *Curr. Opin. Cell Biol.* 6: 34–40, 1994.
118. Eyer, J. and Peterson, A. Neurofilament-deficient axons and perikaryal aggregates in viable transgenic mice expressing a neurofilament-β-galactosidase fusion protein. *Neuron* 12: 389–405, 1994.
119. Monteiro, M. J., Hoffman, P. N., Gearhart, J. D. and Cleveland, D. W. Expression of NF-L in both neuronal and nonneuronal cells of transgenic mice: increased neurofilament density in axons without affecting caliber. *J. Cell Biol.* 111: 1543–1557, 1990.
120. Xu, Z., Cork, L. C., Griffin, J. W. and Cleveland, D. W. Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. *Cell* 73: 23–33, 1993.
121. Collard, J.-F., Côté, F. and Julien, J.-P. Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* 375: 61–64, 1995.
122. Lee, M. K., Marszalek, J. R. and Cleveland, D. W. A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* 13: 975–988, 1994.
123. Pardo, C. A., Xu, Z., Borchelt, D. R., Price, D. L., Sisodia, S. S. and Cleveland, D. W. Superoxide dismutase is an abundant component in cell bodies, dendrites, and axons of motor

neurons and in a subset of other neurons. *Proc. Natl Acad. Sci. U.S.A.* 92: 954–958, 1995.
124. Gurney, M. E., Pu, H., Chiu, A. Y. *et al.* Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264: 1772–1775, 1994.
125. Wang, J., Xu, G. and Borchelt, D. R. High molecular weight complexes of mutant superoxide dismutase 1: age- dependent and tissue-specific accumulation. *Neurobiol. Dis* 9: 139–148, 2002.
126. Wang, J., Xu, G., Gonzales, V. *et al.* Fibrillar inclusions and motor neuron degeneration in transgenic mice expressing superoxide dismutase 1 with a disrupted copper-binding site. *Neurobiol. Dis* 10: 128–138, 2002.
127. Turner, B. J., Lopes, E. C. and Cheema, S. S. Neuromuscular accumulation of mutant superoxide dismutase 1 aggregates in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Neurosci. Lett.* 350: 132–136, 2003.
128. Bruijn, L. I., Beal, M. F., Becher, M. W. *et al.* Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant *in vivo* property of one familial ALS-linked superoxide dismutase 1 mutant. *Proc. Natl Acad. Sci. U.S.A.* 94: 7606–7611, 1997.
129. Watanabe, M., Dykes-Hoberg, M., Culotta, V. C., Price, D. L., Wong, P. C. and Rothstein, J. D. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic laterlal sclerosis neural tissues. *Neurobiology of Disease* 8: 933–941, 2001.
130. Borchelt, D. R., Wong, P. C., Becher, M. W. *et al.* Axonal transport of mutant superoxide dismutase 1 and focal axonal abnormalities in the proximal axons of transgenic mice. *Neurobiol. Dis.* 5: 27–35, 1998.
131. Williamson, T. L. and Cleveland, D. W. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat. Neurosci.* 2: 50–56, 1999.
132. Kang, S. J., Sanchez, I., Jing, N. and Yuan, J. Dissociation between neurodegeneration and caspase-11-mediated activation of caspase-1 and caspase-3 in a mouse model of amyotrophic lateral sclerosis. *J. Neurosci.* 23: 5455–5460, 2003.
133. Terada, S. and Hirokawa, K. Moving on to the cargo problem of microtubule-dependent motors in neurons. *Curr. Opin. Neurobiol.* 10: 566–573, 2000.
134. LaMonte, B. H., Wallace, K. E., Holloway, B. A. *et al.* Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron* 34: 715–727, 2002.
135. Hafezparast, M., Klocke, R., Ruhrberg, C. *et al.* Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science* 300: 808–812, 2003.
136. Schmalbruch, H., Jensen, H.-J. S., Bjærg, M., Kamieniecka, Z. and Kurland, L. A new mouse mutant with progressive motor neuronopathy. *J. Neuropathol. Exp. Neurol.* 50, 192–204, 1991.
137. Pasinelli, P., Borchelt, D. R., Houseweart, M. K., Cleveland, D. W. and Brown, R. H. Caspase-1 is activated in neural cells and tissue with amyotrophic lateral sclerosis-associated mutations in copper-zinc superoxide dismutase. *Neurobiology* 95: 15763–15768, 1998.
138. Klivenyi, P., Ferrante, R. J., Matthews, R. T. *et al.* Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat. Med.* 5: 347–350, 1999.
139. Couillard-Despres, S., Zhu, Q., Wong, P. C., Price, D. L., Cleveland, D. W. and Julien, J. P. Protective effect of neurofilament heavy gene overexpression in motor neuron disease induced by mutant superoxide dismutase. *Proc. Natl Acad. Sci. U.S.A.* 95: 9626–9630, 1998.
140. Groeneveld, G. J., Veldink, J. H., van der Tweel, I. *et al.* A randomized sequential trial of creatine in amyotrophic lateral sclerosis. *Ann. Neurol.* 53: 437–445, 2003.
141. Estévez, A. G., Crow, J. P., Sampson, J. B. *et al.* Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. *Science* 286: 2498–2500, 1999.
142. Schmidt, P. J., Rae, T. D., Pufahl, R. A. *et al.* Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. *J. Biol. Chem.* 274, 1999.
143. Finney, L. A. and O'Halloran, T. V. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* 300: 931–936, 2003.
144. Rothstein, J. D., Dykes-Hoberg, M., Corson, L. B. *et al.* The copper chaperone CCS is abundant in neurons and astrocytes in human and rodent brain. *J. Neurochem.* 72: 422–429, 1999.
145. Williamson, T. L., Bruijn, L. I., Zhu, Q. *et al.* Absence of neurofilaments reduces the selective vulnerability of motor neurons and slows disease caused by a familial amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutant. *Proc. Natl Acad. Sci. U.S.A.* 95: 9631–9636, 1998.
146. Kong, J. and Xu, Z. Overexpression of neurofilament subunit NF-L and NF-H extends survival of a mouse model for amyotrophic lateral sclerosis. *Neurosci. Lett.* 281: 72–74, 2000.
147. Carmeliet, P. and Storkebaum, E. Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Semin. Cell Dev. Biol.* 13: 39–53, 2002.
148. Skene, J. P. and Cleveland, D. W. Hypoxia and Lou Gehrig. *Nat. Genet.* 28: 107–108, 2001.
149. Dopkins, S., Kovner, R., Rich, J. B. and Brandt, J. Access to information about famous individuals in Alzheimer's disease. *Cortex* 33: 333–339, 1997.
150. Yin, C., Knudson, C. M., Korsmeyer, S. J. and Van Dyke, T. Bax suppressestumorigenesis and stimulates apoptosis *in vivo*. *Nature* 385: 637–640, 1997.

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CHAPTER

45

Neurodegenerative α-Synucleinopathies and Tauopathies

Michel Goedert

Maria Grazia Spillantini

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Current concepts of neurodegenerative diseases began to develop a century ago, when the German school of neuropathologists described the salient histological features of what we now know to be the most common of these diseases. In 1907, Alois Alzheimer described the neuritic plaques and neurofibrillary lesions in the disease that was subsequently named after him. In 1911, Alzheimer also described Pick bodies as the characteristic neuropathological lesion of Pick's disease, a form of frontotemporal dementia. In 1912, Friedrich Lewy described the inclusions characteristic of Parkinson's disease, the so-called 'Lewy bodies'. In the 1960s, electron microscopy was used to show that the above inclusions are made of abnormal filaments. At the time, the identification of these lesions was instrumental in establishing the various diseases as discrete entities. However, this work did not establish a role for the lesions in the etiology and pathogenesis of neurodegeneration. Over the past 20 years, a direct correspondence between the formation of the neuropathological lesions and the degenerative process has emerged.

The discovery of this direct relationship was made possible by the merging of two independent lines of research.

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On the one hand, the biochemical study of the neuropathological lesions led to the identification of their main molecular components. On the other hand, the study of rare, familial forms of Alzheimer's disease, frontotemporal dementia and Parkinson's disease led to the identification of gene defects that cause inherited variants of the different diseases. Remarkably, in these cases, the defective genes have been found to encode or increase the expression of the main components of the neuropathological lesions. It has therefore been established that the basis of the familial forms of these diseases is a toxic property conferred by mutations in the proteins that make up the filamentous lesions. A corollary of this insight is that a similar toxic property may also underlie the much more common, sporadic forms of the diseases.

Lewy bodies, neurofibrillary lesions and Pick bodies are intracellular filamentous inclusions. It is now well established that Lewy bodies are made of the protein α-synuclein and both neurofibrillary lesions and Pick bodies of the microtubule-associated protein tau. Mutations in the α-synuclein gene or an increase in its copy number cause autosomal-dominantly inherited forms of Parkinson's disease and dementia with Lewy bodies. Mutations in the tau gene cause a familial form of frontotemporal dementia. Here we review the evidence implicating α-synuclein and tau in these inherited and a number of sporadic neurodegenerative diseases. Collectively, α-synucleinopathies and tauopathies account for the vast majority of cases of late-onset neurodegenerative disease (Tables 45-1 and 45-2).

TABLE 45-1 α-Synuclein diseases

Idiopathic Parkinson's disease
Dementia with Lewy bodies
Pure autonomic failure
REM sleep behavior disorder
Lewy body dysphagia
Incidental Lewy body disease
Inherited Lewy body diseases
Multiple system atrophy

TABLE 45-2 Tau diseases

Alzheimer's disease
Down's syndrome
Progressive supranuclear palsy
Corticobasal degeneration
Pick's disease
Argyrophilic grain disease
Tangle-only dementia
Amyotrophic lateral sclerosis/parkinsonism–dementia complex
Guadeloupean parkinsonism
Frontotemporal dementia and parkinsonism linked to chromosome 17
Postencephalitic parkinsonism
Subacute sclerosing panencephalitis
Niemann–Pick disease type C
Familial British dementia

THE SYNUCLEIN FAMILY

The human synuclein family consists of three members. α-Synuclein, β-synuclein and γ-synuclein range from 127 to 140 amino acids in length, and are 55–62% identical in sequence, with a similar domain organization [1] (Fig. 45-1). They are encoded by three genes located on chromosomes 4q23 (α-synuclein), 5q35 (β-synuclein) and 10q21 (γ-synuclein). More than half of each protein is taken up by six or seven imperfect 11-amino acid repeats with the consensus sequence KTKEGV. This positively charged region is followed by a hydrophobic middle region and a negatively charged region that makes up the carboxy-terminal third of each synuclein. Synucleins are abundant, nervous-system-enriched proteins whose physiological functions are only incompletely understood.

By immunohistochemistry, α- and β-synucleins are concentrated in nerve terminals, with little staining of somata and dendrites. Ultrastructurally, they are found in close proximity to synaptic vesicles. In contrast, γ-synuclein is present throughout nerve cells in many brain regions. In rat, α-synuclein is most abundant throughout telencephalon and diencephalon, with lower levels in more caudal regions. β-Synuclein is distributed fairly evenly throughout the central nervous system, whereas γ-synuclein is most abundant in midbrain, pons and spinal cord, with much lower levels in forebrain areas.

Synucleins are natively unfolded proteins. They have little ordered secondary structure and have only been identified in vertebrates. Experimental studies have shown that α-synuclein can bind to lipid membranes through its amino-terminal repeats, indicating that it may be a lipid-binding protein [2]. It adopts structures rich in α-helical character upon binding to synthetic lipid membranes containing acidic phospholipids. This conformation is taken up by amino acids 1–98, with residues 99–140 being unstructured. In cell lines and primary neurons treated with high fatty acid concentrations, α-synuclein was found to accumulate on phospholipid monolayers surrounding triglyceride-rich droplets. β-Synuclein bound in a similar way, but γ-synuclein failed to bind lipid droplets and remained cytosolic. Accordingly, α-synuclein has been found to bind fatty acids *in vitro*, albeit with a lower affinity than physiological fatty-acid-binding proteins. Both α- and β-synucleins inhibit phospholipase D, suggesting that they may regulate vesicular transport processes. Only little is known about posttranslational modifications of α-synuclein. Serine and tyrosine phosphorylation has been observed in transfected cells, but it remains to be established whether it plays a physiological role in brain. Like some other natively unfolded proteins,

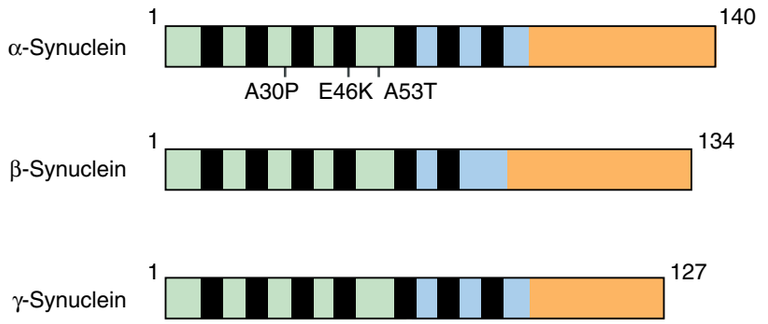

FIGURE 45-1 Schematic diagram of the three human synucleins, which range from 127 to 140 amino acids in length. The amino-terminal repeats are shown as *black* bars. Positively charged regions are indicated in *green*, hydrophobic regions in *blue* and negatively charged regions in *red*. The three known missense mutations (*A30P*, *E46K* and *A53T*) in α-synuclein are shown.

α-synuclein can be degraded by the proteasome in the absence of polyubiquitination.

Inactivation of the α-synuclein gene by homologous recombination results in mice that appear largely normal [3]. Analysis of mice lacking γ-synuclein has similarly failed to reveal any gross abnormalities [4]. In hippocampal slices from mice without α-synuclein, the replenishment of docked vesicles by reserve pool vesicles was slower than in slices from control mice. It suggests a physiological role for α-synuclein in the mobilization of synaptic vesicles.

PARKINSON'S DISEASE AND OTHER LEWY BODY DISEASES

α-Synuclein mutations cause familial Parkinson's disease. In 1990, an autosomal-dominantly inherited form of Parkinson's disease was described in an Italian–American family (the Contursi kindred). It was the first familial form of disease in which Lewy bodies had been shown to be present. In 1997, a missense mutation (A53T) in the α-synuclein gene was identified as the cause of disease in the Contursi kindred [5]. Subsequently, a second mutation (A30P) was identified in a German family with Parkinson's disease. More recently, a third mutation (E46K) was described in a Spanish family with Parkinson's disease and dementia with Lewy bodies. All three missense mutations are located in the repeat region of α-synuclein (Fig. 45-1 and Fig. 45-2).

In addition to changes in the primary structure of α-synuclein, the overexpression of wild-type α-synuclein has been identified as the likely cause of inherited Parkinson's disease–dementia [6] (Fig. 45-2). Thus, duplication or triplication of a 1.6–2.0 Mb region of the long arm of chromosome 4 is responsible for familial forms of disease with abundant and widespread Lewy body pathology. The α-synuclein gene is one of an estimated 17 genes located in this region. Moreover, polymorphic variations in the 5′ non-coding region of the α-synuclein gene have been found to be associated with idiopathic Parkinson's disease in some, but not all, studies. They probably influence the level of α-synuclein expression. Variation in expression levels of α-synuclein may be an important risk factor for the development of Lewy body diseases.

Lewy body filaments are made of α-synuclein. Shortly after the identification of the genetic defect responsible for Parkinson's disease in the Contursi kindred, Lewy bodies and Lewy neurites in the substantia nigra from patients with sporadic Parkinson's disease were shown to be strongly immunoreactive for α-synuclein [7] (Fig. 45-3). Subsequently, Lewy body filaments were found to be

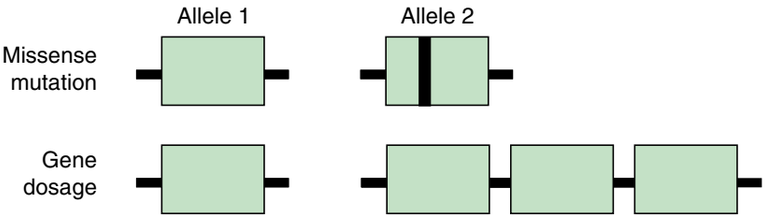

FIGURE 45-2 Missense mutations in the α-synuclein gene and multiplication of the chromosomal region containing the α-synuclein gene cause autosomal-dominantly inherited forms of Parkinson's disease and dementia with Lewy bodies. The α-synuclein gene is shown schematically in *green*.

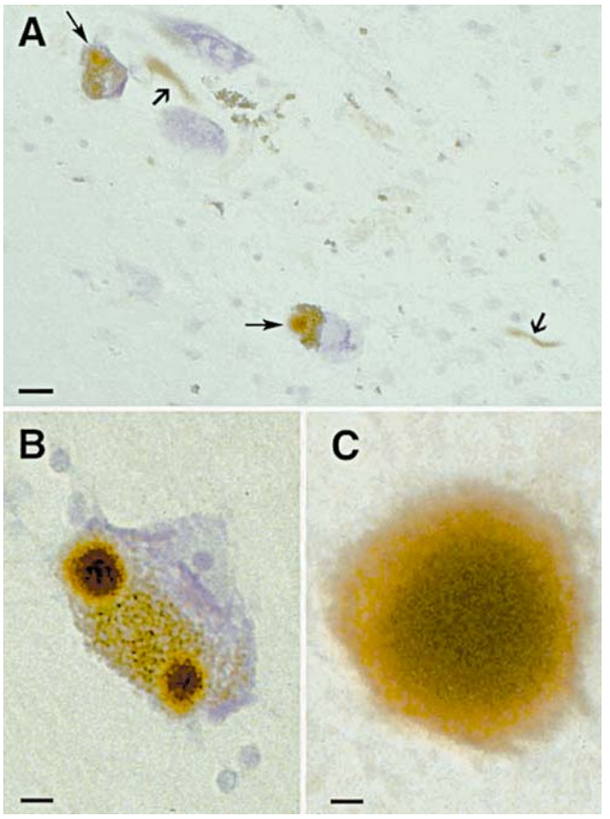

FIGURE 45-3 Substantia nigra from patients with Parkinson's disease immunostained for α-synuclein. (**A**) Two pigmented nerve cells, each containing an α-synuclein-positive Lewy body. Lewy neurites (small arrows) are also immunopositive. Scale bar: 20 μm. (**B**) Pigmented nerve cell with two α-synuclein-positive Lewy bodies. Scale bar: 8 μm. (**C**) α-Synuclein-positive extracellular Lewy body. Scale bar: 4 μm.

decorated by antibodies directed against α-synuclein. The same was true of the Lewy body pathology of dementia with Lewy bodies. This common form of dementia is characterized by large numbers of Lewy bodies and neurites in cortical brain areas, in addition to the substantia nigra.

Filaments associated with Parkinson's disease and dementia with Lewy bodies are unbranched, with a length of 200–600 nm and a width of 5–10 nm. Full-length α-synuclein is present, with its amino- and carboxy-termini being exposed on the filament surface. The core of the filament extends over a stretch of about 70 amino acids that overlaps almost entirely with the lipid-binding region of α-synuclein. Of the three human synucleins, only α-synuclein is associated with the filamentous inclusions of Lewy body diseases.

Prior to this work, ubiquitin staining had been the most sensitive marker of Lewy body pathology. By double-labeling, the number of α-synuclein-positive structures was greater than that stained for ubiquitin, suggesting that the ubiquitination of α-synuclein occurs after assembly. Phosphorylation and nitration are two additional post-translational modifications of filamentous α-synuclein. Phosphorylation at S129 has been documented in Lewy bodies and Lewy neurites and it has been suggested that this may trigger filament assembly [8]. However, it remains to be determined whether phosphorylation at S129 occurs before or after filament assembly in the human brain. Nitration of filamentous α-synuclein was found using antibodies specific for nitrated tyrosine residues. As for ubiquitin, staining for nitrotyrosine was less extensive than staining for α-synuclein, suggesting that nitration of α-synuclein occurs after its assembly into filaments.

Lewy body pathology is also the defining feature of several other, rarer diseases, such as Lewy body dysphagia and pure autonomic failure. In these diseases, Lewy bodies and neurites are largely limited to the enteric and peripheral nervous systems. In Parkinson's disease, Lewy body pathology is also present in the enteric and autonomic nervous systems.

Incidental Lewy body disease describes the presence of small numbers of Lewy bodies and Lewy neurites in the absence of clinical symptoms. It is observed in 5–10% of the general population over the age of 60, and is believed to represent a preclinical form of Lewy body disease. In cases with incidental Lewy body disease, the first α-synuclein-positive structures in the brain form in the dorsal motor nucleus of the glossopharyngeal and vagus nerves, the intermediate reticular zone, the olfactory bulb and the anterior olfactory nucleus [9]. The pathology ascends from vulnerable regions in the medulla oblongata to the pontine tegmentum, midbrain, basal forebrain and cerebral cortex. α-Synuclein deposits may form even earlier in the enteric and peripheral nervous systems, suggesting that Lewy body diseases may originate outside the central nervous system. The first deposits develop in the form of Lewy neurites, suggesting that the filamentous assembly of α-synuclein in axons may precede assembly in cell bodies and dendrites. Incidental Lewy body disease may be at one end of the spectrum of Lewy body diseases, with dementia with Lewy bodies at the other end, and with Lewy body dysphagia, pure autonomic failure and Parkinson's disease in-between. These findings underscore the fact that there is more to Parkinson's disease than the mere degeneration of dopaminergic nerve cells in the substantia nigra.

The Lewy body pathology that is sometimes associated with other neurodegenerative diseases, such as Alzheimer's disease, the parkinsonism–dementia complex of Guam, and neurodegeneration with brain iron accumulation type I, has also been shown to be immunoreactive for α-synuclein. However, it is not an invariable feature of these diseases. Nevertheless, understanding why α-synuclein pathology develops in a proportion of cases in these apparently unrelated conditions, may shed light on the mechanisms operating in diseases defined by the presence of Lewy body pathology.

MULTIPLE SYSTEM ATROPHY

Filamentous α-synuclein deposits characterize this atypical parkinsonian disorder. Lewy body pathology is not generally observed. Instead, glial cytoplasmic inclusions, which consist of filamentous aggregates, are the defining neuropathological feature of multiple system atrophy (Fig. 45-4). They are found mostly in the cytoplasm and, to a lesser extent, the nucleus of oligodendrocytes. Inclusions are also observed in the cytoplasm and nucleus of some nerve cells, and in neuropil threads. The principal brain regions affected are the substantia nigra, striatum, locus coeruleus, pontine nuclei, inferior olives, cerebellum and spinal cord. Typically, nerve cell loss and gliosis are observed. The formation of glial cytoplasmic inclusions may be the primary lesion that will eventually compromise nerve cell function and viability.

Glial cytoplasmic inclusions are strongly immunoreactive for α-synuclein and filaments isolated from the brains of patients with multiple system atrophy are labeled by α-synuclein antibodies [10]. As in dementia with Lewy bodies, assembled α-synuclein is nitrated and phosphorylated at S129, and the number of α-synuclein-positive structures exceeds that stained by anti-ubiquitin antibodies, confirming that the accumulation of α-synuclein precedes ubiquitination. Filament morphologies and their staining characteristics were found to be similar to those of filaments extracted from the brains of patients with Parkinson's disease and dementia with Lewy bodies.

This work revealed an unexpected molecular link between multiple system atrophy and Lewy body diseases. The main difference is that in multiple system atrophy most of the α-synuclein pathology is found in glial cells, whereas in Lewy body diseases most of the pathology is present in nerve cells.

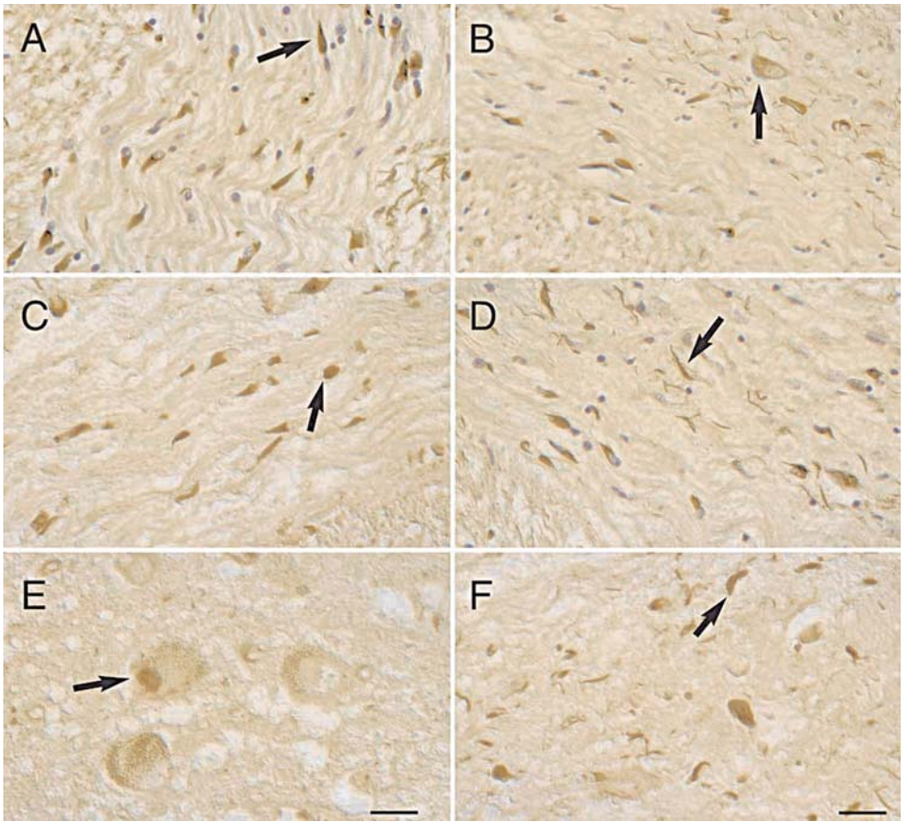

FIGURE 45-4 Brain tissue from patients with multiple system atrophy immunostained for α-synuclein. (**A–D**) α-Synuclein-immunoreactive oligodendrocytes and nerve cells in white matter of pons (**A**, **B**, **D**) and cerebellum (**C**) identified with antibodies recognizing the amino-terminal (**A**, **C**) or the carboxy-terminal (**B**, **D**) region of α-synuclein. (**E**, **F**), α-Synuclein-immunoreactive oligodendrocytes and nerve cells in gray matter of pons (**E**) and frontal cortex (**F**) identified with antibodies recognizing the amino-terminal (**E**) or the carboxy-terminal (**F**) region of α-synuclein. Arrows identify representative examples of each of the characteristic lesions stained for α-synuclein: cytoplasmic oligodendroglial inclusions (**A**, **F**), cytoplasmic nerve cell inclusions (**B**), nuclear oligodendroglial inclusions (**C**), neuropil threads (**D**), and nuclear nerve cell inclusion (**E**). Scale bars: (**E**), 33 μm; (**A–D**, **F**), 50 μm (in **F**).

SYNTHETIC α-SYNUCLEIN FILAMENTS

Recombinant α-synuclein assembles into filaments. They have many of the morphological and ultrastructural characteristics of disease filaments [11, 12] (Fig. 45-5). Assembly is a nucleation-dependent process that occurs through its amino-terminal repeats. The carboxy-terminal region, in contrast, is inhibitory. Assembly is accompanied by the transition from random coil to a β-pleated sheet. By electron diffraction, α-synuclein filaments show a conformation characteristic of amyloid fibers. Under the conditions of these experiments, β- and γ-synucleins failed to assemble, consistent with their absence from the filamentous lesions of the human diseases. When incubated with α-synuclein, β- and γ-synucleins inhibit the fibrillation of α-synuclein, suggesting that they may indirectly influence the pathogenesis of Lewy body diseases and multiple system atrophy.

The E46K and A53T mutations increase the rate of assembly of α-synuclein, indicating that this may be their primary effect. The mechanism of action of the A30P mutation is less clear. One study reported that it causes the accumulation of oligomeric, nonfibrillar α-synuclein, implying that this may be a toxic species. Mutation A30P reduces the binding of α-synuclein to rat brain vesicles, whereas mutation E46K increases lipid binding. Mutation A53T has no significant effect on lipid binding. Many factors have been shown to influence the rate and/or extent of α-synuclein assembly *in vitro*. They include nitration, oxidation, dopamine cytochrome C, polyamines, some pesticides, several di- and trivalent cations and sulfated glycosaminoglycans. Similarly, tau protein has been shown to promote the *in vitro* assembly of α-synuclein. However, the relevance, if any, of these factors for the transition from soluble to filamentous α-synuclein in nerve cells in the brain remains to be established.

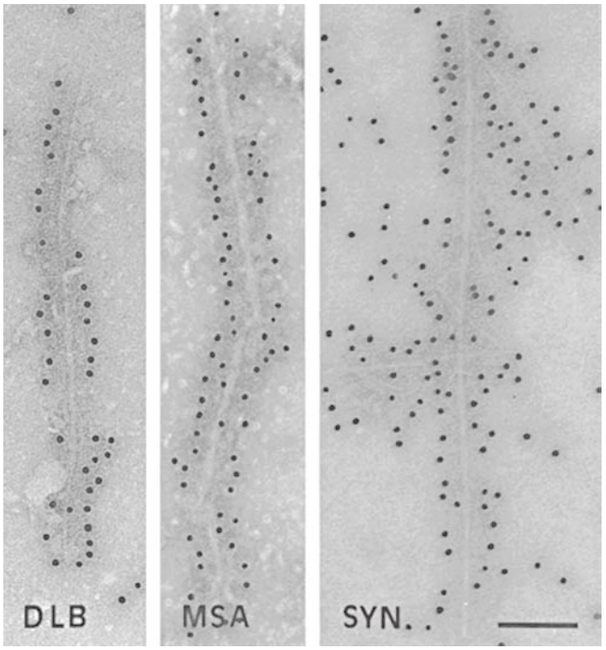

FIGURE 45-5 Filaments extracted from the brains of patients with dementia with Lewy bodies (*DLB*) and multiple system atrophy (*MSA*) or assembled from bacterially expressed human α-synuclein (*SYN*) were decorated by an anti-α-synuclein antibody. The gold particles conjugated to the second antibody appear as black dots. Scale bar: 100 nm.

ANIMAL MODELS OF HUMAN α-SYNUCLEINOPATHIES

Rodents and primates. Mice expressing wild-type and mutant human α-synuclein in nerve cells or glial cells have been shown to develop numerous α-synuclein-immunoreactive cell bodies and processes. However, filament formation and nerve cell loss were a less-consistent feature. One study documented the presence of α-synuclein filaments in brain and spinal cord of mice transgenic for A53T α-synuclein [13]. The formation of inclusions correlated with the appearance of a severe movement disorder [13, 14], suggesting a possible cause-and-effect relationship. A minority of inclusions was ubiquitin-immunoreactive. Signs of Wallerian degeneration were in evidence in ventral roots, but motor neuron numbers were not reduced. A major difference with Parkinson's disease was the absence of significant pathology in dopaminergic nerve cells of the substantia nigra.

Viral vector-mediated gene transfer differs from standard transgenic approaches by being targeted to a defined region of the central nervous system, and by being inducible at any point during the life of the animal. Adeno-associated and lentiviral vectors were used to express human wild-type and mutant α-synuclein in rodent and primate substantia nigra. Lewy-body-like inclusions formed, and a significant proportion of nerve cells degenerated [15]. In the rat, about a quarter of animals were impaired in spontaneous and drug-induced motor behavior.

A neurotoxin model of α-synuclein pathology has been developed in the rat through the chronic administration of the pesticide rotenone, a high-affinity inhibitor of complex I [16]. The rats developed a progressive degeneration of nigrostriatal neurons and Lewy-body-like inclusions that were immunoreactive for α-synuclein and ubiquitin. Behaviorally, they showed bradykinesia, postural instability and resting tremor. Under these conditions, the inhibition of complex I was only partial, indicating that a bioenergetic defect with ATP depletion was probably not involved. Instead, oxidative damage might have played a role, since partial inhibition of complex I by rotenone stimulates the production of reactive oxygen species. It would therefore seem that oxidative stress can lead to the assembly of α-synuclein into filaments.

Flies, worms and yeasts. One of the first reports to describe the overexpression of human α-synuclein made use of *D. melanogaster*, an organism without synucleins [17]. Expression in nerve cells resulted in the formation of filamentous Lewy-body-like inclusions and an age-dependent loss of some dopaminergic nerve cells. The inclusions were α-synuclein- and ubiquitin-positive. The ensuing locomotor defect was reversed by L-DOPA and dopamine agonists. Chaperones modulated these effects. Thus, co-expression of heat shock proteins reduced the toxicity of α-synuclein, whereas a reduction in endogenous chaperones exacerbated nerve cell loss. Overexpression of wild-type and mutant human α-synuclein in *C. elegans* resulted in dopaminergic nerve cell loss and motor deficits, but in the apparent absence of α-synuclein filaments. When expressed in the yeast *S. cerevisiae*, human wild-type and A53T α-synucleins were localized at the plasma membrane, whereas the A30P mutant had a cytosolic distribution, consistent with lipid binding *in vitro* [18]. At 2-fold higher expression levels, inclusions formed and growth was arrested, reminiscent of the gene dosage effects observed in cases of inherited Parkinson's disease–dementia. A genome-wide screen to identify genetic enhancers of α-synuclein toxicity identified genes involved in lipid metabolism and vesicle-mediated transport as the predominant functional class.

α-SYNUCLEINOPATHIES—OUTLOOK

The new work has established that a neurodegenerative pathway leading from soluble to insoluble, filamentous α-synuclein is central to Lewy body diseases and multiple system atrophy. The development of experimental models of α-synucleinopathies has opened the way to the identification of the detailed mechanisms by which the formation of inclusions causes disease. These model systems have also made it possible to identify disease modifiers that may well lead to the development of the first mechanism-based therapies for these diseases. At a conceptual level, it will be important to understand whether α-synuclein has a role to play in disorders, such as autosomal–recessive juvenile forms of parkinsonism caused by mutations in the *Parkin*, *DJ-1* and *PINK-1* genes, or whether there are entirely separate mechanisms by which the dopaminergic nerve cells of the substantia nigra degenerate in Parkinson's disease and in inherited disorders with parkinsonism.

TAU ISOFORMS AND THEIR INTERACTIONS WITH MICROTUBULES

Tau is a microtubule-binding protein that is believed to be important for the assembly and stabilization of microtubules [19]. In nerve cells, tau is normally found in axons, but in the tauopathies it is redistributed to the cell body and dendrites.

Six tau isoforms are expressed in adult human brain. They are produced from a single gene on chromosome 17q21 by alternative mRNA splicing [20] (Fig. 45-6) and differ from one another by the presence or absence of a 29- or 58-amino-acid insert in the amino-terminal half of the protein and by the inclusion, or not, of a 31-amino-acid repeat, encoded by exon 10 of tau in the carboxy-terminal half of the protein. The exclusion of exon 10 leads to the production of three isoforms, each containing three repeats, and its inclusion leads to a further three isoforms, each containing four repeats. The repeats constitute the microtubule-binding region of tau protein, with four-repeat tau being better at promoting microtubule assembly than tau with three repeats. In normal adult human brain, there are similar levels of three-repeat and four-repeat isoforms. In developing human brain, only the shortest tau isoform (three repeats and no amino-terminal inserts) is expressed. In the peripheral nervous system, inclusion of exon 4A in the amino-terminal half results in the expression of high-molecular-weight proteins called *big tau*.

Repeats similar to those in tau are present in the otherwise structurally unrelated high-molecular-weight microtubule-associated proteins MAP2 and MAP4. Repeat sequences are conserved during evolution. Thus, the genomes of *C. elegans* and *D. melanogaster* each encode one protein with tau-like repeats. In all vertebrate species examined, tau is expressed as multiple isoforms that are generated by alternative mRNA splicing. Isoform ratios, however, are not conserved between species. For instance, isoforms with three, four or five repeats are expressed in adult chicken brain, whereas adult rodents only express isoforms with four repeats.

The tau molecule can be subdivided into an amino-terminal domain that projects from the microtubule surface and the carboxy-terminal microtubule-binding domain. Recent structural work has begun to shed light on the way that tau protein interacts with microtubules. When bound to taxol-stabilized microtubules, tau was found to localize along the outer ridges of the microtubule protofilaments. A different conclusion was reached when tau was bound to microtubules in the absence of taxol. The tau repeats were now found to bind to the inner surface of the microtubule, close to the taxol-binding site on β-tubulin. Taxol binds to a site on β-tubulin, where α-tubulin has a conserved extra loop of eight amino acids. Interestingly, this extended loop has significant sequence homology with the tau repeats. Since tubulin cannot have evolved to bind taxol, this work may answer the question of what type of natural substrate binds to this pocket in β-tubulin. In this model, part of the proline-rich region of tau must provide the link between the amino-terminal projection domain on the outside of the microtubule and the repeat motifs on the inside surface. It could thread through one of the holes between protofilaments.

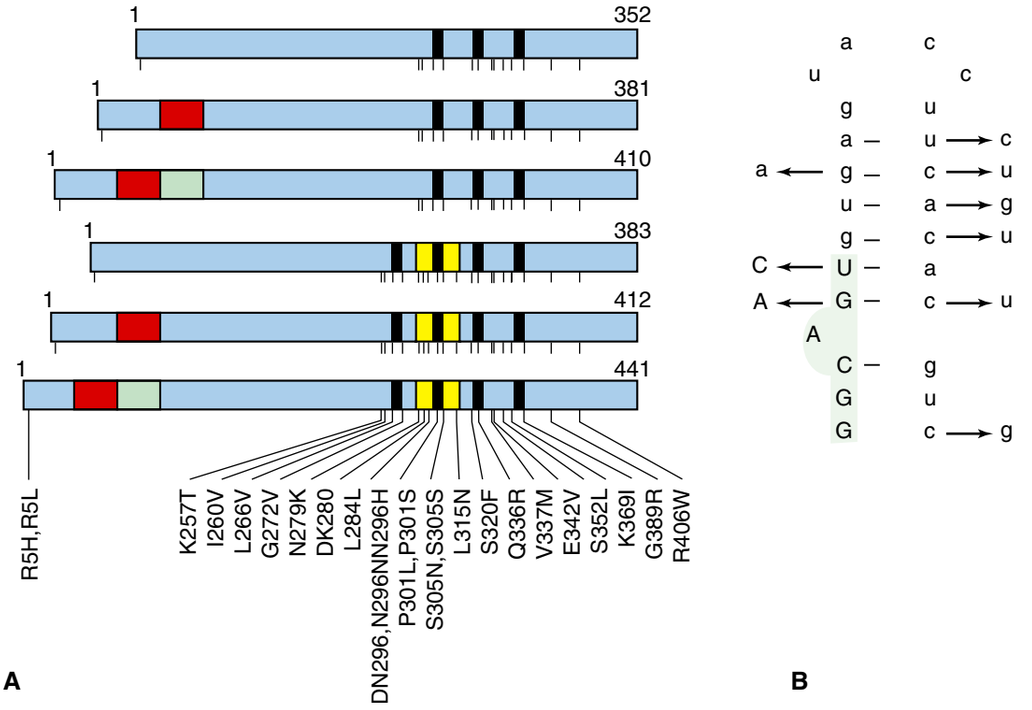

FIGURE 45-6 Mutations in the tau gene in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). (**A**) Schematic diagram of the six tau isoforms (352 to 441 amino acids) that are expressed in adult human brain, with mutations in the coding region indicated using the numbering of the 441 amino acid isoform. Twenty missense mutations, two deletion mutations and three silent mutations are shown. The six tau isoforms are produced by alternative mRNA splicing from a single gene. They differ by the presence or absence of three inserts, shown in *red* (encoded by exon 2), *green* (encoded by exon 3) and *yellow* (encoded by exon 10), respectively. (**B**) Stem-loop structure in the pre-mRNA at the boundary between exon 10 and the intron following exon 10. Nine mutations are shown, two of which (*S305N* and *S305S*) are located in exon 10. Exon sequences are shown against a *green background* in *CAPITALS*, with intron sequences being shown in *lower-case* letters.

Tau is a phosphoprotein. Phosphorylation negatively regulates the ability of tau to interact with microtubules [21]. Many of the phosphorylated sites are serine/threonine–prolines, 17 of which are present in the longest human brain tau isoform. Most of these sites flank the microtubule-binding repeats. Proline-directed protein kinases, such as glycogen synthase kinase-3 (GSK3), cyclin-dependent kinase 5 (CDK5), mitogen-activated protein (MAP) kinase and stress-activated protein (SAP) kinases, phosphorylate these sites *in vitro*. Moreover, lithium chloride treatment has been shown to reduce the level of tau phosphorylation in rodent brain, suggesting that GSK3 is a physiological tau kinase. In addition, other sites in tau are phosphorylated *in vitro* by cyclic AMP-dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinase II (CamKII) and microtubule-affinity-regulating kinase (MARK). Some of these sites are located within the repeat region. Protein phosphatase 2A (PP2A) is the major phosphatase activity in brain towards tau phosphorylated by proline-directed and other kinases.

Phosphorylation is developmentally regulated, such that in fetal brain, tau is more heavily phosphorylated than in adult brain. An increase in tau phosphorylation reminiscent of that present during development was observed in the brain of the hibernating ground squirrel. In adult mouse brain, hyperphosphorylation of tau was present following cold-water stress and starvation. Inhibition of PP2A activity rather than activation of protein kinases appeared to be responsible for this hyperphosphorylation of tau.

Inactivation of the tau gene by homologous recombination results in mice that are largely normal [22], indicating that tau is a nonessential protein. This may reflect functional redundancy. Thus, mice doubly deficient in tau and the microtubule-associated protein MAP1B exhibit nervous system defects that are more severe than those in the MAP1B single knockout line.

TAU AND ALZHEIMER'S DISEASE

Abundant neuritic plaques and neurofibrillary lesions define Alzheimer's disease. Plaques are extracellular deposits made of the fibrillar β-amyloid peptide. The paired helical filament (PHF) makes up the bulk of the intraneuronal neurofibrillary pathology of Alzheimer's disease, with the straight filament (SF) being a minority species.

The paired helical filament is made of tau protein. By the early 1990s, it was clear that tau protein is the major component of PHFs and SFs and that all six brain isoforms are present, each full-length and hyperphosphorylated [23–27]. The microtubule-binding repeat region of tau forms the core of the filament, with the amino- and carboxy-terminal regions forming a fuzzy coat around the filament. PHFs and SFs have a cross-β structure characteristic of amyloid fibers. Following assembly, tau is truncated at the amino-terminus, which is necessary for its subsequent ubiquitination. Following the death of neurofibrillary tangle-bearing cells, the pathological material stays around in the extracellular space, in the form of so-called 'ghost tangles' that consist largely of the ubiquitinated repeat region of tau. It thus appears that in Alzheimer's disease the ubiquitination of tau is a late, secondary event.

Filamentous tau is hyperphosphorylated. This is an early event that appears to precede filament assembly. It also renders tau unable to interact with microtubules. Much effort has gone into the mapping of phosphorylation sites and the identification of candidate protein kinases and phosphatases. For sites that are also phosphorylated in normal brain tau, a higher proportion of tau molecules is phosphorylated in filamentous tau. In addition, filamentous tau is phosphorylated at more serine and threonine residues than tau from normal adult brain. Phosphorylation-dependent anti-tau antibodies were instrumental for the study of many phosphorylation sites. In particular, phosphorylation of S214 and S422 was found to be specific for assembled tau.

The mechanisms underlying filament formation in neurons are still unclear, but it is possible that hyperphosphorylation disengages tau from microtubules, thereby increasing the pool of unbound tau, which may be more resistant to degradation and more prone to aggregate than microtubule-bound tau. It suggests that an increased ability of pathological tau to interact with microtubules may be beneficial. This could in principle be achieved by protein kinase inhibitors and/or activation of PP2A. Organic osmolytes, such as trimethylamine *N*-oxide (TMAO) and betaine have been shown to restore the ability of phosphorylated tau to interact with microtubules, probably through a conformational change in tubulin and/or tau. Similarly, the prolyl isomerase Pin1, which binds to phosphothreonine 231 in tau, has been reported to restore the ability of phosphorylated tau to interact with microtubules and to facilitate its dephosphorylation by PP2A.

The development of the neurofibrillary pathology is not random. It follows a stereotyped pattern with regard to affected nerve cell types, cellular layers and brain regions, with little inter-individual variation. This has been used to define six neuropathological stages of Alzheimer's disease [28]. The very first nerve cells in the brain to develop neurofibrillary lesions are located in pre-α layer of the transentorhinal region, thus defining stage I. Stage II shows a more severe involvement of this region, as well as a mild involvement of the pre-α layer of the entorhinal cortex. Patients with this pathology are cognitively unimpaired, indicating that stages I and II may represent clinically silent stages of Alzheimer's disease. Mild impairment of cognitive function becomes apparent in stages III and IV. Stage III is characterized by severe neurofibrillary lesions in the pre-α layers of both entorhinal and transentorhinal regions. During stages III and IV, mild changes are also present in layer I of Ammon's horn of the hippocampus and in a number of subcortical nuclei, such as the basal forebrain magnocellular nuclei and the anterodorsal thalamic nucleus. The major feature of stages V and VI is the massive development of neurofibrillary pathology in isocortical association areas. They meet the criteria for a neuropathological diagnosis of Alzheimer's disease and are found in patients who were severely demented.

The stereotyped nature of the spatial and temporal development of neurofibrillary pathology contrasts with the development of Aβ deposits. They show a density and distribution that are subject to great individual variation. In general, the first Aβ deposits occur in isocortical areas of the frontal, temporal and occipital lobes, where they tend to precede neurofibrillary deposits. This contrasts with the entorhinal and hippocampal regions, where Aβ deposits appear later than the neurofibrillary pathology. These findings are difficult to explain by the amyloid cascade hypothesis for the pathogenesis of Alzheimer's disease, which proposes that the deposition and fibrillization of Aβ peptides to form extracellular plaques is the central event that causes formation of the neurofibrillary pathology and nerve cell loss.

SPORADIC TAUOPATHIES

They include progressive supranuclear palsy, corticobasal degeneration and Pick's disease. Evidence in support of a role for tau protein in neurodegeneration has been provided through the study of these diseases, which exhibit abundant filamentous tau inclusions, in the absence of extracellular amyloid deposits. Clinically, progressive supranuclear palsy leads to supranuclear gaze palsy and postural instability. Neuropathologically, atrophy of the basal ganglia, subthalamus and brainstem are much in evidence. Like progressive supranuclear palsy, corticobasal degeneration is an atypical parkinsonian disorder. It leads to depigmentation of the substantia nigra and an asymmetric frontoparietal atrophy. Pick's disease, by contrast, is a variant of frontotemporal dementia that is characterized by a frontotemporal lobar and limbic atrophy. Other tauopathies include argyrophilic grain disease, parkinsonism–dementia complex of Guam and Guadeloupean parkinsonism.

Filament morphologies differ to some extent between these diseases, but hyperphosphorylation of tau is a common feature. The phosphorylated sites are similar to those seen in Alzheimer's disease. However, the isoform composition in the filaments varies. Like Alzheimer's disease, parkinsonism–dementia complex of Guam is characterized by the presence of all six tau isoforms. In Pick's disease filaments, a relative preponderance of tau isoforms with three repeats is commonly observed. By contrast, filaments from progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease and Guadeloupean parkinsonism consist almost entirely of tau isoforms with four repeats. This correlates with the presence of tau inclusions, not only in nerve cells, but also in glial cells, chiefly astrocytes and oligodendrocytes.

MUTATIONS CAUSING TAUOPATHY—THE FTDP-17 SYNDROMES

Frontotemporal dementias occur as familial forms and, more commonly, as sporadic diseases. They are characterized by a remarkably circumscribed atrophy of the frontal and temporal lobes of the cerebral cortex, often with additional, subcortical changes. In 1994, an autosomal-dominantly inherited form of frontotemporal dementia with parkinsonism was linked to chromosome 17q21.2. Subsequently, other forms of frontotemporal dementia were linked to this region, resulting in the denomination 'frontotemporal dementia and parkinsonism linked to chromosome 17' (FTDP-17) for this class of disease. All cases of FTDP-17 have so far shown a filamentous pathology made of hyperphosphorylated tau protein (Fig. 45-7). In 1998, mutations in tau were reported in FTDP-17 patients [29–31]. Since then, more than 30 different mutations have been described in over 80 families with FTDP-17 (Fig. 45-6).

Mutations are either exonic or intronic. Missense, deletion and silent mutations are found in the coding region, with intronic mutations being located close to the splice-donor site of the intron following the alternatively

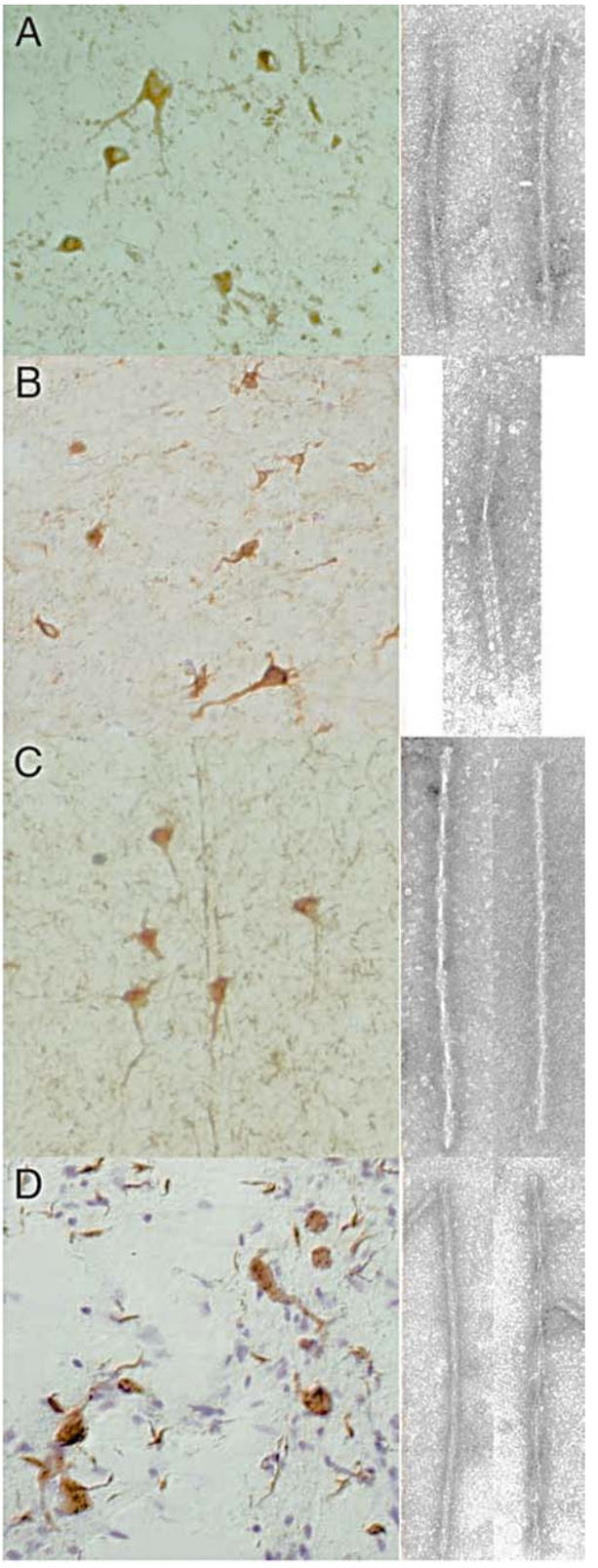

FIGURE 45-7 Pathologies of FTDP-17, as revealed by immunohistochemistry for hyperphosphorylated tau protein and the morphologies of isolated tau filaments. (**A**) The P301L mutation in exon 10 gives rise to a neuronal and glial tau pathology. Filaments consist of narrow twisted ribbons (*left*) as the majority species and rope-like filaments (*right*) as the minority species. (**B**) Mutations in the intron following exon 10 give rise to a neuronal and glial tau pathology. Filaments consist of wide twisted ribbons. (**C**) The V337M mutation in exon 12 gives rise to a neuronal tau pathology. Filaments consist of paired helical filaments (*left*) and straight filaments (*right*), like the tau filaments of Alzheimer's disease. (**D**) The G389R mutation in exon 13 gives rise to a neuronal tau pathology. Filaments consist of straight filaments (*left*) as the majority species and twisted filaments (*right*) as the minority species. The tau pathology resembles that of Pick's disease.

spliced exon 10. Functionally, they fall into two largely nonoverlapping categories — those whose primary effect is at the protein level, and those that influence the alternative splicing of tau pre-mRNA. Most missense mutations reduce the ability of tau protein to interact with microtubules. A number of mutations may cause FTDP-17, at least in part, by promoting the aggregation of tau protein. Most of these mutations lead to the formation of nerve cell inclusions that consist of filaments made of all six brain tau isoforms. Mutations P301L and P301S in exon 10 are exceptions, since they lead to the formation of neuronal and glial inclusions that are made of tau isoforms with four repeats.

The intronic mutations and most coding region mutations in exon 10 increase the splicing of exon 10, thus changing the ratio between three- and four-repeat tau isoforms, resulting in the overproduction of four-repeat tau. This leads to the formation of filaments made of four-repeat tau in both nerve cells and glial cells. Approximately half of the known tau mutations have their primary effect at the RNA level. They affect splicing enhancer or silencer sequences in exon 10 or destabilize a stem-loop structure located at the boundary between exon 10 and the intron that follows it. Thus, to a considerable degree, FTDP-17 is a disease of the alternative mRNA splicing of exon 10 of the tau gene. It follows that a correct ratio of three-repeat to four-repeat tau isoforms is essential for preventing neurodegeneration and dementia in midlife.

RELEVANCE FOR THE SPORADIC TAUOPATHIES

The study of FTDP-17 has established that dysfunction or misregulation of tau protein can cause neurodegeneration and dementia. It follows that tau protein is probably also of central importance in the pathogenesis of diseases, such as Alzheimer's disease, progressive supranuclear palsy, corticobasal degeneration and Pick's disease. This is further underlined by the fact that the aforementioned diseases are partially or completely phenocopied by cases of FTDP-17.

Eight missense mutations in tau have been shown to give rise to a clinical and neuropathological phenotype that is closely related to Pick's disease. The finding that overproduction of four-repeat tau causes disease and leads to the assembly of tau isoforms with only four repeats in nerve cells and glial cells may shed light on the pathogenesis of progressive supranuclear palsy and corticobasal degeneration. Both diseases are characterized by a neuronal and glial tau pathology, with the filaments comprising only four-repeat tau. An association between progressive supranuclear palsy and a dinucleotide repeat polymorphism in the intron between exons 9 and 10 of tau has been described [32]. The alleles at this locus carry 11–15 repeats. The A0 allele, with 11 repeats, has a frequency of over 90% in patients with progressive supranuclear palsy, and about 70% in controls. Subsequently, two common tau haplotypes that differ at the nucleotide level, but not at the level of the protein coding sequence, were identified. Homozygosity of the more common allele H1 predisposes to progressive supranuclear palsy and corticobasal degeneration, but not to Alzheimer's disease or Pick's disease. This work strongly suggests that dysfunction of tau protein is of central importance in progressive supranuclear palsy and corticobasal degeneration, as it is in Pick's disease. Surprisingly, homozygosity for the tau H1 haplotype is also associated with an increased risk of Parkinson's disease, a disorder without significant tau pathology. It remains to be seen whether this association can be explained by the interactions between tau and α-synuclein that have been observed *in vitro*.

SYNTHETIC TAU FILAMENTS

Initial experiments had shown that PHF-like filaments can be assembled *in vitro* from bacterially expressed fragments of tau comprising three repeats. These experiments lent strong support to the view that the repeat region of tau is the only component necessary for the morphological appearance of the PHF. However, they failed to provide insight into filament formation *in vivo*, because the tau filaments were obtained only with truncated tau under nonphysiological conditions. This contrasts with PHFs from Alzheimer's disease brain, which consist of full-length tau protein.

Sulfated glycosaminoglycans induce the assembly of full-length tau into filaments. Assembly of individual three-repeat tau isoforms gave filaments with a typical paired helical filament-like morphology, when incubated with heparin or heparan sulfate, whereas assembly of individual four-repeat tau isoforms gave filaments with a straight appearance [33, 34]. Paired helical-like filaments were decorated by antibodies directed against the amino- and carboxy-termini of tau, but not by an antibody against the microtubule-binding region. A short amino acid sequence (VQIVYK) in the third microtubule-binding repeat of tau has been reported to be essential for heparin-induced filament assembly [35]. Further work revealed that RNA and fatty acids, such as aracidonic acid, can also induce the bulk assembly of full-length recombinant tau into filaments. More recently, α-synuclein was reported to induce tau filament formation, suggesting a direct mechanistic link between the two proteins. These findings were obtained using bacterially expressed, nonphosphorylated tau. The relevance of phosphorylation in these experiments is unclear, with reported effects ranging from no influence to stimulatory and inhibitory effects.

ANIMAL MODELS OF HUMAN TAUOPATHIES

Rodents. Mice expressing wild-type and mutant human tau in nerve cells or glial cells have been found to develop numerous tau-immunoreactive cell bodies and processes. Abundant filaments made of hyperphosphorylated tau protein and neurodegeneration were present in some lines expressing single isoforms of mutant human tau protein [36, 37] (Fig. 45-8 and Fig. 45-9). Hyperphosphorylation of tau appeared to precede filament assembly and an increase in the phosphorylation of soluble tau resulted in increased filament formation [37], suggesting that phosphorylation of tau can drive filament assembly. The presence of extracellular β-amyloid deposits was reported to promote tau filament formation. Filamentous tau deposits were also observed in a mouse line expressing all six wild-type human brain tau isoforms in the absence of endogenous mouse tau [38]. The observed imbalance between levels of three- and four-repeat isoforms may have caused tau pathology in this line. In rodent brain, adeno-associated virus-mediated expression of human P301L tau protein

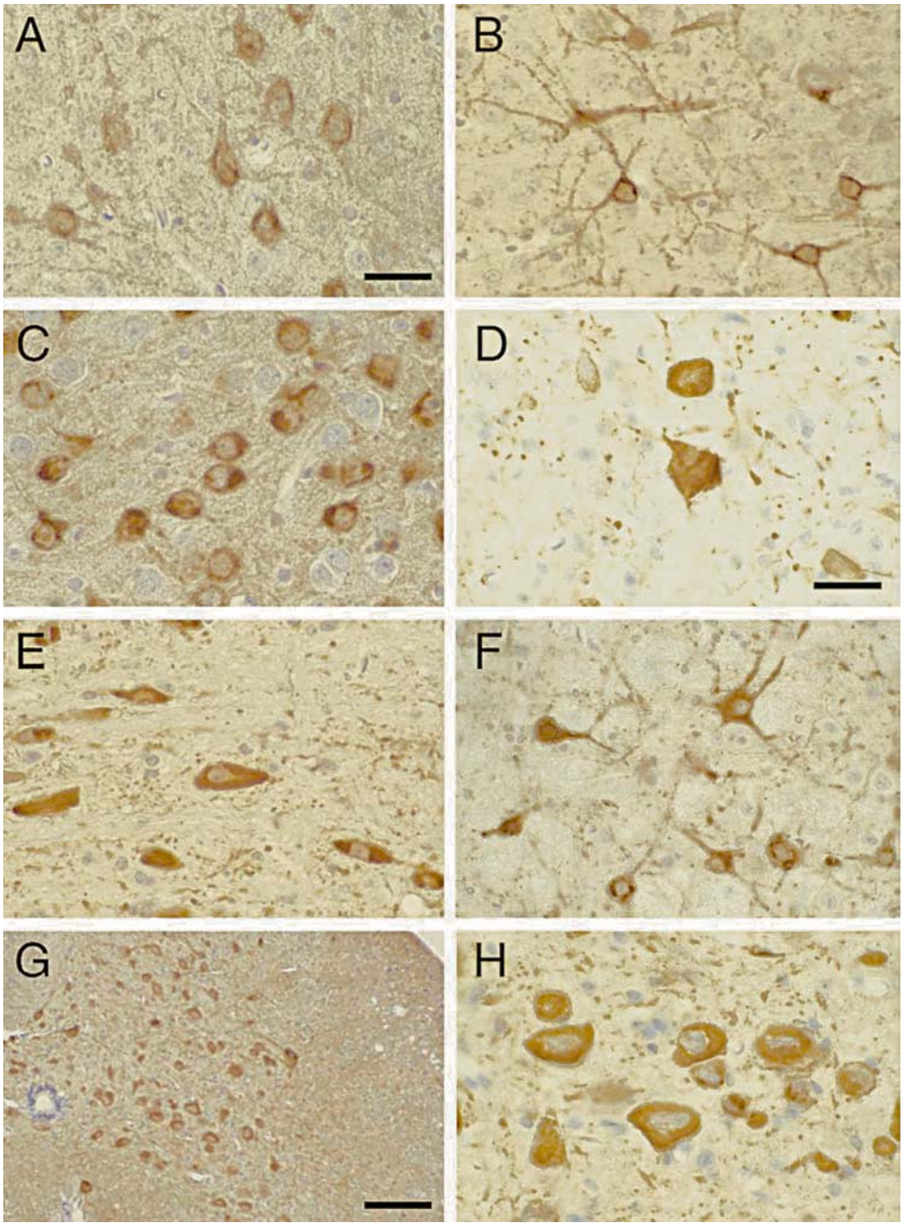

FIGURE 45-8 Tau protein immunoreactivity in brains and spinal cords from mice transgenic for mutant (P301S) human tau protein. (**A**, **B**) Cerebral cortex. (**C**) Amygdala. (**D**) Dentate nucleus of the cerebellum. (**E**, **F**) Brainstem. (**G**, **H**) Spinal cord. Scale bars: (**A–C**, **E**, **F**), 40 μm (in **A**); (**D**, **H**) 60 μm (in **D**); (**G**), 250 μm.

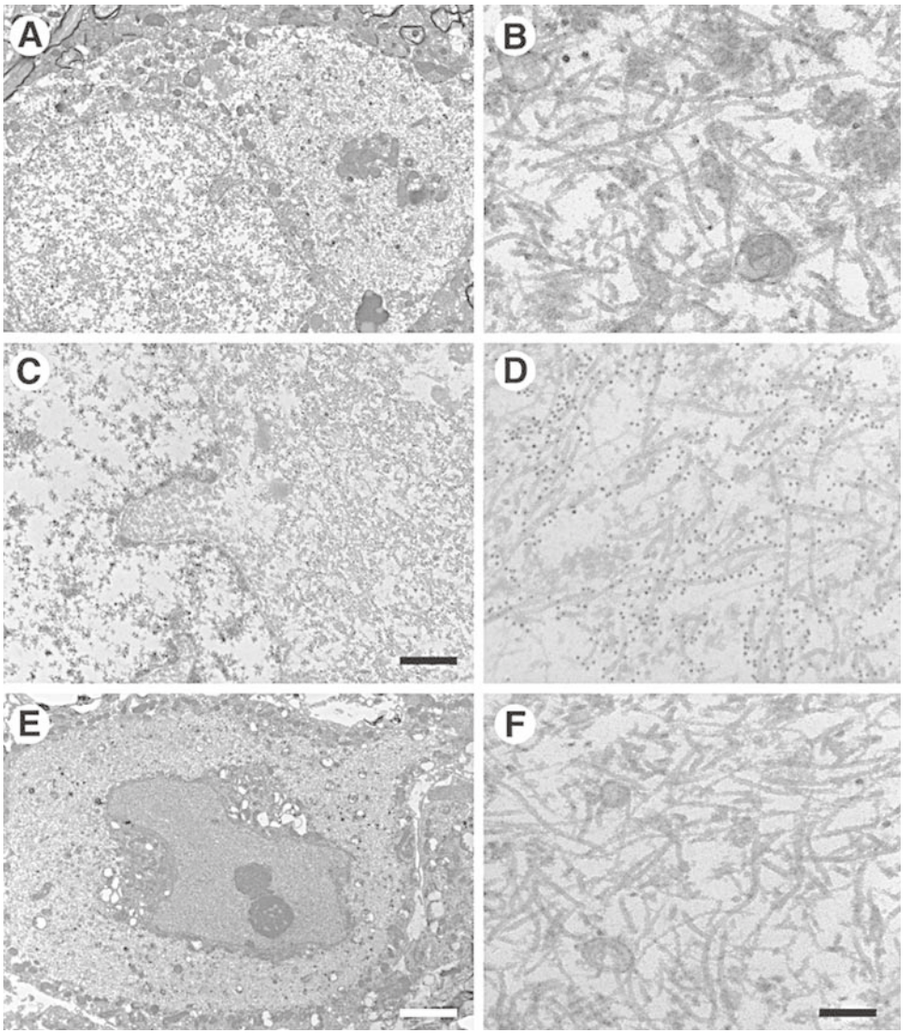

FIGURE 45-9 Tau filaments in brains and spinal cords from mice transgenic for mutant (P301S) human tau protein. (**A**,**B**) Cerebral cortex. (**C**, **D**) Brainstem. (**E**, **F**) Spinal cord. (**B**, **D**, **F**) Higher magnification of parts of the cytoplasmic regions from (**A**, **C**, **E**). The electron micrographs in (**C**, **D**) show immunogold labeling of filaments using a phosphorylation-dependent anti-tau antibody. Scale bars: (**C**), 1.5 μm; (**A**, **E**), 5.5 μm (in **E**); (**B**, **D**, **F**), 300 nm (in **F**).

led to the formation of filamentous deposits made of hyperphosphorylated tau protein.

Loss of function of the prolyl isomerase Pin1 by homologous recombination has been reported to lead to the formation of abundant filaments made of hyperphosphorylated mouse tau and neurodegeneration [39].

Flies and worms. Expression of wild-type and mutant human tau proteins in nerve cells of *D. melanogaster* and *C. elegans* led to a reduced lifespan and the loss of nerve cells, in the apparent absence of tau filaments [40, 41]. Phosphorylation of tau was more extensive in the fly than in the worm. In *Drosophila*, phosphorylation of S262 and S356 in tau by PAR-1 kinase, the fly homologue of MARK, appeared to be necessary for the subsequent phosphorylation at other sites, indicating the existence of a hierarchical and temporally ordered phosphorylation process. Co-expression of human tau with the fly homolog of GSK3-β resulted in accelerated neurodegeneration and the formation of tau-immunoreactive inclusions. In contrast to what has been described in FTDP-17 and mouse models of tauopathies, tau-induced neurodegeneration involved programmed cell death. Taken together, it appears that conformationally altered, nonfilamentous human tau protein is neurotoxic in invertebrates.

TAUOPATHIES — OUTLOOK

It is now well established that a neurodegenerative pathway leading from soluble to insoluble, filamentous tau protein is central to the neurodegenerative process in the human tauopathies. The availability of animal models that exhibit the essential molecular and cellular features

of the human diseases has opened the way to a detailed understanding of the neurodegenerative process and the identification of genetic and pharmacological modifiers. In particular, it will be possible to test the relevance of the hyperphosphorylation of tau. Specific protein kinase inhibitors are widely believed to represent a major class of the drugs of the future. Such compounds may well constitute the first mechanism-based therapies for the human tauopathies.

REFERENCES

1. Goedert, M. Alpha-synuclein and neurodegenerative diseases. *Nature Rev. Neurosci.* 2: 492–501, 2001.
2. Davidson, W.S. *et al.* Stabilization of α-synuclein secondary structure upon binding to synthetic membranes. *J. Biol. Chem.* 273: 9443–9449, 1998.
3. Abeliovich, A. *et al.* Mice lacking α-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25: 239–252, 2000.
4. Ninkina, N. *et al.* Neurons expressing the highest levels of γ-synuclein are unaffected by targeted inactivation of the gene. *Mol. Cell. Biol.* 23: 8233–8245, 2003.
5. Polymeropoulos, M.H. *et al.* Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045–2047, 1997.
6. Singleton, A.B. *et al.* α-Synuclein locus triplication causes Parkinson's disease. *Science* 302: 841, 2003.
7. Spillantini, M.G. *et al.* α-Synuclein in Lewy bodies. *Nature* 388: 839–840, 1997.
8. Fujiwara, H. *et al.* α-Synuclein is phosphorylated in synucleinopathy lesions. *Nature Cell Biol.* 4: 160–164, 2002.
9. Braak, H. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* 24: 197–211, 2003.
10. Spillantini, M.G. *et al.* Filamentous α-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. *Neurosci. Lett.* 251: 205–208, 1998.
11. Crowther, R.A. *et al.* Synthetic filaments assembled from C-terminally truncated α-synuclein. *FEBS Lett.* 436: 309–312, 1998.
12. Conway, K.A., Harper, D.J. and Lansbury, P.T. Accelerated *in vitro* fibril formation by a mutant α-synuclein linked to early-onset Parkinson's disease. *Nature Med.* 4: 1318–1320, 1998.
13. Giasson, B.I. *et al.* Neuronal α-synucleinopathy with severe movement disorder in mice expressing A53T human α-synuclein. *Neuron* 34: 521–533, 2002.
14. Lee, M.K. *et al.* Human α-synuclein-harboring familial Parkinson's disease-linked Ala-53 to Thr mutation causes neurodegenerative disease with α-synuclein aggregation in transgenic mice. *Proc. Natl. Acad. Sci. USA* 99: 8968–8973, 2002.
15. Kirik, D. and Björklund, A. Modeling CNS neurodegeneration by overexpression of disease-causing proteins using viral vectors. *Trends Neurosci.* 26: 386–392, 2003.
16. Betarbet, R. *et al.* Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature Neurosci.* 3: 1301–1306, 2000.
17. Feany, M.B. and Bender, W. A *Drosophila* model of Parkinson's disease. *Nature* 404: 394–398, 2000.
18. Outeiro, T.F. and Lindquist, S. Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science* 302: 1772–1775, 2003.
19. Lee, V.M.-Y., Goedert, M. and Trojanowski, J.Q. Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24: 1121–1159, 2001.
20. Goedert, M. *et al.* Multiple isoforms of human microtubule-associated protein tau: Sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* 3: 519–526, 1989.
21. Lindwall, G. and Cole, R.D. Phosphorylation affects the ability of tau protein to promote microtubule assembly. *J. Biol. Chem.* 259: 5301–5305, 1984.
22. Harada, A. *et al.* Altered microtubule organization in small-calibre axons of mice lacking tau protein. *Nature* 369: 488–491, 1994.
23. Brion, J.P. *et al.* Mise en évidence immunologique de la protéine tau au niveau des lésions de dégénérescence neurofibrillaire de la maladie d'Alzheimer. *Arch. Biol. (Bruxelles)* 95: 229–235, 1985.
24. Grundke-Iqbal, I. *et al.* Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. USA* 83: 4913–4917, 1986.
25. Goedert, M. *et al.* Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: Identification as the microtubule-associated protein tau. *Proc. Natl. Acad. Sci. USA* 85: 4051–4055, 1988.
26. Wischik, C.M. *et al.*, Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 85: 4506–4510, 1988.
27. Lee, V.M.-Y. *et al.* A68: a major subunit of paired helical filaments and derivatized forms of normal tau. *Science* 251: 675–678, 1991.
28. Braak, H. and Braak, E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 82: 239–259, 1991.
29. Poorkaj *et al.* Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Ann. Neurol.* 43: 815–825, 1998.
30. Hutton, M. *et al.* Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature* 393: 702–705, 1998.
31. Spillantini, M.G. *et al.* Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc. Natl. Acad. Sci. USA* 95: 7737–7741, 1998.
32. Conrad, C. *et al.* Genetic evidence for the involvement of tau in progressive supranuclear palsy. *Ann. Neurol.* 41: 277–281, 1997.
33. Goedert, M. *et al.* Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. *Nature* 338: 550–553, 1996.
34. Pérez, M. *et al.* Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau–tau interaction. *J. Neurochem.* 67: 1183–1190, 1996.
35. Von Bergen, M. *et al.* Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif [^{306}QIVYK^{311}] forming beta structure. *Proc. Natl. Acad. Sci. USA* 97: 5129–5134, 2000.

36. Lewis, J. *et al.* Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nature Genet.* 25: 402–405, 2000.
37. Allen, B. *et al.* Abundant tau filaments and nonapoptotic neurodegeneration in transgenic mice expressing human P3015 tau protein. *J. Neurosci.* 22: 9340–9351, 2002.
38. Andorfer, C. *et al.* Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. *J. Neurochem.* 86: 582–590, 2003.
39. Liou, Y.-C. *et al.* Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration. *Nature* 424: 556–561, 2003.
40. Wittmann, C.W. *et al.* Tauopathy in *Drosophila*: Neurodegeneration without neurofibrillary tangles. *Science* 293, 711–714, 2001.
41. Kraemer, B.C. *et al.* Neurodegeneration and defective neurotransmission in a *Caenorhabditis elegans* model of tauopathy. *Proc. Natl. Acad. Sci. USA* 100: 9980–9985, 2003.

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CHAPTER

46

Neurotransmitters and Disorders of the Basal Ganglia

Thomas Wichmann

Mahlon R. DeLong

ANATOMY AND PHYSIOLOGY OF THE BASAL GANGLIA 761
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DISORDERS THAT INVOLVE BASAL GANGLIA DYSFUNCTION 766
Parkinson's disease (PD) is a hypokinetic movement disorder 766
Huntington's disease is a hyperkinetic movement disorder 771
Wilson's disease is a disease of copper accumulation 773
Dystonia is characterized by sustained muscle contractions 775
Many drugs and toxins induce movement disorders 776

The basal ganglia are a group of subcortical nuclei which are components of modular circuits involved in many cortical functions. They have received considerable attention from basic scientists and clinicians alike because of their prominent involvement in movement disorders, a spectrum of diseases including disorders which are characterized by poverty of movement (hypokinetic disorders), as well as disorders characterized by excess movement (hyperkinetic disorders). It has become clear in recent years that most basal ganglia disorders are not restricted to motor disturbances, but involve cognitive and emotional features as well.

ANATOMY AND PHYSIOLOGY OF THE BASAL GANGLIA

The basal ganglia are parts of larger circuits. The basal ganglia are a group of related subcortical nuclei, including the neostriatum (caudate nucleus and putamen), the ventral striatum, the external and internal segments of the globus pallidus (GPe, GPi, respectively), the subthalamic nucleus (STN), and the substantia nigra pars reticulata and compacta (SNr, SNc, respectively). These structures are components of circuits involving portions of the cerebral cortex, thalamus and brain stem (Fig. 46-1, left). Based on their cortical site of origin, these loops can be grouped into skeletomotor, oculomotor, associative, and limbic based on their connectivity and presumed functions.

Due to its relevance to an understanding of movement disorders, the 'motor circuit' has received the most attention. This circuit is centered on somatosensory, motor and premotor cortices, which send projections to the 'motor portions' of striatum. The connections between the striatum and the basal ganglia output nuclei (GPi/SNr) are organized into direct and indirect pathways [1]. The direct pathway is a monosynaptic projection between striatum and GPi/SNr, while the indirect pathway is a polysynaptic connection that involves intercalated neurons in GPe and STN. Some striatofugal neurons may also collateralize more extensively, reaching GPe, GPi/SNr and STN. Other 'motor'-related inputs to striatum and STN arise from the intralaminar thalamic nuclei, i.e. the centromedian and parafascicular nuclei (CM/Pf).

Basal ganglia output is directed from GPi and SNr to the thalamus. Movement-related basal ganglia output projects from GPi almost exclusively to the ventrolateral nucleus of the thalamus which, in turn, projects to the primary motor cortex, the cortical supplementary motor area, and other premotor cortical areas. Movement-related output from SNr terminates in the ventral anterior and in

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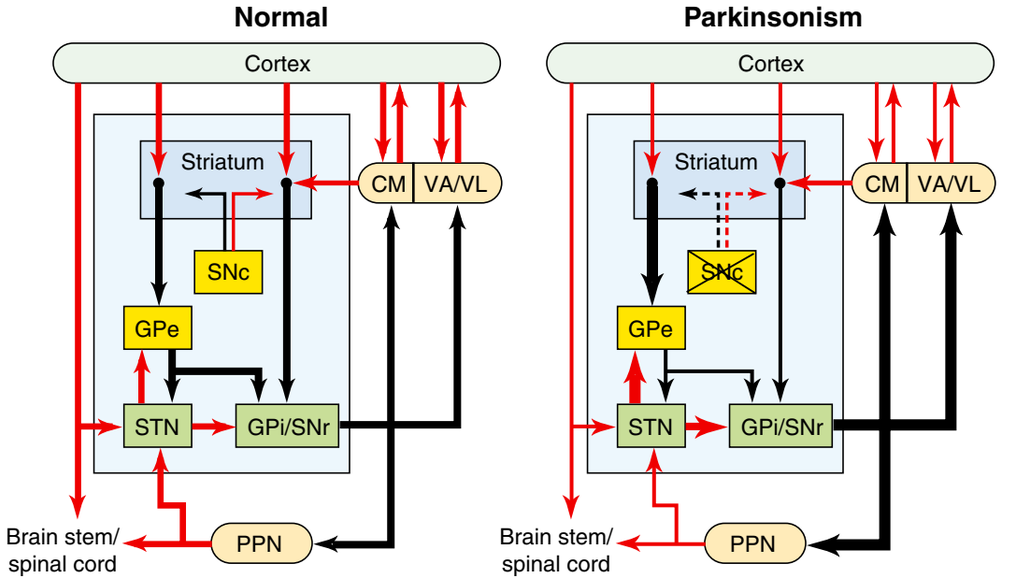

FIGURE 46-1 Simplified diagram demonstrating the anatomical connections within the basal ganglia circuitry, and changes in the activity of basal ganglia nuclei associated with the development of parkinsonism. *GPe*, external pallidal segment; *STN*, subthalamic nucleus; *GPi*, internal pallidal segment; *SNr*, substantia nigra pars reticulata; *SNc*, substantia nigra pars compacta; *PPN*, pedunculopontine nucleus; *CM*, centromedian nucleus of the thalamus; *VA*, ventral anterior nucleus of the thalamus; *VL*, ventrolateral nucleus of the thalamus. Red arrows denote excitatory connections, *black* arrows identify inhibitory (GABAergic) connections. Changes in width of arrows indicate activity changes.

the mediodorsal nuclei of thalamus, which, in turn, innervate premotor (and prefrontal) regions of the frontal lobe. GPi and SNr also project to noncholinergic neurons in the pedunculopontine nucleus (PPN) in the brainstem, and to CM/Pf. Additional projections from the SNr reach the superior colliculus. This connection may play a critical role in the control of saccades and orienting head and eye movements.

Multiple neurotransmitter systems are found in the basal ganglia. The basal ganglia contain most of the classical neurotransmitters, and many additional neuropeptides which may participate in the modulation of information transfer in the basal ganglia. Some of the more important systems will be discussed in the following paragraphs.

GABA is the predominant intrinsic transmitter of the basal ganglia. Inhibition and disinhibition are considered to be the most important modes of information transfer in the basal ganglia. Ninety-five percent of all neurons in the striatum are GABAergic medium spiny neurons. These neurons are the striatal output neurons. The medium spiny neurons which give rise to the direct pathway also contain substance P or dynorphin as a co-transmitter, while those striatal output neurons that give rise to the indirect pathway contain enkephalin as a co-transmitter. Most striatal interneurons, as well as neurons in GPe, GPi and SNr are also GABAergic. Because striatal and GPe efferents end in the SNr, GPe and GPi, GABA is found in high concentrations in these nuclei (Fig. 46-2).

Two GABA receptor subtypes have been characterized as GABA-A and GABA-B (see Ch. 16). GABA-A receptors are inhibitory ionotropic receptors (forming a chloride channel whose conductance is rapidly modulated by ligand binding), found mostly on postsynaptic membranes. GABA-B receptors are pre- and postsynaptic G-protein-coupled receptors.

Glutamate. Inputs to the basal ganglia, from the cortex, PPN and CM/Pf, as well as intrinsic projections from the STN, utilize glutamate as a neurotransmitter (Fig. 46-1).

Glutamate receptors are grouped into ionotropic and metabotropic receptors (mGluRs). Ionotropic glutamate receptors, i.e. NMDA, kainate and AMPA receptors, are present throughout the basal ganglia, and are mostly located postsynaptically. They are the primary receptors used for information transfer from cortex to striatum and STN, and STN output. In the striatum, these receptors may be involved learning, through long-term potentiation (LTP) and long term depression (LTD). In addition to the ionotropic glutamate receptors, there are eight different types of metabotropic glutamate receptors (mGluR1–8) which are classified into three groups based on their genetic and pharmacologic properties. These receptors are present at striatal and extrastriatal sites (Ch. 15).

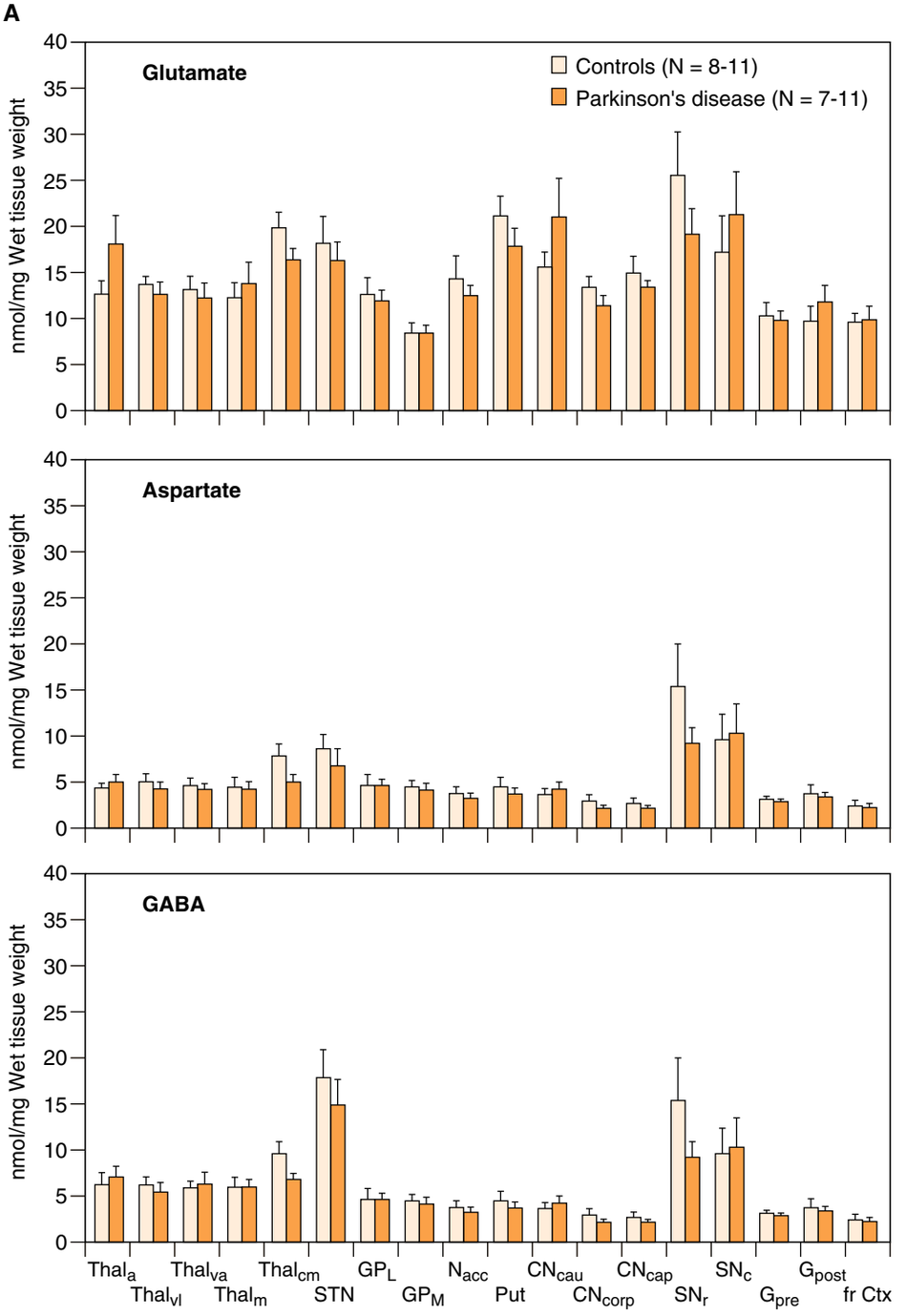

FIGURE 46-2 Concentrations in the basal ganglia in brains from normal controls and from patients with Parkinson's. (**A**) Glutamate, aspartate and GABA. Values are presented as mean ± SEM. *Thal$_a$*, anterior thalamus; *Thal$_{vl}$*, ventral lateral thalamus; *Thal$_{va}$*, ventral anterior thalamus; *Thal$_m$*, medial thalamus; *Thal$_{cm}$*, centromedial thalamus; *STN*, subthalamic nucleus; *GP$_L$*, lateral globus pallidus; *GP$_M$*, medial globus pallidus; *N$_{acc}$*, nucleus accumbens; *Put*, anterior putamen; *CN$_{cau}$*, tail of caudate nucleus; *CN$_{corp}$*, body of the caudate nucleus; *CN$_{cap}$*, head of caudate nucleus; *SN$_r$*, substantia nigra pars reticulata; *SN$_c$*, substantia nigra pars compacta; *G$_{pre}$*, precentral gyrus; *G$_{post}$*, postcentral gyrus; *fr Ctx*, frontal cortex.

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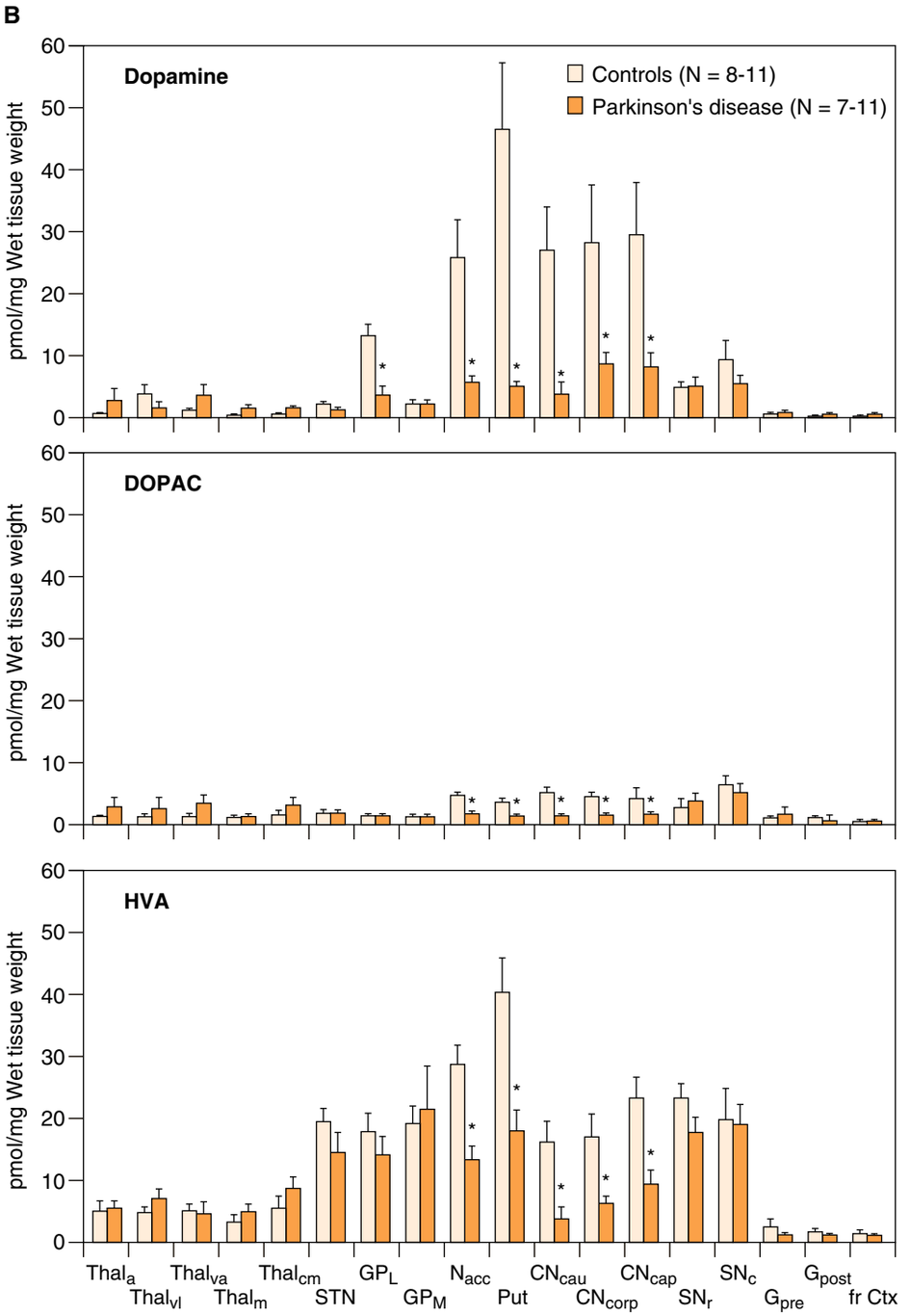

FIGURE 46-2—cont'd (**B**) Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Values are presented as mean ± SEM, *, $p<0.05$. $Thal_a$, anterior thalamus; $Thal_{vl}$, ventral lateral thalamus; $Thal_{va}$, ventral anterior thalamus; $Thal_m$, medial thalamus; $Thal_{cm}$, centromedial thalamus; *STN*, subthalamic nucleus; GP_L, lateral globus pallidus; GP_M, medial globus pallidus; N_{acc}, nucleus accumbens; *Put*, anterior putamen; CN_{cau}, tail of caudate nucleus; CN_{corp}, body of the caudate nucleus; CN_{cap}, head of caudate nucleus; SN_r, substantia nigra pars reticulata; SN_c, substantia nigra pars compacta; G_{pre}, precentral gyrus; G_{post}, postcentral gyrus; *fr Ctx*, frontal cortex.

Acetylcholine. Most of the acetylcholine in the basal ganglia is found in the striatum, as the neurotransmitter of the large spiny interneurons, which account for about 3% of all striatal neurons. Both muscarinic and nicotinic cholinergic receptors are found in the striatum. Postsynaptic muscarinic receptors may inhibit transmitter release from glutamatergic terminals, whereas nicotinic receptor activation may enhance transmitter release.

Dopamine. Dopamine is present in high concentration in the striatum (Fig. 46-2), in densely aborized terminals of projections that originate in the SNc. Neurons in the

ventral tegmental area contribute to the dopamine supply to the ventral striatum as well as the cortex. Perhaps the most important site of extrastriatal dopamine release is the SNr, where the transmitter is released from dendrites of SNc neurons.

Dopamine synthesis in dopaminergic terminals (Fig. 46-3) requires tyrosine hydroxylase (TH) which, in the presence of iron and tetrahydropteridine, oxidizes tyrosine to 3,4-dihydroxyphenylalanine (levodopa, l-DOPA). Levodopa is decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC), an enzyme which requires pyridoxyl phosphate as a coenzyme (see also in Ch. 12).

Dopamine acts on G-protein-coupled receptors belonging to the D1-family of receptors (so-called 'D1-like receptors', or D1LRs, comprised of D1- and D5-receptors), and the D2-family of receptors ('D2-like receptors', or D2LRs comprised of D2-, D3- and D4-receptors). D1LRs stimulate adenylate cyclase activity and, possibly, also phosphoinositide hydrolysis, while D2LRs reduce adenylate cyclase activity. In the striatum, D1LRs are predominately associated with medium spiny neurons of the direct pathway, while D2LRs have been found as autoreceptors on dopaminergic terminals, as heteroreceptors on cholinergic interneurons, and on indirect pathway neurons. In the SNr, D1LRs are located on terminals of the direct pathway projection, while D2LRs appear to function as autoreceptors.

The actions of dopamine are terminated through presynaptic reuptake. Some of the dopamine is then re-incorporated into vesicles, while the rest is metabolized (Fig. 46-3). Dopamine and its O-methyl derivative are both subject to the action of monoamine oxidase (MAO), a flavoprotein present in the outer membrane of the mitochondria. MAO exists in two forms: MAO type A (MAO-A) is predominantly present in catecholaminergic neurons, while MAO type B (MAO-B) predominates in serotonin-containing neurons and in astrocytes. Products of the MAO reaction include the aldehyde corresponding to the amine substrate, hydrogen peroxide and ammonia. Most of the aldehyde undergoes further dehydrogenation to form, in the case of dopamine, DOPAC, which is the substrate for catechol-O-methyltransferase (COMT), to generate homovanillic acid (HVA).

The basal ganglia are involved in multiple functions. The motor circuit of the basal ganglia has received the greatest attention from researchers, because of its perceived involvement in movement disorders. Due to the modular organization of the basal ganglia, many of the findings discussed below for the motor circuit may be applicable to the other circuits as well.

A basic understanding of basal ganglia function can be gleaned from considering the effects of cortical activation on the excitatory (glutamatergic) and inhibitory (GABAergic) projections in the basal ganglia (Fig. 46-1). Voluntary movements appear to be initiated at the cortical level of the motor circuit. Activation of the direct pathway neurons in the striatum will inhibit basal ganglia output neurons, which, in turn, will disinhibit related thalamocortical neurons, and ultimately facilitate movement. In contrast, activation of indirect pathway neurons in the striatum will lead to increased basal ganglia output and, presumably, to suppression of movement. Because most GPi neurons

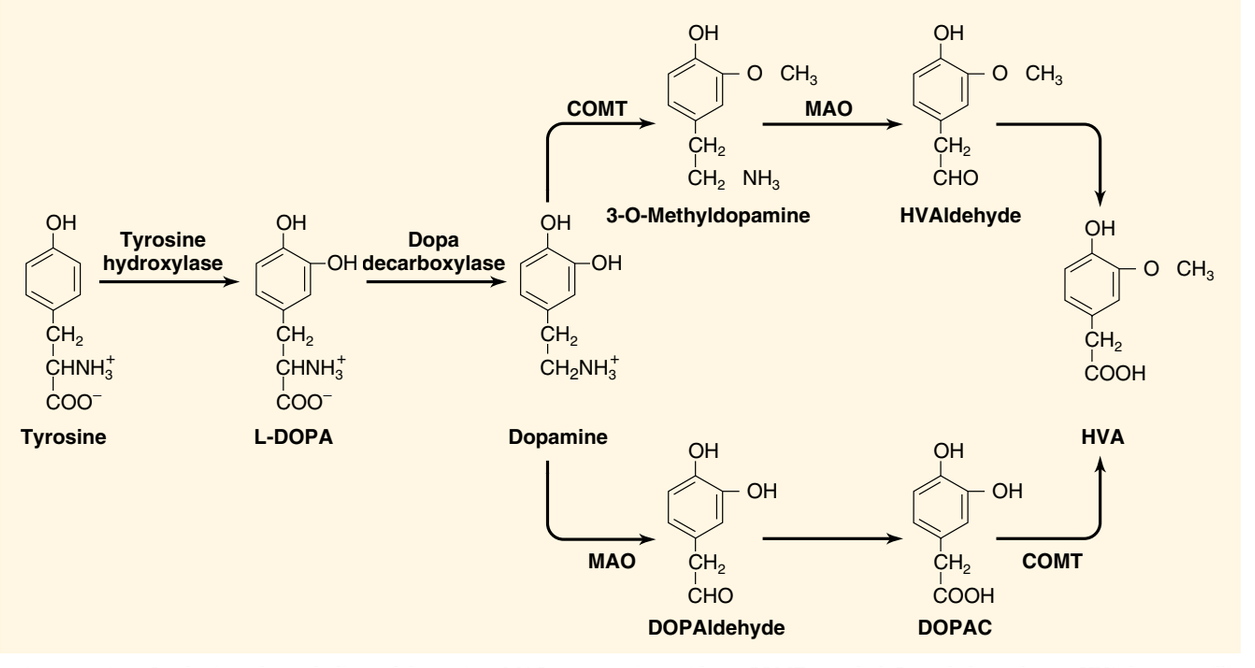

FIGURE 46-3 Synthesis and metabolism of dopamine. *MAO*, monoamine oxidase; *COMT*, catechol-O-methyltransferase; *HVA*, homovanillic acid; *DOPAC*, 3,4-dihydroxyphenylacetic acid.

increase their firing rate with movement, the main role of the basal ganglia motor circuit is to inhibit and to stabilize the activity of the thalamocortical neurons.

The basal ganglia motor circuit may also play a role in the control of the amplitude or velocity of movement, and may focus movements, allowing intended movements to proceed while suppressing other movements (see discussion in ref. [2]). The basal ganglia have also been implicated in self-initiated (internally generated) movements, procedural learning, and movement sequencing [e.g. ref. 3]. Learning may involve modifications of the strength of glutamatergic synapses onto medium spiny neurons in the striatum through dopamine–glutamate interactions which are timed to salient external events. It is thought that phasic dopamine release in the striatum signals whether or not reward-related phenomena in the environment are present. These dopamine signals may affect LTP and LTD at corticostriatal synapses (see also in Ch. 53).

DISORDERS THAT INVOLVE BASAL GANGLIA DYSFUNCTION

Parkinson's disease (PD) is a hypokinetic movement disorder. PD manifests itself in most patients with prominent movement abnormalities, including a 4–6 Hz tremor at rest, muscular rigidity, slowness of movement (bradykinesia), and a failure of movement initiation (akinesia). Many of these signs are initially unilateral, but later progress to involve the opposite side. Depression occurs in up to 50% of patients. PD is a progressive disorder. Non-motor signs and symptoms, such as autonomic insufficiency and disturbances of gait and posture, develop later, along with psychiatric, cognitive and sleep complications.

Between 5 and 24 new cases per 100,000 persons are reported each year (20.5 per 100,000 in the USA). The prevalence of the disease is 57–371 patients per 100,000 persons worldwide (300 per 100,000 for the USA and Canada). With the increasing age of the population, a four-fold increase in the prevalence of PD is expected over the next 20 years. The age of onset differs substantially between patients (average age of onset is 62 years). Young-onset patients commonly suffer from one of the genetic forms of the disease (see below). Males appear to be affected slightly more frequently than females.

Several environmental factors may have an impact on the occurrence of the disease. Living in rural areas, drinking well water, pesticide exposure and head trauma are associated with an increased risk of developing PD, while caffeine consumption, taking nonsteroidal anti-inflammatory medications, and smoking appear to protect from it.

Pathology. The earliest degenerative changes in PD occur in structures outside of the basal ganglia [4], in the olfactory bulb, and brainstem structures, such as the dorsal motor nucleus of vagus, the locus coeruleus and the raphe nuclei. The prominent motor abnormalities in PD appear to arise in large part from degeneration of neurons in the SNc, with resulting loss of dopamine in the basal ganglia. Degenerating dopaminergic cells leave characteristic eosinophilic inclusions in their wake, the so-called Lewy neurites and Lewy bodies. Recent studies have also shown that low-level inflammatory responses may accompany the loss of dopaminergic cells, and may contribute to cell death. Recent imaging studies have suggested that the striatal dopamine concentrations steeply decline, preceding the onset of clinical Parkinsonism, which is usually only seen when more than 70% of striatal dopamine is lost.

In early phases of PD, dopamine loss affects primarily the posterior putamen (the striatal motor area) but later spreads to involve other nigrostriatal regions. In later stages, more widespread dopamine loss and neuronal degeneration in non-dopaminergic systems, such as the locus coeruleus and the raphe nuclei may account for some of the non-motor aspects of PD.

Etiology. Most cases of PD are 'sporadic' and appear to arise from a combination of genetic predisposition and environmental or toxic factors. Purely genetic forms of the disease probably account for less than 10% of cases, but the risk of family members of an affected patient to develop PD is significantly increased even in 'sporadic' PD.

Cells in the SNc are among the most vulnerable cells in the brain due to a relative deficiency in neuroprotective factors, such as the antioxidant glutathione, and because they are exposed to a high level of oxidative stress due to the presence of dopaminergic metabolism and other factors. This environment may render these neurons particularly vulnerable to nonspecific genetic or environmental insults that, by themselves, would not be sufficient to induce cell death in other cells. Interestingly, many of the factors known to be involved in neuronal damage in PD appear to interfere with the cell's ability to eliminate damaged or mutated proteins through the ubiquitin proteasome system [5].

One example of a genetically determined form of parkinsonism, which results directly in degeneration of dopaminergic neurons are mutations of the gene coding for α-synuclein [6]. α-Synuclein is a normal brain protein that is found in synaptic vesicles and membranes along axons and terminals in many neurons. Mutated α-synuclein tends to aggregate as the result of a conformational change of the molecule from its usual unfolded, soluble form into a β-pleated sheath. Aggregated α-synuclein is a prominent component of Lewy bodies.

The mechanism by which the mutated α-synuclein produces toxicity is not clear, but it is thought that the mutated protein forms fibrils, and the oligomers or fibrils formed from this protein exert a toxic gain of function leading to disruption of proteasomal function and the formation of Lewy bodies. However, as evidenced by the

discovery of several families in which triplication of the α-synuclein gene leads to four normal copies of the gene associated with autosomal dominant Parkinson's disease, overproduction of normal α-synuclein may also be causal. Dopaminergic neurons appear particularly sensitive to toxic effects of excessive or fibrillar α-synuclein. Other mutated genes possibly associated with autosomal dominant parkinsonism include UCHL1, NR4A2 and synphilin-1(see ref. [7]).

A number of genetic mutations have been associated with autosomal recessive juvenile Parkinson's disease (ARJP). The most frequent of these is the gene *PARK2*, coding for parkin, which accounts for about half of patients with juvenile onset, below the age of 40. Parkin is an E3 ubiquitin ligase that is necessary for ubiquitination of proteins for their subsequent degradation in proteasomes. Loss of function of the mutated enzyme may result in failure to direct its substrates to the proteasome system for degradation. Of interest is that Lewy bodies are not found in these subjects. Other genes for which there is strong evidence of linkage to ARJP include *DJ-1* (*PARK7*) and *PINK1* (*PARK6*). *DJ-1*, which is translocated to the outer mitochondrial membrane under conditions of oxidative stress, is therefore thought to have a role in protecting neurons from oxidative stress. *PINK1* encodes a putative serine/threonine protein kinase. *PINK1* is located primarily in mitochondria, and evidence from cell cultures suggests that *PINK1* protects cells from stress-induced mitochondrial damage and apoptosis. At present, there is no postmortem neuropathologic information regarding the *DJ-1* and *PINK1* mutations. While the mentioned genetic mutations all lead to parkinsonism with progressive bradykinesia, rigidity, tremor and levodopa-responsiveness, the biochemical processes are different. Key questions related to all of the genetic mutations found are:

- Why is the dopaminergic system so uniquely vulnerable to their effects?
- What are the relationships of Lewy body formation, proteasomal protein degradation and mitochondrial dysfunction to parkinsonism (see ref. [8] for a detailed review).

Animal models. A variety of models can be employed to disturb dopaminergic functions in the brain. Attempts to model parkinsonism by treatment with the dopamine-depleting agent reserpine, or with dopamine-receptor blockers are fairly nonspecific. Models produced by dopaminergic toxins are more faithful reproductions of the pathology of the human disorder. In rodents, injections of 6-hydroxydopamine (6-OHDA) into the SNc, or treatment with mitochondrial complex I inhibitors, such as rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can be used to cause oxidative damage to dopaminergic neurons. Genetic models of parkinsonism in rodents are based on overexpression of α-synuclein. In primates, the most faithful model in terms of behavioral and pathological changes is produced by treatment with MPTP.

Pathophysiology. Metabolic and microelectrode recordings of neuronal activity in animal models of parkinsonism show that spontaneous neuronal discharge in STN, GPi and SNr is increased, as, compared with normal controls, neuronal discharge in GPe is decreased (Fig. 46-4). These results and the known anatomical connections between the different structures have been used to develop a circuit model of parkinsonism (Fig. 46-1, right) in which striatal dopamine loss results in reduced activity in the direct pathway, resulting in disinhibition of the basal ganglia output nuclei. Striatal dopamine loss also results in greater inhibition of GPe, and, consequently, in disinhibition of STN and GPi. The net effect of these changes is an increase in basal ganglia output to brainstem and thalamus, which has been postulated to result in greater inhibition of thalamocortical neurons, and to reduce the responsiveness of brainstem and cortical mechanisms involved in motor control. Brainstem areas such as the PPN may also be involved in the development of akinesia, most likely secondary to excessive inhibitory input from GPi and SNr.

Additional support for this model comes from inactivation studies. For instance, GPi or STN lesions have been shown to reverse all the cardinal motor signs of parkinsonism, presumably by reducing the inhibitory basal ganglia output. However, some results of these lesion studies are not compatible with the simple model shown in Figure 46-1. Thus, surprisingly, lesions of the 'basal ganglia-receiving' areas of the thalamus (VA/VL) do not lead to parkinsonism, and lesions of GPi do not result in excessive movement. These findings suggest that the simple model shown in Figure 46-1(right) may not accurately describe all of the circuit abnormalities in PD.

Other factors that may play a role include specific alterations in information processing, alterations in discharge patterns and changes in the level of synchronization between neighboring neurons. All of these changes have been documented in parkinsonian patients undergoing electrophysiologic recording in the context of neurosurgical treatment of PD, or in animal models of the disorder, but it remains unclear whether there is a causal relationship between any of these abnormalities and the appearance of specific parkinsonian motor signs. The strongest argument for this can be made in the case of a relationship of tremor to the frequently seen oscillatory burst activity in the basal ganglia. However, it has proved to be extremely difficult to identify a single source of oscillatory discharge in the basal ganglia–thalamocortical circuitry.

Within the basal ganglia, abnormal oscillations may arise from changes in local pacemaker networks, through loss of extrastriatal dopamine, or as the result of intrinsic membrane properties of basal ganglia neurons. Increased inhibitory basal ganglia output may also act to induce

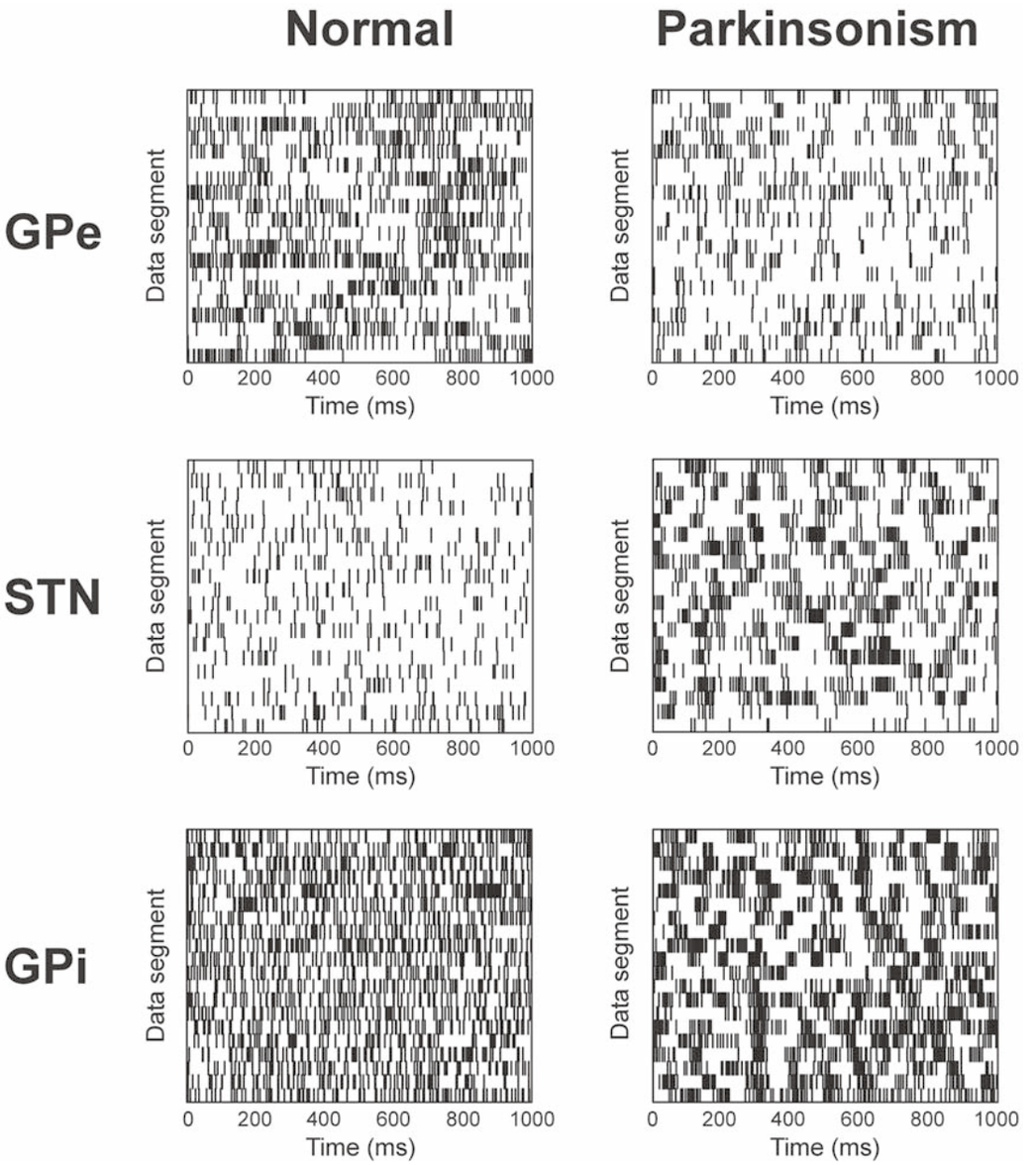

FIGURE 46-4 Activity changes in the basal ganglia in primate parkinsonism. The data are shown as raster diagrams, where each individual line represents a single neuronal discharge. Each diagram shows 20 consecutive 1000 ms segments of data from GPe, STN and GPi. The neuronal activity is reduced in GPe, and increased in STN and GPi. In addition to the rate changes, there are also obvious changes in the firing patterns of neurons in all four structures, with a marked prominence of burstiness and oscillatory discharge patterns in the parkinsonian state.

oscillatory discharge in the thalamus, which may then be transmitted to cortex. Besides tremor, certain aspects of akinesia or bradykinesia may also be related to oscillatory discharge in the basal ganglia. Thus, based on results in patients in whom deep brain stimulation (DBS) leads in the STN were used to record local field potentials from that nucleus, a theory has been proposed according to which the presence of coherent low frequency oscillations (< 30 Hz) in the cortico–basal ganglionic interaction may act to interfere with movement.

Nonoscillatory, phasic activity in the basal ganglia may also be deleterious to normal movement, because it may erroneously signal excessive movement or velocity to precentral motor areas, leading to a slowing or premature arrest of ongoing movements and to greater reliance upon external clues during movement. Any alteration of discharge

in the basal ganglia may also simply introduce noise into thalamic output to the cortex that is detrimental to cortical operations.

Pharmacotherapy. The currently available treatments for PD are symptomatic, and do not alter the course of the disease. The earliest treatment that is still in limited use today, is with the anticholinergic medications, such as trihexiphenidyl or benzotropine. These drugs are useful, particularly for tremor. However, their use is often problematic because of unpleasant side-effects, such as memory disturbances, blurred vision, sedation, dry mouth, or urinary retention, particularly in older patients.

After its introduction in the early 1960s, oral dopamine replacement therapy quickly became the mainstay of treatment of the disorder. The first agent to be introduced was the dopamine precursor levodopa, an amino acid which is metabolized into dopamine both peripherally and centrally. It quickly became apparent that the peripheral conversion into dopamine results in gastrointestinal and autonomic side-effects which significantly limit the usefulness of this agent. This problem was partially remedied by combining levodopa with peripheral blockers of dopa-decarboxylase (carbidopa or benserazide). These combination treatments have had an enormous impact on the outlook for patients with PD, greatly ameliorating many of the clinical features of the disorder and virtually normalizing life expectancy. More recently, blockers of another enzyme which is involved in peripheral metabolism of levodopa, catechol-O-methyl-transferase (COMT), was added to the therapeutic arsenal. The two available COMT-inhibitors, tolcapone and entacapone, are particularly useful in patients who suffer significant motor fluctuations with 'wearing off' of the drug effects, by slowing the metabolism of levodopa. However, problems with liver toxicity forced almost complete withdrawal of tolcapone from the market. The key to the effectiveness of the inhibitors, both carbidopa and entacapone, is that they do not cross the blood–brain barrier, so that their effects are strictly peripheral.

Levodopa therapy has a number of troubling side-effects. The most prevalent short-term problems include nausea and autonomic disturbances, such as orthostatic hypotension, and nausea. However, more troublesome are the development of motor fluctuations (wearing off and failed responses to dosing), drug-induced dyskinesias, and hallucinations. These are the primary reasons why many physicians delay the use of this agent. It also remains a theoretical possibility that levodopa may contribute to the degeneration of dopaminergic cells through free radical production. The results of recent trials suggest, however, that levodopa treatment does not hasten the clinical progression of the disease.

The most important alternatives to levodopa therapy are direct-acting dopamine receptor agonists, such as ropinirole, pramipexole, or pergolide (Fig. 46-5). A number of studies have shown that use of these agents may help to delay the need for use of levodopa/carbidopa. This has prompted the recommendation that dopamine receptor agonists should be used as first-line agents in patients with PD. It appears that these agents may also have a mild neuroprotective effect, which has further bolstered the case for their early use as monotherapy. Because the agonists have a longer half-life than levodopa and carbidopa, they are also often useful in later stages of the disease, when the response to levodopa and carbidopa may become erratic. Another dopamine agonist, apomorphine, has recently reached the market. This agent is only available in injectable form, and has a very short half-life. Its main use is in 'rescue' from episodes of unpredictable worsening in the severity of parkinsonian signs (sudden freezing episodes), sometimes seen in patients with long-standing PD.

The side-effects of agonists are similar to those of levodopa, including nausea, hallucinations and dyskinesias. Although also described for levodopa, recent studies have shown that daytime sedation with sudden 'sleep attacks' is a significant side-effect of these compounds. In addition, pergolide is an ergot derivative, and, like other ergot compounds, may result in ergotism, and may rarely induce cardiac fibrosis and valvular lesions. Ropinirole and pramipexole, both non-ergot compounds, do not have these side-effects.

Neuroprotective treatments. The first controlled clinical trial for neuroprotection in PD evaluated the MAO-B inhibitor selegiline and vitamin E vs. placebo control. Selegiline was selected on the premise that PD might be caused by an (unknown) environmental toxin, akin to MPTP, which requires an MAO-B dependent toxification step [9]. The initial studies with this medication were complicated by the fact that selegiline also has modest symptomatic effects, which were not completely accounted for in the washout phase of these experiments. Although this drug remains in wide use, its neuroprotective benefit remains controversial. It is now thought that mechanisms independent of its MAO-B-blocking actions may contribute to its (rather modest) neuroprotective actions. Initial use of dopamine receptor agonists such as ropinirole or pramipexole appear to be effective in reducing the development of dyskinesias. Coenzyme Q-10, an agent that may help to augment mitochondrial function, has been shown in preliminary trials to slow progression in loss of activities of daily living in PD.

Based on recent insights into the pathogenesis and pathophysiology of PD, a large number of additional compounds have been suggested for neuroprotection in this disease. Drugs that reduce α-synuclein aggregation or inhibit apoptosis (propargylamine, caspase inhibitors), antioxidants (glutathione enhancers, tocopherol, flavonoids), anti-inflammatory drugs (nonsteroidal anti-inflammatory agents, inhibitors of cyclo-oxygenase-2), as well as glutamate receptor antagonists, given in an effort to reduce excitotoxicity, may all serve to provide some level of neuroprotection. In addition, trophic factors and agents to protect mitochondrial function have also been proposed as neuroprotective agents. Conceivably, cocktails of such drugs may

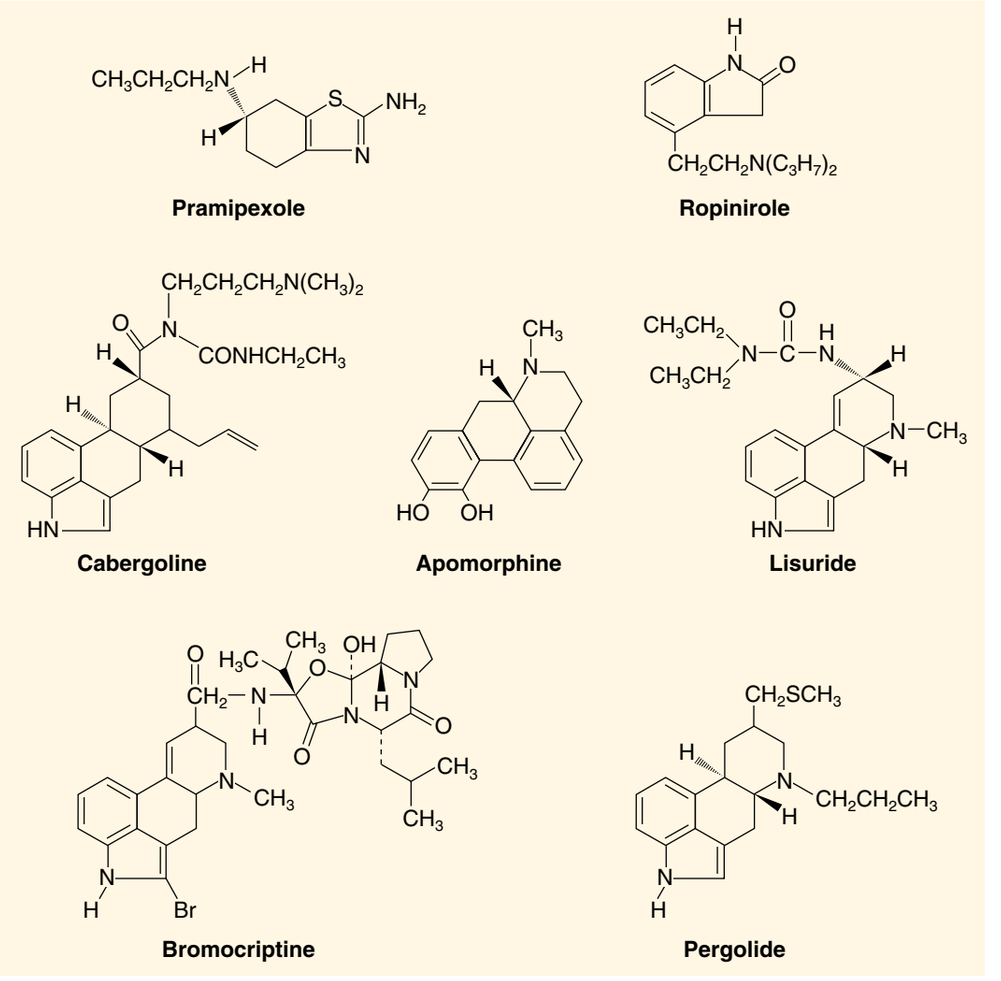

FIGURE 46-5 Chemical structures of dopamine receptor agonists.

be used to provide effects beyond those achievable with single-drug approaches. Finally, in a remarkable series of studies it has been shown, in rodents, that physical exercise can reduce the effects of neurotoxins that induce parkinsonism such as MPTP and 6-OHDA. These effects may be due to exercise-induced production of trophic factors.

Surgical therapy. After 5–10 years, many patients reach a state in which antiparkinsonian drugs are no longer adequate because of the development of intractable motor fluctuations and dyskinesias. For these patients, neurosurgical approaches can be highly beneficial. Neurosurgical procedures were, in fact, widely used in the 1950s and 1960s, but were essentially abandoned with the introduction of levodopa therapy. Following promising results from studies that demonstrated reversal of parkinsonian motor signs through lesioning of the STN in MPTP-treated monkeys [10], there has been renewed interest in surgical treatments of parkinsonism. This was first employed in the form of GPi lesions (pallidotomy) (e.g. ref. [11]). Subsequently, high-frequency deep brain stimulation (DBS) of both the STN and GPi has been shown to reverse parkinsonian signs in a manner similar to ablation. Multiple mechanisms may contribute to these effects, for instance, inhibition of STN neurons through depolarization block, activation of inhibitory afferents, or true activation of STN efferents to the pallidum. Follow-up studies in patients who have been treated with these procedures have demonstrated that although the disease continues to progress in patients with lesions or DBS, patients get significant symptomatic benefit, which may last in excess of five years.

Thalamotomy and thalamic DBS, while effective against parkinsonian tremor, have been largely abandoned since they do not treat akinesia or bradykinesia. Interventions at the level of the STN or GPi are effective against akinesia, bradykinesia rigidity, tremor, as well as disabling drug-induced dyskinesias and dystonia.

Through the use of modern imaging techniques and microelectrode guidance, these procedures have a high success rate and a low incidence of serious complications. Similar to other neurosurgical procedures, they may induce hemorrhage, infection, or cognitive problems (probably due to 'collateral' damage from probe passage through frontal areas), or stroke-like symptoms due to vascular or mechanical damage.

In developed countries, ablative procedures have largely been replaced by DBS, because the latter are equally effective or somewhat superior to ablation, are less invasive, and are reversible and adjustable. In addition, bilateral pallidal lesions have an increased risk of causing speech and swallowing dysfunction, while bilateral DBS procedures can be done without significantly increased risk. However, the stimulators require battery replacements, are prone to mechanical lead difficulties and sometimes require frequent adjustment of stimulation parameters.

One of the persistent paradoxes in basal ganglia research remains that these invasive procedures seem to have very little effect on motor performance in normal animals. Thus, no clear motor deficits and only subtle cognitive side-effects have been demonstrated. Mood disturbances, such as depression or manic episodes have been described particularly after STN DBS procedures, but are also relatively uncommon. It appears that the brain can tolerate or compensate for a lack of basal ganglia function much more readily than for the abnormal basal ganglia output that occurs in PD. In fact, although the mechanism of action of DBS is controversial, it now appears that it may act by replacing the abnormal output of the basal ganglia with a more tolerable pattern of discharge.

New treatment options. A variety of new treatment options are currently under development, and may become available in the near future. Among these are transdermal application of short-acting dopamine agonists. Selective D1-receptor agents, such as dihydrexidine also offer promise. In addition, non-dopaminergic treatments are under development.

One of the most promising treatments in this category is targeting the A2A adenosine receptor (A2ARs). A2ARs are expressed with the greatest abundance in the striatum, but are also found in other nuclei of the basal ganglia. The majority of striatal A2AR receptors appear to be postsynaptic, on dendrites of medium spiny neurons that give rise to the 'indirect' pathway. They may promote GABAergic signaling in these neurons, thus functionally opposing D2-receptor activation. Another important anatomical feature is that many dendrites carrying A2ARs appear to be contacted by glutamatergic terminals, suggesting that A2AR stimulation may facilitate cortical glutamatergic excitatory input to striatopallidal neurons. In parkinsonism, abnormalities of glutamatergic transmission in the striatum have been linked to the development of dyskinesias. Because of these anatomical and pharmacologic features, the use of A2AR antagonists has become a strategy for treating parkinsonism. Experiments in rodents and primates, and preliminary studies in humans, have shown that an A2AR antagonist (KW-6002) potentiates and prolongs the antiparkinsonian response to low-dose levodopa, and attenuates the induction and expression of dyskinesias and other motor response fluctuations that occur in response to dopaminergic stimulation (e.g. ref. [12]).

There is also substantial interest in drugs that target the glutamatergic system. Initial attempts have focused on antagonists that block ionotropic glutamate receptors. Preclinical and clinical studies with these medications (such as MK-801 or remacemide) have yielded mixed results, mostly because of substantial side-effects due to the fact that such glutamate receptors are ubiquitously distributed throughout the central nervous system [13]. More recently, metabotropic glutamate receptors have emerged as potential targets for therapeutic interventions. These compounds have the advantage of being relatively selectively located in specific basal ganglia loci, so that side-effects may be less likely to occur than with the ionotropic glutamate receptor ligands.

Finally, other surgical procedures are also being tested. For instance, there has been a long history of using cell transplants in parkinsonian patients. Initial transplantation studies have focused on the use of dopaminergic adrenal or fetal mesencephalic donor tissues. Owing to the mixed results of recent studies investigating the use of such grafts in parkinsonian patients, these procedures remain experimental. A particular problem that has become apparent is the appearance of transplantation-induced dyskinesias, which may be the result of unregulated dopamine release from the transplanted tissue [14]. Because of ethical and efficacy concerns, it is likely that graft procedures will increasingly rely upon stem cells or encapsulated genetically modified cells, which may offer the opportunity to regulate the graft's dopamine production.

Huntington's disease is a hyperkinetic movement disorder. Huntington's disease was described in 1872 by George Huntington, a general practitioner from Pomeroy, Ohio, who described a Long Island family with a hereditary form of chorea. The term chorea refers to involuntary arrhythmic jerky movements of the limbs. Early in the disease process, the chorea is often a focal phenomenon, and may manifest itself as increased blinking, grimacing, or fidgetiness. Later, it progresses to involve multiple body parts, reaching its maximum within 10 years, when it is gradually replaced by bradykinesia and rigidity, i.e. symptoms reminiscent of PD. Besides the relentless development of chorea, prominent non-motor disturbances such as depression, behavioral disturbances, and cognitive impairments are also seen, and often represent the most significant source of disability for these patients and their families. Many patients have additional, symptoms such as weight loss and autonomic dysfunction.

Men and women are equally affected with a prevalence of 5–10 per 100,000 people [16]. There are distinct geographic

and ethnic differences in the prevalence of Huntington's disease, with the highest prevalence reported in the Western European population. The disease usually manifests itself after the third decade of life, although juvenile cases have been reported. Most patients die as the result of medical complications of the disease, usually 15–20 years after symptom onset.

Pathology and pathophysiology. Pathologic studies have shown widespread neuronal degeneration throughout the cortex, basal ganglia, thalamus and brainstem. Of these, striatal areas seem to be particularly affected [17]. Striatal degeneration first involves enkephalin-containing output neurons, i.e. a group of cells projecting to GPe. This may lead to reduced inhibition of GPe neurons and, subsequently, to increased inhibition of STN neurons, decreased facilitation of GPi output and disinhibition of thalamocortical neurons. Huntington's disease shares the reduction of basal ganglia output to the thalamus with other 'hyperkinetic' disorders, such as hemiballism, a disorder that most often arises from a stroke in the STN. In later stages of Huntington's disease, however, inhibitory striatal output neurons to GPi begin to degenerate, which may result in disinhibition of GPi neurons and development of parkinsonian features.

Striatal degeneration in Huntington's disease goes far beyond the motor portion of this structure. In fact, the earliest and most prominent anatomic and radiologic features of Huntington's disease are in the caudate nucleus. The involvement of basal ganglia–thalamocortical associative and limbic circuits may contribute to the prominent psychiatric and cognitive abnormalities in this disease. Patients also suffer widespread neural degeneration in non-basal-ganglia structures, including cortex, thalamus and brainstem, which may also play a significant role in the disease.

Genetic and molecular aspects of the disease. Huntington's disease results from a mutation of a highly conserved gene on the short arm of chromosome 4 and is transmitted in an autosomal dominant fashion (see ref. [18]). The gene codes for the cytosolic and nuclear protein huntingtin, which is associated with microtubules and synaptic vesicles, and which is widely expressed throughout the nervous system, and in non-neuronal tissues. Huntingtin appears to serve a critical function early in development. It may have a role in axonal transport, and may be involved in processes counterbalancing apoptosis.

Huntington's disease is a prototypical trinucleotide repeat disorder (see also Ch. 39 and **Box 46-1** for disorders due to polyglutamine repeat mutations) [19] in which a short DNA fragment consisting of repeated segments of the trinucleotide sequence CAG greatly expands, resulting in expansion of a polyglutamine sequence in the related gene product. Within a given family, the age of onset of the disease tends to drop from one generation to the next (*anticipation*), at least in part due to further expansion of the CAG repeat sequence.

The process by which expansion of polyglutamine sections within the huntingtin protein results in the neuropathology of Huntington's disease remains unknown, but probably represents a toxic gain of function. Mutated huntingtin tends to form proteolysis-resistant aggregates, probably because of the formation of cross-links between its polyglutamine sections **(Fig. 46-6)**, but it is unclear whether these aggregates are toxic, or whether they simply represent a metabolic end-product. Mutant huntingtin also increases the expression of caspase-1, which may, through caspase-3 activation, trigger apoptosis. Huntingtin may also act to impair proteasomal function and may lead to transcriptional dysregulation. Mutant huntingtin is also associated with changes in activation of various associated proteins, such as huntingtin-associated protein 1 (HAP1), and huntingtin-interacting proteins, which, in turn, may have far-reaching consequences in terms of intracellular transport pathways, the cellular Ca-homeostasis and other phenomena. Finally, problems with mitochondrial energy metabolism have also been identified in Huntington's disease.

Animal models. Early animal models of the disease were directed at mimicking the loss of striatal neurons in Huntington's disease, by injections of neurotoxins into the striatum [20]. Thus, striatal injection of excitatory neurotoxins, such as kainic acid, a rigid analog of glutamate, causes destruction of intrinsic GABA-containing and cholinergic neurons, but spares glia and afferent axons. Affected neurons are those which possess receptors for excitatory amino acid neurotransmitters. Quinolinic acid, a tryptophan metabolite found in brain and other tissues, has a more restricted neurotoxicity, which fairly closely mimics the chemical pathology of early Huntington's disease.

Injections of the neurotoxin 3-nitropropionic acid, an irreversible inhibitor of complex II of the mitochondria respiratory chain, induce selective striatal degeneration similar to that observed in Huntington's disease. Genetically modified animals which express either full-length mutant human huntingtin gene, or sections of the 5′ end of this gene, which contains the CAG expansion, mimic many of the pathologic and some of the behavioral features of the disease.

Symptomatic treatment. The chorea of Huntington's disease responds (partially) to treatment with neuroleptics, which, through blockade of D2 receptors, may help to increase basal ganglia output to more normal levels. Dopamine-depleting agents, such as reserpine or tetrabenazine have also been used. At best, these agents are only moderately effective and they should only be used if the chorea truly interferes with activities of daily living or produces social embarrassment. Neuroleptics and

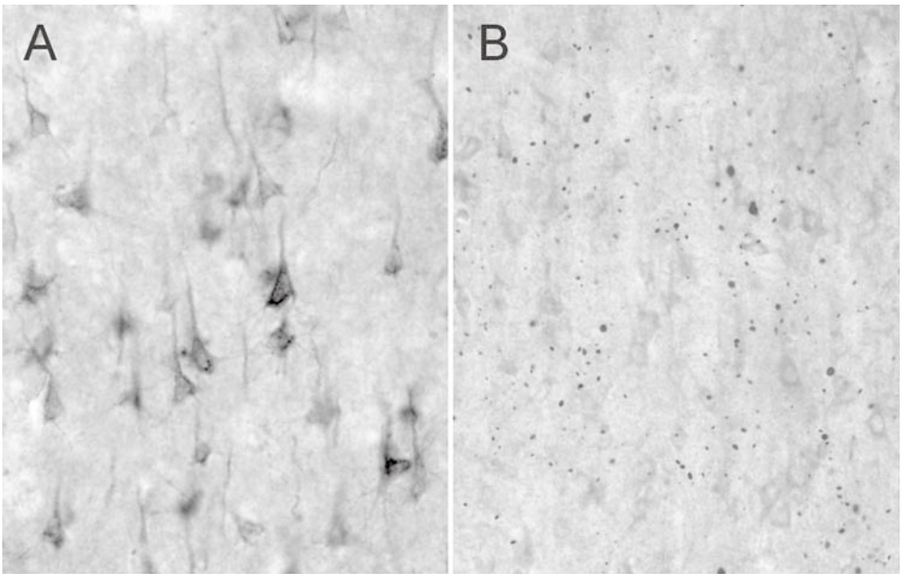

FIGURE 46-6 (**A**) Huntingtin distribution in a normal control in cortex (visualized using an antibody recognizing the internal epitope of the huntingtin molecule). (**B**) Huntingtin aggregates in the cortex of a patient with Huntington's disease (visualized with an antibody recognizing the *N*-terminal epitope of huntingtin). Both images were taken at the same magnification. Courtesy of Dr Claire-Anne Gutekunst (Emory University).

dopamine-depleting agents need to be discontinued in the late akinetic–rigid stage of the disease, because these agents may aggravate parkinsonian symptoms.

Non-motor signs of the disorder are also treatable with symptomatic medications. The frequent mood disorder can be treated with standard antidepressants, including tricyclics (such as amitryptiline) or serotonin reuptake inhibitors (SSRIs, such as fluoxetine or sertraline). This treatment is not without risks in these patients, as it may trigger manic episodes or may even precipitate suicide. Anxiety responds to benzodiazepines, as well as to effective treatment of depression. Long-acting benzodiazepines are favored over short-acting ones because of the lesser abuse potential. Some of the behavioral abnormalities may respond to treatment with the neuroleptics as well. The use of atypical neuroleptics, such as clozapine is preferred over the typical neuroleptics as they may help to control dyskinesias with relatively few extrapyramidal side-effects (Ch. 54).

Finally, it is of utmost importance to provide help with the many social problems these patients and their families and care-givers suffer. At this time there is no proven therapy to prevent the disease; however, genetic testing of patients at risk of the disease, particularly unaffected family members is available. It has been shown that knowing the results of the test helps those at risk of carrying the Huntington gene mutation, regardless of the actual outcome of the test.

Neuroprotective and restorative treatment strategies. Huntington's disease is a disease with a known and testable gene defect, which produces symptoms late in life. This constellation of clinical features makes this disorder an almost ideal candidate for the development of neuroprotective treatments. Various avenues for this have been suggested. Thus, it is known that a continuous influx of the mutant protein is required to maintain inclusions and symptoms, raising the possibility that blockade of the process of polymer formation, which depends on the action of transglutaminases, may be an effective treatment of Huntington's disease. In transgenic mice, use of the competitive transglutaminase inhibitor cystamine extended survival after the appearance of abnormal movements. Various disaccharides (such as trehalose) also inhibit polyglutamine-mediated protein aggregation and may improve motor dysfunction and increase survival in transgenic animals.

Other potential approaches involve the use of anti-apoptotic treatments, such as caspase-1 inhibitors or HDAC inhibitors which may interfere with the transcriptional dysregulation seen in Huntington disease. Both approaches have resulted in encouraging results in animal experiments. The use of growth factors has also been suggested as possible treatment for Huntington's disease.

Finally, cellular transplantation approaches have also been tried. These therapies are aimed at replacing the striatal neurons. However, in view of the widespread pathology, it remains doubtful that this disorder can be treated successfully with these interventions.

Wilson's disease is a disease of copper accumulation. Wilson's disease is an autosomal recessive disorder characterized by the accumulation of copper in liver and brain [21]. Hepatic involvement may result in liver cirrhosis and hepatic cancer. The deposition of copper in the basal ganglia results in a variety of movement disorders, including

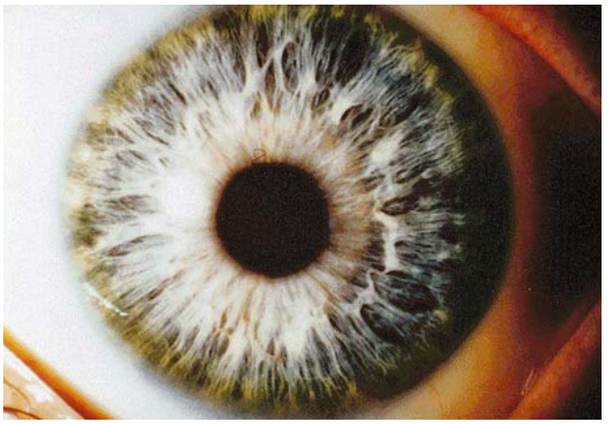

FIGURE 46-7 Kayser–Fleischer ring in a young boy with Wilson's disease. The brown ring at the edge of the cornea is due to corneal copper deposition. Courtesy of Dr Jorge Juncos (Emory University).

tremor, dyskinesias, dystonia and rigidity, as well as behavioral and cognitive abnormalities.

Other organ systems are also involved in the disease. Thus, one of the most striking physical findings in Wilson's disease is the appearance of 'Kayser–Fleischer' rings at the periphery of the cornea. They represent copper deposits, and are seen in about 60% of all patients with Wilson's disease, and in all patients with neurologic manifestations (Fig. 46-7). Less-common signs of the disease are azure lunulae of the fingernails, copper accumulation in joints, resulting in chondrocalcinosis and osteoarthritis, renal hypercalciuria and nephrocalcinosis, perhaps due to a concomitant tubular defect in calcium reabsorption, and cardiomyopathy.

A diagnosis of Wilson's disease should be considered in younger patients presenting with a movement disorder, and in patients of any age who present with a combination of hepatic and neurologic abnormalities. Patients may also first present with behavioral or psychiatric manifestations. The diagnosis should be confirmed by an ophthalmologic assessment to detect Kayser–Fleischer rings, by studies to detect reduced serum levels of the copper-binding protein ceruloplasmin (see below) and elevated urinary copper excretion, and by liver biopsy, if indicated. Imaging techniques such as brain MRI scans to demonstrate basal ganglia abnormalities, cerebral atrophy, and subtle white-matter abnormalities, or echocardiography are also useful.

Several clinically distinct forms of Wilson's disease have been described. Thus, a relatively mild, late-onset form of the disease, has been described in Jewish patients from Eastern Europe. These patients usually present with the neurologic signs of the disease. In contrast, the more common early-onset (childhood) forms of the disease often present first with liver problems, followed by neurologic manifestations.

Molecular, pathophysiologic and genetic aspects. The worldwide prevalence of Wilson disease is estimated to be approximately 3 per 100,000 persons (e.g. ref. [22]). The disease results from mutations within the ATP7B gene on the short arm of chromosome 13 [23, 24]. This gene encodes a protein which appears to be involved in copper transport coupled with the synthesis of ceruloplasmin and other cuproproteins.

Adults require 1–2 mg of copper per day, and eliminate excess copper in bile and feces. Most plasma copper is present in ceruloplasmin. In Wilson's disease, the diminished availability of ceruloplasmin interferes with the function of enzymes that rely on ceruloplasmin as a copper donor (e.g. cytochrome oxidase, tyrosinase and superoxide dismutase). In addition, loss of copper-binding capacity in the serum leads to copper deposition in liver, brain and other organs, resulting in tissue damage. The mechanisms of toxicity are not fully understood, but may involve the formation of hydroxyl radicals via the Fenton reaction, which, in turn initiates a cascade of cellular cytotoxic events, including mitochondrial dysfunction, lipid peroxidation, disruption of calcium ion homeostasis, and cell death.

Animal models. There are several animal models for Wilson's disease which may prove useful for the study of copper metabolism, ceruloplasmin functions, and the pathophysiology of Wilson's disease. One of these models is the Long–Evans Cinnamon rat, a genetically authentic model of Wilson's disease, in which the animals develop hepatitis and liver carcinoma, associated with abnormally high hepatic copper, which can be prevented by treatment with copper-chelating agents. Genetic mouse models have also been studied. Thus, homozygous null mutants for the Wilson's disease gene display a gradual accumulation of copper in liver (resulting in liver cirrhosis), kidney, brain, placenta and lactating mammary glands. However, other features of the phenotype of these animals do not resemble Wilson's disease, but rather suggest a copper-deficiency state. Finally, copper toxicosis, an autosomal recessive disorder in dogs, is also thought to be a genetically faithful model of Wilson's disease.

Treatment. Since the 1950s, the treatment of Wilson's disease has relied on chelating agents [25]. Early attempts to use BAL or EDTA for this purpose were unsuccessful, but penicillamine, triethylene tetramine dihydrochloride (trientine), and tetrathiomolybdate, all in combination with a low-copper diet, have proved to be effective, and result in the urinary excretion of large amounts of copper. The use of penicillamine is complicated by the fact that it may induce a transient worsening of neurologic function due to rapid mobilization of copper, and also has other side-effects, such as the development of nephrosis. Tetrathiomolybdate is an effective alternative with fewer side-effects [26]. In cases in which the dose was rapidly escalated, however, bone marrow suppression or liver function abnormalities have been described.

In addition, treatment with zinc acetate helps to reduce serum copper levels. Zinc acetate is a relatively nontoxic agent which blocks intestinal copper uptake and induces

hepatic metallothionein. It has a slow onset of action compared to penicillamine. Zinc treatment has also been used to prevent the onset of symptoms of Wilson's disease in patients who had been shown to be at risk (for instance, through genetic testing).

In cases in which drug treatment management is not successful, liver transplantation can be used. This is a highly effective treatment to correct the hepatic and metabolic problems, but neurologic symptoms and signs are often irreversible.

Dystonia is characterized by sustained muscle contractions. In patients with dystonia, normal movements are disrupted by cocontraction of agonist and antagonist muscles, and by excessive activation of inappropriate musculature (*overflow*), leading to abnormal postures and slow involuntary twisting movements, which are often associated with movement execution.

Etiology and classification. Dystonia may arise from a variety of disease processes, the majority of which involve the basal ganglia. Dystonia can be classified as a generalized or focal disorder. The most common forms of dystonia are focal in nature (e.g. spasmodic torticollis, blepharospasm, writer's cramp, etc.) and occur in adults, while many of the childhood dystonias are generalized.

Dystonia can also be classified as 'primary' or 'secondary'. In children and young adults, one of the main forms of 'primary' dystonia (i.e. dystonia without a clearly defined pathologic basis) is idiopathic torsion dystonia. The disease is autosomal dominant, occurring with high frequency (1 per 23,000 live births) in Ashkenazi Jews [27]. The penetrance, however, is only 30%. The disorder is caused by a genetic defect in the DYT1 gene on the short arm of chromosome 9 which codes for the ATP-binding protein torsin A. Torsin A is present throughout the brain, but is enriched in SNc neurons, suggesting a tie with dopaminergic transmission. It is located primarily in the nuclear envelope. The disease often begins in childhood or adolescence with involuntary posturing of the limbs, and tends to generalize within a few years.

More than a dozen other determined forms of dystonia have been described. They differ in their mode of inheritance, and the genes involved. A detailed description of these disorders is beyond the scope of this chapter.

Dystonia due to identifiable structural or biochemical abnormalities ('secondary' dystonia) often occurs weeks or months after strokes or other focal lesions, which commonly involve the basal ganglia, but may also involve the thalamus or cerebellum. Dystonia is also seen in children with cerebral palsy and in patients with abnormalities of dopaminergic transmission. For instance, dystonia may develop in the context of Parkinson's disease, either as an early parkinsonian sign, or in response to dopaminergic drugs. A particularly interesting inherited disease results in a combination of dystonia and parkinsonian features at a young age, which responds dramatically to treatment with low-dose levodopa ('dopamine-responsive dystonia'). These patients suffer from a genetic defect of dopamine synthesis, caused by reduced GTP cyclohydrolase activity. This enzyme is rate-limiting in the biosynthesis of tetrahydrobiopterin, a cofactor of the dopamine-synthesizing enzyme tyrosine hydroxylase (see Fig. 40-2).

Pathophysiology. Research into the pathophysiology of primary dystonia has demonstrated that this disorder is associated with widespread loss of inhibition and a loss of specificity in sensorimotor maps at the cortical level. The notion that dystonia may result from aberrant plasticity in susceptible individuals is also indirectly supported by the observation that the beneficial effect of neurosurgical interventions for dystonia, such as pallidotomy or DBS, is typically delayed for several weeks or months.

In cases in which dystonia results from lesions affecting the striatum or its dopaminergic supply, such lesions may affect the affinity or number of dopamine receptors in the unlesioned portion of the striatum, or may lead to reorganization of striatal topography. This will eventually result in altered activity in the basal ganglia output structures. Recent PET studies and results of single-cell recording in human patients with dystonia have suggested a combination of activity changes in the direct and indirect pathways (see below).

Pharmacologic studies suggest that abnormalities in both the indirect and direct pathway contribute to dystonia. Thus, D2LR antagonists have a substantial potential for inducing dystonia, presumably by increasing striatal outflow to GPe via the indirect pathway, whereas D1-receptor antagonists may be beneficial in this regard, presumably by reducing striatal outflow to GPi along the direct pathway. By inference, these data suggest that a relative increase in the activity along the direct pathway (compared to that along the indirect pathway) may strongly contribute to dystonia. Increased activity along the direct pathway may be due to activity changes in feedback loops that regulate GPi activity, for instance the pathway through CM. Interestingly, it has been reported that thalamic lesions were most effective against dystonia if they included the CM nucleus **(Fig. 46-1)**.

Agonist/antagonist co-activation in dystonia may primarily reflect a defect in segregation of 'channels' passing through the basal ganglia output nuclei, i.e. an increased degree of synchronization. The development of dystonia would also depend on the presence of low overall discharge rates in the basal ganglia output nuclei, permitting excess movement. The degree of synchronization is probably determined for the most part by the presence or absence of dopamine in the striatum, but could also be explained by extrastriatal dopamine loss, for instance at the level of the STN. Given the differential effects of dopamine D1 and D2 receptor antagonists in the production of dystonia, the phenomenon of synchronization is likely to be primarily a function of abnormal discharge in the indirect pathway.

Lack of segregation could affect smaller channels within motor subcircuits or could lead to synchronized activity

between different subcircuits. The latter possibility has been favored by the results of recent PET studies in dystonic patients, which have demonstrated widespread changes in the activity of prefrontal areas. Physiologic studies have also provided evidence that dystonia is associated with increased excitability of motor areas (particularly the SMA), probably due to widespread decrease in cortical inhibition.

Treatment. Treatment for dystonia is for the most part symptomatic, except in rare instances where known mechanisms are present and specific therapies are available. The available treatments include support and rehabilitation, pharmacotherapy and, in some cases, functional neurosurgery. Sensory retraining in humans with focal dystonias has resulted in a substantial recovery of function in some patients.

Pharmacotherapy. Anticholinergic drugs, such as trihexyphenidyl, are the most effective forms of treatment for generalized primary dystonia. The use of these medications is often limited by side-effects, such as constipation, dry mouth, blurred vision and urinary retention, as well as impaired short-term memory, confusion and hallucinations. Benzodiazepines, including clonazepam or diazepam, are also effective for dystonia, alone or in combination with anticholinergics. Dosages are raised slowly until benefits are obtained or side-effects, including sedation, ataxia and confusion, prevent further escalation. Baclofen, a GABA-B-receptor agonist, is also effective for treating both focal and generalized dystonia. Relatively high doses are required (60–100 mg); however, side-effects are often limiting. A baclofen pump for intrathecal infusion may be helpful for such cases. Dystonia involving the legs and trunk is most responsive to this form of therapy. Unfortunately, sustained benefits are not the rule, and complications are not infrequent. Dopamine-depleting agents, such as tetrabenazine or reserpine may also be helpful. Dopaminergic drugs are occasionally beneficial in both generalized and focal dystonias, but the most dramatic effects are seen in individuals with DRD, who experience a dramatic and sustained improvement with even a small dose of levodopa. Paradoxically, a fair percentage of patients with generalized dystonia (and craniocervical dystonia) respond to DA *antagonists*, such as haloperidol or pimozide. Sometimes, the combination or 'cocktail' of tetrabenazine, pimozide and trihexyphenidyl is reported to be effective.

In general, the response of focal dystonias to drug treatments with systematically applied drugs is unsatisfactory. However, focal dystonias (or generalized dystonias with prominent focal symptoms) respond dramatically to botulinum toxin injected into the affected muscle groups. The agent acts by preventing fusion of synaptic vesicles with the synaptic membranes in axonal terminals, thereby preventing the release of transmitter into the synaptic cleft. If injected into muscle, this affects mostly cholinergic transmission at neuromuscular junctions, resulting in dose-dependent weakness of the injected muscle. Repeated injections are required every two to five months. A problem with this form of treatment is that some patients develop resistance to the toxin after several injections, usually due to an immune reaction which results in the development of antibodies against the toxin. In these cases, different botulinum toxin serotypes can be used.

Surgical approaches. Prior to the introduction of botulinum toxin, peripheral denervation procedures, such as dorsal or anterior cervical rhizotomy and selective peripheral denervation, were commonly performed, primarily for the treatment of cervical dystonia. These are now performed far less frequently, generally in patients with cervical dystonia who fail botulinum toxin injections. Stereotactic surgery is used primarily for severe generalized dystonia unresponsive to other treatments. In recent years, following the success with PD, pallidotomy and DBS of the pallidum are being used with promising results. The best candidates for surgery appear to be individuals with primary dystonia. Patients with secondary forms of dystonia are less likely to benefit. Bilateral surgery is usually necessary to obtain control of axial dystonia.

Many drugs and toxins induce movement disorders. In addition to MPTP, other drugs that alter the availability of dopamine or that affect its actions at receptors may induce movement disorders with parkinsonian features. Some drugs have an opposite effect, producing hyperactive states with involuntary, abnormal movements. Indeed, an important and distressing adverse effect of levodopa therapy is the development of dyskinesias. Furthermore, the neurons of the basal ganglia and associated structures are uniquely vulnerable to the effects of a variety of toxins, and this sensitivity is a critical factor in the neurological complications that accompany these substances. Generally, toxins produce more extensive neurological damage and a greater variety of clinical deficits than are found in PD. Damage to the nigrostriatal dopaminergic neurons appears to be responsible for the parkinsonian features that occur after exposure to these toxins, whereas involvement of other basal ganglia or associated systems may be responsible for the development of involuntary dyskinesias.

Dopamine depleting agents. Reserpine, a natural alkaloid that blocks vesicular transport of monoamines, depletes stored monoamines, including DA. DA depletion is associated with the emergence of parkinsonism. This effect of reserpine was among the first clues that PD is the result of DA deficiency (see above). Generally, the parkinsonism resulting from reserpine is reversible.

As indicated earlier, α-methyldopa treatment of hypertension sometimes results in the appearance of parkinsonian symptoms. This is presumed to be a consequence of DA depletion by replacement of DA with the relatively inactive false transmitter α-methyldopamine, as well as by inhibition of AADC (Ch. 12).

Dopamine receptor blocking agents. Many of the neuroleptics used in the treatment of schizophrenia frequently produce parkinsonian symptoms as unwanted effects. Neuroleptics block dopamine receptors and their therapeutic effect seems to be related to this action. Although these drugs act on DA systems without distinction, some are more selective. Thioridazine, clozapine and molindone, for example, have electrophysiological effects in the limbic region of the brain but little action in the nigro-striatal area. This selectivity may be related to receptor subtype specificity (see Chs 12 and 54).

Patients who have received neuroleptics for long periods of time may develop a hyperkinetic disorder of the extrapyramidal system characterized by involuntary, purposeless movements affecting many parts of the body. This is known as tardive dyskinesia. Most commonly, these are manifested in a syndrome involving abnormal movements of the tongue, mouth and masticatory muscles. There are also choreoathetoid movements of the extremities. The mechanism by which these symptoms develop remains unknown.

Effects similar to those of the neuroleptics have also been described for other dopamine-blocking agents. Thus, parkinsonism and tardive symptoms may result from use of metoclopramide, a drug which is commonly used to enhance gastric motility, or certain antiemetics, such as perphenazine.

Manganese. A small proportion of miners exposed to manganese dust develop manganism. Manganese is absorbed from the intestine as well as through the pulmonary epithelium, and once in the systemic circulation, it readily enters the brain. After a relatively short interval (months) of exposure to high doses of the dust, the disease is ushered in by psychiatric problems. 'Manganese madness' is characterized by emotional lability, hallucinations, irritability and aggressiveness. When the exposure is to low amounts of manganese, the behavioral changes may be mild and reversible. After more prolonged exposure, behavioral symptoms are replaced by signs of neurological damage. In contrast to PD, the globus pallidus and SNr are the sites of greatest damage; but the striatum, STN, frontal and parietal cortex, cerebellum and hypothalamus may also be involved. Epidemiological studies have shown no risk for the development of PD from drinking of well water rich in manganese. This discrepancy demonstrates the need for further research into the mechanism(s) of manganese-induced movement disorders.

Iron. Iron plays a vital role during development and growth and is an important factor in many metabolic reactions, including protein synthesis as a cofactor of both heme and nonheme enzymes, and in the development of neuronal processes. However, free iron, particularly Fe^{2+}, is highly toxic by virtue of its ability to trigger cellular deleterious effects, including the Fenton reaction, which generates free radical species and lipid peroxidation (Ch. 32). Fe^{3+} (the oxidized form of Fe^{2+}) and total iron are both found in increased concentration in the substantia nigra of patients with PD. It is now clear that exposure to iron does not pose an increased risk for the occurrence of parkinsonism, so that the increase of this metal may be related to some alteration in iron homeostasis.

Carbon disulfide. Carbon disulfide is a volatile, lipid-soluble industrial solvent that enters the body by inhalation or absorption through the skin. The early symptoms of carbon disulfide poisoning resemble those of manganese poisoning; subsequently, neurological deficits are widespread and include peripheral neuropathy as well as encephalopathy with memory loss, incoordination and parkinsonism. Relatively little is known about the pathological changes in humans, but in monkeys exposed to carbon disulfide, damage to the globus pallidus and substantia nigra suggest that similar pathological changes may account for the parkinsonian features of toxicity in humans. Other sulfur compounds, such as sulfuric acid and sulfur hydride, have also been implicated in inducing parkinsonian-like clinical features.

Carbon monoxide. Inhalation of carbon monoxide, which binds avidly to hemoglobin and to cytochromes, is one of the most fatal forms of poisoning. Survivors of acute carbon monoxide poisoning may develop, over several days or weeks, a delayed encephalopathy with memory loss, personality changes and some parkinsonian movement deficits, usually associated with damage to the globus pallidus, which has been reported among the pathological features of this syndrome at autopsy. In spite of all of these detrimental effects, CO is also a putative neurotransmitter (see Ch. 10).

REFERENCES

1. Alexander, G. E. and Crutcher, M. D. Functional architecture of basal ganglia circuits: Neural substrates of parallel processing. *Trends Neurosci.* 13: 266–271, 1990.
2. Wichmann, T. and T. DeLong, T. Functional neuroanatomy of the basal ganglia in Parkinson's disease. *Adv. Neurol.* 91: 9–18, 2003.
3. Schultz, W. The phasic reward signal of primate dopamine neurons. *Adv. Pharmacol.* 42: 686–690, 1998.
4. Braak, H., Del Tredici, K., Rub, U. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* 24: 197–211, 2003.
5. McNaught, K. S., Olanow, C. W., Halliwell, B. *et al.* Failure of the ubiquitin-proteasome system in Parkinson's disease. *Nature Reviews, Neuroscience* 2: 589–594, 2001.
6. Polymeropoulos, M. H., Lavedan, C., Leroy, E. *et al.* Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045–2047, 1997.
7. Healy, D. G., Abou-Sleiman, P. M. and Wood, N. W. PINK, PANK, or PARK? A clinicians' guide to familial parkinsonism. *The Lancet, Neurology* 3: 652–662, 2004.

8. Huang, Y., Cheung, L., Rowe, D. and Halliday, G. Genetic contributions to Parkinson's disease. *Brain Res. Rev.* 46: 44–70, 2004.
9. Anon. Datatop: A multicenter controlled clinical trial in early Parkinson's disease. Parkinson study group. *Arch. Neurol.* 46: 1052–1060, 1989.
10. Bergman, H., Wichmann, T. and DeLong, M. R. Reversal of experimental parkinsonism by lesions of the subthalamic nucleus. *Science* 249: 1436–1438, 1990.
11. Baron, M. S., Vitek, J. L., Bakay, R. A. E. *et al.* Treatment of advanced Parkinson's disease by gpi pallidotomy: 1 year pilot-study results. *Ann. Neurol.* 40: 355–366, 1996.
12. Rosin, D. L., Hettinger, B. D., Lee, A. and Linden, J. Anatomy of adenosine a2a receptors in brain: Morphological substrates for integration of striatal function [Comment]. *Neurology* 61: S12–18, 2003.
13. Greenamyre, J. T., Eller, R. V., Zhang, Z. *et al.* Antiparkinsonian effects of remacemide hydrochloride, a glutamate antagonist, in rodent and primate models of Parkinson's disease [Comment]. *Ann. Neurol.* 35: 655–661, 1994.
14. Freed, C. R., Greene, P. E., Breeze, R. E. *et al.* Transplantation of embryonic dopamine neurons for severe Parkinson's disease[Comment]. *N. Engl. J. Med.* 344: 710–719, 2001.
15. Gash, D. M., Zhang, Z., Ovadia, A. *et al.* Functional recovery in parkinsonian monkeys treated with gdnf. *Nature* 380: 252–255, 1996.
16. Harper, P. S. The epidemiology of Huntington's disease. *Hum. Genet.* 89: 365–376, 1992.
17. Reiner, A., Albin, R. L., Anderson, K. D. *et al.* Differential loss of striatal projection neurons in Huntington disease. *Proc. Natl Acad. Sci. USA* 85: 5733–5737, 1988.
18. Reddy, P. H., Williams, M. and Tagle, D. A. Recent advances in understanding the pathogenesis of Huntington's disease. *Trends Neurosci.* 22: 248–255, 1999.
19. Hs.DCR Group. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72: 971–983, 1993.
20. Brouillet, E., Conde, F., Beal, M. F. and Hantraye, P. Replicating Huntington's disease phenotype in experimental animals. *Prog. Neurobiol.* 59: 427–468, 1999.
21. Mercer, J. F. The molecular basis of copper-transport diseases. *Trends Mol. Med.* 7: 64–69, 2001.
22. Olivarez, L., Caggana, M., Pass, K. A. *et al.* Estimate of the frequency of Wilson's disease in the US Caucasian population: A mutation analysis approach. *Ann. Hum. Genet.* 65: 459–463, 2001.
23. Kooy, R. F., Van der Veen, A. Y., Verlind, E. *et al.* Physical localisation of the chromosomal marker d13s31 places the Wilson disease locus at the junction of bands q14.3 and q21.1 of chromosome 13. *Hum Genet.* 91: 504–506, 1993.
24. Frydman, M., Bonne-Tamir, B., Farrer, L. A. *et al.* Assignment of the gene for Wilson disease to chromosome 13: Linkage to the esterase d locus. *Proc. Natl Acad. Sci. USA* 82: 1819–1821,1985.
25. El-Youssef, M. Wilson disease. *Mayo Clin. Proc.* 78: 1126-1136, 2003.
26. Brewer, G. J., Hedera, P., Kluin, K. J. *et al.* Treatment of Wilson disease with ammonium tetrathiomolybdate: III. Initial therapy in a total of 55 neurologically affected patients and follow-up with zinc therapy. *Arch. Neurol.* 60: 379–385, 2003.
27. Almasy, L., Bressman, S., de Leon, D. and Risch, N. Ethnic variation in the clinical expression of idiopathic torsion dystonia. *Movement Disorders* 12: 715–721, 1997.

BOX 46-1

*Summary of Polyglutamine Repeat Disorders**

Sangram Sisodia

Disease	Reference	Gene	Locus	Parent-of-origin	Repeat size: Normal	Repeat size: Disease	Protein	Repeat location	Regions effected
Huntington's disease	[1, 2]	*HD*	4p16.3	paternal	10–34	40–121	huntingtin	coding region (amino-terminal)	striatum, cerebral cortex
Dentatorubral-pallidoluysian atrophy (Haw River syndrome)	[3, 4, 5]	*DRPLA*	12p13.31	paternal	7–25	49–75	atrophin-1	coding region (amino-terminal)	cerebellum, cerebral cortex, basal ganglia, Luys body
Kennedy's disease/ spinobulbar muscular atrophy	[6,7]	*AR*	Xq13-21	not determined	9–36	38–62	androgen receptor	coding region (amino-terminal)	anterior horn and bulbar neurons, dorsal root ganglia
Spinocerebellar ataxia type 1	[8]	*SCA1*	6p23	paternal	6–39	40–82	ataxin 1	coding (amino-terminal)	cerebellar Purkinje cells, dentate nucleus, brainstem
Spinocerebellar ataxi type a, type 2	[9]	*SCA2*	12q24.1	paternal	13–33	32–200	ataxin-2	coding region (amino-terminal)	cerebellar Purkinje cells, brain-stem, fronto-temporal lobes
Spinocerebellar ataxi type a, type 3	[10]	*SCA3*	14q32.1	paternal	13–44	55–84	ataxin-3	coding region (amino-terminal)	cerebellar dentate neurons, basal ganglia, brainstem, spinal cord
Spinocerebellar ataxi type a, type 6	[11, 12]	*SCA6*	19p13	not determined	4–18	21–33	α1A-voltage-dependent calcium channel subunit	coding region (carboxyl-terminal)	cerebellar Purkinje cells, dentate nucleus, inferior olive
Spinocerebellar ataxi type a, type 7	[13, 14]	*SCA7*	3p12-13	paternal	4–35	37–306	ataxin-7	coding region (amino-terminal)	cerebellum, brainstem, macula, visual cortex

*The mutated gene products in all these cases exhibit toxic gain of function. See Triplet Repeat Disorders in Ch. 39 and Huntington's Disease in Chs 39 and 46 for discussions of genetic pathology.

1. Vonsattel, J. P., Myers, R. H., Stevens, T. J. *et al.* Neuropathological classification of Huntington's disease. *J. Neuropathol. Exp. Neurol.* 44: 559, 1985.
2. Wexler, N. S., Young, A.B., Tanzi, R.E. *et al.* Homozygotes for Huntington's disease. *Nature* 326: 194–197, 1987.
3. Burke, J.R., Wingfield, M.S., Lewis, K.E. *et al.* The Haw River syndrome: dentatorubropalli-doluysian atrophy (DRPLA) in an African-American family. *Nat. Genet.* 7: 521–524, 1994.
4. Koide, R., Ikeuchi, T., Onodera, O. *et al.* Unstable expansion of CAG repeat in hereditary dentatorubralpallidoluysian atrophy (DRPLA). *Nat. Genet.* 6: 9–13, 1994.
5. Ikeuchi, T., Koide, R., Tanaka, H. *et al.* Dentatorubral-pallidoluysian atrophy: clinical features are closely related to unstable expansions of trinucleotide (CAG) repeat. *Ann. Neurol.* 37: 769–775, 1995.
6. Arbizu, T., Santamaria, J., Gomez, J. M. *et al.* A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure. *J. Neurol. Sci.* 59: 371–382, 1983.
7. La Spada, A. R., Wilson, E.M., Lubahn, D.B. *et al.* Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 352: 77–79, 1991.
8. Zoghbi, H. Y. and Ballabio, A. Spinocerebellar ataxia type 1. In *The Metabolic and Molecular Bases of Inherited Disease,* eds. Scriver, C. R. , Beaudet, A. L., Sly, W. S. and Valle, D., New York: McGraw-Hill, 7th edn, 1995, pp. 4559–4567.
9. Dürr, A., Smadja, D., Cancel, G. *et al.* Autosomal dominant cerebellar ataxia type I in Martinique (French West Indies). Clinical and neuropathological analysis of 53 patients from three unrelated SCA2 families. *Brain* 118: 1573–1581, 1995.
10. Dürr, A., Stevanin, G., Cancel, G. *et al.* Spinocerebellar ataxia 3 and Machado–Joseph disease: clinical, molecular and neuropathological features. *Ann. Neurol.* 39: 490–499, 1996.
11. Ikeuchi, T., Takano, H., Koide, R. *et al.* Spinocerebellar ataxia type 6: CAG repeat expan-sion in a1A voltage-dependent calcium channel gene and clinical variations in Japanese population. *Ann. Neurol.* 42: 879–884, 1997.
12. Zhuchenko, O., Bailey, J., Bonnen, P. *et al.* Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the a1A-voltage-dependent calcium channel. *Nat. Genet.* 15: 62–69, 1997.
13. Martin, J.J., Van Regemorter, N., Krols, L. *et al.* On an autosomal dominant form of retinal-cerebellar degeneration: an autopsy study of five patients in one family. *Acta Neuropathol.* 88: 277–286, 1994.
14. David, G., Abbas, N., Stevanin, G. *et al.* Cloning of the SCA7 gene reveals a highly unstable CAG repeat expansion. *Nat. Genet.* 17: 65–70, 1997.

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CHAPTER

47

Neurobiology of Alzheimer's Disease

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ALZHEIMER'S DISEASE IS THE MOST PREVALENT NEURODEGENERATIVE DISORDER 781

The clinical syndrome, ranging from mild cognitive impairment to severe dementia, reflects cellular abnormalities in specific regions of the brain/circuits 782

Advances in laboratory measurements and imaging of amyloid burden in brain will be of value in establishing the diagnosis of Alzheimer's disease (AD) 782

Some cases of familial AD (fAD) are due to mutant genes inherited as autosomal dominants; ApoE4 allele is associated with increased risk 782

Multiple neurotransmitter circuits are damaged by the disease 783

Neuritic plaques, one of the pathological hallmarks of the disease, are composed of swollen neurites, extracellular deposits of Aβ 40–42 peptides, (derived from β- and γ- secretase cleavages of APP) and surrounding astrocytes and microglia 783

Neurofibrillary tangles (NFT) are comprised of bundles of paired helical filaments (PHF) made up of hyperphosphorylated tau 783

Aspartyl proteases carry out the β- and γ-secretase cleavages of APP to generate Aβ peptides 784

Transgenic strategies have been used to create models of Aβ amyloidosis 785

Gene targeting approaches have identified and validated certain genes as targets for therapy 785

Transgenic mouse models are being used to test a variety of novel therapies, including ways of reducing secretase activities and decreasing Aβ burden by Aβ immunotherapy 786

Several of these experimental approaches are moving into clinical trials 787

ALZHEIMER'S DISEASE IS THE MOST PREVALENT NEURODEGENERATIVE DISORDER

Alzheimer's disease (AD), manifesting as progressive memory loss and cognitive impairments [1], affects more than 4 million elderly individuals in the USA [1, 2]. This syndrome results from abnormalities associated with dysfunction and death of specific populations of neurons, particularly those in neural systems involved in memory and cognition [3]. The pathology is characterized by intracellular and extracellular protein aggregates (tau and Aβ related abnormalities) in neurofibrillary tangles (NFT) and neuritic amyloid plaques, respectively [4–6]. Genetic evidence indicates that the inheritance of mutations in several genes causes autosomal dominant familial AD (fAD), while the presence of certain alleles of *ApoE4* are significant risk factors for putative sporadic disease [3, 7, 8].

Due to increased life expectancy and the postwar baby boom, the elderly are the most rapidly growing segment of our society. Thus, over the next several decades, the number of persons with AD in the USA will triple. Because of prevalence, lack of mechanism-based treatments, cost of care, and impact on individuals and caregivers, AD is one of the most challenging diseases in medicine [3, 4, 9, 10]. This review first briefly discusses the clinical syndrome, laboratory tests, genetics, and pathological/biochemical features of the human illness. Subsequently, we describe selected aspects of: the biology of proteins implicated in pathogenesis of disease; the value of transgenic and gene-targeted mouse models; the identification of several therapeutic targets; and results of selected experimental treatments in the models. We demonstrate how information from

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genetics, pathology, and biochemistry has been used to create models of disease (i.e. mice expressing mutant transgenes). In parallel, we demonstrate how results of targeting genes encoding proteins implicated in disease pathways have provided insights of the roles of these proteins in the pathogenesis of AD and the potential of these proteins as therapeutic targets. The value of these targets for new treatment strategies is being tested in model systems. As safety and efficacy are assured, some of these therapeutic approaches will be increasingly used in human trials [10]. The development of effective new therapies will have a significant impact on the health and care of the elderly.

The clinical syndrome, ranging from mild cognitive impairment to severe dementia, reflects cellular abnormalities in specific regions of the brain/circuits. The disease often initially manifests as a syndrome termed mild cognitive impairment (MCI), which is usually characterized by a memory complaint and impairments on formal testing, intact general cognition, preserved daily activities and absence of overt dementia [11]. MCI is regarded by many as a transitional stage between normal aging and early AD [11–13]. The clinical manifestations of symptomatic AD include increasing difficulties with memory and with other cognitive functions (executive functions, language, attention, judgment, etc.) [1, 6, 12–14]. Some patients develop psychotic symptoms. Over time, mental functions and activities of daily living are increasingly impaired. In the late stages, these individuals become profoundly demented and usually die of intercurrent illnesses.

For a diagnosis, clinicians rely on histories, on physical, neurological and psychiatric examinations, on neuropsychological tests [1, 14], and on laboratory studies [15, 16].

Advances in laboratory measurements and imaging of amyloid burden in brain will be of value in establishing the diagnosis of Alzheimer's disease (AD). In cases of AD, the CSF levels of Aβ peptides are often low, and CSF levels of tau may be higher than in controls [15]. Values vary between individuals and single measures may not be great diagnostic value. Over several examinations, the clinical profile, in concert with laboratory assessments, allows the clinician to make a diagnosis of possible or probable AD [17].

In cases of AD, magnetic resonance imaging (MRI) often discloses regional brain atrophy, particularly involving hippocampus and entorhinal cortex [1, 14], and rates of atrophy which correlate with changes in clinical status and may have predictive value for diagnosis. Positron emission tomography (PET) using [^{18}F]-deoxyglucose or single photon emission computerized tomography (SPECT) commonly demonstrate decreased glucose utilization and early reduction in regional blood flow in the parietal and temporal lobes.

The most exciting recent advance in imaging relevant to AD is the use of PET with the radiolabeled Pittsburgh Compound B (PIB) which demonstrates Aβ amyloid burden *in vivo*. This brain penetrant ^{11}C-labeled uncharged thioflavin derivative binds to Aβ with high affinity [16]. The PET patterns of labeled PIB are interpreted to reflect the Aβ burden in the brain [16]. In comparison with controls, subjects with AD show marked retention of label in several areas of brain that usually accumulate amyloid. This approach should eventually prove useful for enhancing accuracy of diagnosis and should allow assessment of the efficacies of new anti-amyloid therapeutics. Further details of brain imaging are found in Ch. 58.

Some cases of familial AD (fAD) are due to mutant genes inherited as autosomal dominants; ApoE4 allele is associated with increased risk. Genetic risk factors for AD include: mutations in APP (chromosome 21); mutations in *presenilin (PS) 1* (chromosome 14) and *PS2* (chromosome 1); and different susceptibility alleles of *ApoE* (chromosome 19) [3, 7, 8, 18]. Autosomal dominant mutations in APP, PS1, or PS2 usually cause disease earlier than occurs in sporadic cases. The majority of mutations in APP, PS1 and PS2 influence BACE1 and γ-secretase cleavages to increase the levels of all Aβ species or the relative amounts of toxic Aβ 42 [7, 8, 18]. Individuals with trisomy 21 or Down's syndrome (DS) have an extra copy of APP (and other genes) in the putative obligate DS region; these individuals develop AD pathology relatively early in life. The presence of ApoE4 predisposes to later onset AD and some cases of late-onset fAD [19]. Recent research has identified other loci that confer risk [8].

Mutations of APP gene. APP, a type I transmembrane protein existing as several isoforms, is abundant in the nervous system, rich in neurons, and transported anterograde in axons to terminals [20–22]. Its specific functions remain to be defined [7, 23, 24]. APP is cleaved by activities of BACE1 (*β*-site *A*PP *c*leaving *e*nzyme *1*) and the γ-secretase complex, which generate the *N*- and *C*- termini of Aβ peptides, respectively [7, 10, 25–32]. The APPswe mutation, a double mutation involving codons 670 and 671, enhances many-fold the BACE1 cleavage at the *N*-terminus of Aβ; the result is substantial elevation in levels of all Aβ peptides. With APP_{717} mutations, γ-secretase cleavage is altered leading to increased secretion of Aβ 42, which is a more toxic peptide. Thus, some of the APP mutations linked to fAD can change the processing of APP and influence the biology of Aβ by increasing the production of Aβ peptides or the amounts of the more toxic Aβ 42; other mutations may promote local fibril formation.

Mutations of PS1 and PS2 genes. PS1 and PS2, two highly homologous and conserved 43- to 50-kDa multipass transmembrane proteins [3, 7, 33, 34], are involved in Notch1 signaling pathways critical for cell fate decisions [7, 28]. They are endoproteolytically cleaved to form an *N*-terminal 28 kDa fragment and a *C*-terminal 18 kDa fragment [33, 35], both of which (along with several other proteins described below) are critical components of the

γ-secretase complex [7, 27–32, 36]. Nearly 50% of cases of early-onset fAD are linked to approximately 90 different mutations in PS1 [3, 7, 8, 34]. A small number of PS2 mutations cause autosomal dominant fAD in several pedigrees [3, 8]. The majority of abnormalities in PS genes are single amino acid missense mutations that enhance γ-secretase activities and increase the levels of the Aβ 42 peptides.

ApoE alleles. ApoE carries cholesterol and other lipids in the blood. In humans, three alleles exist: ApoE2, ApoE3 and ApoE4 [19]. The ApoE3 allele is most common in the general population (frequency of 0.78), whereas the allelic frequency of ApoE4 is 0.14. However, in clinic-based studies, the ApoE4 allelic frequency in patients with late-onset disease (>65 years of age) is 0.50; thus, the presence of ApoE4 increases the risk for AD [8, 19]. Significant differences exist in the abilities of ApoE isoforms to bind Aβ and these features of the individual protein are hypothesized to differentially influence aggregation, deposition and/or clearance of Aβ by different ApoE isoforms.

Multiple neurotransmitter circuits are damaged by the disease. The clinical signs of AD reflect the distribution of abnormalities among different populations of neurons in brain regions/systems critical for memory, learning, and cognitive performance. Circuits damaged by the disease include: the basal forebrain cholinergic system; hippocampus; entorhinal cortex; limbic cortex; and neocortex [13, 37–39]. Neurodegeneration in these regions/circuits are reflected by: NFT and neurites, related to the presence of cytoskeletal abnormalities, particularly involving conformational alterations in phospho-tau leading to PHF [5]; the presence of neuritic Aβ-containing plaques (sites of synaptic disconnection) in brain regions receiving inputs from these neurons [7, 40]; reductions in both generic and transmitter specific synaptic markers in the target fields of these cells [40]; evidence of death of neurons in these regions [37, 40]; and local glial and inflammatory responses, particularly associated with plaques. Disruption of synaptic communication in these circuits, associated with degeneration of neurons, has profound clinical consequences [40, 41]. Abnormalities that damage the circuits involving the entorhinal cortex, medial temporal cortex, and hippocampus, contribute significantly to memory impairments. Pathology in the neocortex is reflected by deficits in higher cognitive functions, such as disturbances in language, calculation, problem solving, and judgment. Alterations in the basal forebrain cholinergic system may contribute to difficulties in memory, arousal and attention, while involvement of the limbic cortex, amygdala, thalamus, and monoaminergic systems result in behavioral and emotional disturbances [1].

Neuritic plaques, one of the pathological hallmarks of the disease, are composed of swollen neurites, extracellular deposits of Aβ 40–42 peptides, (derived from β- and γ-secretase cleavages of APP) and surrounding astrocytes and microglia. Neuritic Aβ plaques are composed of Aβ deposits of β-pleated sheet peptides surrounded by swollen neurites (nerve terminals). Aβ1–40, 42 and 11–40, 42 amyloid peptides are derived by β- and γ-secretase cleavages of APP to generate Aβ1–40, 42 and 11–40, 42 peptides [3, 7, 26, 42]. These Aβ peptides accumulate in the extracellular space of the neuropil of the neocortex and hippocampus. In neurons, APP is delivered by anterograde axonal transport to terminals [20–22], from which, following β- and γ-secretase cleavages, extracellular monomeric Aβ 40 and 42, 43 peptides are released [7, 21, 36, 43]. Aβ multimers assemble into β sheets, into protofilaments, and into fibrils [44]; these aggregates are birefringent when stained with Congo red or thioflavin dyes and viewed in polarized light or fluorescence illumination, respectively. Considerable debate exists concerning the Aβ species/conformational state exhibiting the greatest toxicity, with plaques, fibrils, protofibrils, or oligomers proposed as principal offenders [44, 45]. It now is believed that multimers, sometimes termed *A*β *d*erived *d*iffusible *l*igands (ADDLs), are the principal toxic entities [41, 42, 45, 46].

In one model, APP and pro-amyloidogenic secretases are transported to terminals and thus neurons are thought to be the major source of APP that gives rise to Aβ [43]. At synapses, BACE1 cleaves APP to form amyloidogenic *C*-terminal derivatives [21], which are then cleaved by γ-secretase to generate Aβ 40, 42, 43 peptides. Released normally at terminals, Aβ may influence synaptic functions [28, 40], perhaps behaving as a modulator depressing activity at excitatory, glutamatergic synapses via the NMDA receptor [24]. With increasing accumulations of Aβ 42 multimers at terminals, synaptic functions, including long term potentiation (LTP, see discussion in Ch. 53), are disrupted [47]. In this scenario, neuritic amyloid plaques, a classical feature of AD, are complex structures representing sites of Aβ-mediated damage to synapses associated with degeneration of neurites and disconnection of terminals from targets. Surrounding plaques are astrocytes and microglia which produce cytokines, chemokines and other factors (including complement components) involved in inflammatory processes (see Inflammation in Ch. 33).

Neurofibrillary tangles (NFT) are comprised of bundles of paired helical filaments (PHF) made up of hyperphosphorylated tau. Tangles are fibrillar intracytoplasmic inclusions in cell bodies/proximal dendrites of affected neurons, while neuropil threads and neurites are predominantly swollen, filament-containing dendrites and distal axons/terminals, respectively [5]. These intracellular lesions are rich in PHF comprised of poorly soluble hyperphosphorylated isoforms of tau, a low-molecular-weight microtubule-associated protein [5]. In human brain, alternative splicing from a single gene leads to formation of six tau isoforms, consisting of three isoforms of three repeat (3-R) tau, and three isoforms of four repeat (4-R) tau, the latter derived by inclusion of exon 10 in the transcript [5, 48]. Normally, tau, synthesized in neuronal cell bodies, is transported anterograde in axons, where it acts, via repeat

regions that interact with tubulin, to stabilize tubulin polymers critical for microtubule assembly and stability [5, 48] (see also axonal transport in Ch. 28). The post-translationally modified tau, which exhibits abnormal conformations, differs somewhat in the different tauopathies: in cases of AD, the PHF are composed of six isoforms of tau; in contrast, the inclusions occurring in cases of progressive supranuclear palsy (PSP) and cortical basal degeneration (CBD)are characterized by 4-R tau, while the inclusions seen in individuals with Pick's disease (PD) are enriched in 3-R tau [5] (see also Tauopathies in Ch. 45).

In one hypothetical model linking Aβ and phosphorylated tau, Aβ42 damages terminals leading to synaptic disconnections which, perhaps preferentially in primates with six isoforms of tau, in turn lead to retrograde signals that ultimately trigger the activation of kinases (or the suppression of phosphatases) whose activities lead to excessive phosphorylation of tau at certain residues. Subsequently, conformational changes in the protein cause the formation of PHF. Since the cytoskeleton is essential for maintaining cell geometry and for the intracellular trafficking and transport of proteins and organelles, disturbances of the cytoskeleton can lead to alterations in axonal transport which, in turn, can compromise the functions and viability of neurons (see Chs 8,9 and 28). Eventually, affected nerve cells die (possibly by apoptosis; apoptosis is discussed in Ch. 35) [37, 49] and extracellular tangles remain as 'tombstones' of the nerve cells destroyed by disease.

Aspartyl proteases carry out the β- and γ-secretase cleavages of APP to generate Aβ peptides. APP is processed by β- and γ-secretase enzymes resulting in release of the ectodomain of APP (APPs), the production of a cytosolic fragment termed *A*PP *i*ntracellular *d*omain (AICD), and the generation of several Aβ peptides. In the CNS, Aβ peptides are generated by sequential endoproteolytic cleavages of neuronal APP by two membrane-bound enzymes: as described below, BACE1 cleaves APP at the Aβ +1 and +11 sites to generate APP–β carboxyl terminal fragments (APP–βCTFs) [26, 50]; while γ-secretase complex cleaves, via regulated intramembranous proteolysis, APP–βCTFs at several sites including Aβ 40, 42, 43 to form these peptides [10, 27, 29–32, 32, 49, 51, 52]. The γ-secretase cleavages of APP–βCTF or αCTF release the AICD, which forms a multimeric complex with Fe 65, a nuclear adaptor protein [23]. It has been suggested that the complex of Fe 65 and Aβ or Fe 65 alone (in a new conformation), enters the nucleus and binds to the histone acetyltransferase, Tip60, to influence gene transcription [23]. This signaling mechanism is analogous to that occurring in the Notch1 pathway following the S3 cleavage of NEXT to produce NICD [28, 31]. In other cells in other organs, APP can also be cleaved endoproteolytically within the Aβ sequence through alternative, nonamyloidogenic pathways involving α-secretase (TACE or *T*NF-*a*lpha *c*onverting *e*nzyme) or BACE2 [30, 53]. The α-secretase and BACE2 cleavages, which occur in non-neural tissues, preclude the formation of Aβ peptides and thus are thought to protect these organs from Aβ amyloidosis [43].

BACE1 and BACE2, encoded by genes on chromosomes 11 and 21, respectively, are transmembrane aspartyl proteases that are directly involved in the cleavages of APP [25, 26, 30, 50, 53, 54]. Analyses of cells and brains from BACE1$^{-/-}$ mice [26, 50] discloses that Aβ 1–40/42 and Aβ 11– 40/42 are not secreted in these samples [26, 50]. BACE1 is demonstrable in the CNS and immunoreactivity is visualized in some synaptic regions. BACE1 preferentially cleaves APP at the +11/+1 sites of Aβ in APP [26] and this enzyme is essential for the generation of Aβ [26, 50]. Significantly, APPswe is cleaved perhaps 100-fold more efficiently at the +1 site than is wild type APP. Thus, the presence of this mutation greatly increases BACE1 cleavage and accounts for the elevation of Aβ species in the presence of this mutation. It has been reported that the expression of BACE1 is increased in certain regions of brain from some cases of sporadic AD [55, 56]. Thus, BACE1 is the principal neuronal β-secretase and is responsible for the critical penultimate pro-amyloidogenic cleavages. Although BACE1 mRNA is present in a variety of tissues (particularly the pancreas), levels of this protein are low in most non-neural tissues. In the pancreas, BACE1 mRNA is high, but the transcript is alternatively spliced to produce a smaller protein incapable of cleaving APP.

BACE2 mRNA, present in a variety of systemic organs, is very low in neural tissues, except for scattered nuclei in the hypothalamus and brainstem. BACE2 activity appears to be virtually undetectable in brain regions involved in AD. BACE2 is responsible for generation of anti-amyloidogenic cleavages at +19/+20 of Aβ [53]. Thus, BACE2, an anti-amyloidogenic enzyme, acts like α-secretase or TACE which cleave between residues 16 and 17 of the Aβ peptide [7, 57].

γ-Secretase, essential for the regulated intramembraneous proteolysis of a variety of transmembrane proteins, is a multiprotein catalytic complex, which includes: PS; Nicastrin (Nct), a type I transmembrane glycoprotein [7, 27, 48, 51]; and Aph-1 and Pen-2, two multipass transmembrane proteins [7, 27–32, 32, 51, 52, 58, 59]. PS1 is isolated with γ-secretase under specific detergent soluble conditions; it is selectively crosslinked or photoaffinity-labeled by transition state inhibitors [60, 61]; substitutions of aspartate residues at D257 in TM 6 and at D385 in TM 7 have been reported to reduce secretion of Aβ and cleavage of Notch1 *in vitro*; PS1$^{-/-}$ cells show decreased levels of secretion of Aβ [27, 29, 51, 62]. Aph-1 and Pen-2 [27, 29, 59] are novel transmembrane proteins: Aph-1 has seven predicted transmembrane domains; and Pen-2 has two predicted transmembrane regions [27, 29].

The functions of these protein and their interactions with each other in the complex and in γ-secretase activity are not yet fully defined. PS1 may: act as an aspartyl protease itself; function as a cofactor critical for the activity of

γ-secretase; or play a role in trafficking of APP or proteins critical for enzyme activity to the proper compartment for γ-secretase cleavage [36, 61, 62]. In one model, Aph-1 and Nct form a pre-complex that interacts with PS; subsequently Pen-2 influences the cleavage of PS into two fragments; all proteins are critical for the activities of γ-secretase complex [51, 52].

Significantly, γ-secretase cleaves both Notch1 and APP [28], generating intracellular peptides termed NICD and AICD [23] which influence transcription [23, 28]. As described above, the AICD interacts with FE65, a cytosolic adapter; this interaction leads to a signal which influences transcription [23, 28]. Results of targeting of PS1, Nct and Aph-1 in mice [51, 52, 63, 64], which are described below, are consistent with the concept that PS1, Nct and Aph-1 [27, 29, 30, 65] are critical components of the γ-secretase complex. The phenotypes of targeted PS1, Nct, and Aph-1 mice are the result of impaired Notch1 signaling.

Transgenic strategies have been used to create models of Aβ amyloidosis. In mice, expression of APPswe or APP_{717} minigenes (with or without mutant PS1) leads to an Aβ amyloidosis in the CNS [66], with the severity of pathology influenced by the nature and levels of the expressed transgene and the specific mutation. Mice expressing both mutant APP and PS1 develop accelerated disease. In these animals, levels of Aβ (particularly Aβ 42) in brain are elevated, and diffuse Aβ deposits and neuritic plaques appear in the hippocampus and cortex. In transgenic mice generated at Johns Hopkins University by Dr David Borchelt [67–69], the pathology evolves in stages: levels of Aβ peptides in brain increase with age; over time, Aβ deposits appear to become increasingly abundant; swollen neurites develop in proximity to these deposits; and neuritic plaques are associated with glial responses. In these mice, aggregated tau and tangles are not detectable. The density of synaptic terminals is reduced and several neurotransmitter markers are decreased; in some settings, these abnormalities appear linked to deficiencies in synaptic transmission [47, 70]. Moreover, some lines of mice show degeneration of subsets of neurons. Although amyloid has been detected *in vivo* by invasive techniques [71], it has proved more difficult to detect Aβ with whole brain imaging.

The paucity of tau abnormalities in these various lines of mutant APP and PS1 mice described above may be related to differences in tau isoforms expressed in this species. Early efforts to express mutant tau transgenes in mice did not lead to striking clinical phenotypes or pathology. More recently, mice overexpressing tau show clinical signs, attributed to degeneration of motor axons [5]. When the prion or Thy1 promoters are used to drive tau_{P301L} (a mutation linked to autosomal dominant frontotemporal dementia with parkinsonism; see Ch. 45), tangles develop in neurons of the brain and spinal cord [72]. Mice expressing APPswe/tau_{P301L} exhibit enhanced tangle-like pathology in limbic system and olfactory cortex [73]. Moreover, when Aβ 42 fibrils are injected into specific brain regions of tau_{P301L} mice, the number of tangles is increased in those neurons projecting to sites of Aβ injection.

A triple transgenic mouse (3×Tg-AD) was created by microinjecting APPswe and tau_{P301L} into single cells derived from monozygous $PS1_{M146V}$ knockin mice [74]. These mice develop age-related plaques and tangles as well as deficits in LTP which appear to antedate overt pathology [74]. However, mice bearing both mutant tau and APP (or APP/PS1) or mutant tau mice injected with Aβ are not fully faithful models of fAD because the presence of the tau mutation alone is associated with the development of tangles.

Finally, learning deficits, problems in object recognition memory, and difficulties in performing tasks to assess spatial reference and working memory, have been identified in some of the lines of mutant mice with high levels of mutant transgene expression [70, 75]. Although these mice do not fully recapitulate the complete phenotype of AD, these animals are very useful subjects for research designed to examine disease mechanisms and to test novel therapies.

Gene targeting approaches have identified and validated certain genes as targets for therapy. To begin to understand the functions of some of the proteins thought to play roles in AD, investigators have targeted in mice a variety of genes encoding these proteins, including APP, *a*myloid *p*recursor *l*ike *p*roteins (APLPs), BACE1, PS1, Nct and Aph-1.

Homozygous $APP^{-/-}$ mice are viable and fertile, but appear to have subtle decreases in locomotor activity and forelimb grip strength. The absence of substantial phenotypes in $APP^{-/-}$ mice is thought to be related to functional redundancy of two amyloid-precursor-like proteins, APLP1 and APLP2, homologous to APP. $APLP2^{-/-}$ mice appear relatively normal, while $APLP1^{-/-}$ mice exhibit a postnatal growth deficit [76]. $APLP1^{-/-}$ mice are viable, but $APP/APLP2^{-/-}$ mice and $APLP1/APLP2^{-/-}$ mice do not survive the perinatal period [76]. These observations support the concept that some redundancy exists between members of this interesting family of proteins [76].

$BACE1^{-/-}$ mice are viable and healthy, have no obvious phenotype or pathology, and can mate successfully [26, 50]. Importantly, cortical neurons cultured from $BACE1^{-/-}$ embryos do not show cleavages at the +1 and +11 sites of Aβ, and the secretion of Aβ peptides is abolished even in the presence of elevated levels of exogenous wt or mutant APP [26]. The brains of $BACE1^{-/-}$ mice appear morphologically normal and Aβ peptides are not produced [26, 50]. These results establish that BACE1 is the neuronal β-secretase required to generate the *N*-termini of Aβ [26]. At present, a consensus has not been reached with regards to other substrates cleaved by BACE1 [30]. Moreover, preliminary behavioral studies of the $BACE1^{-/-}$ mice indicate that these animals show altered performance on some tests

of cognition and emotion. Nevertheless, BACE1 appears to be an outstanding target for development of an anti-amyloidogenic therapy.

PS1$^{-/-}$ mice, in contrast to BACE1$^{-/-}$ mice, do not survive beyond the early postnatal period and show severe developmental abnormalities of the axial skeleton, ribs and spinal ganglia; resembling a partial Notch1$^{-/-}$ phenotype [63, 64], these features occur because PS1 (along with Nct, Aph-1 and Pen-2) are components of the γ-secretase complex that carries out the S3 intramembranous cleavage of Notch1 [27–29, 48, 51, 77, 78]. Without this cleavage, NICD is not released from the plasma membrane and, therefore, the signal does not reach the nucleus to initiate transcriptional processes essential for cell fate decisions. PS2$^{-/-}$ mice are viable and fertile, although they develop age-associated mild pulmonary fibrosis and hemorrhage. Mice lacking PS1 and PS2 die midway through gestation showing a Notch1$^{-/-}$ phenotype.

Nct$^{-/-}$ embryos die by embryonic day 10.5 and exhibit several developmental patterning defects, including abnormal segmentation of somites; the phenotype closely resembles that seen in embryos lacking Notch1 or both PS. Importantly, secretion of Aβ peptides is abolished in Nct$^{-/-}$ fibroblasts, whereas it is reduced by ~50% in NctT$^{+/-}$ cells [51]. The failure to generate Aβ peptides in NctT$^{-/-}$ cells is accompanied by destabilization of the β-secretase complex and by accumulation of APP *C*-terminal fragments. Moreover, analysis of APP trafficking in NctT$^{-/-}$ fibroblasts reveals a significant delay in the rate of APP re-internalization compared with that of control cells.

To determine the contributions of mammalian Aph-1 homologs in formation of functional γ-secretase complexes, we generated Aph-1a$^{-/-}$ mice [52]. As compared to littermate controls, the development of Aph-1a$^{-/-}$ embryos was dramatically retarded by embryonic day 9.5 and exhibited patterning defects that resemble, but are not identical to those of Notch1, Nct or PS$^{-/-}$ embryos. Moreover, in immortalized Aph-1a$^{-/-}$ fibroblasts, the levels of Nct, PS fragments and Pen-2 are dramatically decreased. Consequently, deletion of Aph-1a results in significant reductions in levels of the high molecular weight γ-secretase complex and in the secretion of Aβ. Three murine Aph-1 alleles, termed Aph-1a, Aph-1b and Aph-1c, encode four distinct Aph-1 isoforms: Aph-1aL and Aph-1aS derived from differential splicing of Aph-1a, Aph-1b and Aph-1c [52]. Importantly, complementation analysis reveals that all mammalian Aph-1 isoforms are capable of restoring the levels of Nct, PS and Pen-2 in Aph-1a$^{-/-}$ cells. Taken together, the findings establish that Aph-1a is the major mammalian Aph-1 homolog present in PS-dependent γ-secretase complexes during embryogenesis, and support the view that mammalian Aph-1 isoforms define a set of functional γ-secretase complexes.

Transgenic mouse models are being used to test a variety of novel therapies, including ways of reducing secretase activities and decreasing Aβ burden by Aβ immunotherapy. Many experimental therapeutic efforts have focused on influencing Aβ production (by inhibiting or modulating secretase activities), the aggregation of Aβ, its clearance, and the neurotoxicity of Aβ 42 [9, 10, 79]. Although mutant transgenic mice do not recapitulate the full phenotype of AD, they represent excellent models of Aβ amyloidosis and are highly suitable for identification of therapeutic targets, and for testing new treatments *in vitro*. Because it is not possible to discuss all treatment efforts in transgenic mice here, we focus on a few selected studies to illustrate experimental therapeutic strategies.

BACE1 is the principal β-secretase in neurons *in vitro*, BACE1-deficient neurons fail to secrete Aβ even when co-expressing the APPswe and mutant PS1 genes [25, 26, 36, 43, 50]. Significantly, BACE1$^{-/-}$, APPswe, PS1ΔE9 mice do not develop the Aβ deposits and age-associated abnormalities in working memory that occur in the APPswe, PS1ΔE9 model of Aβ amyloidosis [67]. Similarly, BACE1$^{-/-}$ TG2576 mice appear to be rescued from age dependent memory deficits and physiological abnormalities [80]. These data indicate that BACE1 is a very attractive therapeutic target. However, BACE1$^{-/-}$ mice develop abnormalities in performance of tasks assessing cognition and emotion, which can be rescued if APP is overexpressed in brain. In trials, it will be important to critically examine the influences of BACE1 inhibitors on memory, cognition and emotion.

The γ-secretase complex catalyzes the final cleavage of APP, which liberates the Aβ peptide into the extracellular space (amyloid deposits) [9, 10, 27, 30–32, 51, 52]. As demonstrated by the gene targeting strategies described above, this complex is critically dependent upon the presence of PS1 and 2, Nct, Aph-1a and Pen-2. Because reductions in these components decrease levels of Aβ, γ-secretase activity is a significant target for therapy. Inhibition of γ-secretase decreases production of Aβ in cell-free and cell-based systems and in levels of Aβ mutant mice with Aβ amyloidosis. However, γ-secretase activity is also essential for Notch processing critical for lineage specification and cell growth during embryonic development. Inhibitors could influence these activities. Significantly, LY-411, 575 not only reduced production of Aβ, but it also had profound effects on T and B cell development and on the appearance of intestinal mucosa (proliferation of goblet cells, increased mucin in gut lumen and crypt necrosis). Thus, several adverse effects could occur following inhibition of this enzyme, and it will be important for investigators to be alert for these effects.

Many pharmaceutical and biotechnology companies, and some academic laboratories, are using high throughput screen and molecular modeling strategies to discover compounds that inhibit these enzyme activities [9, 10, 32, 36]. Once lead compounds are identified, medicinal chemists will modify these compounds to enhance efficacy, allow passage through the blood–brain barrier, and reduce any potential toxicities.

Aβ immunotherapy has been used in both prevention and treatment trials in mutant mice. Both Aβ immunization (with Freund's adjuvant) and passive transfer of Aβ antibodies reduce levels of Aβ and plaque burden in

mutant APP and APP/PS1 transgenic mice [79, 81–87]. Efficacy seems to be related to antibody titer. The mechanisms of enhanced clearance are not certain, but two, not mutually exclusive, hypotheses have been suggested:

1. A small amount of Aβ antibody reaches the brain, binds to Aβ peptides, promotes the disassembly of fibrils, and, via the Fc antibody domain, encourages activated microglia to enter the affected region and remove Aβ [86]; and/or
2. Serum antibodies serve as a sink to draw the amyloid peptides from the brain into the circulation, thus changing the equilibrium of Aβ in different compartments and promoting removal of Aβ from the brain [82, 84, 85, 88].

Significantly, immunotherapy in transgenic mice is successful in partially clearing Aβ, in attenuating learning and behavioral deficits in at least two cohorts of mutant APP mice, and in reducing tau abnormalities in the triple transgenic mice [83–85].

Several of these experimental approaches are moving into clinical trials. Presently available therapies for patients include: cholinesterase inhibitors; agents that influence glutamate neurotransmission; neuroprotective and anti-inflammatory approaches; pharmacological agents useful for behavioral disturbances [1]; and statins that reduce brain amyloid burden [89]. However, these approaches do not directly influence the disease process, and new disease-modifying treatments are the major unmet need in AD. In addition to the investigations described, a variety of other treatments are being tested in model systems.

The identification of genes mutated or deleted in the inherited forms of AD has allowed investigators to create *in vivo* and *in vitro* model systems relevant to this illness. In this review, we have emphasized the value of transgenic and gene-targeted models and the lessons they provided for understanding mechanisms of AD, for identifying therapeutic targets and for testing new treatments in models.

First, for illustrative purposes, we focused on studies of the progeny of BACE1$^{-/-}$ mice and mutant APP/PS1 transgenic mice, which provides a dramatic example of the impact of reducing activity of secretases. New models using conditional expression systems, or RNAi silencing, will allow investigators to examine the pathogenesis of diseases and to assess the degrees of irreversibility of the disease processes. The results of these approaches will provide us with a better understanding of the mechanisms that lead to diseases and help us to design new treatments.

Second, we described the successful outcomes of Aβ immunotherapy in mutant mice with Aβ amyloidosis. Unfortunately, in humans, although Phase 1 trials with Aβ peptide and adjuvant vaccination were not associated with any adverse events, Phase 2 trials were suspended because of severe adverse reactions (meningoencephalitis) in a subset of patients [79, 90]. The pathology in a single case, consistent with T-cell meningitis [90], was interpreted to show some clearance of Aβ deposits, yet these regions contained a relatively high density of tangles, neuropil threads and vascular amyloid deposits [90]. In some regions, Aβ immunoreactivity was associated with microglia. T-cells were conspicuous in subarachnoid space and around some vessels [90]. The trial was truncated. The events occurring in this subset of patients illustrate the challenges of extrapolating outcomes in mutant mice to human trials. Interestingly, assessment of cognitive functions in a small subset of patients (30) who received vaccination and booster immunizations, disclosed that patients who generated Aβ antibodies as measured by a new assay, had a slower decline in several functional measures over time [91]. Investigators have continued to pursue the passive immunization approach and attempting to make antigens that do not stimulate T-cell-mediated immunologic attacks [79].

The lines of research described above have greatly enhanced our understanding of AD. We are now on the threshold of implementation of novel treatments, based on an understanding of the neurobiology and biochemistry of the illness. We anticipate that future discoveries will lead to the design of new mechanism-based therapies that can be tested in models of AD, and, eventually, these approaches can be introduced into clinical settings for the benefit of patients with this devastating disease. We are confident that parallel strategies will provide similar benefits for the treatment of other diseases of the nervous system.

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REFERENCES

1. Cummings, J. L. Alzheimer's disease. *N. Engl. J. Med.* 351: 56–67, 2004.
2. Brookmeyer, R., Gray, S. and Kawas, C. Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. *Am. J. Public Health* 88: 1337–1342, 1998.
3. Price, D. L., Tanzi, R. E., Borchelt, D. R. and Sisodia, S. S. Alzheimer's disease: Genetic studies and transgenic models. *Annu. Rev. Genet.* 32: 461–493, 1998.
4. Wong, P. C., Cai, H., Borchelt, D. R. and Price, D. L. Genetically engineered mouse models of neurodegenerative diseases. *Nature Neurosci.* 5: 633–639, 2002.
5. Lee, V. M., Goedert, M. and Trojanowski, J. Q. Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24: 1121–1159, 2001.

6. Morris, J. C. and Price, J. L. Pathologic correlates of nondemented aging, mild cognitive impairment, and early-stage Alzheimer's disease. *J. Mol. Neurosci.* 17: 101–118, 2001.
7. Sisodia, S. S. and George-Hyslop, P. H. γ-Secretase, Notch, Aβ and Alzheimer's disease: where do the presenilins fit in? *Nat. Rev. Neurosci.* 3: 281–290, 2002.
8. Tanzi, R. E. and Bertram, L. New frontiers in Alzheimer's disease genetics. *Neuron* 32: 181–184, 2001.
9. Walsh, D. M. and Selkoe, D. J. Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron* 44: 181–193, 2004.
10. Citron, M. Strategies for disease modification in Alzheimer's disease. *Nat. Rev. Neurosci.* 5: 677–685, 2004.
11. Petersen, R. C. Mild cognitive impairment clinical trials. *Nat. Rev. Drug Discov.* 2: 646–653, 2003.
12. Petersen, R. C., Doody, R., Kurz, A. *et al.* Current concepts in mild cognitive impairment. *Arch. Neurol.* 58: 1985–1992, 2001.
13. Morris, J. C., Storandt, M., Miller, J. P. *et al.* Mild cognitive impairment represents early-stage Alzheimer disease. *Arch. Neurol.* 58: 397–405, 2001.
14. Nestor, P. J., Scheltens, P. and Hodges, J. R. Advances in the early detection of Alzheimer's disease. *Nature Med.* 10 Suppl: S34–S41, 2004.
15. Sunderland, T., Linker, G., Mirza, N. *et al.* Decreased beta-amyloid 1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. *JAMA* 289: 2094–2103, 2003.
16. Klunk, W. E., Engler, H., Nordberg, A. *et al.* Imaging brain amyloid in Alzheimer's disease using the novel positron emission tomography tracer, Pittsburgh Compound-B. *Ann. Neurol.* 55: 1–14, 2004.
17. McKhann, G., Drachman, D., Folstein, M. *et al.* Clinical diagnosis of Alzheimer's disease: report of the NINCDS–ADRDA Work Group under the auspices of the Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34: 939–944, 1984.
18. Ghiso, J. and Wisniewski, T. An animal model of vascular amyloidosis. *Nature Neurosci.* 7: 902–904, 2004.
19. Corder, E. H., Saunders, A. M., Risch, N. J. *et al.* Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nature Genetics* 7: 180–184, 1994.
20. Koo, E. H., Sisodia, S. S., Archer, D. R. *et al.* Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. *Proc. Natl Acad. Sci. USA* 87: 1561–1565, 1990.
21. Buxbaum, J. D., Thinakaran, G., Koliatsos, V. *et al.* Alzheimer amyloid protein precursor in the rat hippocampus: transport and processing through the perforant path. *J. Neurosci.* 18: 9629–9637, 1998.
22. Sisodia, S. S., Koo, E. H., Hoffman, P. N. *et al.* Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. *J. Neurosci.* 13: 3136–3142, 1993.
23. Cao, X. and Sudhof, T. C. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* 293: 115–120, 2001.
24. Kamenetz, F., Tomita, T., Hsieh, H. *et al.* APP processing and synaptic function. *Neuron* 37: 925–937, 2003.
25. Vassar, R., Bennett, B. D., Babu-Khan, S. *et al.* β-secretase cleavage of Alzheimer's amyloid precusor protein by the transmembrane aspartic protease BACE. *Science* 286: 735–741, 1999.
26. Cai, H., Wang, Y., McCarthy, D. *et al.* BACE1 is the major β-secretase for generation of Aβ peptides by neurons. *Nature Neurosci.* 4: 233–234, 2001.
27. De Strooper, B. Aph-1, Pen-2, and nicastrin with presenilin generate an active gamma-secretase complex. *Neuron* 38: 9–12, 2003.
28. Selkoe, D. and Kopan, R. Notch and presenilin: regulated intramembrane proteolysis links development and degeneration. *Annu. Rev. Neurosci.* 26: 565–597, 2003.
29. Takasugi, N., Tomita, T., Hayashi, I. *et al.* The role of presenilin cofactors in the gamma-secretase complex. *Nature* 422: 438–441, 2003.
30. Haass, C. Take five—BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *EMBO J.* 23: 483–488, 2004.
31. Iwatsubo, T. The gamma-secretase complex: machinery for intramembrane proteolysis. *Curr. Opin. Neurobiol.* 14: 379–383, 2004.
32. Marjaux, E., Hartmann, D. and De Strooper, B. Presenilins in memory, Alzheimer's disease, and therapy. *Neuron* 42: 189–192, 2004.
33. Doan, A., Thinakaran, G., Borchelt, D. R. *et al.* Protein topology of presenilin 1. *Neuron* 17: 1023–1030, 1996.
34. Sherrington, R., Rogaev, E. I., Liang, Y. *et al.* Cloning of α-gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375: 754–760, 1995.
35. Thinakaran, G., Harris, C. L., Ratovitski, T. *et al.* Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J. Biol. Chem.* 272: 28415–28422, 1997.
36. Wolfe, M. S. Therapeutic strategies for Alzheimer's disease. *Nat. Rev. Drug Discov.* 1: 859–866, 2002.
37. Whitehouse, P. J., Price, D. L., Clark, A. W. *et al.* Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann. Neurol.* 10: 122–126, 1981.
38. Hyman, B. T., Van Hoesen, G. W., Damasio, A. R. and Barnes, C. L. Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. *Science* 225: 1168–1170, 1984.
39. Braak, H. and Braak, E. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol.* 82: 239–259, 1991.
40. Price, D. L. and Sisodia, S. S. Mutant genes in familial Alzheimer's disease and transgenic models. *Annu. Rev. Neurosci.* 21: 479–505, 1998.
41. Selkoe, D. J. Alzheimer's disease is a synaptic failure. *Science* 298: 789–791, 2002.
42. Iwatsubo, T., Odaka, A., Suzuki, N. *et al.* Visualization of $A\beta 42^{43}$ and Aβ 40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is $A\beta 42^{43}$. *Neuron* 13: 45–53, 1994.
43. Wong, P. C., Price, D. L. and Cai, H. The brain's susceptibility to amyloid plaques. *Science* 293: 1434–1435, 2001.
44. Caughey, B. and Lansbury, P. T. Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* 26: 267–298, 2003.
45. Gong, Y., Chang, L., Viola, K. L. *et al.* Alzheimer's disease-affected brain: presence of oligomeric Aβ ligands (ADDLs) suggests a molecular basis for reversible memory loss. *Proc. Natl Acad. Sci. USA* 100: 10417–10422, 2003.
46. Lue, L. F., Kuo, Y. M., Roher, A. E. *et al.* Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am. J. Pathol.* 155: 853–862, 1999.

47. Hsia, A. Y., Masliah, E., McConlogue, L. *et al.* Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc. Natl Acad. Sci. USA* 96: 3228–3233, 1999.
48. Edbauer, D., Winkler, E., Haass, C. and Steiner, H. Presenilin and nicastrin regulate each other and determine amyloid beta- peptide production via complex formation. *Proc. Natl Acad. Sci. USA* 99: 8666–8671, 2002.
49. Koo, E. H. and Kopan, R. Potential role of presenilin-regulated signaling pathways in sporadic neurodegeneration. *Nature Med.* 10 Suppl; S26–S33, 2004.
50. Luo, Y., Bolon, B., Kahn, S. *et al.* Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nature Neurosci.* 4: 231–232, 2001.
51. Li, T., Ma, G., Cai, H. *et al.* Nicastrin is required for assembly of presenilin/gamma-secretase complexes to mediate notch signaling and for processing and trafficking of beta-amyloid precursor protein in mammals. *J. Neurosci.* 23: 3272–3277, 2003.
52. Ma, G., Li, T., Price D. L. and Wong, P. C. APH-1a is the principal mammalian APH-1 isoform present in γ-secretase complexes during embryonic development. *J. Neurosci.* In Press, 2004.
53. Farzan, M., Schnitzler, C. E., Vasilieva, N. *et al.* BACE2, a β-secretase homolog, cleaves at the β site and within the amyloid-β region of the amyloid-β precursor protein. *Proc. Natl Acad. Sci. USA* 97: 9712–9717, 2000.
54. Yan, R., Bienkowski, M. J., Shuck, M. E. *et al.* Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* 402: 533–537, 1999.
55. Yang, L. B., Lindholm, K., Yan, R. *et al.* Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nature Med.* 9: 3–4, 2003.
56. Li, R., Lindholm, K., Yang, L. B. *et al.* Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. *Proc. Natl Acad. Sci. USA* 101: 3632–3637, 2004.
57. Sisodia, S. S., Koo, E. H., Beyreuther, K. T. *et al.* Evidence that β-amyloid protein in Alzheimer's disease is not derived by normal processing. *Science* 248: 492–495, 1990.
58. Steiner, H., Winkler, E., Edbauer, D. *et al.* PEN-2 is an integral component of the gamma-secretase complex required for coordinated expression of presenilin and nicastrin. *J. Biol. Chem.* 277: 39062–39065, 2002.
59. Goutte, C., Tsunozaki, M., Hale, V. A. and Priess, J. R. APH-1 is a multipass membrane protein essential for the Notch signaling pathway in *Caenorhabditis elegans* embryos. *Proc. Natl Acad. Sci. USA* 99: 775–779, 2002.
60. Li, Y. M., Xu, M., Lai, M. T. *et al.* Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* 405: 689–694, 2000.
61. Esler, W. P., Kimberly, W. T., Ostaszewski, B. L. *et al.* Transition-state analogue inhibitors of gamma-secretase bind directly to presenilin-1. *Nature Cell Biol.* 2: 428–434, 2000.
62. Naruse, S., Thinakaran, G., Luo, J. J. *et al.* Effects of PS1 deficiency on membrane protein trafficking in neurons. *Neuron* 21: 1213–1221, 1998.
63. Wong, P. C., Zheng, H., Chen, H. *et al.* Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. *Nature* 387: 288–292, 1997.
64. Shen, J., Bronson, R. T., Chen, D. F. *et al.* Skeletal and CNS defects in presenilin-1-deficient mice. *Cell* 89: 629–639, 1997.
65. Capell, A., Steiner, H., Romig, H. *et al.* Presenilin-1 differentially facilitates endoproteolysis of the beta-amyloid precursor protein and Notch. *Nature Cell Biol.* 2: 205–211, 2000.
66. Sturchler-Pierrat, C., Abramowski, D., Duke, M. *et al.* Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proc. Nat. Acad. Sci. USA* 94: 13287–13292, 1997.
67. Borchelt, D. R., Thinakaran, G., Eckman, C. B. *et al.* Familial Alzheimer's disease-linked presenilin 1 variants elevate Aβ 1-42/1-40 ratio *in vitro* and *in vivo*. *Neuron* 17: 1005–1013, 1996.
68. Borchelt, D. R., Ratovitski, T., Van Lare, J. *et al.* Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 19: 939–945, 1997.
69. Jankowsky, J. L., Fadale, D. J., Anderson, J. *et al.* Mutant presenilins specifically elevate the levels of the 42 residue β-amyloid peptide *in vivo*: evidence for augmentation of a 42-specific γ-secretase. *Hum. Mol. Genet.* 13: 159–170, 2004.
70. Chapman, P. F., White, G. L., Jones, M. W. *et al.* Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nature Neurosci.* 2: 271–276, 1999.
71. Bacskai, B. J., Hickey, G. A., Skoch, J. *et al.* Four-dimensional multiphoton imaging of brain entry, amyloid binding, and clearance of an amyloid-beta ligand in transgenic mice. *Proc. Natl Acad. Sci. USA* 100: 12462–12467, 2003.
72. Gotz, J., Chen, F., Van Dorpe, J. and Nitsch, R. M. Formation of neurofibrillary tangles in P3011 tau transgenic mice induced by Aβ fibrils. *Science* 293: 1491–1495, 2001.
73. Lewis, J., Dickson, D. W., Lin, W.-L. *et al.* Enhanced neurofibrillary degeneration in transgenic mice expressing mutat tau and APP. *Science* 293: 1487–1491, 2001.
74. Oddo, S., Caccamo, A., Shepherd, J. D. *et al.* Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. *Neuron* 39: 409–421, 2003.
75. Chen, G., Chen, K. S., Knox, J. *et al.* A learning deficit related to age and β-amyloid plaques in a mouse model of Alzheimer's disease. *Nature* 408: 975–979, 2000.
76. Heber, S., Herms, J., Gajic, V. *et al.* Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. *J Neurosci.* 20: 7951–7963, 2000.
77. Esler, W. P., Kimberly, W. T., Ostaszewski, B. L. *et al.* Activity-dependent isolation of the presenilin-γ-secretase complex reveals nicastrin and a γ substrate. *Proc. Natl Acad. Sci. USA* 14: 1–6, 2002.
78. De Strooper, B., Annaert, W. G., Cupers, P. *et al.* A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* 398: 518–522, 1999.
79. Monsonego, A. and Weiner, H. L. Immunotherapeutic approaches to Alzheimer's disease. *Science* 302: 834–838, 2003.
80. Ohno, M., Sametsky, E. A., Younkin, L. H. *et al.* BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron* 41: 27–33, 2004.
81. Bard, F., Cannon, C., Barbour, R. *et al.* Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nature Med.* 6: 916–919, 2000.
82. DeMattos, R. B., Bales, K. R., Cummins, D. J. *et al.* Brain to plasma amyloid-beta efflux: a measure of brain amyloid

burden in a mouse model of Alzheimer's Disease. *Science* 295, 2264–2267, 2002.
83. Kotilinek, L. A., Bacskai, B. J., Westerman, M. *et al.* Reversible memory loss in a mouse transgenic model of Alzheimer's disease. *J. Neurosci.* 22: 6331–6335, 2002.
84. Morgan, D., Diamond, D. M., Gottschall, P. E. *et al.* Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. *Nature* 408: 982–985, 2000.
85. Dodart, J. C., Bales, K. R., Gannon, K. S. *et al.* Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. *Nature Neurosci.* 5: 452–457, 2002.
86. Schenk, D., Barbour, R., Dunn, W. *et al.* Immunization with amyloid-beta attenuates Alzheimer disease-like pathology in the PDAPP mouse. *Nature* 400: 173–177, 1999.
87. Chauhan, N. B. and Siegel, G. J., Efficacy of anti-Aβ antibody isotypes used for intracerebroventricular immunization in TgCRND8. *Neurosci. Lett.* 375: 143–147, 2005.
88. Cirrito, J. R., May, P. C., O'Dell, M. A. *et al. In vivo* assessment of brain interstitial fluid with microdialysis reveals plaque-associated changes in amyloid-beta metabolism and half-life. *J. Neurosci.* 23: 8844–8853, 2003.
89. Chauhan, N. B., Siegel, G. J. and Feinstein, D. L., Effects of lovostatin and pravastatin on amyloid processing and inflammatory response in TgCRND8 brain, *Neurochem. Res.* 29: 1897–1911, 2004.
90. Nicoll, J. A., Wilkinson, D., Holmes, C. *et al.* Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. *Nature Med.* 9: 448–452, 2003.
91. Hock, C., Konietzko, U., Streffer, J. R. *et al.* Antibodies against β-amyloid slow cognitive decline in Alzheimer's disease. *Neuron* 38: 547–554, 2003.

CHAPTER

48

Molecular Basis of Prion Diseases

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INTRODUCTION

The prion diseases are a closely related group of neurodegenerative conditions which affect both humans and animals. They have previously been described as the subacute spongiform encephalopathies, slow virus diseases and transmissible dementias, and include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and the human prion diseases, Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI) and kuru. Prion diseases are

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unique in biology in that they manifest as sporadic, genetic and infectious disorders. Although historically rare in humans, affecting about one person per million worldwide per annum, the appearance of a novel human prion disease, variant CJD (vCJD), and the experimental confirmation that this is caused by the same prion strain as that causing BSE in cattle, has raised the possibility that a major epidemic of vCJD will occur within the U. K. and other European countries as a result of dietary or other exposure to BSE prions. These concerns, together with those of potential iatrogenic transmission of preclinical vCJD via medical and surgical procedures, have led to intense efforts to understand the molecular basis of prion propagation and to develop rational therapies.

PRION DISEASES ARE BIOLOGICALLY UNIQUE

Discovery of the prion protein. The demonstration of transmission of prion disease by inoculation of scrapie brain isolates into sheep, and kuru, CJD and GSS brain isolates into primates, formed the basis for the concept of 'transmissible dementias'; however, the nature of the transmissible agent has been a subject of heated debate for many years. The understandable initial assumption that the agent must be some form of virus was challenged by the failure to directly demonstrate such a virus (or indeed any immunological response to it) and by evidence indicating that the transmissible agent showed remarkable resistance to treatment expected to inactivate nucleic acids. Such findings led to suggestions that the transmissible agent may be devoid of nucleic acid [1], and might be a protein [2]. Subsequently, the progressive enrichment of brain homogenates for prion infectivity resulted in the isolation of a protease resistant sialoglycoprotein, designated the prion protein (PrP), by Prusiner and co-workers in 1982. This protein was the major constituent of infective fractions and was found to accumulate in affected brains and sometimes to form amyloid deposits. The term prion (from ***pro***teinaceous ***in***fectious particle) was proposed by Prusiner to distinguish the infectious pathogen from viruses or viroids. Prions were defined as 'small proteinaceous infectious particles that resist inactivation by procedures which modify nucleic acids' [3].

Prion protein is host encoded. At the time of its isolation, PrP was assumed to be encoded by a gene within the putative slow virus thought to be responsible for these diseases. However, amino-acid sequencing of part of PrP, and the subsequent recovery of cognate cDNA clones using an isocoding mixture of oligonucleotides, led to the realization that PrP was encoded by a single-copy chromosomal gene, rather than by a putative nucleic acid in fractions enriched for scrapie infectivity [4].

Aberrant metabolism of the prion protein is the central feature of prion diseases. It is now clear that the central and unifying hallmark of the prion diseases is the aberrant metabolism of PrP, which exists in at least two conformational states with different physicochemical properties. The normal cellular form of the protein, referred to as PrP^{C}, is a highly conserved cell surface glycosylphosphatidylinositol (GPI)-anchored sialoglycoprotein that is sensitive to protease treatment and soluble in detergents. The disease-associated scrapie isoform, referred to as PrP^{Sc} is found only in prion infected tissue as aggregated material, is partially resistant to protease treatment and insoluble in detergents. Due to its physiochemical properties the precise atomic structure of the infectious particle or prion is still undetermined but considerable evidence argues that prions are composed largely, if not entirely, of an abnormal isoform of PrP. The essential role of host PrP for prion propagation and pathogenesis is demonstrated by the fact that knockout mice lacking the PrP gene ($Prnp^{0/0}$ mice) are entirely resistant to prion infection, and that reintroduction of the murine PrP transgene restores susceptibility to infection [4]. For a comprehensive review of prion disease biology see [5].

ANIMAL PRION DISEASES

Scrapie and BSE. Scrapie is the prototypic prion disease, and has been recognized as an enzootic disease of sheep and goats for more than 250 years. The etiology of natural scrapie has been the subject of intense debate for many years, but it is now clear that it is an infectious disease, for which susceptibility is genetically modulated by the host [6]. Following its discovery in 1985, BSE reached epidemic proportions in cattle within the U. K., with smaller numbers in many other European countries. Epidemiologic studies point to contaminated offal used in the manufacture of meat and bone meal and fed to cattle as the source of prions responsible for BSE, although the nature and source of the original prion contamination remains unknown [7]. The host range of BSE appears to be unusually wide, affecting many other animal species. Foodstuffs contaminated with BSE appear to have caused prion disease in several other animal species in the U. K., including domestic cats, exotic ungulates and captive cats in zoos. In addition to these 'natural' infections, BSE has been experimentally transmitted to a wide variety of species.

Other animal prion diseases. Outbreaks of transmissible mink encephalopathy and chronic wasting disease in captive populations of mink, mule deer and elk in certain regions of the U.S.A. have also been attributed to prion-infected foodstuffs, although the origin of prion infection is unclear [8]. Epidemiological studies suggest lateral transmission as the most plausible explanation for the spread of chronic wasting disease in captive populations

of Rocky Mountain elk. Chronic wasting disease has also been diagnosed in free-ranging mule deer, Rocky Mountain elk and white-tailed deer from north-central Colorado [8].

HUMAN PRION DISEASES

Human prion diseases encompass the three etiological types of prion disease: inherited, sporadic and acquired forms.

Human prion disease most commonly presents with a sporadic etiology. Approximately 85 % of cases of human prion disease occur sporadically as Creutzfeldt–Jakob disease at a rate of roughly one case per million population per year across the world, with an equal incidence in men and women. The etiology of sporadic CJD is unknown, although hypotheses include somatic mutation in the human prion protein gene (*PRNP*), or the spontaneous conversion of PrP^{C} into PrP^{Sc} as a rare stochastic event. Homozygosity at a common coding polymorphism at codon 129 of *PRNP* encoding either methionine or valine predisposes to the development of sporadic CJD [9]. Additionally, a *PRNP* susceptibility haplotype has been identified indicating further genetic susceptibility to sporadic CJD at or near to the *PRNP* locus [5, 10].

Pathogenic mutations in the prion protein gene cause inherited prion disease. Approximately 15% of human prion diseases are associated with autosomal dominant pathogenic mutations in *PRNP* (Fig. 48-1). The identification of one of the pathogenic *PRNP* mutations in a case with neurodegenerative disease allows diagnosis of an inherited prion disease and subclassification according to mutation, and also permits presymptomatic genetic testing in affected families [5, 10, 11]. Over 50 pathogenic mutations have been described in two groups:

1. Point mutations resulting in amino acid substitutions in PrP or, in two cases, production of a stop codon resulting in expression of a truncated PrP;
2. Insertions encoding additional integral copies of an octapeptide repeat present in a tandem array of five copies in the normal protein (Fig. 48-1).

How pathogenic mutations in *PRNP* cause prion disease has yet to be resolved. However, in most cases the mutation is thought to lead to an increased tendency of PrP^{C} to form PrP^{Sc}, although there is evidence to suggest that this may not be solely attributable to decreased thermodynamic stability of mutated PrP^{C}. Experimentally manipulated mutations of the prion gene can lead to spontaneous neurodegeneration without the formation

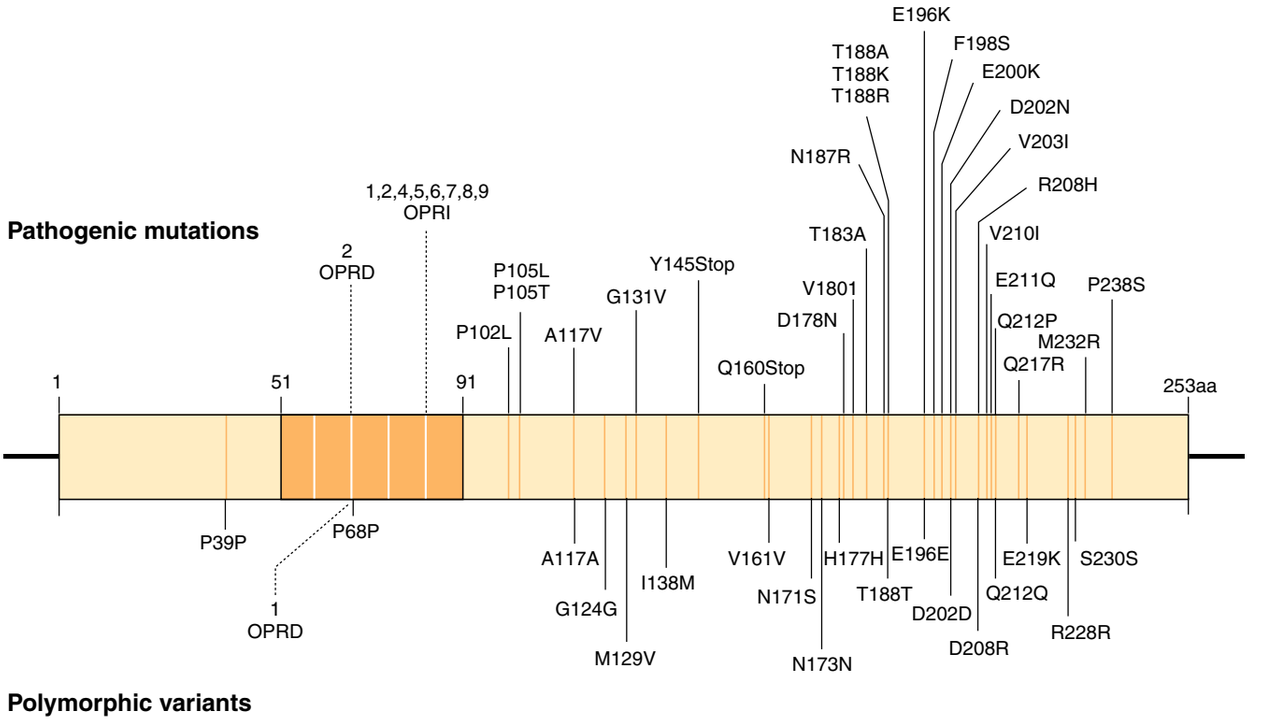

FIGURE 48-1 Pathogenic mutations and polymorphisms in the human prion protein. The pathogenic mutations associated with human prion disease are shown above the human PrP coding sequence. These consist of 1, 2 or 4-9 octapeptide repeat insertions (*ORPI*) within the octapeptide repeat region between codons 51 and 91, a 2-octapeptide repeat deletion (*ORPD*), and various point mutations causing missense or stop amino-acid substitutions. Point mutations are designated by the wild-type amino acid preceding the codon number, followed by the mutant residue, using single-letter amino-acid nomenclature. Polymorphic variants are shown below the PrP coding sequence.

of detectable protease resistant PrP. These findings raise the question of whether all inherited forms of human prion disease invoke disease through the same mechanism, and in this regard it is currently unknown whether all are transmissible by inoculation [5].

Acquired human prion diseases include kuru and variant CJD. Although the human prion diseases are transmissible diseases, acquired forms have, until recently, been confined to rare and unusual situations. The two most frequent causes of iatrogenic CJD occurring through medical procedures have arisen as a result of implantation of dura mater grafts, and treatment with human growth hormone derived from the pituitary glands of human cadavers [12]. Less-frequent incidences of human prion disease have resulted from iatrogenic transmission of CJD during corneal transplantation, contaminated electroencephalographic (EEG) electrode implantation and surgical operations using contaminated instruments or apparatus [12]. The most well-known incidences of acquired prion disease in humans resulting from a dietary origin have been kuru that was transmitted by cannibalism among the Fore linguistic group of the Eastern Highlands in Papua New Guinea [5, 13, 14], and more recently the occurrence of vCJD in the U. K. and other European countries which is causally related to BSE in cattle [5]. Incubation periods of acquired prion diseases in humans can be extremely prolonged, and it remains to be seen if a substantial epidemic of vCJD will occur within the U. K. and elsewhere [15].

Prion protein polymorphism contributes genetic susceptibility to prion disease. *PRNP* codon 129 polymorphism plays a key role in contributing genetic susceptibility to acquired human prion disease [5, 14, 16]. In iatrogenic CJD caused by exposure to contaminated pituitary hormones, *PRNP* codon 129 heterozygotes have a longer mean incubation period [12]. All cases of variant CJD to date have been methionine homozygotes while homozygotes of either allele have an earlier age of onset for kuru [5, 14]. Kuru imposed strong balancing selection on the Fore population essentially eliminating *PRNP* 129 homozygotes. Elderly survivors of the kuru epidemic, who had multiple exposures at mortuary feasts, are, in marked contrast to younger unexposed Fore, predominantly *PRNP* 129 heterozygotes. Worldwide *PRNP* haplotype diversity and coding allele frequencies suggest that kuru-like epidemics imposed strong balancing selection at this locus during the evolution of modern humans [14].

Human prion diseases are clinically heterogeneous. All forms of human prion disease are invariably fatal following clinical onset; however, there is marked phenotypic variability depending upon etiology. Progressive dementia, cerebellar ataxia, pyramidal signs, chorea, myoclonus, extrapyramidal features, pseudobulbar signs, seizures and amyotrophic features are seen in variable combinations. The clinical presentation in classical CJD is a rapidly progressive dementia accompanied by myoclonus, followed by decline to akinetic mutism and death often within 3–4 months. Traditionally, inherited prion diseases have been classified by the presenting clinical syndrome, falling into three main subdivisions of either GSS, CJD or FFI. GSS classically presents as a chronic cerebellar ataxia with pyramidal features, with dementia occurring much later in a clinical course that is typically longer than that seen in classical CJD. FFI is characterized by progressive untreatable insomnia, dysautonomia, dementia and selective thalamic degeneration and is most commonly associated with a missense mutation at codon 178 of *PRNP*, although sporadic FFI with no causative mutation in *PRNP* has been reported. The availability of direct gene markers for inherited prion diseases has enabled identification of highly atypical cases and has widened the known phenotypic range for these disorders.

Some families show remarkable phenotypic variability that can encompass both CJD- and GSS- like cases as well as other cases that do not conform to CJD, GSS or FFI phenotypes. Because of the extensive phenotypic variability seen in inherited prion disease and its ability to mimic other neurodegenerative conditions, notably Alzheimer's disease, prion protein gene analysis should be considered in the investigation of all presenile ataxias and dementias, even in the absence of an apparent family history of neurodegenerative illness. The clinical features of kuru consist of a progressive cerebellar ataxia accompanied by dementia in the later stages and death, which usually occurs within 12 months. Iatrogenic prion disease arising from intracerebral or optic inoculation usually manifests clinically as classical CJD, while those arising from a peripheral route of inoculation, such as pituitary growth hormone commonly present with a progressive ataxia. The early clinical presentation of vCJD resembles kuru more than classical CJD and consists of behavioral and psychiatric disturbances, peripheral sensory disturbance and cerebellar ataxia. For reviews on human prion disease see [5, 10, 17].

PRION DISEASE PATHOLOGY AND PATHOGENESIS

Peripheral pathogenesis involves the lymphoreticular system. Although the pathological consequences of prion infection occur in the central nervous system, and experimental transmission of these diseases is most efficiently accomplished by intracerebral inoculation, most natural infections do not occur by these means. Indeed, administration to sites other than the central nervous system is known to be associated with much longer incubation periods, which may extend to 20 years or more [5, 12]. Experimental evidence suggests that this latent period is associated with clinically silent prion replication in

lymphoreticular tissue, whereas neuroinvasion takes place later. The *M* cells in the intestinal epithelium mediate prion entry from the gastrointestinal lumen into the body, and follicular dendritic cells (FDCs) are thought to be essential for prion replication and for accumulation of disease-associated PrP^{Sc} within secondary lymphoid organs. B-cell deficient mice are resistant to intraperitoneal inoculation with prions probably because of their involvement with FDC maturation and maintenance. The interface between FDCs and sympathetic nerves represents a critical site for the transfer of lymphoid prions into the nervous system; however, the mechanism by which this is achieved remains unknown. Distinct forms of prion disease show differences in lymphoreticular involvement that may be related to the etiology of the disease or to divergent properties of distinct prion strains. For a review of prion disease peripheral pathogenesis see [18].

Prion disease produces characteristic pathology in the central nervous system. The brains of patients or animals with prion disease frequently show no recognizable abnormalities on gross examination at necropsy; however, microscopic examination of the central nervous system typically reveals characteristic histopathologic changes, consisting of neuronal vacuolation and degeneration, which gives the cerebral gray matter a microvacuolated or 'spongiform' appearance, and a reactive proliferation of astroglial cells. For a review see [19]. Although spongiform degeneration is frequently detected, it is not an obligatory neuropathologic feature of prion disease; astrocytic gliosis, although not specific to the prion diseases, is more constantly seen. The lack of an inflammatory response is also an important characteristic. Demonstration of abnormal PrP immunoreactivity, or more specifically biochemical detection of PrP^{Sc} in brain material by immunoblotting techniques is diagnostic of prion disease and some forms of prion disease are characterized by deposition of amyloid plaques composed of insoluble aggregates of PrP [19]. Amyloid plaques are a notable feature of kuru and GSS, but they are less frequently found in the brains of patients with sporadic CJD which typically show a diffuse pattern of abnormal PrP deposition (Fig. 48-2).

The histopathologic features of vCJD are remarkably consistent and distinguish it from other human prion diseases with large numbers of PrP-positive amyloid plaques that differ in morphology from the plaques seen in kuru and GSS, in that the surrounding tissue takes on a microvacuolated appearance, giving the plaques a florid appearance (Fig. 48-2). vCJD is distinct in its pathogenesis from other human prion diseases, as PrP^{Sc} is readily detectable in lymphoreticular tissues in vCJD, and not in classical CJD or inherited cases of human prion disease. Tonsil biopsy is used for antemortem diagnosis of vCJD and, to date, has shown 100% sensitivity and specificity for diagnosis of vCJD at an early clinical stage. The demonstration of extensive lymphoreticular involvement in the peripheral pathogenesis of vCJD raises concerns that iatrogenic transmission of vCJD prions through medical procedures may be a major public health issue [5, 10].

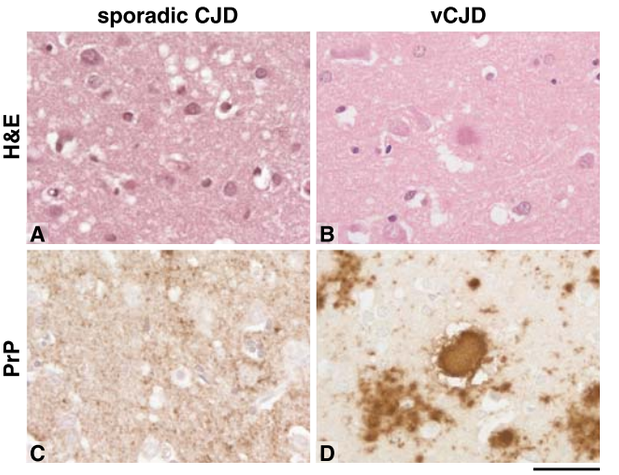

FIGURE 48-2 Prion disease pathology. Serial brain sections from sporadic CJD (**A**, **C**) and vCJD (**B**, **D**) show spongiform neurodegeneration following hematoxylin and eosin staining (*H&E*) and abnormal PrP immunoreactivity following immunohistochemistry using an anti-PrP monoclonal antibody (*PrP*). Abnormal PrP deposition in sporadic CJD most commonly presents as diffuse, synaptic staining, whereas vCJD is distinguished by the presence of florid PrP plaques consisting of a round amyloid core surrounded by a ring of spongiform vacuoles. Scale bar: 50 μm. Figure courtesy of Dr Sebastian Brandner.

THE PROTEIN-ONLY HYPOTHESIS OF PRION PROPAGATION

A wide body of data now supports the idea that infectious prions consist principally or entirely of an abnormal isoform of PrP. Disease associated PrP, designated PrP^{Sc}, is derived from PrP^{C} by a post-translational mechanism and neither amino acid sequencing nor systematic study of known covalent post-translational modifications have shown any consistent differences between PrP^{C} and PrP^{Sc} [5, 20].

Prion propagation involves conversion of PrP^{C} to PrP^{Sc}. The protein-only hypothesis, in its current form, argues that prion propagation occurs through PrP^{Sc} acting to replicate itself with high fidelity by recruiting endogenous PrP^{C}, and that this conversion involves only conformational change [5;20]. However, the underlying molecular events during infection that lead to the conversion of PrP^{C} to PrP^{Sc} and how PrP^{Sc} accumulation leads to neurodegeneration remain poorly defined. The most coherent and general model thus far proposed is that the protein PrP fluctuates between a dominant native state, PrP^{C}, and a series of minor conformations, one or a set of which can self-associate in an ordered manner to produce a stable supramolecular structure, PrP^{Sc}, composed of misfolded

PrP monomers. Once a stable 'seed' structure is formed, PrP can then be recruited leading to an explosive, autocatalytic formation of PrP^{Sc}. Such a mechanism could underlie prion propagation and account for the transmitted, sporadic and inherited etiologies of prion disease. Initiation of a pathogenic self-propagating conversion reaction, with accumulation of aggregated PrP, may be induced by exposure to a 'seed' of aggregated PrP following prion inoculation, or as a rare stochastic conformational change, or as an inevitable consequence of expression of a pathogenic PrP^{C} mutant which is predisposed to form misfolded PrP (Fig. 48-3). Such a system would be extremely sensitive to three factors:

1. Overall PrP^{C} concentration;
2. The equilibrium distribution between the native conformation and the self-associating conformation;
3. Complimentarity between surfaces which come together in the aggregation step.

All three of these predictions from this minimal model are manifest in the etiology of prion disease: an inversely proportional relationship between PrP^{C} expression and prion incubation period in transgenic mice; predisposition by relatively subtle mutations in the protein sequence; and a requirement for molecular homogeneity between PrP^{Sc} and PrP^{C} for efficient prion propagation [4, 5, 20]. It is clear that a full understanding of prion propagation will require knowledge both of the structure of PrP^{C} and PrP^{Sc} and of the mechanism of conversion between them.

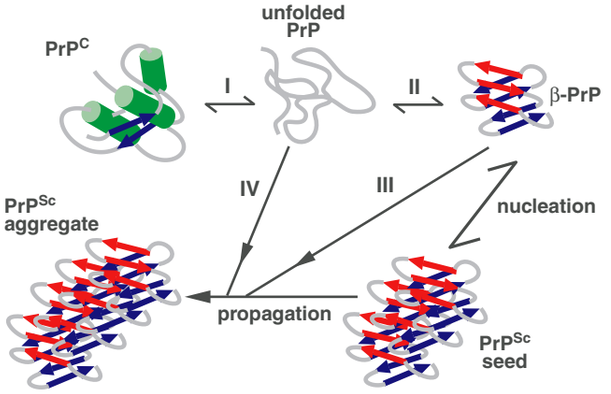

FIGURE 48-3 The protein-only model of prion propagation. The schematic shows a plausible mechanism that could account for prion propagation. α-helical cellular isoforms of prion protein (*PrP^{C}*) proceed via an unfolded state (*I*) to refold into a predominantly β-sheet rich form, *β-PrP* (*II*). Aggregation of β-PrP may form a critical 'seed' of PrP^{Sc} which then propagates, essentially irreversibly, through further recruitment of β-PrP monomers (*III*) or unfolded PrP monomers (*IV*). This mechanism can account for the transmitted, sporadic and inherited etiologies of prion disease. Initiation of a pathogenic self-propagating conversion reaction may be induced by exposure to a 'seed' of PrP^{Sc} following prion inoculation, or as a rare stochastic conformational change in wild-type PrP^{C}, or as an inevitable consequence of expression of a pathogenic PrP^{C} mutant that is predisposed to form β-PrP.

CHARACTERIZATION OF PrP^{C}

PrP^{C} has a predominantly α-helical conformation. The conformation of the cellular isoform of PrP was first established by NMR measurements made on the recombinant mouse protein. Since then, NMR measurements on recombinant hamster and human PrP show that they have essentially the same conformation, and these data have been supported by crystallographic determination of the structure of a human PrP dimer. For reviews see [5, 21, 22]. Following cleavage of an *N*-terminal signal peptide, and removal of a *C*-terminal peptide on addition of a GPI anchor, the mature PrP^{C} species consists of an *N*-terminal region of about 100 amino acids, which is unstructured in the isolated molecule in solution, and a *C*-terminal segment, also around 100 amino acids in length. The *C*-terminal domain is folded into a largely α-helical conformation (three α-helices and a short anti-parallel β-sheet) and stabilized by a single disulfide bond linking helices 2 and 3 (Fig. 48-4). There are two asparagine-linked glycosylation sites (Fig. 48-4). The *N*-terminal region contains a segment of five repeats of an eight-amino acid sequence (the octapeptide-repeat region), expansion of which by insertional mutation leads to inherited prion disease. While unstructured in the isolated molecule, this region contains a tight binding site for a single Cu^{2+} ion, with a second tight Cu^{2+} binding site present upstream of the octa-repeat region but before the structured *C*-terminal domain. Both high affinity Cu^{2+} binding sites can also bind Zn^{2+} ions with binding constants of physiological relevance, and it thus seems likely that the *N*-terminal region of PrP may acquire coordinated structure *in vivo* through coordination of either Cu^{2+} or Zn^{2+} ions [5].

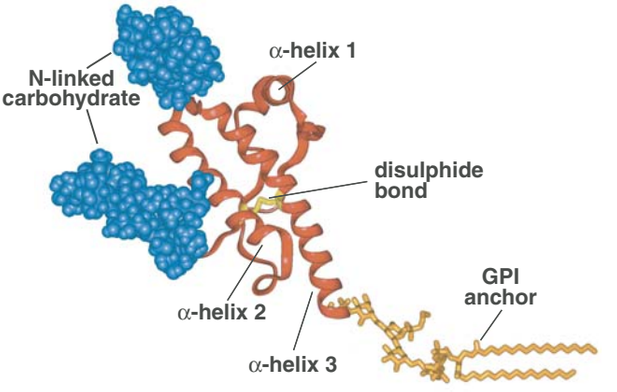

FIGURE 48-4 Model structure of PrP^{C}. The conformation of recombinant mouse PrP (residues 124–231) determined by NMR is shown in *red* ribbon. The single disulfide bridge linking α-helixes 2 and 3 is shown in *yellow*. *N*-linked carbohydrate groups are shown as space-filling structures in *blue*. The glycosylphosphatidylinositol (*GPI*) anchor that attaches PrP to the outer surface of the cell membrane is shown in *gold*.

The structured *C*-terminal domain folds and unfolds reversibly in response to chaotropic denaturants, and it appears there are no populated intermediates in the folding reaction and that the protein displays unusually rapid rates of folding and unfolding (for a review see [5]). These data suggest that PrP^{Sc} is unlikely to be formed from a kinetic folding intermediate, as has been hypothesized in the case of amyloid formation in other systems, and is more likely to be formed from the unfolded state of PrP. Pathogenic mutations in *PRNP* seen in inherited prion diseases may produce disease by destabilizing PrP^{C} which would predispose the molecule to aggregate. Alternatively, a mutation could facilitate the interaction between PrP^{C} and PrP^{Sc} or affect the binding of a ligand or co-protein.

Reverse genetics approaches to studying PrP^{C}. While $Prnp^{0/0}$ mice are completely resistant to prion infection, reconstitution of such mice with PrP transgenes restores susceptibility to prion infection in a species-specific manner, and allows a reverse genetics approach to study structure–function relationships in PrP (for reviews see [4, 23]). Expression of PrP *N*-terminal deletion mutants to residue 106 are tolerated and support prion propagation; however, deletion beyond this leads to severe ataxia and neuronal loss in the granular cell layer of the cerebellum [4]. This has led to the hypothesis that PrP and a structural homologue compete for the same receptor or ligand. An additional gene (*Prnd*) has been discovered downstream of the *Prnp* locus, that encodes a 179-residue protein with between 20% and 24% identity to PrP (depending upon the species), that is a plausible candidate for the putative competitor [4]. PrP in its entirety is unnecessary for prion propagation. Not only can the unstructured *N*-terminal 90 amino acids be deleted, but also the first α-helix, the second β-strand and part of helix 2. In transgenic animals, a 106 amino acid fragment of the protein comprising PrPΔ23–88Δ141–176 conferred susceptibility to and propagation of prions (for a review see [4]).

The function of PrP^{C} remains unknown. PrP is highly conserved among mammals, has been identified in marsupials, birds, amphibians and fish, and may be present in all vertebrates. It is expressed during early embryogenesis and is found in most tissues in the adult with the highest levels of expression in the central nervous system, in particular in association with synaptic membranes. PrP is also widely expressed in cells of the immune system. As a GPI-anchored cell surface glycoprotein, it has been speculated that PrP may have a role in cell adhesion or signaling processes, but its precise cellular function has remained obscure [5, 24]. Newly synthesized PrP^{C} is transported to the cell surface, from which it may cycle via either clathrin-coated vesicles or caveolae-dependent endocytic pathways. A range of PrP^{C} interacting molecules have been identified; however, despite a growing body of evidence suggesting that PrP^{C} may play a role in cell survival through interaction with apoptotic pathways, the *in vivo* relevance of such interactions has yet to be convincingly demonstrated. A role for PrP in copper or zinc metabolism or transport seems possible, and disturbance of this function by the conformational transition from normal to disease related isoforms of PrP may be involved in prion-related neurotoxicity.

PrP knockout mice have subtle abnormalities of synaptic transmission. Mice lacking PrP as a result of gene knockout ($Prnp^{0/0}$ mice) show no gross phenotype; however, these mice are completely resistant to prion disease following inoculation and do not replicate prions [4]. $Prnp^{0/0}$ mice do, however, show subtle abnormalities in synaptic physiology, in circadian rhythms and in sleep (for reviews see [4, 5]). In particular, abnormalities of inhibitory synaptic transmission in hippocampal slices with a reduced amplitude of the maximal inhibitory postsynaptic current have been observed, and this phenotype can be rescued by a reintroduction of a PrP transgene. A second physiological phenotype for $Prnp^{0/0}$ mice manifests as a reduction of slow afterhyperpolarizations evoked by trains of action potentials in hippocampal neurons, and is due to disruption of calcium-activated potassium currents. While the relative normality of $Prnp^{0/0}$ mice was thought to result from effective adaptive changes during development, data from *Prnp* conditional knockout mice suggest this is not the case [25]. These mice undergo ablation of neuronal PrP expression at 9 weeks of age and remain healthy without evidence of neurodegeneration or an overt clinical phenotype. These findings demonstrate that acute loss of neuronal PrP in adulthood is tolerated, and that the pathophysiology of prion diseases is not due to loss of normal PrP function in neurons. Notably, slow afterhyperpolarizations in hippocampal CA1 cells are affected by the conditional knockout of PrP, suggesting that this phenotype is specifically due to the absence of PrP, reflecting loss of a differentiated neuronal function, rather than a developmental deficit arising from congenital knockout of PrP [25].

CHARACTERIZATION OF PrP^{SC}

PrP^{Sc} has a predominantly β-sheet conformation. PrP^{Sc} is extracted from affected brains as highly aggregated, detergent-insoluble, material that is not amenable to high-resolution structural techniques. However, Fourier transform infrared spectroscopic methods show that PrP^{Sc}, in sharp contrast to PrP^{C}, has a high β-sheet content. PrP^{Sc} is covalently indistinguishable from PrP^{C} but can be distinguished from PrP^{C} by its partial resistance to proteolysis and its marked insolubility in detergents (for reviews see [20, 22]). Under conditions in which PrP^{C} exists as a detergent-soluble monomer and is completely degraded by the nonspecific protease, proteinase K, PrP^{Sc} exists in an aggregated form with the *C*-terminal two-thirds of the protein showing marked resistance to proteolytic degradation

leading to the generation of amino terminally truncated fragments of di-, mono- and non-glycosylated PrP (Fig. 48-5). For a particular PrP^{Sc} isoform all three PrP^{Sc} glycoforms show equivalent accessibility to proteinase K and are cleaved at the same point in the *N*-terminus of the protein (Fig. 48-5).

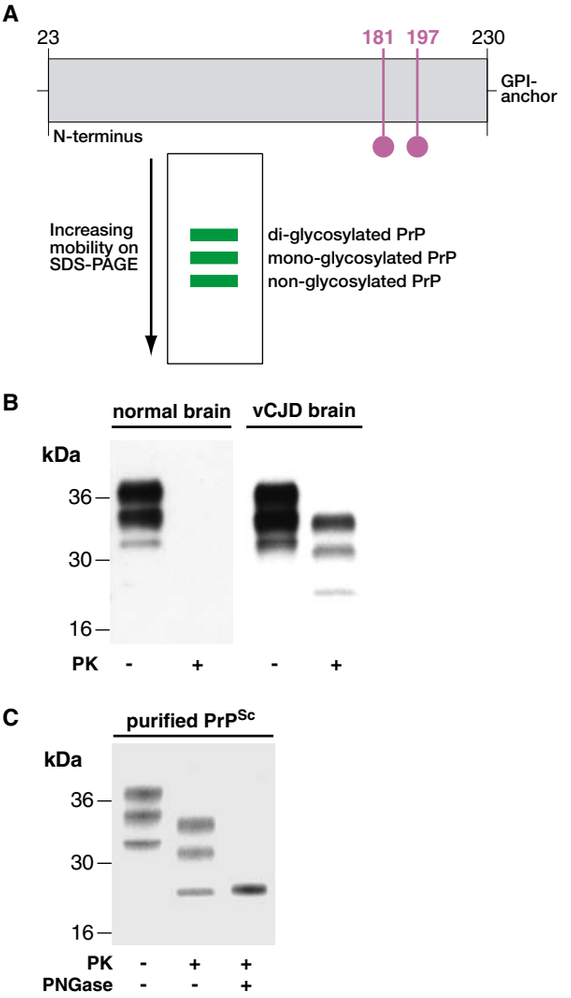

FIGURE 48-5 Western blot analysis of PrP. (**A**) Following cleavage of an *N*-terminal signal peptide, and removal of a *C*-terminal peptide on addition of a GPI anchor, mature human PrP^{C} consists of a 208-residue polypeptide that contains two sites for *N*-linked glycosylation at asparagine residues 181 and 197. PrP^{C} is expressed as di-, mono- and non-glycosylated forms giving rise to three principal PrP bands following sodium dodecyl sulfate polyacrylamine gel electrophoresis (SDS–PAGE). (**B**) Western blot analysis of normal human brain and vCJD brain homogenate before and after treatment with proteinase K (*PK*). PrP^{C} in both normal and vCJD brain is completely degraded by proteinase K, whereas PrP^{Sc} present in vCJD brain shows resistance to proteolytic degradation leading to the generation of amino terminally truncated fragments of di-, mono- and non-glycosylated PrP. (**C**) Western blot analysis of purified PrP^{Sc} from vCJD brain before and after treatment with proteinase K (*PK*) or after consecutive treatment with proteinase K and Peptide-*N*-glycosidase F (*PNGase*). Proteinase K cleaves at the same point in the *N*-terminus of all three PrP^{Sc} glycoforms as removal of *N*-linked carbohydrate with PNGase results in the generation of a single band corresponding to the non-glycosylated proteolytic fragment of PrP^{Sc}.

***In vitro* conversion of PrP^{C} to alternative conformations.** Defining the precise molecular events that occur during the conversion of benign PrP^{C} to the infectious isoform of PrP is of paramount importance, as this process is a prime target for therapeutic intervention. Direct *in vitro* mixing experiments have been performed in an attempt to produce PrP^{Sc}. In such experiments PrP^{Sc} is used as a seed to convert PrP^{C} to a protease resistant form, designated PrP^{Res}. While there are now many examples in the literature of conditions that generate PrP^{Res}, to date such reactions have proved inefficient and have not yet demonstrated *de novo* production of prion infectivity [26]. Despite the obvious limitations of such experiments they may represent an initial step in the generation of the infectious isoform of PrP, which requires additional, as yet unknown, co-factors for the acquisition of infectivity [26]. Seeded conversion has been used successfully to identify compounds that inhibit the *in vitro* generation of PrP^{Res}; however, none of these compounds has yet shown major therapeutic potential for inhibiting prion infectivity *in vivo*. More recently, immunotherapeutic approaches to treating prion disease have shown more promise. For a review of therapeutic approaches to treating prion diseases see [27].

Conversion of recombinant α-PrP to β-PrP as a model of prion propagation. The difficulty in performing structural studies on native PrP^{Sc} has led to attempts to produce soluble β-sheet rich forms of recombinant PrP, which may be amenable to NMR or crystallographic structure determination. Conditions have now been identified in which the PrP polypeptide can be converted between alternative folded conformations representative of PrP^{C} and PrP^{Sc}. At neutral or basic pH PrP adopts an α-helical fold representative of PrP^{C} and this conformation is locked by the presence of the native disulfide bond. Upon reduction of the disulfide bond, PrP^{C} rearranges to a predominantly β-sheet structure. This alternative conformation, designated β-PrP, is only populated at acidic pH with the PrP^{C} conformation predominating at neutral pH. Importantly, β-PrP shares overlapping properties with PrP^{Sc} , including partial resistance to proteolysis and a propensity to aggregate into fibrils [28]. This work suggests β-PrP may be an intermediate on the pathway to formation of PrP^{Sc}, and the conditions required to form β-PrP may be relevant to the conditions PrP^{C} would encounter within the cell during internalization and recycling. The low pH and reducing environment of the endosomal pathway would be a likely candidate, and, although this remains to be demonstrated, there is evidence implicating either late-endosome-like organelles or lysosomes as sites for PrP^{Sc} replication. It remains to be demonstrated whether alternative conformational states of recombinant PrP are sufficient to cause prion disease in an experimental host, or whether other cellular co-factors are also required.

THE MOLECULAR BASIS OF PRION STRAIN DIVERSITY

A major problem for the 'protein-only' hypothesis of prion propagation has been how to explain the existence of multiple isolates, or strains, of prions. Prion strains are distinguished by their biological properties: they produce distinct incubation periods and patterns of neuropathological targeting (so-called lesion profiles) in defined inbred mouse lines. For a review see [29]. As they can be serially propagated in inbred mice with the same *Prnp* genotype, they cannot be encoded by differences in PrP primary structure. Usually, distinct strains of conventional pathogen are explained by differences in their nucleic acid genome. However, in the absence of such a scrapie genome, alternative possibilities must be considered. Weissmann proposed a 'unified hypothesis' where, although the protein alone was argued to be sufficient to account for infectivity, it was proposed that strain characteristics could be encoded by a small cellular nucleic acid, or 'co-prion' [30]. Although this hypothesis leads to the testable prediction that strain characteristics, unlike infectivity, would be sensitive to UV irradiation, no such test has been reported. At the other extreme, the protein-only hypothesis would have to explain how a single polypeptide chain could encode multiple disease phenotypes. Clearly, understanding how a protein-only infectious agent could encode such phenotypic information is of considerable biological interest.

Prion strain diversity may be encoded by PrP itself. Support for the contention that strain specificity may be encoded by PrP itself was provided by study of two distinct strains of transmissible mink encephalopathy prions, which can be serially propagated in hamsters, designated 'hyper' and 'drowsy'. These strains can be distinguished by differing physiochemical properties of the accumulated PrP^{Sc} in the brains of affected hamsters. Following limited proteolysis, strain-specific migration patterns of PrP^{Sc} on western blots are seen, which relate to different *N*-terminal ends of PrP^{Sc} following protease treatment implying differing conformations of PrP^{Sc} [31]. Similarly, different human PrP^{Sc} isoforms have been found to propagate in the brain of patients with phenotypically distinct forms of CJD (for reviews see [5, 10]).

Distinct PrP^{Sc} types are seen in human prion disease. The different fragment sizes seen on western blots, following treatment with proteinase K, suggests that there are several different human PrP^{Sc} conformations, referred to as molecular strain types (Fig. 48-6). These types can be further classified by the ratio of the three PrP bands seen after protease digestion, corresponding to amino-terminally truncated cleavage products generated from di-, mono-, or non-glycosylated PrP^{Sc}. Four types of human PrP^{Sc} have now been commonly identified using molecular strain typing [5, 10, 32] (Fig. 48-6), although much greater

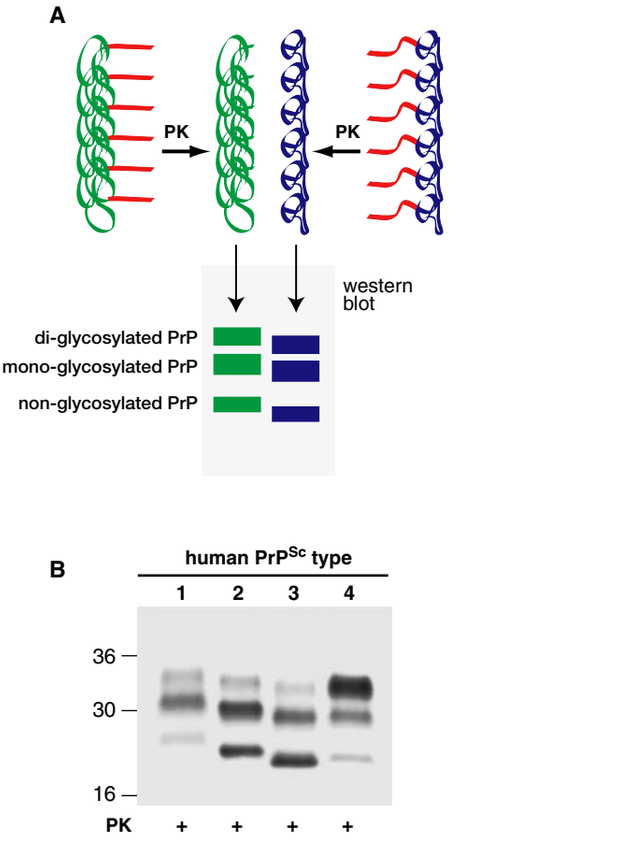

FIGURE 48-6 Molecular analysis of PrP^{Sc} isoforms. (**A**) The schematic demonstrates the principle of molecular stain typing of PrP^{Sc} isoforms by limited proteolytic digestion and western blotting. Two distinct aggregates of PrP^{Sc} with differing conformations (shown in *green* or *blue*) present different accessibilities to proteinase K within the *N*-terminal region of the protein (shown in *red*). Disparity in the most *C*-terminal scissile bond accessible to proteinase K results in a different mobility of *C*-terminal PrP^{Sc} proteolytic fragments generated from of di-, mono- and non-glycosylated PrP^{Sc} that can be visualized by western blotting. (**B**) Western blot showing proteinase K digestion products from distinct human PrP^{Sc} conformers designated PrP^{Sc} types 1–4. PrP^{Sc} types 1–3 are seen in the brain of classical forms of CJD (either sporadic or iatrogenic CJD), PrP^{Sc} type 4 is uniquely seen in vCJD brain and differs markedly in the proportions of di- and mono-glycosylated glycoforms.

heterogeneity seems likely [10]. Sporadic and iatrogenic CJD are associated with PrP^{Sc} types 1–3, while type 4 PrP^{Sc} is uniquely associated with vCJD, and is characterized by a fragment size and glycoform ratio that is distinct from PrP^{Sc} types 1–3, but similar to PrP^{Sc} seen in BSE and BSE when transmitted to several other species. PrP^{Sc} types 1 and 2 associated with clinically distinct subtypes of sporadic CJD have different *N*-terminal conformations determined by metal-ion binding, and such post-translational modification of PrP through coordination of copper and zinc ions provides a mechanism for the generation of multiple PrP^{Sc} conformers [10]. Polymorphism at *PRNP* residue 129 appears to place constraints upon the propagation of distinct PrP^{Sc} types in humans [5, 10], and it has

now become clear that prion strain selection and the propagation of distinct PrP^{Sc} types may be crucially influenced by other genetic loci of the host genome [33–35].

The hypothesis that alternative conformations or assembly states of PrP^{Sc} provide the molecular substrate for clinicopathological heterogeneity seen in human prion diseases (and that this relates to the existence of distinct human prion strains) has been strongly supported by transmission experiments to conventional and transgenic mice. Transgenic mice expressing human PrP with either valine or methionine at residue 129 have shown that this polymorphism constrains both the propagation of distinct human PrP^{Sc} conformers and the occurrence of associated patterns of neuropathology [5, 35]. These data strongly support the 'protein-only' hypothesis of infectivity, by suggesting that prion strain variation is encoded by a combination of PrP conformation and glycosylation [5]. These data also provide a molecular basis for *PRNP* codon 129 as a major locus influencing both prion disease susceptibility and phenotype in humans [5, 14].

Molecular strain typing facilitates investigation of prion disease etiology. The identification of strain-specific PrP^{Sc} structural properties now allows an etiology-based classification of human prion disease by typing of the infectious agent itself. Molecular strain typing of prion isolates is being applied in the molecular diagnosis of sporadic and variant CJD, and to produce a new classification of human prion diseases with implications for epidemiological studies investigating the etiologies of sporadic CJD and BSE-related human prion disease [5, 35]. Stratification of all human prion disease cases by PrP^{Sc} type will enable rapid recognition of any change in relative frequencies of particular PrP^{Sc} sub-types in relation to either BSE exposure patterns or iatrogenic sources of vCJD prions. Such methods allow strain typing to be performed in days rather than the 1–2 years required for classical biological strain typing. This technique may also be applicable to determining whether BSE has been transmitted to other species, thereby posing a threat to human health [5].

PRION TRANSMISSION BARRIERS

Prion transmission between species is limited by a barrier. A 'species barrier' restricts transmission of prion disease between different mammalian species. On primary passage of prions from species A to species B, usually not all inoculated animals of species B develop disease, and those that do have much longer and more variable incubation periods than those that are seen with transmission of prions within the same species. On second passage of infectivity to further animals of the species B, transmission parameters resemble within-species transmissions, with most if not all animals developing the disease with short and consistent incubation periods. Species barriers can therefore be quantified by measuring the fall in mean incubation period on primary and second passage, or, perhaps more rigorously, by a comparative titration study. The effect of a very substantial species barrier is that few, if any, animals succumb to clinical prion disease at all on primary passage, and then at incubation periods approaching the natural lifespan of the species concerned [5, 36].

Both PrP sequence and prion strain type influence prion transmission barriers. Early studies of the molecular basis of the species barrier argued that it principally resided in differences in PrP primary structure between the species from which the inoculum was derived and the inoculated host. However, it has been clear for many years that prion strain type also has a crucial effect on species barriers. For example, the natural transmission of BSE to a wide variety of hosts, and the subsequent demonstration that transmission of BSE from these different species (all with varied PrP primary amino acid sequences) to mice results in the maintenance of a 'BSE-like' strain, provides a key example of where effects from a prion strain appear more important than PrP primary sequence homology to such barriers [37]. Similarly, following transmission to transgenic mice expressing only human PrP there is disparity in the behavior of sporadic or vCJD prions that have originated in humans expressing wild-type PrP of identical primary sequence. Whereas sporadic CJD prions have transmission characteristics consistent with the complete absence of a species barrier, vCJD prions have transmission properties that are completely distinct from other human prions but closely similar to those of cattle BSE, and that are consistent with the presence of a barrier [5, 35]. The term 'species barrier' does not seem appropriate to describe such effects and the general term 'transmission barrier' now seems more preferable [5, 15, 36].

A conformational selection model of prion transmission barriers. Recently, a conformational selection model of prion transmission barriers has been proposed that encompasses contributions from both PrP sequence and strain-specific PrP^{Sc} structural properties [5, 15, 36]. Both PrP amino-acid sequence and strain type will affect the three-dimensional structure of glycosylated PrP, which will presumably, in turn, affect the efficiency of the protein–protein interactions thought to determine prion propagation. Mammalian PrP genes are highly conserved, and presumably only a restricted number of different PrP^{Sc} conformations (that are highly stable and can therefore be serially propagated) will be permissible thermodynamically, and will constitute the range of prion strains seen. PrP glycosylation may be important in stabilizing particular PrP^{Sc} conformations. While a significant number of different PrP^{Sc} conformations may be possible among the range of mammalian PrPs, only a subset of these would be allowable for a given single mammalian PrP sequence. Substantial overlap between the favored conformations for PrP^{Sc} derived from species A and species B might

therefore result in relatively easy transmission of prion diseases between these two species, while two species with no preferred PrP^{Sc} conformations in common would have a large barrier to transmission (and indeed transmission would necessitate a change of strain type). According to such a model of a prion transmission barrier, BSE may represent a thermodynamically highly favored PrP^{Sc} conformation that is permissive for PrP expressed in a wide range of different species, accounting for the remarkable promiscuity of this strain in mammals [5,36]. Contribution of other components to the species barrier are possible, and may involve interacting co-factors that mediate the efficiency of prion propagation, although no such factors have yet been convincingly identified [26].

Subclinical forms of prion disease pose a risk to public health. Recent data have further challenged our understanding of prion transmission barriers. The assessment of species barriers has historically relied on the development of a clinical disease in inoculated animals; however, during infection with prion diseases, infectious titres in the brain rise progressively throughout prolonged, clinically silent, incubation periods. Therefore, asymptomatic animals can have significant infectious titres in the brain and other tissues. However, subclinical forms of prion infection that are distinct from preclinical forms have now been recognized, in which animals become asymptomatic carriers of infectivity and do not develop clinical disease during a normal lifespan. Such carrier states are well recognized in other infectious diseases. The high incidence of subclinical prion infection seen in BSE- and vCJD-inoculated transgenic mice expressing human PrP [35], together with other recent data demonstrating extensive subclinical prion infection in other rodent models of prion disease [36], indicate that current definitions of transmission barriers (conventionally assessed on the basis of occurrence of clinical disease in inoculated animals), must be re-assessed.

Subclinical or carrier states of prion disease have major implications for public health, most notably iatrogenic transmission from apparently healthy individuals. The existence of subclinical prion infections also raise the possibility that other species (such as sheep, pigs and poultry), exposed to BSE prions by contaminated feed, might be able to develop subclinical carrier states. Given that BSE prions are pathogenic in a wide variety of species, and that the strain characteristics of BSE prions are retained upon transmission to new species, it must be considered possible, if not probable, that BSE in animals other than cattle will retain pathogenicity for humans.

CELL DEATH IN PRION DISEASE

The precise structure of the infectious agent and the cause of neuronal cell death in prion disease remain unclear. The current working hypothesis is that an abnormal isoform of PrP is the infectious agent. To date, the most highly enriched preparations contain one infectious unit per 10^5 PrP monomers; however, it is unclear whether this indicates that a large aggregate is necessary for infectivity, or at the other extreme, whether only a single one of these PrP^{Sc} molecules is actually infectious. The relationship of PrP^{Sc} molecules to infectivity could simply, however, relate to the rapid clearance of prions from the brain known to occur on intracerebral challenge.

The mechanism of prion-mediated neurodegeneration is unknown. Various hypotheses have been proposed to explain the mechanism of spongiform change and neuronal cell loss [38]. These have included direct neurotoxic effects from a region of the prion protein encompassing residues 106–126 (although this is controversial), to increased oxidative stress in neurones as a result of PrP^{C} depletion, which has been proposed to function as an antioxidant molecule. It has also been suggested that PrP^{C} plays a role in regulating apoptosis, with disturbance of normal cellular levels of PrP during infection leading to cell death. Although PrP^{C} expression is required for susceptibility to the disease, a number of observations argue that PrP^{Sc} and indeed prions (whether or not they are identical) may not themselves be highly neurotoxic. Prion diseases in which PrP^{Sc} is barely or not detectable have been described, and different strains of prions are known to differ in their degree of protease resistance [5]. Mice with reduced levels of PrP^{C} expression have extremely high levels of PrP^{Sc} and prions in the brain, and yet remain well for several months after their wild-type counterparts succumb. Conversely, transgenic mice, with high levels of murine PrP^{C}, have short incubation periods and yet produce low levels of PrP^{Sc} after inoculation with mouse prions [4]. In addition, wild-type brain grafts producing high levels of PrP^{Sc} do not damage adjacent tissue in $Prnp^{0/0}$ mice [38]. The demonstration that animals with subclinical prion infection can harbor high levels of prion infectivity and detectable PrP^{Sc} without exhibiting any clinical signs of prion disease challenges our understanding of the pathogenic mechanisms involved in these diseases [5, 36].

Depletion of neuronal PrP during infection prevents prion neurotoxicity. While the requirement for PrP to mediate neurodegeneration in prion disease remains absolute, conditional knockout of PrP indicates that prion neurodegeneration is unlikely to be related to gross loss of PrP^{C} function in neurons [25]. Based upon existing data it is possible to hypothesize that the neurotoxic prion molecule may not be PrP^{Sc} itself, but a toxic-intermediate that is produced in the process of conversion of PrP^{C} to PrP^{Sc}, with PrP^{Sc}, present as highly aggregated material, being a relatively inert end-product [5, 36]. The steady-state level of such a toxic monomeric or oligomeric PrP intermediate, designated PrP^{L} (for lethal), could determine the rate of neurodegeneration. Subclinical prion infection states may

generate the toxic intermediate at extremely low levels below the threshold required for neurotoxicity [5, 36].

Recently, direct support for this hypothesis has been demonstrated by depleting endogenous neuronal PrP^{C} in mice with established neuroinvasive prion infection. This depletion of PrP^{C} reverses early spongiform change and prevents neuronal loss and progression to clinical prion disease despite the accumulation of extraneuronal PrP^{Sc} to levels seen in terminally ill wild-type mice [39]. These data establish that propagation of non-neuronal PrP^{Sc} is not pathogenic, but arresting the continued conversion of PrP^{C} to PrP^{Sc} within neurons during scrapie infection prevents prion neurotoxicity.

FUTURE PERSPECTIVES

Prion diseases appear to be disorders of protein conformation, and elucidating their precise molecular mechanisms may be of far wider pathological significance. It is of considerable interest that many of the commoner neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease are also associated with abnormal protein aggregates. The apparent ability of a single polypeptide chain to encode information and specify distinctive phenotypes is unprecedented, and it seems unlikely that evolution will not have used this mechanism in many other ways. While the protein-only hypothesis of prion propagation is supported by compelling experimental data, and now appears also able to encompass the phenomenon of prion strain diversity, the goal of producing prions *in vitro* remains. Success in producing disease in experimental animals that can be serially propagated following inoculation with PrP^{Sc}-like forms derived from recombinant PrP would not only prove the protein-only hypothesis, but would provide an essential model by which the mechanism of prion propagation could be understood in molecular detail.

REFERENCES

1. Alper, T. *et al.* Does the agent of scrapie replicate without nucleic acid? *Nature* 214: 764–766, 1967.
2. Griffith, J. S. Self replication and scrapie. *Nature* 215: 1043–1044, 1967.
3. Prusiner S. B. Novel proteinaceous infectious particles cause scrapie. *Science* 216: 136–144, 1982.
4. Weissmann, C. and Flechsig, E. PrP knock-out and PrP transgenic mice in prion research. *Br. Med. Bull* 66: 43–60, 2003.
5. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. *Annu. Rev. Neurosci.* 24: 519–550, 2001.
6. Hunter N. Scrapie and experimental BSE in sheep. *Br. Med. Bull.* 66: 171–183, 2003.
7. Smith, P. G. and Bradley, R. Bovine spongiform encephalopathy (BSE) and its epidemiology. *Br. Med. Bull.* 66: 185–198, 2003.
8. Sigurdson, C. J. and Miller, M. W. Other animal prion diseases. *Br. Med. Bull.* 66: 199–212, 2003.
9. Palmer, M. S. *et al.* Homozygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease. *Nature* 352: 340–342,1991.
10. Wadsworth, J. D. F. *et al.* Molecular and clinical classification of human prion disease. *Br. Med. Bull.* 66: 241–254, 2003.
11. Collinge, J. *et al.* Presymptomatic detection or exclusion of prion protein gene defects in families with inherited prion diseases. *Am. J. Hum. Genet.* 49: 1351–1354, 1991.
12. Brown, P. *et al.* Iatrogenic Creutzfeldt–Jakob disease at the millennium. *Neurology* 55: 1075–1081, 2000.
13. Alpers, M. P. Kuru. In *Human biology in Papua New Guinea: the small cosmos.* Eds Attenborough, R. D. and Alpers, R. D. Oxford: Clarendon Press, 1992, pp 313–334.
14. Mead, S. *et al.* Balancing selection at the prion protein gene consistent with prehistoric kuru-like epidemics. *Science* 300: 640–643, 2003.
15. Collinge J. Variant Creutzfeldt–Jakob disease. *Lancet* 354: 317–323, 1999.
16. Collinge, J., Palmer, M. S. and Dryden, A. J. Genetic predisposition to iatrogenic Creutzfeldt–Jakob disease. *Lancet* 337: 1441–1442, 1991.
17. Collinge, J. Prion disease. In *New Oxford Textbook of Psychiatry*. Eds Gelder, M.G., Lopez-Ibor, J. J. and Andreasen N. C. Oxford: Oxford University Press, 2000, pp 404–415.
18. Aguzzi, A. Prions and the immune system: a journey through gut, spleen, and nerves. *Adv. Immunol.* 81: 123–171, 2003.
19. Budka, H. Neuropathology of prion diseases. *Br. Med. Bull.* 66: 121–130, 2003.
20. Prusiner S. B. Prions. *Proc. Natl Acad. Sci. USA.* 95: 13363–13383, 1998.
21. Wuthrich, K. and Riek, R. Three-dimensional structures of prion proteins. *Adv. Protein Chem.* 57: 55–82, 2001.
22. Riesner, D. Biochemistry and structure of PrP^{C} and PrP^{Sc}. *Br. Med. Bull.* 66: 21–33, 2003.
23. Asante, E. and Collinge, J. Transgenic studies of the influence of the PrP structure on TSE diseases. In *Advances in Protein Chemistry*. London: Academic Press, 2000, pp 273–311.
24. Lasmezas, C. I. Putative functions of PrP^{C}. *Br. Med. Bull.* 66: 61–70, 2003.
25. Mallucci, G. R. *et al.* Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J.* 21: 202–210, 2002.
26. Caughey, B. Prion protein conversions: insight into mechanisms, TSE transmission barriers and strains. *Br. Med. Bull.* 66: 109–120, 2003.
27. Dormont, D. Approaches to prophylaxis and therapy. *Br. Med. Bull.* 66: 281–292, 2003.
28. Jackson, G. S. *et al.* Reversible conversion of monomeric human prion protein between native and fibrilogenic conformations. *Science* 283: 1935–1937, 1999.
29. Bruce, M. E. TSE strain variation. *Br. Med. Bull.* 66: 99–108, 2003.
30. Weissmann, C. A 'unified theory' of prion propagation. *Nature* 352: 679–683, 1991.
31. Bessen, R. A. and Marsh, R. F. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *J. Virol.* 68: 7859–7868, 1994.

32. Collinge, J. *et al.* Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* 383: 685–690, 1996.
33. Lloyd, S. E. *et al.* Identification of multiple quantitative trait loci linked to prion disease incubation period in mice. *Proc. Natl Acad. Sci. USA* 98: 6279–6283, 2001.
34. Jackson, G. S. i. HLA-DQ7 antigen and resistance to variant CJD. *Nature* 414: 269–270, 2001.
35. Asante, E. A. *et al.* BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J.* 21: 6358–6366, 2002.
36. Hill, A. F. and Collinge, J. Subclinical prion infection. *Trends Microbiol.* 11: 578–584, 2003.
37. Bruce, M. *et al.* Transmission of bovine spongiform encephalopathy and scrapie to mice: Strain variation and the species barrier. *Philos. Trans. R. Soc. Lond. [Biol.]* 343: 405–411, 1994.
38. Brandner, S. CNS pathogenesis of prion diseases. *Br. Med. Bull.* 66: 131–139, 2003.
39. Mallucci, G. *et al.* Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science* 302: 871–874, 2003.

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PART

–VII–

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CHAPTER

49

Molecular Biology of Vision

Hitoshi Shichi

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PHYSIOLOGICAL BACKGROUND

The eye is a remarkable photosensor that can detect a single photon and transmit its signal to the brain. In the vertebrate eye the receptors for light are the visual, or photoreceptor, cells of the retina. Each visual cell comprises two principal parts: the outer segment, which contains light-absorbing visual pigments, and the inner segment, which contains the nucleus, mitochondria, endoplasmic reticulum, and other subcellular organelles and which metabolically supports the functions of the outer segment (Fig. 49-1). The segments are connected by the ciliary process, or cilium. The inner segments of visual cells have terminals that synapse with horizontal cells and bipolar cells. The bipolar cells, in turn, form synapses with ganglion and amacrine cells.

Light-absorbing pigments differentiate rod cells for black–white vision and three types of cone cells for color vision. Visual cells are of two types. Rod cells, which have elongated outer segments, contain rhodopsin, with a light wavelength for maximal absorption (λ_{max}) of ~500 nm, the visual pigment responsible for dim-light vision, also termed black-and-white or scotopic vision. Cone cells, possessing cone-shaped outer segments, are the photoreceptors for daylight vision, also termed color or photopic vision. In the human retina, there are three types of cone cells, each containing one of three pigments: λ_{max} = 420, 530 and 560 nm. According to one estimate, the human eye contains 120 million rod cells in the peripheral region of the retina and 6.5 million cone cells concentrated mainly in the central, or foveal, region.

Absorption of light causes *inhibition* of vertebrate photoreceptor cells, which then initiate programs of responses among retinal neurons. The plasma membrane of each

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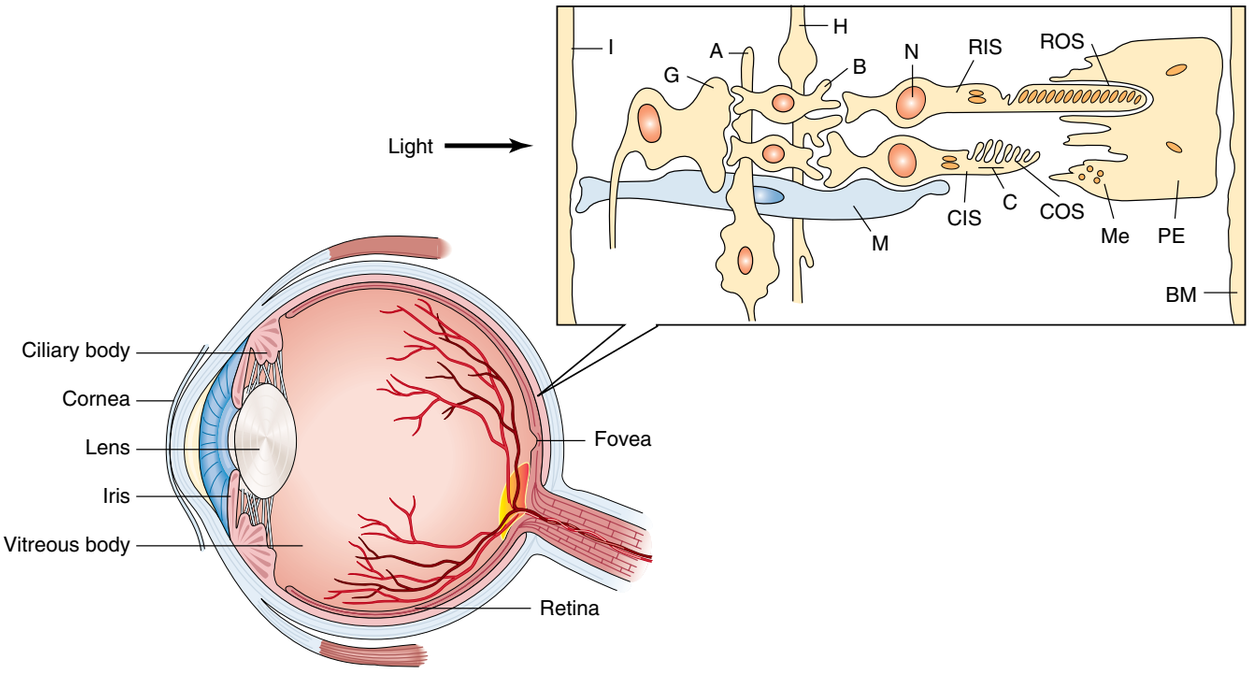

FIGURE 49-1 The vertebrate retina: *A*, amacrine cell; *B*, bipolar cell; *BM*, Bruch's membrane; *C*, ciliary process (cilium); *CIS*, cone inner segment; *COS*, cone outer segment; *G*, ganglion cell; *H*, horizontal cell; *I*, inner limiting membrane; *M*, Muller cell (glial cell); *Me*, melanin granule; *MC*, mitochondrion; *N*, nucleus; *PE*, retinal pigmented epithelium; *RIS*, rod inner segment; *ROS*, rod outer segment.

rod outer segment possesses several thousand cation channels through which Na^+, Ca^{2+} and Mg^{2+} enter the cell at a rate of about 30,000 cations per channel per second *in the dark*. Na^+ accounts for more than 80% of this current. The plasma membrane of the rod inner segment has ATP-dependent Na^+/K^+ pumps that pump Na^+ out of the cell. Thus, in the dark the plasma membrane of the vertebrate rod cell shows a membrane potential of about 40 mV (inside negative) and is depolarized. Photoreception by the visual pigment rhodopsin triggers an enzymatic cascade that leads to closure of the cation channel and, due to a resultant decrease in intracellular Na^+, hyperpolarizes the membrane. Therefore, 'visual excitation' in the vertebrate photoreceptor means hyperpolarization of the visual cell plasma membrane caused by inhibition of its conductance. The neurotransmitter, glutamate, released continuously in the dark at the synaptic terminal of a visual cell, is thus inhibited by light. The retina, which is part of the CNS, contains numerous neurotransmitters, including acetylcholine, dopamine, GABA, glycine, glutamate, and neuropeptides and their receptors [1–3]. Glutamate is released by photoreceptors, as well as bipolar cells and ganglion cells. Ionotropic glutamate receptors account for most of the fast excitatory synaptic transmission in the retina. Bipolar cells have both ionotropic and metabotropic glutamate receptors. Half of the amacrine cells release the inhibitory neurotransmitters, glycine and GABA, whereas the ganglion cells and bipolar cells express receptors for both glycine and GABA. Amacrine cells are diverse and some also release acetylcholine, dopamine, or serotonin. These cells also contain various neuropeptides, including substance P, enkephalin, glucagon, somatostatin, neurotensin, neuropeptide Y, vasoactive intestinal peptide and cholecytokinin, which co-localize with other neurotransmitter substances and function as neuromodulators. The effects of neuromodulators generally are slower and last longer than those of neurotransmitters.

Retinal responses to different light frequencies are encoded in the retina and conveyed to the thalamus and visual cortex. Absorption of photons by visual cells triggers a series of events that result in a particular pattern of retinal stimulation and, eventually, stimulation of cerebral neurons. In dim-light vision, the magnitude of the neural response in rod cells is directly related to the perception of brightness. In color vision, absorption of light by at least two cone cell pigments with different absorption maxima is essential to discriminate color. The ratio of the magnitudes of the induced photoresponses determines the color perceived. The arrays of visual signals generated by the photoreceptor cells and programmed by retinal neurons, principally bipolar cells, are transmitted to the lateral geniculate bodies via ganglion cell axons, which make up the optic nerves and tracts. Impulses are conveyed by the geniculocalcarine radiations to the visual cortex of the brain, where the signals representing light intensity and wavelength are decoded separately by different neurons. The coding and decoding of visual signals,

an important area of neurophysiology, is not covered here. This chapter deals primarily with the structure and function of photoreceptors and the molecular events that take place after photon absorption by the visual cells [4–6].

PHOTORECEPTOR MEMBRANES AND VISUAL PIGMENTS

Rod outer segment membranes are arranged in stacks of disks containing rhodopsin. The outer segment of a rod cell consists of a stack of 1,000–2,000 disks encased in a sack of plasma membrane. It is presumed that the disks are formed by evagination of the plasma membrane in the proximal region of the outer segment, followed by fusion of two adjacent evaginates and detachment from the plasma membrane. This is probably controlled by the ciliary process. This repeated evagination and disk formation greatly increases the area of membrane within each rod cell. This increases the amount of visual pigment, a transmembrane protein, in each cell. As new disks are formed, older disks are pushed toward the apex of the outer segment. Disks that eventually reach the apex are shed from the tip and phagocytized by the pigmented epithelial cells. In higher-order animals, rod-disk shedding follows a circadian rhythm: shedding occurs soon after the onset of light [7]. The life cycle of a single disk may last from a few days to months, depending on the species. Disk membrane components, such as proteins, carbohydrates and lipids, are synthesized in the inner segment and transported to the basal region of the ciliary process, where membrane assembly occurs. In contrast to rod disks, cone disks generally remain contiguous with the plasma membrane, and shedding occurs in the dark rather than in the light.

Rod outer segment membranes, which are comprised of >95% disk membranes and <5% plasma membrane, consist of 60% protein and 40% phospholipids. In vertebrate photoreceptors, phosphatidylethanolamine and phosphatidylcholine account for about 80% of the phospholipids. High concentrations of polyunsaturated fatty acids make rod membranes as fluid as olive oil at physiological temperatures, allowing the integral membrane protein rhodopsin to rotate freely and diffuse within the membrane. This fluidity may be important in allowing photoactivated rhodopsin to collide quickly with so many molecules of the peripheral membrane proteins, such as G protein and rhodopsin kinase. The most abundant polyunsaturated fatty acid is docosahexaenoic acid, consisting of 22 carbons and six unsaturated bonds, linked to the middle carbon of the glycerol moiety of phospholipids.

Rhodopsin is a transmembrane protein linked to 11-*cis*-retinal, which, on photoabsorption, decomposes to opsin and all-*trans*-retinal. Rhodopsin has a molecular weight of about 40,000. Its *C*-terminus is exposed on the cytoplasmic surface of the disk, and its sugar-containing *N*-terminal sequence is exposed on the intraluminal surface. Half of the mass is embedded in the hydrophobic region of the membrane lipid bilayer, with the remaining half distributed equally on both sides of the membrane. The primary structures of about 100 visual pigment proteins of different species are known, including human rhodopsin and cone pigments (Fig. 49-2). Based on their amino acid sequences, these pigments have been grouped into five distinct molecular subfamilies [5]. All visual pigments possess seven segments of hydrophobic sequences separated by segments of hydrophilic sequences. The seven hydrophobic sequences, designated helices 1–7 (see bovine rhodopsin shown as an example in Fig. 49-3), are α-helical and form a bundle that spans the membrane. The cytoplasmic sequence between the end of helix 7 and cysteine-322 forms a short helix (helix 8). Near the *N*-terminus, two sugar moieties, each composed of three mannoses and three *N*-acetylglucosamines, are linked to asparagine-2 and asparagine-15 in bovine rhodopsin, whereas near the *C*-terminus cysteine-322 and cysteine-323 carry palmitoyl groups which probably penetrate the membrane, thereby forming an additional polypeptide loop. The *C*-terminal sequence contains three phosphorylatable serine residues (Ser-334, -338, -343). All of the rod and cone opsins are members of the G-protein coupled receptor (GPRC) family ([8] and Ch. 10). A three-dimensional structure of bovine rhodopsin has been determined by x-ray crystallography [8, 9].

The chromophore, 11-*cis*-retinal, is linked to the α-amino group of lysine-296 in helix 7 via a protonated Schiff base. Protonated Schiff bases usually absorb light maximally at around 440 nm, but the λ_{max} of rhodopsin is near 500 nm. This 60 nm 'red shift' of the λ_{max} can be explained by the strength of interaction of the positive charge of the protonated Schiff base with a counter-ion. The more distant the counter-ion is from the Schiff base, the greater is the red shift. In the case of rhodopsin, the counter-ion is the carboxylate group of glutamic acid-113 (Fig. 49-3). When the helices are bundled together, the acidic residue in helix 3 moves to the vicinity of the chromophore in helix 7 and donates the necessary negative charge. On absorption of light, rhodopsin decomposes to the opsin protein and all-*trans*-retinal. The reaction occurs through several spectrally distinct intermediates (Fig. 49-4). Formation of bathorhodopsin involves photoisomerization of the 11-*cis*-retinylidene chromophore to a constrained all-*trans* form and, at physiological temperature, occurs within a few picoseconds after light absorption. Bathorhodopsin then undergoes thermal relaxation, giving rise first to lumirhodopsin and then to metarhodopsin I. Current evidence suggests that the formation of metarhodopsin II is of particular significance because this intermediate transmits the photosignal to a G protein.

Bleached rhodopsin must be regenerated to maintain normal vision. Regeneration occurs by several mechanisms. The major pathway occurs in the retinal pigmented

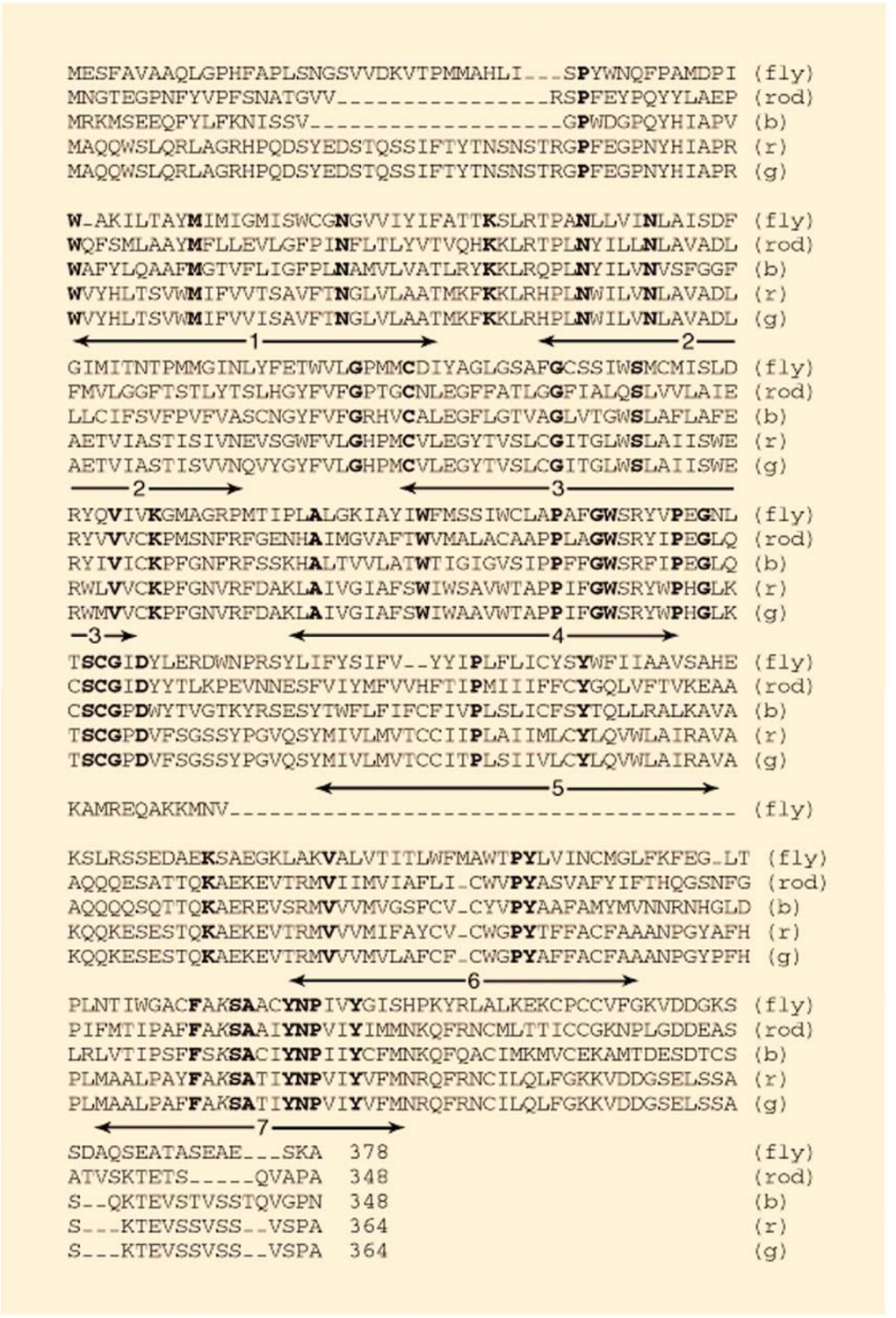

FIGURE 49-2 The primary structures of visual pigments. The fly sequence is from *Drosophila*. All other sequences are human. The lower three are the sequences of cone pigments deduced from cDNA sequences: *b*, blue-sensitive; *r*, red-sensitive; and *g*, green-sensitive. Residues identical in all sequences are shown in **boldface**. Gaps necessary to maintain the alignment are connected by *dashed lines*. The transmembrane segments shown in Figure 49-3 are indicated by the *numbered arrows* below the corresponding sequences. The lysine to which retinal is linked to form the chromophore is indicated as *K* near the center of transmembrane segment 7.

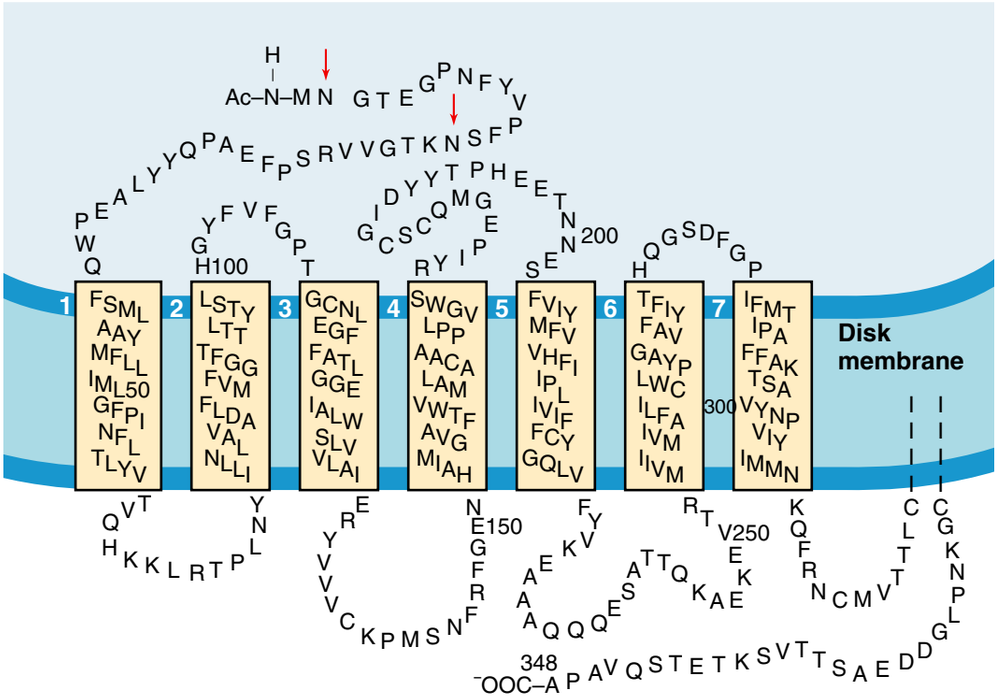

FIGURE 49-3 Proposed transmembrane disposition of bovine rhodopsin. Sugar moieties at asparagine-2 and asparagine-15 are shown with *red arrows*. Palmitoyl groups at cysteine-322 and cysteine-323 are indicated with *broken lines*. Hydroxyamino acid residues that may be phosphorylated by rhodopsin kinase are clustered in a *C*-terminal domain of the molecule.

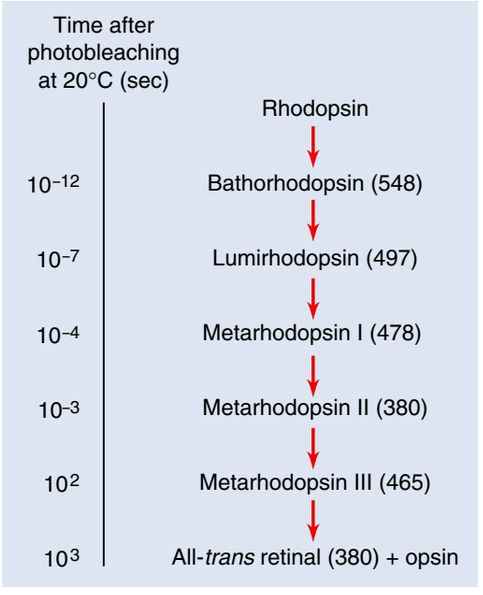

FIGURE 49-4 Intermediates formed after photic bleaching of vertebrate rhodopsin. Numbers in parentheses are wavelengths of light (in nanometers) absorbed maximally by the individual intermediates.

epithelium and involves isomerization of all-*trans*- to 11-*cis*-retinal, followed by transport of 11-*cis*-retinal to the outer segment where it is recombined with opsin [10]. All-*trans*-retinol produced by reduction of retinal in the outer segment is transported to retinal pigmented epithelium. The pigmented epithelium contains the enzyme, lecithin:retinal acyltransferase, that reversibly transfers acyl group (primarily palmityl) from lecithin to all-*trans*-retinol forming all-*trans* esters. According to a current hypothesis, in the pigmented epithelium all-*trans*-retinyl esters bind to a carrier protein called RPE65, and this complex interacts with an isomerohydrolase to produce and quickly hydrolyze the ester to form 11-*cis*-retinol [11]. The free energy of hydrolysis of the ester is coupled to drive the isomerization process. Shuttling of retinoids between the photoreceptor and pigmented epithelium may involve several additional retinoid-binding proteins. A second regeneration mechanism involves photoconversion of thermal intermediates, such as the *meta*-rhodopsins, to rhodopsin. For example, regeneration of squid rhodopsin occurs mainly by photoisomerization of opsin-linked retinal at pH 10 and subsequent dark adaptation [12]. In a third mechanism, the photoisomerization to the 11-*cis* form may take place in the rod outer segment membrane after all-*trans*-retinal has formed complexes with phospholipids. For example, all-*trans*-*N*-retinylidene

phosphatidylethanolamine is photoisomerized to the 11-*cis* form within the disk membrane and reacts with opsin [13].

Cone-cell pigments contain different opsins that have high sequence homology. The primary structures of the three human cone pigments were deduced from analysis of the genomic and complementary DNA clones encoding them [14] (Fig. 49-2). These cone-pigment sequences have >41% identity with rhodopsin. In the case of red and green pigments only 15 of the 364 residues are different. Genetic analysis locates the human rhodopsin gene on chromosome 3 and the blue pigment gene on chromosome 7. Although none of the human cone pigments has been isolated in pure form, the chicken cone pigment, iodopsin, has been purified and characterized [15]. Iodopsin (λ_{max} 571 nm) forms batho-iodopsin on light absorption at −196°C. This intermediate reverts back to iodopsin on warming and, if illuminated by 600 nm light at −183°C, iodopsin is converted to lumiiodopsin, which thermally decays to *meta*-iodopsin I and *meta*-iodopsin II. Cone pigments respond to light faster and, after bleaching, regenerate faster than rhodopsin.

PHOTOTRANSDUCTION

Light absorption by rhodopsin leads to closure of Na^+-conductance channels via a chemical messenger system. Light is known to close the cation channels on the outer segment plasma membrane and to induce hyperpolarization of the membrane within 100 ms. The magnitude of light response reaches a maximum by absorption of several hundred photons per rod cell. Below the saturation light intensity, the relationship between the magnitude of light response *(I)* and the energy of irradiating light *(A)* is given by

$$I = I_{max} A/(A + K)$$

Where I_{max} is the magnitude of response at the saturation light intensity and K is light energy required for 50% of the maximum response [16]. This equation has the same form as the Michaelis–Menten equation of enzyme kinetics. By analogy to enzyme kinetics, we may assume that rhodopsin reacts with light to form a photoactivated rhodopsin, probably *meta*-rhodopsin II, which, in turn, gives rise to an intracellular messenger, X, that links the photochemical reaction of rhodopsin to the plasma membrane channels. It is logical to postulate such a messenger, because the disk membrane, where rhodopsin is localized, is not continuous with the plasma membrane that contains the cation channels. The messenger must satisfy at least two properties. First, its concentration in the outer segment cytoplasm must change rapidly on light irradiation and return to the original value after light is turned off. Second, the messenger must mimic the effect of light when introduced into the outer segment cytoplasm in the dark.

A G-protein/cGMP system in outer segments is responsive to photoactivated rhodopsin. Exposure of an outer segment membrane fragment to cGMP in patch-clamp experiments increases its Na^+-conductance in the dark [17]. This effect is reversible. The concentration of cGMP in dark-adapted rod outer segments is high and decreases rapidly when the rod is irradiated at low Ca^{2+}. The decrease of cGMP is proportional to the log of light intensity. These results suggested that cGMP is messenger X . The crucial evidence for this cGMP hypothesis of vertebrate phototransduction is that the cation channel is, in fact, kept open by cGMP and closes when cGMP is hydrolyzed in light. It should be noted that the amount of messenger X that is expected to 'accumulate' in light is represented by the amount of cGMP hydrolyzed. Several components that participate include rhodopsin, G protein (transducin), arrestin, phosphodiesterase, rhodopsin kinase, guanylate cyclase, guanylate cyclase activating protein, and cGMP-gated cation channel. Relevant properties of these components are described below (see also ref. [5]).

Photoactivated rhodopsin (R*) activates a G protein which in turn mediates activation of a cGMP phosphodiesterase (PDE) (Fig. 49-5). A single R* molecule, before it is inactivated, may produce hundreds of active G protein molecules but this is the rate-limiting step in rod vision. Rhodopsin has an ERY motif in helix 3 at the cytoplasmic surface (Fig. 49-3), that is thought to be involved in the interaction with G proteins because most G-protein-coupled receptors contain the homologous DRY sequence at this location [9]. The polypeptide loops between helices 3 and 4 and helices 5 and 6 and a cytoplasmic loop (helix 8) between helix 7 and the palmitoyl anchors are probably also involved in G-protein binding. The rod G-protein, transducin, consists of three subunits α, β and γ, with GDP bound to the α subunit in the inactive form. R* catalyzes exchange of GTP for GDP, and the G_α-GTP thus formed dissociates from $G_{\beta\gamma}$, which remains membrane-bound. Rods and cones express distinct G proteins. Two GTP-activated G complexes are required to activate one phosphodiesterase molecule by forming a PDE-$\alpha\beta\gamma_2$ complex by successively dissociating two G-protein γ subunits. In the case of rods, PDEα and PDEβ designate two different isoforms expressed in these cells — sometimes called PDE6A and PDE6B, *not* to be confused with the G-protein subunits. Cones appear to express a single phosphodiesterase isoform, 'cone PDEβ'or PDE6C (see Table 21-1, Fig. 21-6). A single PDE molecule may hydrolyze 10^5–10^6 cGMP molecules during the lifetime of one R*. Because of this large gain, the cGMP hypothesis is also called the cGMP cascade hypothesis.

Visual pigment kinases belong to the G-protein-coupled receptor kinase (GRK) family ([18], Ch. 23). Rhodopsin kinase or RK and cone-pigment kinase are designated GRK1 and GRK7, respectively. The catalytic site of RK is located in the middle of the polypeptide chain and is highly conserved. The kinase also has an ATP binding sequence, GXGXXGX and a GRK-specific signature sequence, D (L/M)G. At high

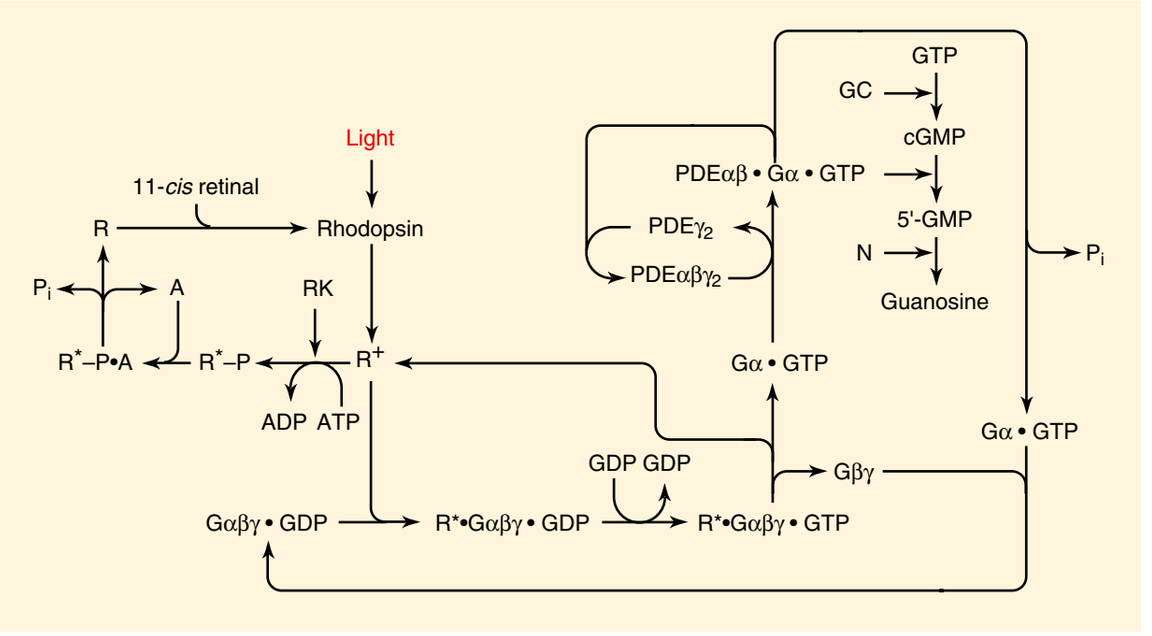

FIGURE 49-5 Light-elicited biochemical reactions leading to cGMP hydrolysis: *Gαβγ*, guanine nucleotide-binding protein (transducin); *PDEβγ2*, phosphodiesterase; *GC*, guanylyl cyclase; *N*,5′-nucleotidase; *P*$_i$, inorganic phosphate; *RK*, rhodopsin kinase; *R**, photoactivated rhodopsin; *R*, inactive form of rhodopsin; *A*, arrestin. cGMP binds to the cation channel protein of the plasma membrane and opens the channel.

levels of photobleaching where 20–40% of rhodopsin is converted to R*, RK phosphorylates only one serine residue (Ser^{343}, Ser^{338} or Ser^{334} at the *C*-terminal sequence of R*. At low bleaching levels (<1% R*), multiple sites appear to be phosphorylated. Phosphorylation of R* partially inhibits its ability to activate G protein, whereas binding of R* with arrestin, a 48 kDa protein blocks binding of G protein to R*. In the rods, cGMP is steadily synthesized by guanylate cyclase (GC) and hydrolyzed by PDE. Retinal GCs are members of the membrane-bound GC family (Ch. 21) and two forms, GC-E (ret GC-1) and GC-F (ret GC-2), are present in both rods and cones [19, 20]. GC functions as a dimer. Two GC activating proteins are known which have three Ca^{2+}-binding EF hands [21]. They activate GC at low Ca^{2+} and inhibit GC at high Ca^{2+}. The cyclic-nucleotide-gated (CNG) cation channel of rods is a tetrameric complex composed of three CNGA1 subunits and one CNGB1 subunit [22]. While the homomeric channel formed by CNGA1 subunits is opened by cGMP, CNGB1 subunits do not form functional channels and are considered to have modulatory roles including inhibition of the channel by Ca^{2+}-calmodulin. Cone channels are more permeable to Ca^{2+} than rod channels and little affected by Ca^{2+}-calmodulin. Even after the closure of CNG channels, cation concentrations in the outer segment continue to change due to a $Na^+/Ca^{2+}/K^+$ exchange protein located in the plasma membrane. The exchanger exists as a dimer. It exchanges four Na^+ (inward) for one Ca^{2+} and one K^+ (outward) and becomes inactive at low Ca^{2+} concentrations (see Ch. 5). The rate of exchange is faster in cones than in rods.

Although abundant evidence supports the cGMP cascade hypothesis for vertebrate vision, whether cGMP plays a key role in invertebrate vision remains controversial. Because invertebrate visual cells depolarize in light, a rise in cGMP is expected upon light absorption. However, light does not increase cGMP in invertebrate photoreceptors [23]. A mechanism proposed for invertebrate phototransduction involves light-stimulated hydrolysis of phosphatidyl inositol 4,5-biophosphate (PIP_2). Reception of an external signal by a variety of receptors is known to evoke hydrolysis of PIP_2 to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) by activating phospholipase C. In this mechanism, both IP_3 and DAG can serve as intracellular messengers. IP_3 increases intracellular Ca^{2+}, and DAG stimulates protein kinase C. Injection of IP_3 into Limulus ventral photoreceptors in the dark mimics the light effect, evoking membrane depolarization without latency and an increase in intracellular Ca^{2+} [24].

Visual photoreceptors have an ability to adapt to a wide range of light intensities. The retina can respond to a broad range of light intensities due to its property of adaptation. Light adaptation (or background adaptation) refers to photosensitivity changes observed in background illumination that causes only a low level of pigment bleaching. On the other hand, photoreceptor desensitization by high light intensities, that produce significant amounts of

bleached pigment, is called bleaching adaptation. Changes in intracellular Ca^{2+} play an important role in both types of adaptation in rods and cones. In background light adaptation, GC modulation by Ca^{2+} is important for photoregulation at low light intensities, while PDE modulation by Ca^{2+} plays a dominant role in photosensitivity regulation at high light intensities. In bleaching adaptation, low levels of fully bleached pigments (i.e. decay products of R*) activate G protein, and consequently PDE regulation seems to be responsible for changes in light sensitivity. The recovery process from bleaching adaptation is known as dark adaptation and is important for resetting the photoreceptor systems. As described above and in Fig. 49-5, R* is inactivated by phosphorylation and binding of arrestin and releases all-*trans*-retinal. During dark adaptation, the opsin protein is dephosphorylated, dissociated from arrestin, and reconjugated with 11-*cis*-retinal. GTP bound to the α-subunit of G protein is hydrolyzed to GDP, a reaction probably accelerated *in vivo* by a GTPase accelerating protein. The Gα subunit then dissociates from PDE-αβ and reassociates with G protein βγ subunits. PDE-αβ becomes inactive by association with its inhibitory γ subunits.

COLOR BLINDNESS

Red–green color blindness is explained by unequal intragenic recombination between a pair of X chromosomes. The blue pigment gene is located on chromosome 7, whereas both red and green pigment genes reside on the X chromosome [14]. Amino-acid sequences of the three cone pigments of human retina (Fig. 49-2) indicate that red and green pigments are very similar, showing 96% homology, but blue pigment differs more. Peculiarly, in color normals each X chromosome has one red pigment gene and either one, two or three green pigment genes. A hypothesis to explain the variability of the green pigment gene is that the red pigment gene occurs upstream of the 5′ end of the green pigment gene, and a pair of homologous red–green gene arrangements has undergone unequal recombinations during evolution. Red–green color blindness is explained by abnormalities in the unequal intragenic recombination as described below.

Approximately 8% of Caucasian men inherit a color abnormality: one type, dichromats, require only two primary wavelengths to match all colors. Another type, anomalous trichromats, require three primary wavelengths but make color matches different from the normal. Loss of one of the three color pigments or systems explains dichromats, while a shift in the spectral sensitivity of one of the three pigments or systems may be responsible for anomalous trichromatism. Blue cone abnormalities are inherited as autosomal traits and are rare. Therefore, abnormalities in the red and green cone systems have been most extensively studied. From psychophysical studies, defects in the red and green cone systems are mapped to two tightly linked loci on the X chromosome which are most likely the loci of red and green pigment genes.

What are the underlying genetic mechanisms that produce dichromats and anomalous trichromats? Nathans [25] hypothesizes that visual abnormalities are caused by alterations in red and green pigment genes by unequal intragenic recombination. Red and green pigment genes are similar and tightly linked on the X chromosome, as described above. If, during the development of ova and sperm cells, a pair of chromosomes joins by crossing over in the region encoding red and green pigments and breaks between the two genes of one of the chromosomes, one chromosome may lose the green pigment gene and the other chromosome may have two. However, breaking within a gene may result in a hybrid gene composed of part of the green pigment gene and part of the red pigment gene. If the X chromosome lacking the green pigment gene is inherited, the carrier is a true dichromat. Depending on the location of breakpoints, a variety of hybrid genes are generated; a man with a hybrid gene producing a nonfunctional pigment similar to the normal pigment will be a dichromat. However, the carriers of hybrid genes producing pigments with spectral sensitivities intermediate between those of red and green pigments will be anomalous trichromats.

RETINITIS PIGMENTOSA

Mutations in rhodopsin and other photoreceptor proteins are linked to retinitis pigmentosa. Retinitis pigmentosa (RP) is a group of inherited retinopathies that affects about 1 in 4,000 humans [26]. RP may be classified into four types: autosomal dominant (19%), autosomal recessive (19%), X-linked (8%) and allied diseases (54%). RP is characterized by loss of night vision in the early stage, followed by loss of peripheral vision. Chromosomal loci for numerous RP genes have been mapped and mutations characterized [27].

The first gene suspected to be linked to dominant-type RP was located on the long arm of chromosome 3, and its mutant product was found to be a rhodopsin: replacement of proline 23 by histidine [26]. About 100 point mutations within the rhodopsin gene have so far been reported, including mutations causing autosomal recessive RP and congenital stationary night blindness. Another gene of dominant-type RP, localized on the short arm of chromosome 6, was recently identified to be peripherin, a structural protein with a M_r of 35,000, present in the rim of disks of rod outer segments [28]. A defect in the peripherin gene is involved in a hereditary retinopathy of the mouse, termed 'retinal degeneration slow', or rds [29]. In another form of mouse retinopathy, rd, a defect is in the β subunit of cGMP-PDE [30]. Mutations in an oxygen-regulated photoreceptor protein called RP1 cause nearly 10% of autosomal dominant RP [31]. Up to 15% of X-linked RP is accounted for by mutations in RP2 gene [32]. RP2 protein is a dual-acylated protein that has sequence similarity to a tubulin-specific chaperone cofactor. These findings suggest that defects causing photoreceptor degenerations in

different forms of RP could be in any structural and functional proteins associated with rod outer segments. Very little is known about how mutations in these proteins bring about degenerations of the rod cells. It is possible that various elements released into the subretinal space via the retinal vasculature normally protect the photoreceptors and that mutations forfeit the protection. In RCS rats, a strain with inherited retinal dystrophy, in which the ability of the retinal pigmented epithelium to phagocytize photoreceptor outer segments is impaired, subretinal or intravitreal injection of basic fibroblast growth factor causes a significant delay in retinal degeneration [33].

Choroideremia can be classified, broadly, as an X-linked form of RP. A study of lymphoblasts from subjects with this disorder has disclosed deficient activity of geranylgeranyl transferase [34]. This enzyme catalyzes the transfer of geranylgeranyl groups to guanine nucleotide-binding proteins. This modification is necessary for these proteins to participate in membrane fusion processes. Both the biogenesis of photoreceptor disks and their subsequent phagocytosis by the pigmented epithelial cells involve very active membrane fusion and turnover. Thus, it is quite possible that the pathology of RP may arise from an enzyme deficiency of this type.

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is a common cause of central vision impairment in the elderly, affecting more than 11 million people in the U. S. A.. A juvenile form of macular degeneration, called Stargardt's disease, is caused by mutations in a gene encoding an ATP-binding cassette transporter (ABCR, see Ch. 5) expressed in the retina. Environmental factors, such as cigarette smoking and diet, as well as genetic factors contribute to age-related degeneration. In order to examine whether ABCR mutations are associated with the disease, 167 unrelated patients were screened [35]. Alterations in the ABCR gene were found among as many as 16% of patients. Age-related macular degeneration is clinically subdivided into 'dry' and 'wet' forms. The dry form is characterized by the accumulation of cellular debris, called drusen, in and under the retinal pigmented epithelium, and the wet form involves choroidal neovascularization. ABCR gene mutations were found to be more frequent among patients with dry than among those with wet macular degeneration. There is evidence suggesting that ABCR transports retinal (or *N*-retinylidene phosphatidylethanolamine (NRPE)) from the luminal side to the cytosolic side of the disk membrane [36]. NRPE is produced by photobleaching of rhodopsin in the outer segment [13]. If the ABCR protein is defective and not able to transport the retinoid across the disk membrane, its buildup may become toxic and cause retinal degeneration. Beside the genetic risk, environmental and metabolic factors also contribute to AMD. The retinal pigmented epithelial cells of aged human eyes accumulate a compound formed by condensation of NRPE with a second molecule of all-*trans*-retinal and hydrolysis of the adduct by phospholipase D. The compound, designated A2E [37], makes RPE cells sensitive to blue light that induces oxidative stress and cell apoptosis. A possible role of A2E in the pathogenesis of dry form of AMD has been suggested [38].

REFERENCES

1. Pourcho, R. G. Neurotransmitters in the retina. *Curr. Eye Res.* 15: 797–803, 1996.
2. Wässle, H., Koulen, P., Brandstätter, H., Fletcher, E. L. and Becker, C.-M. Glycine and GABA receptors in the mammalian retina. *Vision Res.* 38: 1411–1430, 1998.
3. Brandstätter, J. H. Glutamate receptors in the retina: the molecular substrate for visual signal processing. *Curr. Eye Res.* 25: 327–331, 2002.
4. Stryer, L., Dowling, J. and Wiesel, T. (organizers). A collection of papers presented at a colloquium entitled 'Vision from Photon to Perception'. *Proc. Natl Acad. Sci. USA* 93: 557–639, 1996.
5. Ebrey, T. and Koutalos, Y. Vertebrate photoreceptors. *Prog. Retina. Eye Res.* 20: 49–94, 2001.
6. Fain, G. L., Matthews, H. R., Cornwell, M. C. and Koutalos Y. Adaptation in vertebrate photoreceptors. *Physiol. Rev.* 81: 117–151, 2001.
7. Bok, D. Retinal photoreceptor–pigment epithelium interactions. *Invest. Ophthalmol. Vis. Sci.* 26: 1659–1694, 1985.
8. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M. and Shichida, Y. Functional role of internal water molecules in rhodopsin revealed by x-ray crystallography. *Proc. Natl Acad. Sci. USA* 99: 5982–5987, 2002.
9. Filipek, S., Stenkamp, R. E., Teller, D. C. and Palczewski, K. G protein-coupled receptor rhodopsin: a prospectus. *Annu. Rev. Physiol.* 65: 851–879, 2003.
10. Crouch, R. K., Chader, G. J., Wiggert, B. and Pepperberg, D. R. Retinoids and the visual process. *Photochem. Photobiol.* 64: 613–621, 1996.
11. Mata, N.L., Moghrabi, W.N., Lee, J.S., Bui, T.V., Radu, R.A., Horwitz, J. and Travis, G.H. Rpe65 is a retinyl ester binding protein that presents insoluble substrate to the isomerase in retinal pigment epithelial cells. *J. Biol. Chem.* 279: 635–643, 2004.
12. Suzuki, T., Sugahara, M. and Kito, Y. An intermediate in the photoregeneration of squid rhodopsin. *Biochem. Biophys. Acta* 275: 260–270, 1972.
13. Shichi, H. and Somers, R. L. Possible involvement of retinylidene phospholipids in photoisomerization of all-*trans* to 11-*cis* retinal. *J. Biol. Chem.* 249: 6570–6577, 1974.
14. Nathans, J., Thomas, D. and Hogness, D. S. Molecular genetics of human color vision: The genes encoding blue, green, and red pigments. *Science* 232: 193–202, 1986.
15. Yoshizawa, T. and Kuwata, O. Iodopsin, a red sensitive cone visual pigment in the chicken retina. *Photochem. Photobiol.* 54: 1061–1070, 1991.
16. Korenbrot, J. I. Signal mechanisms of phototransduction in retinal rod. *CRC Crit. Rev. Biochem.* 17: 223–256, 1985.
17. Fesenko, E., Kolesnikov, S. S. and Lyubarsky, A. L. Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature* 313: 310–313, 1985.

18. Maeda, T., Imanishi, Y. and Palczewski, K. Rhodopsin phosphorylation: 30 years later. *Prog. Retina. Eye Res.* 22: 417–434, 2003.
19. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L. and Hurley, J. B. Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (Ret GC), Ret GC-2. *Proc. Natl Acad. Sci. USA* 92: 5535–5539, 1995.
20. Yang, R. B., Foster, D. C., Garbers, D. L. and Fulle, H. J. Two membrane forms of guanylyl cyclase found in the eye. *Proc. Natl Acad. Sci. USA* 92: 602–606, 1995.
21. Palczewski, K., Polans, A. S., Baehr, W. and Ames, J. B. Ca^{2+}-binding proteins in the retina: structure, function, and the etiology of human visual diseases. *BioEssays* 22: 337–350, 2000.
22. Trudeau, M. C. and Zagotta, W. N. Calcium/calmodulin modulation of olfactory and rod cyclic nucleotide-gated ion channels. *J. Biol. Chem.* 278: 18705–18708, 2003.
23. Brown, J. E., Faddis, M. and Combs, A. Light does not induce an increase in cyclic-GMP content of squid or *Limulus* photoreceptors. *Exp. Eye Res.* 54: 403–410, 1992.
24. Fein, A. Excitation and adaptation of *Limulus* photoreceptors by light and inositol 1,4,5-triphosphate. *Trends Neurosci.* 9: 110–114, 1986.
25. Nathans, J. Molecular biology of visual pigments. *Ann. Rev. Neurosci.* 10: 163–194, 1987.
26. Berson, E. L. Retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 34: 1659–1676, 1993.
27. Saleem, R. A. and Walter, M. A. The complexities of ocular genetics. *Clin. Genet.* 61: 79–88, 2002.
28. Humphries, P., Kenna, P. and Farrar, G. J. On the molecular genetics of retinitis pigmentosa. *Science* 256: 804–808, 1992.
29. Arikawa, K., Molday, L. L., Molday, R. S. and Williams, D. S. Localization of peripherine/rds in the disk membranes of cone and rod photoreceptors: relationship to disk membrane morphogenesis and retinal degeneration. *J. Cell Biol.* 116: 659–667, 1992.
30. Farber, D. B. From mice to men: the cyclic GMP phosphodiesterase gene in vision and disease. *Invest. Ophthalmol. Vis. Sci.* 36: 263–275, 1995.
31. Berson, E. L., Grimsby, J. L., Adams, S. M., McGee, T. L., Sweklo, E., Pierce, E. A., Sandberg, M. A. and Dryja, T. P. Clinical features and mutations in patients with dominant retinitis pigmentosa-1 (RP1). *Invest. Ophthalmol. Vis. Sci.* 42: 2217–2224, 2001.
32. Chapple, J. P., Grayson, C., Hardcastle, A. J., Bailey, T. A., Matter, K., Adamson, P., Grahams, C. H., Willison, K. R. and Cheetham, M. E. Organization on the plasma membrane of the retinitis pigmentosa protein RP2: investigation of association with detergent-resistant membranes and polarized sorting. *Biochem. J.* 372: 427–433, 2003.
33. Faktorovich, E. G., Steinberg, R. H., Yasumura, D., Matthes, M. and LaVail, M. M. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature* 347: 83–86, 1990.
34. Seabra, M. C., Brown, M. S. and Goldstein, J. L. Retinal degeneration in choroideremia: deficiency of rat geranylgeranyl transferase. *Science* 259: 377–381, 1993.
35. Allikmets, R., Shroyer, N. F., Singh, N., Seddon, J. M., Lewis, R. A., Bernstein, P. S., Peiffer, A., Zabriskie, N. A., Li, X., Hutchinson, A., Dean, M., Lupski, J. R. and Leppert, M. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Science* 277: 1805-1807, 1997.
36. Sun, H. and Nathans, J. Mechanistic studies of ABCR, the ABC transporter in photoreceptor outer segments responsible for autosomal recessive Stargardt disease. *J. Bioenerg. Biomemb.* 33: 523–530, 2001.
37. Sakai, N., Decatur, J. and Nakanishi, K. Ocular age pigment 'A2-E': an unprecedented pyridinium bisretinoid. *J. Am. Chem. Soc.* 118: 1559–1560, 1996.
38. Shaban, H., and Richter, C. A2E and blue light in the retina: the paradigm of age-related macular degeneration. *Biol. Chem.* 383: 537–545, 2002.

CHAPTER

50

Molecular Biology of Olfaction and Taste

Steven D. Munger

OLFACTION 817
The mammalian olfactory system possesses enormous discriminatory power 817
The initial events in olfaction occur in a specialized olfactory neurepithelium 818
Odor discrimination could involve a very large number of different odorant receptors, each specific for one or a small set of odorants 818
The information generated by hundreds of different receptor types must be organized to achieve a high level of olfactory discrimination 820
Zonal expression of olfactory receptors 821
Convergence of sensory neurons onto a few glomeruli in the olfactory bulb 821
The sensitivity of the olfactory system is likely to derive from the capacity of the olfactory transduction apparatus to effectively amplify and rapidly terminate signals 821
Odorant recognition initiates a second-messenger cascade leading to the depolarization of the neuron and the generation of action potentials 821
Negative-feedback processes mediate adaptation of the olfactory transduction apparatus to prolonged or repetitive stimulation 823
Alternative second-messenger pathways may be at work in olfactory transduction 823
The vomeronasal organ is an accessory chemosensing system that plays a major role in the detection of conspecific chemical cues, also known as pheromones 824
Chemosensory neurons of the vomeronasal system are narrowly tuned to specific chemical cues, and utilize a unique mechanism of sensory transduction 824

TASTE 825
Multiple senses, including taste, contribute to our total perception of food 825
Taste receptor cells are organized into taste buds 825
Sensory afferents within three cranial nerves innervate the taste buds 826
Information coding of taste is not strictly according to a labeled line 826
Taste cells have multiple types of ion channels 826
Salts and acids are transduced by direct interaction with ion channels 826
Taste cells contain receptors, G proteins and second-messenger-effector enzymes 827
Gustducin is a taste-cell-specific G protein closely related to the transducins 827
Sweet, bitter and umami taste involve receptor-coupled second-messenger pathways that are differentially expressed across the gustatory epithelium 827

The mammalian olfactory and taste (gustatory) systems are extraordinarily accomplished in the detection of odor, pheromone and taste stimuli that may number in the millions [1]. Utilizing a number of specialized cells, a vast array of chemosensory receptors, and highly regulated signal transduction mechanisms, the olfactory and taste systems are able to specifically encode a range of chemically diverse stimuli so that quality food can be identified, predators can be avoided, and appropriate mating partners can be found. Enormous strides have been made in understanding the molecular mechanisms that underlie olfaction and taste in vertebrates. The application of innovative experimental techniques, as well as the availability of several sequenced genomes, has allowed for the identification and characterization of molecules involved in the initial events in perception in these two sensory systems, and has provided fresh insight into the processes by which olfactory and gustatory discriminations are achieved.

OLFACTION

The mammalian olfactory system possesses enormous discriminatory power. It is claimed that humans can perceive many thousands of different odorous molecules, termed odorants. Even slight alterations in the structure of an odorant can lead to profound changes in perceived odor quality. One commonly cited example is carvone, whose L- and D-stereoisomers are perceived as spearmint and caraway, respectively. However, more subtle molecular alterations can also generate striking changes in perception.

The fine discriminatory power of the mammalian olfactory system is likely to derive from information-processing events that occur at several distinct anatomical

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sites [2]: the olfactory epithelium of the nasal cavity, where odors are first sensed by olfactory sensory neurons; the olfactory bulb, where information received from the sensory neurons is first processed; and the cortex, where information received from the olfactory bulb is thought to be further refined to allow for the discrimination of thousands of different odors.

The initial events in olfaction occur in a specialized olfactory neurepithelium. In mammals, this structure lines the posterior nasal cavity. The olfactory epithelium contains three predominant cell types: the olfactory sensory neuron (OSN); the supporting, or sustentacular, cell; and the basal cell (Fig. 50-1). OSNs turn over throughout life. They are continuously replaced from progenitor populations within the basal cells. Two morphologically distinguishable types of dividing basal cell have been identified in mammals, the horizontal basal cell and the globose basal cell. There is evidence for both basal cell types containing multipotent precursor cell populations, although the precise lineage relationships of the cells of the olfactory epithelium remain controversial.

The OSN is a bipolar cell that extends a single dendrite to the apical surface of the epithelium. From the dendritic terminus, numerous nonmotile cilia project into the layer of mucus that lines the nasal cavity. The cilia contain the molecular machinery of olfactory transduction, which includes receptors, synthetic enzymes and ion channels. The details of signal generation, adaptation and propagation will be discussed in a later section.

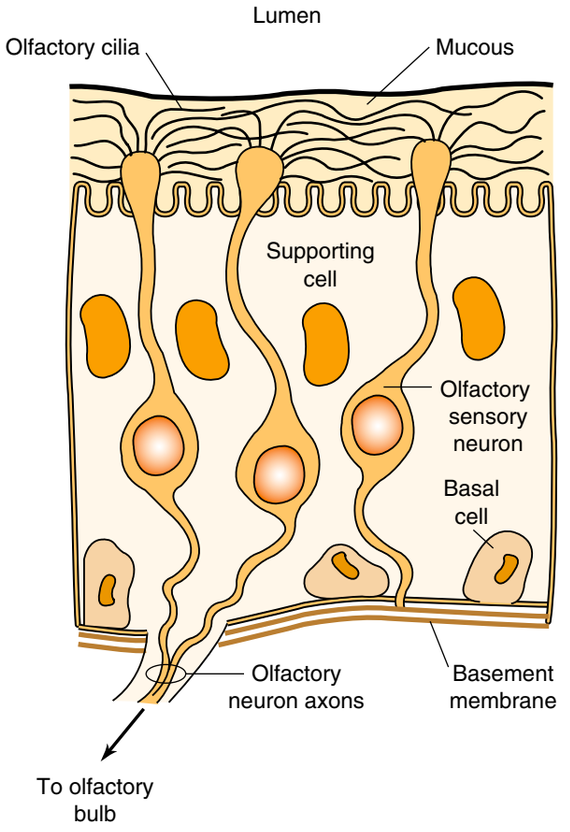

FIGURE 50-1 A schematic diagram of the olfactory epithelium. The initial events in odor perception occur in the olfactory epithelium of the nasal cavity. Odorants interact with specific odorant receptors on the lumenal cilia of olfactory sensory neurons. The signals generated by the initial binding events are transmitted along olfactory neuron axons to the olfactory bulb of the brain.

Each OSN projects an unbranched axon to the olfactory bulb, where it forms synapses within specialized regions of neuropil, called glomeruli. OSNs form synapses with both projection neurons and local interneurons. The major output neurons of the bulb, the mitral and tufted cells, project to higher olfactory areas in the brain, including the olfactory cortex. Bulbar interneurons, such as periglomerular cells and granule cells, help to shape sensory input and bulbar output in several ways, including creating an interglomerular center-surround inhibitory network that may enhance some features of odor signals [3].

Electrophysiological experiments demonstrate that most OSNs recognize multiple odorants. It is thought that the patterns of axonal convergence in the olfactory bulb by OSNs that recognize different odorants might constitute elementary odor codes. It is not clear how many different types of odorant receptor would be required to accurately encode a single odorant, nor is it obvious how narrowly or broadly tuned they might be or how their expression might be organized to achieve a high level of sensory discrimination.

Odor discrimination could involve a very large number of different odorant receptors, each specific for one or a small set of odorants. At the other extreme, there could be a relatively small number of different odorant receptors (ORs), each of low specificity and capable of interacting with a wide assortment of different odorous ligands. Individual OSNs could each express only a single OR type or multiple different odorant receptors.

The identification and cloning of genes encoding ORs has made it possible to begin to answer these questions. Nearly 15 years ago, Buck and Axel made a seminal discovery when they identified the first members of a large multigene family in mammals encoding hundreds of diverse ORs [4]. ORs are members of the G-protein-coupled receptor (GPCR) superfamily, and share with them a number of stereotypical motifs, including an apparent seven transmembrane domain topology. However, ORs share unique sequence motifs not seen in other GPCRs, and which, along with their expression in OSNs, help to define them as a unique group of receptor proteins [5].

Analyses of the human and mouse genomes has revealed ~350 functional OR genes in humans (with an equivalent number of OR pseudogenes) and ~1,200 in mice [6–9], making this the largest mammalian gene family. OR genes are found in clusters within the genome on nearly every chromosome, suggesting a large number of local tandem duplications during evolution [9]. While the accepted numbers of OR genes may change in the coming years with greater analysis of the genomes and of OR transcripts, it is clear even today that the large number of

mammalian ORs has enormous implications for the way odorants are detected and encoded by the olfactory system.

Olfactory receptors can be grouped based on conservation of protein sequences. First, mammalian olfactory receptor genes can be divided into two phylogenetic classes (Fig. 50-2). Class I genes are more ancient, and are more closely related to those found in fish. Class II genes are an evolutionarily more recent family, and appear restricted to terrestrial vertebrates. It is reasonable to hypothesize that these two phylogenetic classes of OR subserve different olfactory roles, and this possibility is a focus of current interest to both neurobiologists and evolutionary biologists. Second, OR genes can be further divided into subfamilies based on sequence similarity (Fig. 50-2). Members of a subfamily exhibit a sequence conservation of >40% amino-acid identity [8], as compared to 37% average amino-acid similarity across the OR family [1]. It is tempting to propose that the members of divergent subfamilies recognize different structural classes of odorants. The highly related members of a subfamily might either recognize the same odorants, or detect subtle differences between structurally related odorants.

A striking feature of ORs is that some regions or the protein are highly variable, while others are relatively conserved. As seen in Figure 50-3 [5], transmembrane domain 4 (TM4), TM5 and parts of TM3 are highly variable across the OR repertoire. As it would be expected that variation in amino-acid sequence would be a hallmark of a ligand-binding pocket, enabling divergent ORs to recognize

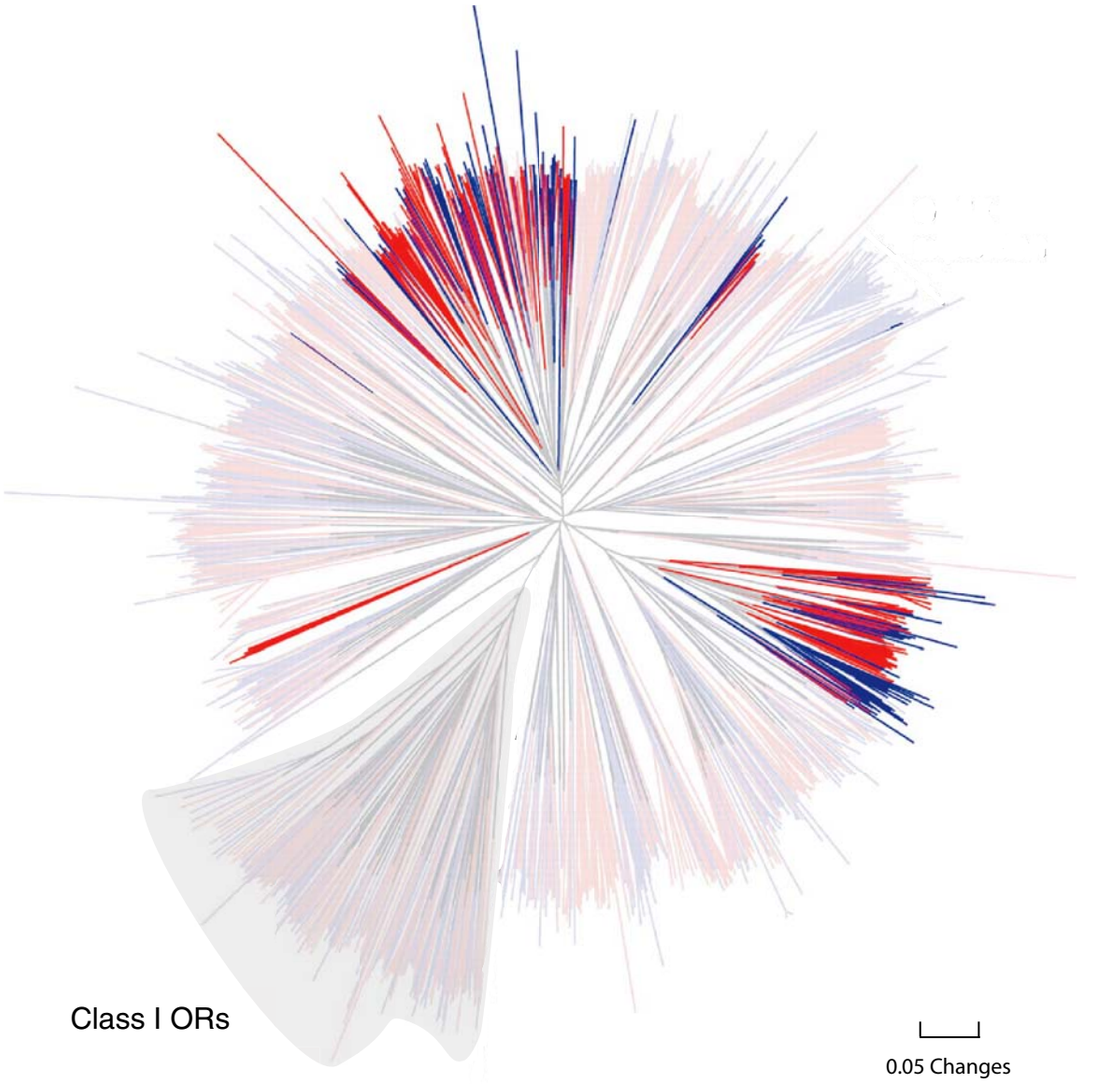

FIGURE 50-2 Phylogenetic relationships between human and mouse OR genes. This unrooted tree represents all full-length mouse and human OR proteins. Class I ORs, which most closely resemble those found in fish and amphibians, are *shaded*. Class II ORs are restricted to the mammalian lineage and are *unshaded*. Human sequences are colored *blue* and mouse sequences *red*. Human and mouse sequences found in syntenic genomic locations on human chromosome 11 and mouse chromosome 2 are highlighted in *darker colors*. Clustering of these receptors on the tree support their identification as orthologous groups. Clustering of human (*blue*) or mouse (*red*) receptors within clusters suggests that the OR gene repertoire has arisen largely through gene duplication. Modified from [71], with permission.

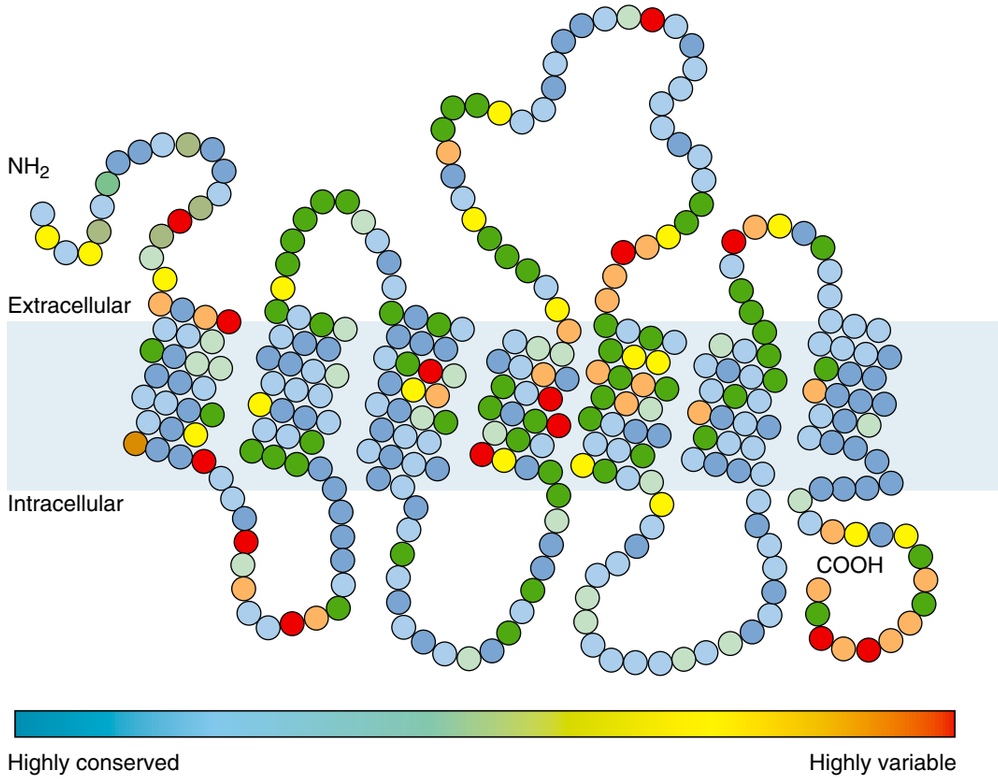

FIGURE 50-3 Amino acid sequence conservation across mammalian odorant receptors. ORs pass through the plasma membrane (*blue box*) seven times, with the *N*-terminus located extracellularly and the *C*-terminus intracellularly. The degree of conservation of each amino acid in this consensus OR is indicated by a colored ball, with *dark blue* being most highly conserved and *red* most highly variable. Modified from [5], with permission.

chemically diverse odorants, these regions have been proposed as the site of ligand interaction [4]. This idea is further supported by two observations: (1) many odorants are hydrophobic, and are likely to partition within the plane of the lipid bilayer, and (2) many other Class A GPCRs, which include the ORs along with rhodopsin (Ch. 49), the β-adrenergic receptor (Ch. 12), and many others, have a ligand-binding pocket formed by the transmembrane helices.

Unfortunately, technical hurdles have slowed progress on pairing individual receptors with their cognate odor ligands, making a systematic structure–function analysis of ORs problematic. Using a number of approaches, such as viral-mediated overexpression of individual receptors *in vivo*, isolation of receptor cDNAs from single olfactory neurons with characterized responses to a panel of odors, or the coupling of expressed receptors to endogenous cellular signaling pathways in heterologous cells, researchers have identified ligands for only a small number of receptors [1]. However, even this small number has revealed some important features of OR function. First, ORs can have a broad receptive range; that is, they respond to more than one odorant, and often to odorants of more than one chemical class (e.g. aldehydes vs. alcohols). Second, more than one OR can be activated by the same odorant. For example, it is estimated that up to 55 ORs in the mouse genome can respond to the aliphatic aldehyde octanol [10]. Third, not every OR activated by a particular odor responds with the same efficacy to that odor. Together, these observations indicate a repertoire of ORs with overlapping odorant profiles. The idea that the identity of an individual odor is encoded by several differentially tuned ORs has been called a combinatorial odor code [11]. As will be discussed in a later section, studies of odor coding in the olfactory bulb largely support this model.

The information generated by hundreds of different receptor types must be organized to achieve a high level of olfactory discrimination. The visual, auditory (Chs 49 and 51) and somatosensory systems localize environmental information in space and possess neural topographical maps of that spatial information. The olfactory system does not perceptually localize environmental information in external space. However, it could use spatial determinants within the nervous system to encode information. If so, topographical maps or spatial codes for odors might be evident within the olfactory epithelium or olfactory bulb. For example, olfactory neurons that express a particular odorant receptor gene, and therefore recognize the same odorants, might be clustered in one region of the olfactory epithelium or might all form synapses at a

discrete site in the olfactory bulb. On the other hand, neurons that express the same odorant receptor gene could be broadly distributed in the olfactory epithelium or form synapses at many different bulbar sites, encoding information by a nontopographical strategy. The identification of olfactory receptors has provided an important tool for dissecting how olfactory information is coded in neural space.

Zonal expression of olfactory receptors. In the olfactory epithelium, different OR genes are expressed within restricted anatomical zones [12, 13]. These zones exhibit bilateral symmetry in the two nasal cavities and are virtually identical across individuals. Within each zone, many different members of the OR gene family are expressed. However, each individual gene may be expressed only within a single zone. Although the zonal assignment of each gene seems to be strictly regulated, though perhaps not absolute, within each zone neurons that express a particular receptor gene are broadly distributed. It thus appears that when an olfactory neuron or its progenitor chooses which odorant-receptor gene(s) to express, it is restricted to a single zonal gene set but may choose a receptor gene or set of genes to express from among the zonal set via a stochastic mechanism. Recently, *cis*-acting regulatory elements have been identified near one OR gene cluster that participates in this choice [14]. This work and others [15] also suggest a poorly understood negative feedback mechanism for a phenomenon that is accepted, but not proven: in most circumstances, OSNs express only one OR gene.

Convergence of sensory neurons onto a few glomeruli in the olfactory bulb. The odorant receptor expression zones in the olfactory epithelium exhibit a pronounced dorsal–ventral and medial–lateral organization. Interestingly, the axonal projection from the olfactory epithelium to the olfactory bulb is also organized along the dorsal–ventral and medial–lateral axes [16]. Comparisons of the locations of the expression zones with the topography of projections between the olfactory epithelium and olfactory bulb indicate that the organization of odorant receptor gene expression in the epithelium is preserved in the axonal projection to the olfactory bulb. Indeed, the map is even more specific than this. Axons from olfactory sensory neurons expressing the same receptor gene converge on just a few glomeruli in the bulb, usually two [17–19]. Thus, it appears that all of the cells expressing a particular receptor are somehow targeted to just a few among approximately 1,900 glomeruli in the mouse olfactory bulb. Interestingly, the olfactory receptor itself seems to play a critical role in this specific pattern of axonal targeting, although whether this is a direct or indirect role is unclear.

These results suggest that an initial organization of olfactory sensory information occurs in the olfactory epithelium, and that this organization is maintained in the patterns of signals transmitted to the olfactory bulb. This conclusion is consistent with the observation that responses in mitral cells to odors applied to the epithelium are narrowly tuned and that sensitivity to particular types of odor, determined by either functional group or carbon chain length, is spatially organized in the bulb [20]. Thus, the organization of receptors into broad zones in the epithelium is further refined in the bulb, where receptors find common targets.

This pattern of organization in the bulb argues that the glomerulus, not the receptor, is the fundamental unit of olfactory coding. Given the overlapping odor specificities of individual olfactory receptors, one would then expect that an individual odor would activate more than just one or two glomeruli, an expectation that has been supported by a number of functional studies. It is now believed that some version of a combinatorial code for odors is present in the bulb, where the identity of a particular odorant is represented by the pattern of glomerular activity in the whole bulb. As more researchers turn their focus to higher brain centers, such as the olfactory cortex, it will be interesting to see the extent to which the glomerular pattern of odor activity is maintained.

The sensitivity of the olfactory system is likely to derive from the capacity of the olfactory transduction apparatus to effectively amplify and rapidly terminate signals. The olfactory system responds to extremely low concentrations of odorants and has a fast recovery of perceptual sensitivity. In recent years, significant advances have been made in the understanding of the signal-transduction events that take place when an olfactory sensory neuron is exposed to an odorant. Although olfactory perception is believed to be extremely sensitive, the degree of sensitivity is controversial [2]. Human olfactory thresholds of 10^{-11} M have been measured by psychophysical methods. In such studies, however, the actual concentration of odorant at the odorant receptor cannot be known definitively. Due to factors such as odor molecule interactions, diffusion, adsorption into mucus, and anatomical structures which may trap and concentrate odor molecules, these estimates could be as much as two to four orders of magnitude too low. It can be shown from the law of mass action that with significant amplification mechanisms, such that occupation of only a few receptors would be sufficient to elicit a response, thresholds as much as five or six orders of magnitude lower than the K_d can be attained. Thus, K_d values of 10^{-6} could, in theory, produce threshold responses at odor concentrations as low as 10^{-11}, permitting the olfactory system to maintain the broad specificity seen in physiological recordings, and the high threshold sensitivity measured in psychophysical experiments. Several amplification mechanisms that could serve this purpose are described below.

Odorant recognition initiates a second-messenger cascade leading to the depolarization of the neuron and the generation of action potentials. Stimulation of single OSNs with odorants causes the membrane to depolarize,

leading to the generation of action potentials (Fig 50-4). Underlying the initial depolarization is the influx of cations through a conductance in the specialized cilia extending from the distal end of the cell. The biochemical elements coupling odorant receptor binding to the opening of a cation channel are now understood in some detail [2, 21]. A consensus view is presented in Figure 50-5.

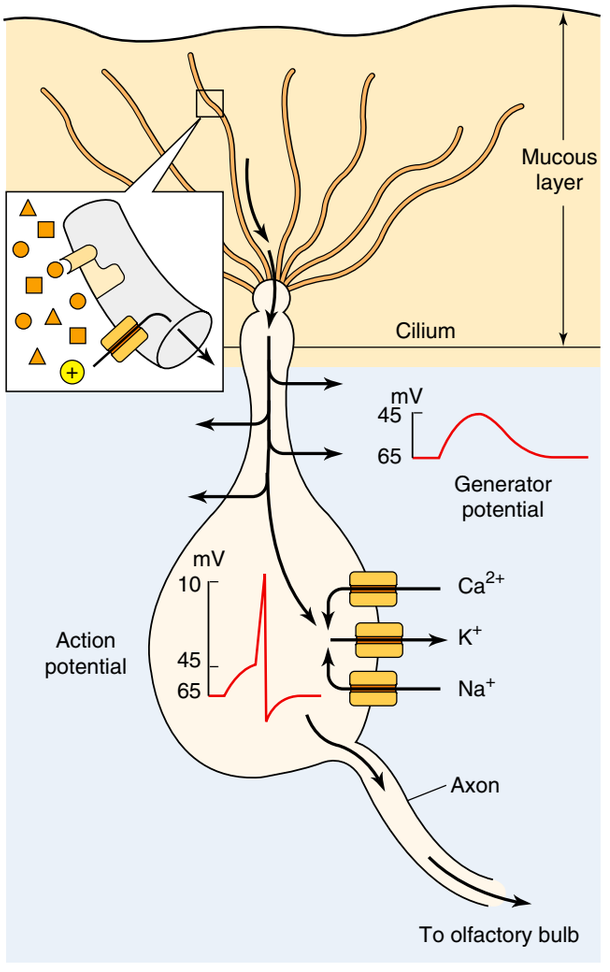

FIGURE 50-4 Schematic drawing of an olfactory neuron showing the single bipolar morphology, with a single thick dendrite ending in a knob-like swelling and an unbranched axon projecting from the proximal soma centrally to the olfactory bulb. The cell is highly compartmentalized into transduction and signaling regions. Transduction occurs in the cilia, which extend from the distal dendritic knob into the mucous layer. A receptor-coupled second-messenger system (see Fig. 50-5) results in the opening of a cation-selective channel in the ciliary membrane. The influx of cations depolarizes the cell membrane from its resting level near −65 to −45 mV, in a graded manner. This depolarization spreads by passive current flow through the dendrite to the soma. A depolarization that reaches −45 mV is sufficient to activate voltage-gated Na^+ channels and initiate impulse generation. This Na^+ current along with several varieties of voltage-dependent K^+ currents and a small Ca^{2+} current produce one or more action potentials that are propagated down the axon to the brain.

The second-messenger cascade of olfactory transduction is a classic cyclic nucleotide-based system with some interesting modifications. A G protein (Ch. 19) that is likely to couple odorant receptors to other intracellular elements of the cascade, $G\alpha_{olf}$, has been identified [22]. An isoform of $G\alpha_s$, it is highly enriched in OSN cilia. Deletion of this gene in mice abolishes nearly all odor responses in OSNs and odor-mediated behaviors, confirming its central role in olfactory transduction. Since $G\alpha_{olf}$ can interact with GPCRs other than odorant receptors, it has been proposed that the existence of a separate gene for $G\alpha_{olf}$ may, in some manner, permit the specific and high level of expression of this protein observed in olfactory neurons.

OSNs also contain an adenylyl cyclase isoform (Ch. 21), type III (ACIII) that is highly enriched in olfactory cilia [23]. An important characteristic of this isozyme is that, when expressed in a mammalian cell line, its basal activity is extremely low, while in its stimulated state it has a catalytic rate higher than other known cyclases. These properties could confer a high signal-to-noise ratio on the system, being quiescent in the absence of stimulus but able to rapidly generate large amounts of cAMP upon odor exposure. Deletion of the gene encoding ACIII elicits a general anosmia in mice [24]. ACIII is also inhibited by Ca^{2+}, providing an opportunity for negative feedback upon intracellular elevations of this signaling molecule.

In 1987, Nakamura and Gold [25] recorded a cAMP-activated conductance from OSN cilia. This was the final link between the biochemical cascade and the electrical signal. It was subsequently determined that the channel responsible is comprised of three distinct olfactory-specific subunits, CNGA2, CNGA4 and CNGB1b; all are members of the cyclic-nucleotide-gated (CNG) channel family (Ch. 21) that also contains the cGMP-activated channel found in photoreceptors. These channels are homologous with voltage-sensitive channels, such as K^+ and Ca^{2+} channels [26]. CNGA2 is obligatory for channel function both *in vitro* and *in vivo*, while CNGA4 and CNGB1b serve important modulatory roles [26]. The olfactory CNG channel is selective for cations; is sensitive to both cAMP, with a K_d of 20 μM, AND cGMP WITH A K_d of 5 μM; requires at least three molecules of cyclic nucleotide to bind for activation, and is required for odor signaling in OSNs [26, 27].

The activation of tens to hundreds of these channels and the subsequent influx of cations, including both Na^+ and Ca^{2+}, leads to depolarization of the cell membrane. Olfactory neurons have one additional level of amplification that is rather unusual. A Ca^{2+}-activated Cl^- conductance is also present on the cilia and is opened during the odor response by the Ca^{2+} ions flowing into the cilia through the cAMP-gated channel [28]; this channel has not been cloned. Curiously, olfactory neurons maintain a very high intracellular Cl^- concentration, perhaps as high as 125 mM, so that the driving force for Cl^- ions is outward and, therefore, further depolarizes the cell. This additional current may be important for preserving ionic

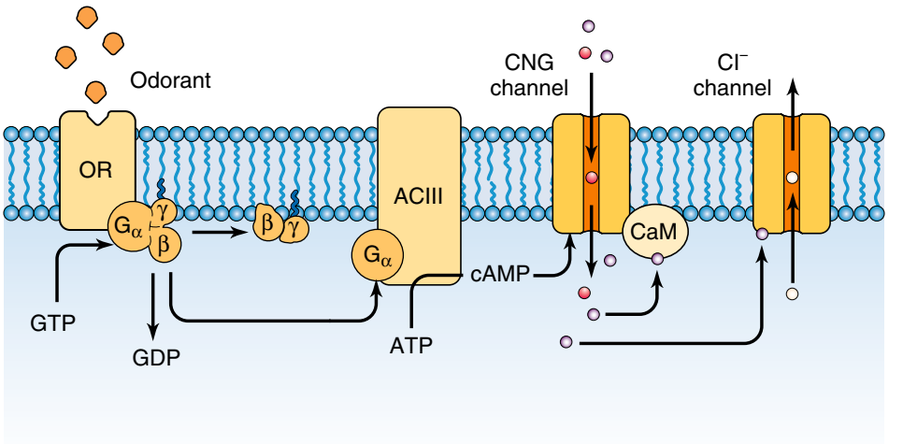

FIGURE 50-5 A model for the transduction of odors in OSNs. The individual steps are detailed in the text. Note that several feedback loops modulate the odor response, including inhibition of the CNG channel by Ca^{2+} ions (*purple balls*) that permeate the channel, and a Ca^{2+}/calmodulin (CaM)-mediated desensitization of the channel that underlies rapid odor adaptation. Several other mechanisms, including phosphodiesterase-mediated hydrolysis of the second messenger, cAMP, and phosphorylation of the OR by various kinases, have also been described.

driving forces across a membrane in a compartment that is not highly regulated, that is, the mucus. Changing ion concentrations in the mucus could affect the driving forces on ions, so the olfactory neuron supplies its own driving force by maintaining a high intracellular concentration of Cl^-.

This pathway provides several amplification steps between odorant binding and signal generation. Due to the electrically compact structure of the cell, it is possible for the activation of only a few tens of channels to drive the membrane to the threshold for action-potential generation. Thus, it is theoretically possible that the limit of olfactory detection is a single molecule, although this has not yet been conclusively demonstrated.

Negative-feedback processes mediate adaptation of the olfactory transduction apparatus to prolonged or repetitive stimulation. Upon application of a sustained odor stimulus to an olfactory neuron, the current response is transient, falling back to baseline within 4–5 s. The termination of a response in the continued presence of agonist is characteristic of many signaling systems, and is variously known as adaptation or desensitization, depending on the putative site of the off mechanism. Typically, a negative-feedback process is at work such that accumulation of a product of the agonist response serves to turn off an upstream link in the signal-generating cascade. In the olfactory neuron, two feedback messengers have been identified, and, as might be expected, they are Ca^{2+} and cyclic nucleotides.

The cyclic-nucleotide-gated channel is permeable to cations, especially Ca^{2+}. Thus, increased channel activation results in influx of Ca^{2+} and a transient rise in the intracellular Ca^{2+} concentration. Intracellular Ca^{2+} concentrations of 1–3 μM have been found to lead to a decrease in the open probability of the ion channel, even in the presence of high concentrations of cAMP [29]. Mediated by the intermediate Ca^{2+}-binding protein calmodulin, this Ca^{2+}-dependent desensitization of the channel accounts for virtually all short-term adaptation, and is dependent on the presence of the CNGA4 subunit, which increases the kinetics of Ca^{2+}/calmodulin-mediated desensitization of the native channel nearly 70-fold [30]. This is an attractive mechanism for mediating a rapid but short-lasting form of adaptation since it is dependent on the influx of Ca^{2+} during the response to an odor.

Other mechanisms have also been implicated in odor adaptation, including cAMP-dependent phosphorylation of ciliary proteins via protein kinase A; G-protein-receptor kinase activity (GRK3), possibly via phosphorylation of the OR; Ca^{2+}/calmodulin kinase II (CaMKII) phosphorylation of ACIII; cGMP; and carbon monoxide [31]. These latter three mechanisms have been particularly linked to longer-lasting forms of adaptation, on the order of tens of seconds (for CaMKII) or minutes (CO/cGMP). Together with the short-term adaptation described above, these various molecular mechanisms provide the OSN with a number of ways to fine-tune odor responses over time.

Alternative second-messenger pathways may be at work in olfactory transduction. The role of cAMP in olfactory transduction is well established. Are there alternative pathways, such as those involving phospholipids and Ca^{2+}? Several groups have reported that certain odorants can elicit an increase in the phosphoinositde second messenger inositol 1,4,5,-trisphosphate (IP_3) (Ch. 20). However, there is no clear evidence that IP_3 directly mediates an electrical response in OSNs, nor is there a clear rationale for two parallel excitatory odor transduction cascades. However, more recent data support the idea that phosphoinositides or enzymes related to their metabolism may play a modulatory role, shaping the OSN output by

interactions with the cAMP-signaling cascade or the CNG channel [32]. It should be noted, however, that among invertebrates there is good evidence that IP_3, as well as cAMP, play important roles in olfactory signal transduction.

There is one small subset of cells in the main olfactory epithelium that clearly does not utilize a cAMP-dependent transduction cascade. These cells, which may represent less than 0.1% of the ciliated neurons in the epithelium, do not express G_{olf}, ACIII or CNGA2. They do, however, express components of a putative cGMP signaling mechanism: a transmembrane isoform of guanylyl cyclase called GC-D (Ch. 21); a splice variant of the cGMP-sensitive channel subunit CNGA3, the principal subunit found in cone photoreceptors; and cGMP-sensitive phosphodiesterase 2A [21]. These cells, which project their axons to the caudal olfactory bulb, terminating in ~9 'necklace' glomeruli, are of unknown chemosensory function.

The vomeronasal organ is an accessory chemosensing system that plays a major role in the detection of conspecific chemical cues, also known as pheromones. These cues are important in rearing, territorial, courtship and, in particular, sexual behaviors. The vomeronasal organ (VNO) is separate from the main epithelium in mammals, comprising a thin epithelial tissue within a bony capsule in the lateral wall of the nasal cavity. It is probably vestigial in humans. The VNO epithelium contains at least two populations of microvillar chemosensory neurons; one is in the more apical aspects of the epithelium, while the other lies in the more basal region. These two populations of vomeronasal neurons (VNs) are defined by the differential expression of several genes. For example, the apical VNs express the G-protein subunit $G\alpha_{i2}$, while the basal neurons express $G\alpha_o$. Apical and basal VNs also express different putative chemosensory receptors, the V1Rs and V2Rs, respectively. There are ~150 V1Rs, and a similar number of V2Rs. These receptors, while both GPCRs, are otherwise unrelated to each other: V1Rs are Class A GPCRs, while V2Rs are members of Class C (similar to metabotropic glutamate receptors; Ch. 15)(Fig. 50-6). They also bear no sequence homology to ORs. Although the V1Rs have been linked to pheromone responses, only a single V1R, and no V2R, has been paired with a cognate ligand [1]. The basal VNs also specifically express several major histocompatibility complex class Ib genes. While their specific function is still unclear, they do appear to play an important role in V2R trafficking to, or stabilization in, the plasma membrane [33].

Chemosensory neurons of the vomeronasal system are narrowly tuned to specific chemical cues, and utilize a unique mechanism of sensory transduction. Pheromones, odorants released by one member of a species that elicit behavioral or hormonal changes in another member, have been identified in a number of mammals, and are often found in urine. Leinders-Zufall and colleagues first demonstrated the specific activation of VNs by odorants with pheromone properties in mice [34]. In doing so, two unique properties of VNs were identified that functionally differentiate them from OSNs. First, VNs are extremely sensitive to their ligands, with activation thresholds as low as 10^{-11} M. Second, in contrast to the broadly tuned OSNs, VNs are highly selective, seemingly responsive to only single stimuli even at high stimulus concentrations. These properties suggest a different coding strategy for these two types of olfactory neurons: broadly tuned OSNs that can respond to novel odorants, and narrowly tuned, highly sensitive VNs that specifically respond to conspecific odorants of particular behavioral relevance.

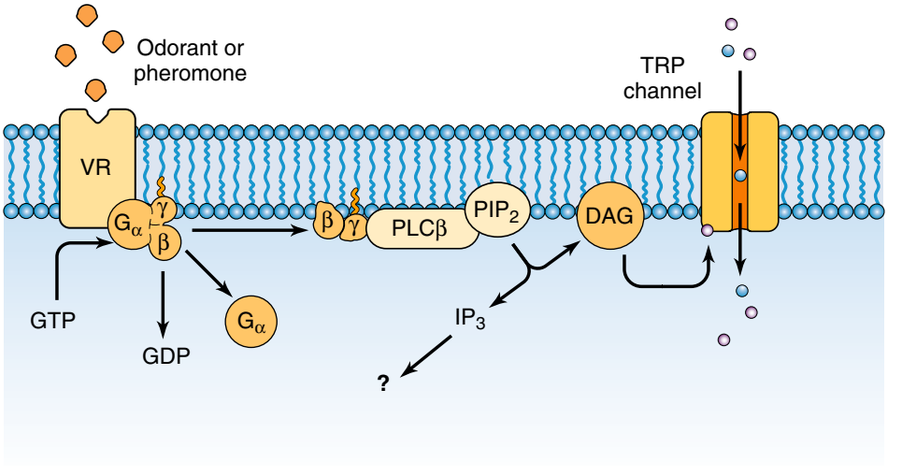

FIGURE 50-6 A model for chemosensory transduction in vomeronasal sensory neurons. The individual steps are detailed in the text. In contrast to the transduction cascade in OSNs, the mechanism of vomeronasal transduction is less well characterized. Vomeronasal neurons express either V1R or V2R receptors and either $G\alpha_{i2}$ or $G\alpha_o$, respectively. The TRPC2 channel subunit is expressed in all vomeronasal neurons, and may be part of a multimeric channel complex. Ca^{2+} ions are represented as *purple balls*, Na^+ ions as *blue balls. VR*, vomeronasal receptor (V1R or V2R); *PIP_2*, phosphatidylinositol 4,5-bishphosphate; *IP_3*, inositol 1,4,5-trisphosphate; *DAG*, diacylglycerol.

The biochemical cascade responsible for the transduction of VN stimuli is not fully understood. However, recent findings implicate a phospholipid signaling pathway at work in both VN populations (Fig. 50-6). All VNs express an ion channel subunit, TRPC2, that is a member of the transient receptor potential (TRP) family [35]. The essential role of TRPC2 in VN function, and the critical role of VNs in some pheromone-dependent behaviors, was conclusively demonstrated when the *Trpc2* gene was deleted in mice: VN activity in response to pheromones was nearly or completely abolished, and null mice exhibited altered sexual and social behaviors [36, 37]. The native VN channel containing TRPC2 is directly gated by the lipid messenger diacylglyerol in a pheromone-dependent manner [38]. However, the activation of vomeronasal receptors has still not been directly linked to diacylglycerol production via any G protein or phospholipase isoforms.

TASTE

Multiple senses, including taste, contribute to our total perception of food. Our perception of the flavor of food is a complex experience based upon multiple senses: taste *per se*, which includes sweet, sour, salty and bitter; olfaction, which includes aromas; touch, also termed 'mouth feel', that is, texture and fat content; and thermoreception and nociception caused by pungent spices and irritants. Taste proper is commonly divided into four categories of primary stimuli: sweet, sour, salty and bitter. One other primary taste quality, termed umami (the taste of L-glutamate), is still somewhat controversial. Mixtures of these primaries can mimic the tastes of more complex foods.

The chemical complexity of taste stimuli suggests that taste receptor cells utilize multiple molecular mechanisms to detect and distinguish among these compounds. Our sense of taste can detect and discriminate various ionic stimuli, for example, Na^+ as salty, H^+ as sour, sugars as sweet and alkaloids as bitter. While a number of recent studies have begun to identify the molecular players in taste transduction, the relative inaccessibility of the sensory cells, the diversity of sensory cell across the epithelium, and a number of functional and anatomical differences between animal models, even amongst rodents, mean that the mechanisms of taste transduction are still poorly understood.

Taste receptor cells are organized into taste buds. The chemical detection of taste stimuli resides in specialized epithelial cells, taste receptor cells (TRCs), which in vertebrates are present as ovoid clusters, or taste buds, each containing 50 to 100 cells (Fig. 50-7). The taste buds are embedded within the nonsensory lingual epithelium of the tongue, and they are housed within connective tissue specializations called papillae, which in humans are fungiform, foliate and circumvallate. Taste buds are also found in the palate, pharynx and upper portion of the esophagus.

The taste bud is a polarized structure with a narrow apical opening, termed the taste pore, and basolateral synapses with afferent nerve fibers. Solutes in the oral cavity make contact with the apical membranes of the TRCs via the taste pore. There is a significant amount of lateral connectedness between taste cells within a bud: both electrical synapses between TRCs and chemical synapses between TRCs and Merkel-like basal cells have been demonstrated to occur [39]. Furthermore, there are symmetrical synapses between TRCs and Merkel-like basal cells [39]. In addition, these basal cells synapse with the afferent nerve fiber, suggesting that they may function in effect as interneurons [39]. The extensive lateral interconnections

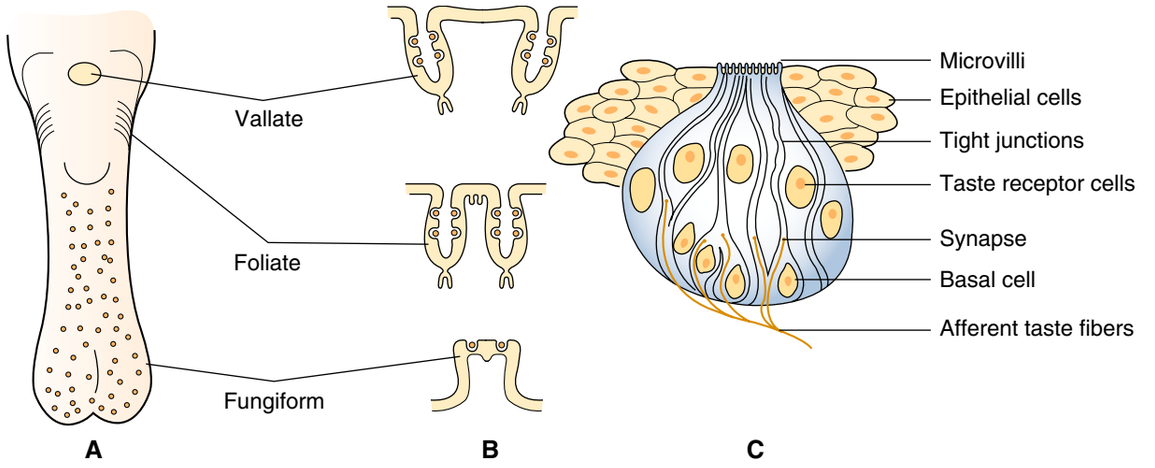

FIGURE 50-7 Rat tongue, taste papillae and taste buds. (**A**) Surface of the rat tongue showing location of the taste papillae. (**B**) Cross-section of the three main types of taste papillae: fungiform, foliate and vallate. (**C**) The taste bud contains 50–100 taste cells, including receptor cells and basal cells.

within a bud along with the branching of afferent fibers to synapse with several receptor cells within a given bud, suggest that much signal processing occurs peripherally within the taste bud itself.

Sensory afferents within three cranial nerves innervate the taste buds. The sensory fibers that innervate the taste buds travel in cranial nerves VII, IX and X. The chorda tympani carries fibers from VII to innervate taste buds on the anterior portion of the tongue, within the fungiform papillae and the anterior foliate papillae. The lingual branch of the glossopharyngeal nerve (IX) innervates buds within the circumvallate papillae and the posterior foliate papillae, while the superior laryngeal branch of the vagus (X) innervates taste buds of the epiglottis and the esophagus. These nerves relay taste information both to forebrain taste areas and to local circuits involved in oromotor reflexes.

Information coding of taste is not strictly according to a labeled line. Two different models have been proposed to account for information coding in the gustatory system: (1) labeled line and (2) across-fiber pattern code. The labeled-line model predicts that individual TRCs will respond to only a single taste quality; separate afferent pathways then transmit information about each taste quality to the gustatory cortex via the medulla and the thalamus. Thus, in the labeled-line model, the function of any one neuron in an afferent pathway is to signal its particular encoded taste quality. The across-fiber pattern-coding model proposes that individual taste cells respond to multiple taste qualities. Information about taste quality is then transmitted to the brain by afferent fibers that have broadly overlapping response spectra. Thus, the code for a particular quality is determined by the pattern of activity across all of the afferent nerve fibers, rather than by activity in any single nerve fiber.

Experimental results suggest that gustatory information coding utilizes both types of mechanisms. For example, single chorda tympani fibers respond to multiple taste qualities, such as salt and sour or sour and bitter, although they will often show a preferential response for a single taste quality. This implies that while some type of pattern-recognition processing must occur in taste coding, there is likely also some specificity of inputs from cells in the taste buds. Recent molecular biological experiments support this more nuanced view of taste processing. For example, *in vivo* expression in TRCs that normally express sweet taste receptors of a non-native receptor responsive to a synthetic ligand, results in mice that prefer the taste of this previously 'tasteless' stimulus [40].

Taste cells have multiple types of ion channels. TRCs are electrically excitable and capable of generating action-potentials: voltage-dependent channels for Na^+, Ca^{2+} and K^+, similar to those in neurons, have been detected in vertebrate TRCs. The surface distribution of these channels may reflect differences in their functional activities, but the consequences of this are not well understood. Ion channels also play more direct roles in the transduction of salts and acids [41]. These ionic stimuli can either directly enter taste cells via channels in the apical membranes, or can modulate the activity of channels. Interestingly, some ions can diffuse through the tight junctions of the taste epithelium to interact with channels in the basolateral membranes of the TRCs, the so-called paracellular taste pathway.

Salts and acids are transduced by direct interaction with ion channels. Salt taste in mammals is mediated, at least in part, by a voltage-independent, amiloride-blockable, apical Na^+ channel. Micromolar concentrations of amiloride block this Na^+ conductance in isolated taste cells and reduce Na^+ salt stimulation of gustatory nerve fibers [42]. The presumed mechanism for taste cell response to salt is direct influx of Na^+ through this amiloride-blockable channel, leading to taste-cell depolarization, action-potential generation and neurotransmitter release. These Na^+ channels share a number of physiological and pharmacological properties with ENaC channels. Members of the degenerin (DEG)/ENaC ion channel superfamily, ENaCs are commonly found in epithelial cells involved in solute transport, such as renal epithelial cells. DEG/ENaC family members display a common topology, including two putative transmembrane helices, a large extracellular loop and a smaller pore-forming loop (Fig. 50-8). Although α, β and γ subunits of the ENaC channel have been localized to the apical membranes of TRCs (ENaCs function as heteroligomeric complexes), they have still not been definitively linked to salt taste. Furthermore, other mechanisms must contribute to the transduction of Na^+ taste, since about 20% of the afferent nerve response to NaCl is

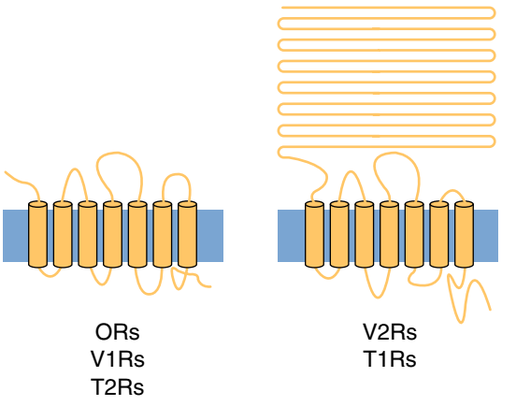

FIGURE 50-8 Topology of GPCR-type chemosensory receptors. Chemosensory receptors are found in two major GPCR classes: Class A (rhodopsin-like) and Class C (mGluR-like). ORs, V1R vomeronasal receptors and T2R bitter taste receptors are all Class A, though they share no amino-acid sequence. V2R vomeronasal receptors and T1R taste receptors are Class C GPCRs, and have the long, extracellular *N*-terminus stereotypical of that class. V2Rs and T1Rs also exhibit no amino-acid sequence similarity.

amiloride-insensitive. One of these mechanisms is believed to involve permeation of Na^+ through the tight junctions that connect taste cells at the apex of the taste bud; the Na^+ presumably enters taste cells through basolateral Na^+ channels [43]. Recently, a TRPV1-like channel has been implicated in amiloride-insensitive salt taste in mice [44].

Sour taste is a function of the acidity of a solution, depending primarily on the proton concentration and to a lesser extent on the particular anion involved. As with salty taste, sour taste is perceived as a result of the direct interaction of protons with ion channels of taste cells. Sour stimuli can depolarize TRCs by either activating cation channels or directly permeating ion channels. Several mechanisms have been found to contribute to sour transduction. As for salt taste, DEG/ENaC channels may be involved in sour taste transduction. For example, in hamsters, protons enter the cell through an amiloride-sensitive Na^+ channel; in the absence of Na^+, protons permeate the channel, depolarizing taste receptor cells [45]. Clearly, other mechanisms must be involved or we could not distinguish salts from acids. ASICs (acid sensing ion channels) and MDEG1/BnaC1 (mammalian degenerin-1/brain-type sodium channel-1) are directly activated by protons and are expressed in taste tissue. Other mechanisms of acid transduction could include proton permeation of the tight junctions at the apex of the taste bud or acidification of potassium channels. The basolateral targets of protons have not been identified.

Taste cells contain receptors, G proteins and second-messenger-effector enzymes. Research on the biochemistry and molecular biology of taste has long lagged behind that on vision or olfaction, because of the relative inaccessibility of taste receptor cells and the lack of high-affinity ligands specific for taste receptors. However, more recently, significant progress has been made in identifying important players in the transduction of sweet, umami and bitter taste. It is now clear that these tastants are transduced predominantly, if not exclusively, by G-protein-coupled, second-messenger cascades.

Gustducin is a taste-cell-specific G protein closely related to the transducins. The first heterotrimeric G protein subunit to be implicated in taste transduction was α-gustducin. This novel G protein α subunit was first cloned from rat taste tissue [46] and is expressed in about 25–30% of TRCs of the circumvallate, foliate and fungiform papillae [47]. Gustducin is most closely related to the transducins (Ch. 49), the rod-and-cone-photoreceptor G proteins, which have also been found in taste cells. Gustducin has been implicated in the transduction of bitter tasting stimuli through the activation of a phosphodiesterase [47]. In TRCs, α-gustducin associates with the G protein $\beta\gamma$ subunits $G\beta_3$ and $G\gamma_{13}$. Interestingly, these subunits activate a phosphoinositide-mediated signaling pathway through phospholipase C-β_2 (PLCβ_2) [48]. PLCβ_2 is also critical for the transduction of bitter tastants both *in vitro* [48] and *in vivo* [49]. It remains unclear whether a cyclic nucleotide cascade may contribute to the transduction of bitter tastants through the modulation of phophoinositide signaling, or via a more direct mechanism.

Gustducin plays a role in sweet and umami taste, as well. As expected, gustducin null mice, in which the gene encoding gustducin has been deleted by gene targeting, exhibit normal responses to salts and acids but reduced responses to bitter stimuli [50]. Surprisingly, these null mice also had reduced responses to sweet and umami stimuli [50, 51]. Highlighting the fact that taste transduction mechanisms may subtly vary across the gustatory epithelium (see below), gustducin is important for umami detection from anterior, but not posterior taste buds. Further studies will be required to determine the precise role of gustducin in the sweet transduction cascade.

Sweet, bitter and umami taste involve receptor-coupled second-messenger pathways that are differentially expressed across the gustatory epithelium. Though sweet and bitter transduction may share some common downstream signaling mechanisms, it was hypothesized that each must utilize different groups of receptor proteins. Though the first taste-specific GPCRs were identified several years ago [52], for many years they remained orphan receptors (i.e. their physiological ligands were unknown). Now called T1R1 and T1R2, these receptors are Class C GPCRs and contain a large extracellular *N*-terminus that is thought to contain the ligand-binding domain (Fig. 50-8). Recently, several groups have utilized a combination of molecular biological and genetic approaches to identify the gene encoding a third T1R, T1R3 [53–59]. The T1Rs are differentially distributed across the gustatory epithelium: T1R1 and T1R2 are expressed in distinct populations of TRCs (T1R1 is found predominantly on the anterior tongue and T1R2 mostly on the posterior tongue), but are always coexpressed with T1R3. This differential pattern of expression suggested varied functions of T1R1 and T1R2, a possibility born out by subsequent *in vitro* and *in vivo* studies (see below).

Certain mutations in the mouse *Tas1r3* gene (which encodes T1R3) correlate with a reduced sensitivity to sweet compounds, including saccharin and many natural sugars [60]. The identification of *Tas1r3* as the saccharin-sensitivity gene *Sac* provided the first evidence that the T1Rs might be involved in sweet taste. The T1Rs function as heteromeric receptors (probably dimers), with T1R2 and T1R3 combining to form a receptor for sweet-tasting compounds including sugars, sweeteners, and some D-amino acids [55] (T1R3 can also form homomeric receptors responsive to high concentrations of natural sugars). In contrast, T1R1/T1R3 heteromers are insensitive to sweet-tasting stimuli but do respond to umami stimuli, including many L-amino acids [61, 62]. Furthermore, T1R1/T1R3 responses are potentiated by 5′-ribonucleotides, a characteristic of umami taste. Although it is now accepted that the T1Rs are essential for normal sweet and umami

taste sensitivity in mammals, some studies have implicated T1R-independent mechanisms, as well [63,64]. Resolution of this controversy awaits further experimentation.

A distinct receptor family is involved in the detection of bitter tastants. Bitter agents are very structurally diverse, suggesting multiplicity of receptors and/or multiplicity of detection pathways. Many bitter compounds are lipophilic and membrane-permeant, and may act on intracellular or integral membrane targets. However, it is clear that GPCRs play a central role in the detection of bitter stimuli. The T2R family comprises ~30 Class A GPCRs (Fig. 50-8) whose genes (the *Tas2rs*) were first identified based on their proximity to known genetic loci involved in bitter taste [65]. Subsequent *in vitro* experiments have paired several of these receptors to a number of bitter ligands [1], confirming the role of T2Rs in bitter taste. Unlike the T1Rs, which show a somewhat differential pattern of expression, it appears that T2R-containing TRCs express most, if not all, of the *Tas2r* genes. This overlapping pattern of expression is consistent with bitter-taste behavior, as animals do a poor job of discriminating bitter compounds and may be more dependent on high taste-sensitivity to these compounds, which are often toxic.

How T1Rs and T2Rs are coupled to intracellular signaling cascades is still unclear, but a model for the major molecular players is presented in Figure 50-9. Gustducin is critical for bitter, sweet and umami taste, but is unlikely to account for all T1R or T2R-mediated signaling as gustducin knockout animals retain residual taste responses [50]. Other G-protein isoforms have been implicated in taste, including transducin, $G\beta_3$ and $G\gamma_{13}$, more may play a role, as well [47].

Both cyclic-nucleotide and phosphoinositide-second-messenger signaling mechanisms have been implicated in bitter, sweet and umami taste [47]. The strongest evidence supports a role of PLCβ2 in the transduction of stimuli of these three taste qualities. Deletion of the gene encoding PLCβ2 from mice abolishes behavioral responses to bitter, sweet or umami stimuli, while restoration of PLCβ2 expression in only T2R-expressing cells rescues bitter taste alone [49].

In many cells, phosphoinositide signaling leads to an elevation in intracellular calcium levels through the release of calcium from intracellular stores in response to IP_3-dependent gating of channels in the endoplasmic epithelium (Ch. 20). It is not known if IP_3 plays a critical role in TRC transduction, but such a role would be consistent with recent findings that a Ca^{2+}-activated cation channel, TRPM5, is essential for normal sweet, bitter and umami taste function [49, 66–69].

Many textbooks continue to state incorrectly that taste sensitivities are strictly localized on the gustatory epithelium. However, there is some regionalization of sensitivity that is consistent with the observation that taste receptors and intracellular signaling components are differentially localized across the gustatory epithelium [70] (Fig. 50-10). For example, gustducin is only expressed in a subset of TRCs, although it is found across the epithelium. ENaC is heavily expressed in the anterior tongue and palate, but at lower levels in the posterior tongue. Gustducin is implicated in umami detection from the anterior, but not the posterior tongue. A number of gustducin-expressing cells do not express TRPM5. These differential patterns of expression highlight the functional and molecular

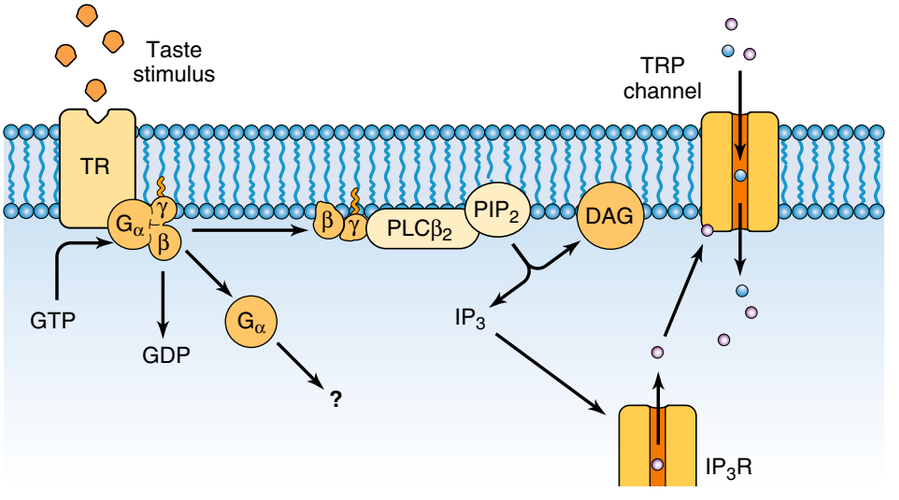

FIGURE 50-9 A model for the major signaling mechanisms for the transduction of bitter, sweet and umami stimuli. The individual steps are detailed in the text. Note that stimuli of each of these taste qualities interact with GPCRs: bitter stimuli with T2Rs and sweet and umami stimuli with T1Rs. α-gustducin has been implicated in the transduction of all three types of stimuli, but other α-subunits may couple to T1Rs and T2Rs in some TRC subpopulations (e.g. Fig. 50-10). PLCβ2 and the Ca^{2+}-activated TRP channel subunit TRPM5 are essential for normal bitter, sweet and umami taste, but other mechanisms may play some role as well. For example, some bitter stimuli may diffuse through the plasma membrane to activate intracellular proteins directly, and cyclic nucleotide-dependent signaling has been implicated in both bitter and sweet taste [47, 70]. The role of IP_3 and the IP_3R in the stimulus-dependent increase in intracellular Ca^{2+} as depicted is speculative.

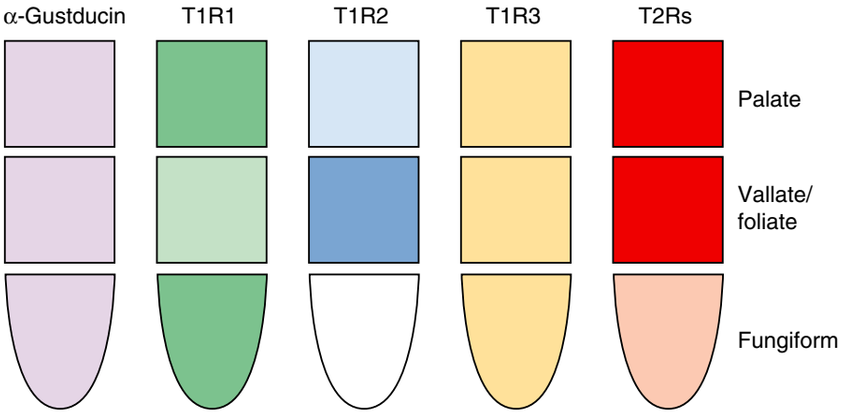

FIGURE 50-10 Many taste transduction elements are differentially expressed across the gustatory epithelium. Three areas of epithelium are represented: the anterior tongue (with fungiform papillae), the posterior tongue (with vallate and foliate papillae) and the soft palate. *Darker colors* represent relatively greater numbers of TRCs expressing that protein. It is important to note that within a given region, only a subset of TRCs will express a given protein. For example, α-gustducin is expressed in 20–30% of TRCs, and α-gustducin is co-expressed with T2Rs but is found in only subsets of T1R-expressing TRCs. The specifics of these expression patterns also probably vary between species. Modified from [70], with permission.

complexity of taste transduction. A major effort in taste transduction research over the coming years will focus on understanding how subtle differences in transduction mechanisms across TRC subpopulations may have an impact on taste function.

ACKNOWLEDGMENTS

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REFERENCES

1. Mombaerts, P. Genes and ligands for odorant, vomeronasal and taste receptors. *Nat. Rev. Neurosci.* 5: 263–278, 2004.
2. Firestein, S. How the olfactory system makes sense of scents. *Nature* 413: 211–218, 2001.
3. Aungst, J. L. *et al.* Centre-surround inhibition among olfactory bulb glomeruli. *Nature* 426: 623–629, 2003.
4. Buck, L. and Axel, R, A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65: 175–187, 1991.
5. Liu, A. H. *et al.* Motif-based construction of a functional map for mammalian olfactory receptors. *Genomics* 81: 443–456, 2003.
6. Glusman, G. *et al.* The complete human olfactory subgenome. *Genome Res* 11: 685–702, 2001.
7. Zozulya, S., Echeverri, F. and Nguyen, T. The human olfactory receptor repertoire. *Genome Biol.* 2: research0018. 1–research0018. 12, 2001.
8. Zhang, X. and Firestein, S. The olfactory receptor gene superfamily of the mouse. *Nat. Neurosci.* 5: 124–133, 2002.
9. Young, J. M. *et al.* Different evolutionary processes shaped the mouse and human olfactory receptor gene families. *Hum. Mol. Genet.* 11: 535–546, 2002.
10. Araneda, R. C. *et al.* A pharmacological profile of the aldehyde receptor repertoire in rat olfactory epithelium. *J. Physiol.* 555: 743–756, 2004.
11. Malnic, B. *et al.* Combinatorial receptor codes for odors. *Cell* 96: 713–723, 1999.
12. Ressler, K. J., Sullivan, S. L. and Buck, L. B. A zonal organization of odorant receptor gene expression in the olfactory epithelium. *Cell* 73: 597–609, 1993.
13. Vassar, R., Ngai, J. and Axel, R. Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell* 74: 309–318, 1993.
14. Serizawa, S. *et al.* Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. *Science* 302: 2088–2094, 2003.
15. Lewcock, J. W. and Reed, R. R. A feedback mechanism regulates monoallelic odorant receptor expression. *Proc. Natl Acad. Sci USA* 101: 1069–1074, 2004.
16. Astic, L., Saucier, D. and Holley, A. Topographical relationships between olfactory receptor cells and glomerular foci in the rat olfactory bulb. *Brain Res.* 424: 144–152, 1987.
17. Ressler, K. J., Sullivan, S. L. and Buck, L. B. Information coding in the olfactory system : evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell* 79: 1245–1255, 1994.
18. Vassar, R. *et al.* Topographic organization of sensory projections to the olfactory bulb. *Cell* 79: 981–991, 1994.
19. Mombaerts, P. *et al.* Visualizing an olfactory sensory map. Cell 87: 675–686, 1996.
20. Mori, K. and Yoshihara, Y. Molecular recognition and olfactory processing in the mammalian olfactory system. *Prog. Neurobiol.* 45: 585–619, 1995.
21. Zufall, F. and Munger, S. D. From odor and pheromone transduction to the organization of the sense of smell. *Trends Neurosci.* 24: 191–193, 2001.
22. Jones, D. T. and Reed, R. R. G_{olf}: an olfactory neuron specific-G protein involved in odorant signal transduction. *Science* 244: 790–795, 1989.

23. Bakalyar, H. A. and Reed, R. R. Identification of a specialized adenylyl cyclase that may mediate odorant detection. *Science* 250: 1403–1406, 1990.
24. Wong, S. T. *et al.* Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. *Neuron* 27: 487–497, 2000.
25. Nakamura, T. and Gold, G. H. A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* 325: 442–444, 1987.
26. Kaupp, U. B. and Seifert, R. Cyclic nucleotide-gated ion channels. *Physiol. Rev.* 82: 769–824, 2002.
27. Brunet, L. J., Gold, G. H. and Ngai, J. General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. *Neuron* 17: 681–693, 1996.
28. Kurahashi, T. and Yau, K. W. Olfactory transduction. Tale of an unusual chloride current. *Curr. Biol.* 4: 256–258, 1994.
29. Chen, T. Y. and Yau, K. W. Direct modulation by Ca^{2+}-calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. *Nature* 368: 545–548, 1994.
30. Munger, S. D. *et al.* Central role of the CNGA4 channel subunit in Ca^{2+}-calmodulin-dependent odor adaptation. *Science* 294: 2172–2175, 2001.
31. Zufall, F. and Leinders-Zufall, T. The cellular and molecular basis of odor adaptation. *Chem. Senses* 25: 473–481, 2000.
32. Spehr, M. *et al.* 3-phosphoinositides modulate cyclic nucleotide signaling in olfactory receptor neurons. *Neuron* 33: 731–739, 2002.
33. Loconto, J. *et al.* Functional expression of murine V2R pheromone receptors involves selective association with the M10 and M1 families of MHC class Ib molecules. *Cell* 112: 607–618, 2003.
34. Leinders-Zufall, T. *et al.* Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* 405: 792–796, 2000.
35. Liman, E. R., Corey, D. P. and Dulac, C. TRP2 : a candidate transduction channel for mammalian pheromone sensory signaling. *Proc. Natl Acad. Sci. USA* 96: 5791–5796, 1999.
36. Stowers, L. *et al.* Loss of sex discrimination and male–male aggression in mice deficient for TRP2. *Science* 295: 1493–1500, 2002.
37. Leypold, B. G. *et al.* Altered sexual and social behaviors in trp2 mutant mice. *Proc. Natl Acad. Sci. USA* 99: 6376–6381, 2002.
38. Lucas, P. *et al.* A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone transduction. *Neuron* 40: 551–561, 2003.
39. Roper, S. D. The microphysiology of peripheral taste organs. *J. Neurosci.* 12: 1127–1134, 1992.
40. Zhao, G. Q. *et al.* The receptors for mammalian sweet and umami taste. *Cell* 115: 255–266, 2003.
41. Bigiani, A., Ghiaroni, V. and Fieni, F. Channels as taste receptors in vertebrates. *Prog. Biophys. Mol. Biol.* 83: 193–225, 2003.
42. Heck, G. L., Mierson, S. and DeSimone, J. A. Salt taste transduction occurs through an amiloride-sensitive sodium transport pathway. *Science* 223: 403–405, 1984.
43. Ye, Q., Heck, G. L. and DeSimone, J. A. The anion paradox in sodium taste reception: resolution by voltage-clamp studies. *Science* 254: 724–726, 1991.
44. Lyall, V. *et al.* The mammalian amiloride-insensitive nonspecific salt taste receptor is a vanilloid receptor-1 variant. *J. Physiol.* 558: 147–159, 2004.
45. Gilbertson, T. A. *et al.* Proton currents through amiloride-sensitive Na channels in hamster taste cells. Role in acid transduction. *J. Gen. Physiol.* 100: 803–824, 1992.
46. McLaughlin, S. K., McKinnon, P. J. and Margolskee, R. F. Gustducin is a taste-cell-specific G protein closely related to the transducins. *Nature* 357: 563–569, 1992.
47. Margolskee, R. F. Molecular mechanisms of bitter and sweet taste transduction. *J. Biol. Chem.* 277: 1–4, 2002.
48. Huang, L. *et al.* Ggamma13 colocalizes with gustducin in taste receptor cells and mediates IP3 responses to bitter denatonium. *Nat. Neurosci.* 2: 1055–1062, 1999.
49. Zhang, Y. *et al.* Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* 112: 293–301, 2003.
50. Wong, G. T., Gannon, K. S. and Margolskee, R. F. Transduction of bitter and sweet taste by gustducin. *Nature* 381: 796–800, 1996.
51. He, W. *et al.* Umami taste responses are mediated by alpha-transducin and alpha-gustducin. *J. Neurosci.* 24: 7674–7680, 2004.
52. Hoon, M. A. *et al.* Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell* 96: 541–551, 1999.
53. Max, M. *et al.* Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. *Nat. Genet.* 28: 58–63, 2001.
54. Montmayeur, J. P. *et al.* A candidate taste receptor gene near a sweet taste locus. *Nat. Neurosci.* 4: 492–498, 2001.
55. Nelson, G. *et al.* Mammalian sweet taste receptors. *Cell* 106: 381–390, 2001.
56. Sainz, E. *et al.* Identification of a novel member of the T1R family of putative taste receptors. *J. Neurochem.* 77: 896–903, 2001.
57. Li, X. *et al.* High-resolution genetic mapping of the saccharin preference locus (Sac) and the putative sweet taste receptor (T1R1) gene (Gpr70) to mouse distal Chromosome 4. *Mamm. Genome.* 12: 13–16, 2001.
58. Bachmanov, A. A. *et al.* High-resolution genetic mapping of the sucrose octaacetate taste aversion (Soa) locus on mouse Chromosome 6. *Mamm. Genome.* 12: 695–699, 2001.
59. Kitagawa, M. *et al.* Molecular genetic identification of a candidate receptor gene for sweet taste. *Biochem. Biophys. Res. Commun.* 283: 236–242, 2001.
60. Reed, D. R. *et al.* Polymorphisms in the taste receptor gene (Tas1r3) region are associated with saccharin preference in 30 mouse strains. *J. Neurosci.* 24: 938–946, 2004.
61. Nelson, G. *et al.* An amino-acid taste receptor. *Nature* 416: 199–202, 2002.
62. Li, X. *et al.* Human receptors for sweet and umami taste. *Proc. Natl Acad. Sci. USA* 99: 4692–4696, 2002.
63. Chaudhari, N., Landin, A. M. and Roper, S. D. A metabotropic glutamate receptor variant functions as a taste receptor. *Nat. Neurosci.* 3: 113–119, 2000.
64. Damak, S. *et al.* Detection of sweet and umami taste in the absence of taste receptor T1r3. *Science* 301: 850–853, 2003.
65. Adler, E. *et al.* A novel family of mammalian taste receptors. *Cell* 100: 693–702, 2000.

66. Perez, C. A. *et al.* A transient receptor potential channel expressed in taste receptor cells. *Nat. Neurosci.* 5: 1169–1176, 2002.
67. Hofmann, T. *et al.* TRPM5 is a voltage-modulated and Ca^{2+}–activated monovalent selective cation channel. *Curr. Biol.* 13: 1153–1158, 2003.
68. Liu, D. and Liman, E. R. Intracellular Ca^{2+} and the phospholipid PIP2 regulate the taste transduction ion channel TRPM5. *Proc. Natl Acad. Sci. USA* 100: 15160–15165, 2003.
69. Prawitt, D. *et al.* TRPM5 is a transient Ca^{2+}-activated cation channel responding to rapid changes in Ca^{2+} i. *Proc. Natl Acad. Sci. USA* 100: 15166–15171, 2003.
70. Gilbertson, T. A. and Boughter, J. D. Jr, Taste transduction: appetizing times in gestation. *Neuroreport* 14: 905–911, 2003.
71. Young, J. M. and Trask, B. J. The sense of smell: genomics of vertebrate odorant receptors. *Hum. Mol. Genet.* 11: 1153–1160, 2002.

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CHAPTER

51

Molecular Biology of Hearing and Balance

Peter G. Gillespie

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A unified model for mechanotransduction allows comparison of mechanoreceptors from many organisms and cell types 833

MODEL SYSTEMS 834
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Hair cells are specialized mechanoreceptors located in the inner ear; these cells transduce mechanical forces transmitted by sound and head movement, and permit an organism to sense features of the external world. Well-characterized biophysically, a molecular description of hair-cell transduction has finally begun to emerge. Although many key molecules remain to be identified, striking molecular and functional correspondences between vertebrate hair cells and invertebrate mechanoreceptors have indicated that some types of mechanoreceptors probably share a common ancestor. The continued application of genetic, molecular, biological and biophysical approaches should lead to a more thorough understanding of this critical sensory cell.

GENERAL FEATURES OF MECHANORECEPTORS

Mechanotransduction is of great utility for all organisms. Most multicellular organisms exploit mechanoreceptors — specialized cells that detect external mechanical forces — to help construct their internal view of the external world. The senses of hearing, balance and touch all rely on mechanoreceptors, as do the proprioceptive sensations that tell an organism how it is situated in the environment. Mechanoreception is probably one of the most ancient of the senses.

A unified model for mechanotransduction allows comparison of mechanoreceptors from many organisms and cell types. Mechanoreceptors nearly universally use ion channels for transducing sensory information. Mechanoreceptors are either neurons or are neuroepithelial cells with synapses, and the currency of the nervous system is the membrane potential. Opening an ion channel allows a cell quickly and extensively to modulate its membrane potential, and hence neurotransmitter release, the final step in mechanoreception at the cellular level.

Ion channels open or close upon relative movement of internal domains, movements that can be elicited by

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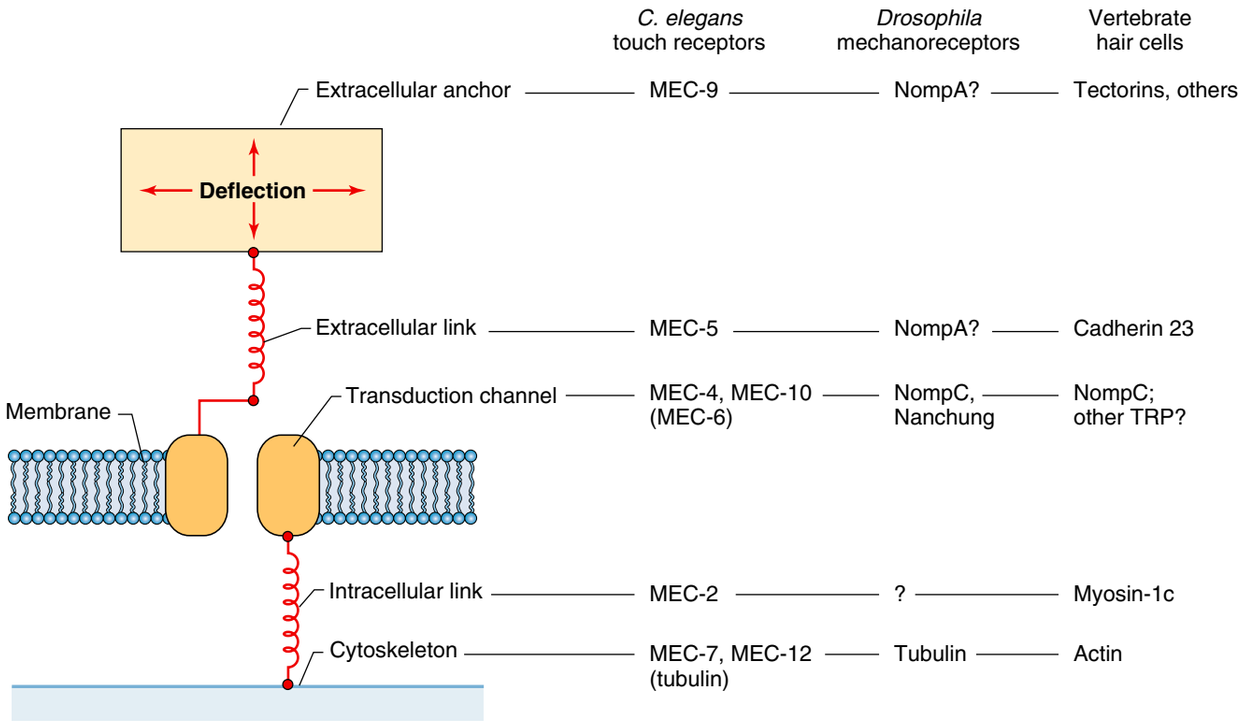

FIGURE 51-1 General model for mechanotransduction. **Left**, a transduction channel, embedded in the plasma membrane, is anchored mechanically to the cytoskeleton and to an extracellular structure that can move relative to the cell. Movements of the extracellular structure relative to the cytoskeleton cause tension to develop throughout the system, which is delivered to the gate of the channel. Increased gating tension causes channels to open. **Right**, candidate proteins are named from the best-characterized mechanoreceptor systems for each of the elements indicated on the left.

voltage, ligand binding, or force (see Ion Channels in Ch. 6). How can force influence domain movements within ion channels? In some cases, domains can be moved by tension within the plasma membrane. Bacterial osmosensors are thought to work this way; as membrane tension increases, the channels gate to reduce tension [1]. However, most mechanoreceptors apparently do not sense membrane tension. Instead, independent investigation of hair cells, worm touch receptors and fly bristles have converged on a single general model for how transduction channels are gated (Fig. 51-1). In this model, the ion channel is anchored on both sides of the membrane. The intracellular anchor is the cytoskeleton, actin in the case of hair cells and microtubules in the case of characterized mechanoreceptors in worms and flies. The extracellular anchor varies substantially from cell type to cell type. The critical feature of this model is that external forces cause a net displacement of the two anchors; the force is transmitted to the channel, and net domain movement is triggered [1].

MODEL SYSTEMS

Understanding the general features of mechanoreceptors has been greatly assisted by the use of model systems. Genetically tractable organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* have permitted a much more thorough inventory of the parts required for mechanotransduction than is possible in most vertebrates; the recent ascent of the zebrafish *Danio rerio* as a model has extended the advantages of a genetic organism to vertebrate mechanotransduction.

Genes responsible for touch detection in *C. elegans* have been identified and suggest a model for mechanical transduction. Worms like *C. elegans* respond to a gentle touch on the nose by turning away. Mechanical deformation of the worm's cuticle stimulates specific cells (six body-touch sensory neurons), which transmit sensory information to neural circuits that control locomotion. Genetic experiments have revealed ~12 genes involved in this response, and the identities of these genes have suggested a specific molecular model for the transduction apparatus (reviewed in [2]). Extracellular matrix molecules (MEC-5, MEC-9) presumably are mechanically connected to ion channels formed by MEC-4 and MEC-10 subunits, which belong to the DEG/ENaC family of channels. MEC-6 also contributes to this channel complex. These ion channels are connected (probably by MEC-2) to an underlying microtubule network, which consists of unique 15-protofilament microtubules (tubulin subunits encoded by the *mec-7* and *mec-12* genes). Movement of the cuticle relative to the microtubule

network mechanically stimulates the transduction channel, which admits cations that depolarize the cell. Recent work has shown that excitation of the mechanosensory neurons leads rapidly to Ca^{2+} influx, apparently through MEC-4-type channels, which can be detected in live worms subject to touch stimuli [3]. Although direct mechanical gating of the DEG/ENaC channels has not been observed in intact neurons or cultured cells, the preponderance of the evidence supports this model.

Is this system relevant for hair cells? It does not appear so. Indeed, there appear to be at least two broad classes of mechanoreceptors, one like those of *C. elegans* touch neurons that rely on DEG/ENaC channels, and another class that apparently relies on *transient receptor potential* (TRP) channels. As we will see later, circumstantial evidence suggests that hair cells use TRP channels. Other mechanoreceptors in vertebrates may be related to *C. elegans* touch cells.

Polymodal sensory neurons in *C. elegans* are also mechanoreceptors. Polymodal sensory neurons in *C. elegans* seem to fall into the TRP class of mechanoreceptors as they apparently rely on several TRP channels for mechanosensation [4]. These sensory neurons respond to nose touch, hyperosmolarity and volatile repellents by triggering a backward response in the worm; the OSM-9 and OCR-2 channels apparently mediate these responses, and the pathways differ for each sensory modality. However, little is known about the transduction mechanism in these cells.

***Drosophila* bristle receptors and chordotonal organs are surface mechanoreceptors.** These surface mechanoreceptors on insects have been studied for many years. Progress on identifying molecules responsible for insect mechanoreception received a big boost in 1994, when Kernan *et al.* developed a screen for mechanoreceptor mutants in *Drosophila* [5]. They identified several dozen mutants that were deficient in mechanoreception and, with the assistance of their colleagues, have begun to identify them.

The most important molecules so far identified from this screen include a likely transduction channel, an extracellular molecule that could gate channels, and several molecules known to be important for axonemal structure and function. Although the set of molecules is less complete than that identified for *C. elegans* touch receptors, the diversity of mechanotransduction in *Drosophila* and the apparent similarity of these receptors to those in vertebrates, including hair cells (see below), demonstrates the significance of this model system.

The protein NompC is a TRP channel and appears to be the major transduction channel in fly bristles [6]. Bristle mechanotransduction resembles hair-cell transduction remarkably in its speed, polarity and adaptation [6], suggesting the possibility of a close evolutionary relationship between these mechanoreceptors. NompC is not the only bristle transduction channel, however, as a residual transduction current remains in *nompC* null flies. Moreover, NompC plays a relatively small role in fly hearing [7], where the TRP channel *nanchung* is essential [8]. These results have intensified the search for TRP channels that could mediate transduction in hair cells, as discussed below.

The gene *nompA* encodes a multi-domain extracellular protein that is expressed by support cells that cradle the mechanically sensitive neuron [9]. NompA is localized to the dendritic cap, which is covers the mechanically sensitive outer segment of the neuron. NompA is a good candidate for a protein that couples mechanically sensitive channels to external stimuli.

nompB is the gene for a protein involved in intraflagellar transport, the basic mechanism by which axonemal structures like mechanoreceptor dendrites are maintained by the cell [10]. We certainly expect that this screen will identify genes like *nompB*, just as deafness screens have identified proteins thought to be involved in hair-cell structural integrity.

Insect mechanoreceptors and hair cells share evolutionary relationships. In flies, the proneural gene *atonal* controls development of mechanoreceptors; *Math1* plays a similar role in mice. Indeed, *atonal* can fully substitute for *Math1* in mice and *Math1* can fully substitute for *atonal* in flies [11], suggesting that these two disparate systems might share a common genetic program used for development. As another example, as we will discuss later, *Drosophila* bristle mechanoreceptors and zebrafish hair cells each rely on the TRP channel NompC for mechanotransduction.

HAIR CELLS

Hair cells are the sensory cells of the auditory and vestibular systems. Hair cells are the sensory cells of the internal ear, essential for the senses of sound and balance. The hair cell's transduction apparatus, the molecular machinery that converts forces and displacements into electrical responses, can respond to mechanical stimuli of less than 1 nm in amplitude, and of tens or even hundreds of kilohertz in frequency. Indeed, our hearing is ultimately limited by Brownian motion of water molecules impinging on the transduction apparatus.

Even though well-characterized at a biophysical level, the mechanical transduction mechanism of hair cells is still not fully understood in molecular terms. This discrepancy is in part due to the extreme scarcity of hair cells; instead of the millions or even hundreds of millions of receptor cells that the olfactory and visual systems possess, only a few tens of thousands of hair cells are found in the internal ears of most vertebrate species. The small number of hair cells and the direct transduction mechanism has greatly impeded molecular biological and

biochemical characterization. Consequently, molecular description of hair-cell transduction has lagged well behind description of vision and olfaction.

Hair cells are neuroepithelial cells; their large basolateral surface includes synaptic contacts with afferent and efferent nerve fibers, while the mechanically sensitive hair bundle is located on their apical surface. The hair bundle is an ensemble of 30–300 actin-filled stereocilia and a single axonemal kinocilium (Fig. 51-2). The kinocilium, present during development in all hair cells, degenerates in those cells sensitive to high auditory frequencies. Stereocilia range in height from 1 µm, for very high frequency auditory detection, to 50 µm or more, in some vestibular systems. Stereocilia contain hundreds of cross-linked actin filaments throughout their length; as the stereocilium approaches the apical surface of the hair cell, that number systematically declines to a dozen or two. Stereocilia are thus mechanically stiff throughout most of their length, but are flexible at the insertion point. Stereocilia do not flex independently, however, as they are cross-linked together by a variety of linkages, including ankle links, lateral links and tip links.

The consequence of the mechanical properties of the stereocilia and their interconnection by flexible linkages is that, when the bundle is deflected by a mechanical stimulus, the bundle moves as a whole. Moreover, individual stereocilia slide with respect to each other, a movement that underlies mechanical transduction (Fig. 51-3).

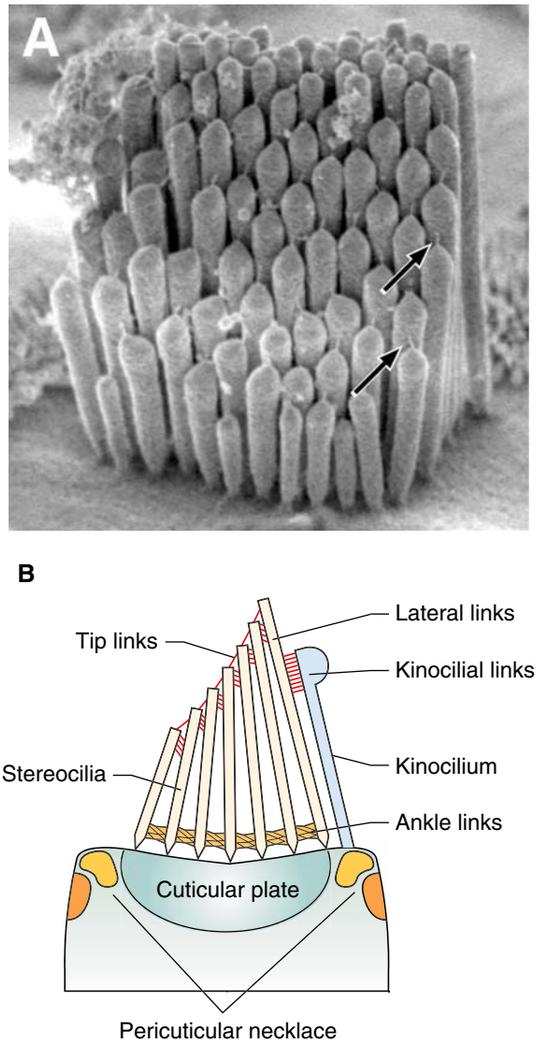

FIGURE 51-2 Hair-bundle structure. (**A**). The hair bundle, consisting of ~100 stereocilia, protrudes from the apical surface of this chicken-cochlea hair cell. Deflection of the bundle towards the taller stereocilia imparts tension onto tip links (indicated by *arrows*), which gate transduction channels. (**B**) Schematic depiction of a hair bundle. Actin-rich structures (e.g. stereocilia and cuticular plate) are indicated in *red*. The axonemal kinocilium, a microtubule-based cilium, is indicated in *blue*. The pericuticular necklace, a location of membrane vesicles bearing proteins targeted to the bundle, is indicated in *green*. Links between the stereocilia and between the tallest stereocilia and the kinocilium are also indicated.

Hair cells are exposed to unusual extracellular fluids and potentials. The apical surfaces of hair cells are exposed to an unusual extracellular fluid called endolymph. Endolymph is relatively similar in ionic composition to cytoplasm: it is high in K^+ (~150 mM), low in Na^+ (~2 mM), and relatively low in Ca^{2+} (~100 µM). Endolymph may have evolved to separate from hair cells the energy expenditure required to remove ions from hair cells. Normally, an excitatory cell like a neuron must pump out all of the ions that entered during an excitatory response (see Ch. 5). The ear separates this pumping task away from the hair cell. K^+, the major current-carrying ion, enters hair cells down an electrical gradient (hair cells are typically −60 mV) through transduction channels; when channels are closed, however, K^+ readily leaves through basolateral K^+ channels down the normal K^+ electrochemical gradient. The energy expenditure required for transduction is thus passed on to the stria vascularis cells, which, via the Na/K-ATPase, establish the high K^+ concentration in the endolymph.

The endolymphatic compartment of the auditory system is at an elevated potential (about +80 mV); this endocochlear potential increases the driving force on K^+ yet more, producing additional transduction current. Some mutations that cause deafness affect either K^+ levels in the endolymph or the endolymphatic potential itself.

Mechanical transduction depends on activation of ion channels linked to extracellular and intracellular structures. A comprehensive model for hair-cell transduction has emerged, derived primarily from biophysical and morphological investigations. Residing in the mechanoreceptive organelle of a hair cell, the hair bundle, the transduction apparatus consists of at least three components: the *transduction channel*, a mechanically gated ion channel; the *tip link*, an extracellular filament that transmits force to the channel's gate; and the *adaptation motor*,

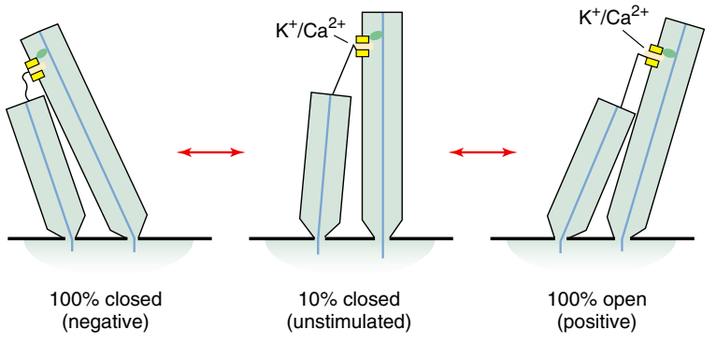

FIGURE 51-3 Hair-cell transduction. Only two stereocilia out of a bundle are shown. The transduction channel (*yellow*) is gated by the tip link. At rest, channels spend ~10% of the time open. An excitatory deflection moves the bundle towards the taller stereocilia; increased tension in the tip link leads to channel opening and entry of K^+ and Ca^{2+}. Moving the bundle towards the shorter stereocilia is inhibitory; this movement causes tension in the tip link to drop, and the channels then close.

a mechanism that maintains an optimal tension in the tip link so that the channel can respond to displacements of atomic dimensions (Fig. 51-3). The tip link is probably connected in series with the *gating spring*, an elastic element through which stimulus energy can affect the transduction channel. Although highly specialized for the internal ear, the channel, gating spring, and adaptation mechanism are likely to be general requirements for any mechanical transduction apparatus; detailed characterization of the transduction apparatus of the hair cell may therefore eventually illuminate other transduction systems.

In this model, deflection of the hair bundle in the excitatory direction — towards the tallest stereocilia — causes a shorter stereocilia to slide down its neighbor's side, stretching the gating spring. Tension in the gating spring is transmitted to the transduction channel, which responds by increasing the frequency and the duration of the openings.

Transduction channels do not remain open, even if a deflection is maintained; using two independent processes, the hair cell adapts to a sustained mechanical stimulus [12]. Rapid channel reclosure (sometimes called fast adaptation) occurs on a time-scale of a few milliseconds or less; Ca^{2+} entering open transduction channels binds to a site on or nearby the channel, causing the channel to close (Fig. 51-4). Because closing channels exert a negative-directed force on the hair bundle, the fast adaptation mechanism can mechanically amplify an input; if the negative force occurs during the negative phase of a sinusoidal stimulus, the forces add and bundle movement is enhanced.

A slower mechanism, operating on a time-scale of tens of milliseconds, adjusts the transduction apparatus so that the open probability at rest is optimal and fast adaptation is enhanced. Slow adaptation is mediated by a cluster of myosin molecules [13]; the myosins exert a resting tension, but the oppositely directed force in a stimulus overcomes the resting tension, dragging the myosin complex down the stereocilium (Fig. 51-5). As the motors descend the stereocilium, tension in the gating spring is reduced, and the transduction channels close. When the bundle is moved in the negative direction, reducing tension in the gating spring and hence tension applied to the motors, the adaptation motor climbs the actin of the stereocilium until the motors generate enough tension in the gating spring to stall themselves (Fig. 51-5).

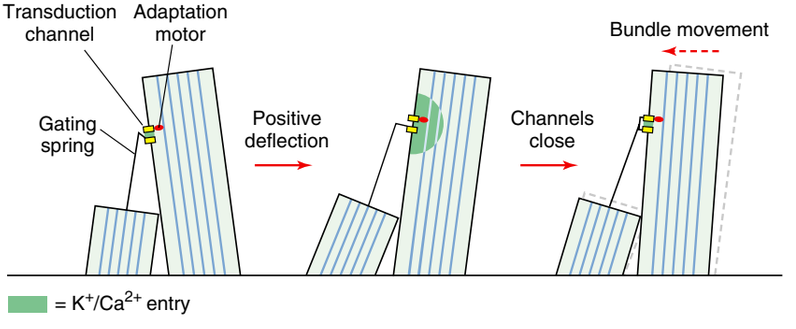

FIGURE 51-4 Fast adaptation by hair cells. After a positive (excitatory) deflection, Ca^{2+} enters transduction channels, binds to a site at the channel, yanking the channel shut. The movement of the channel's gate increases tension in the tip link, and the bundle is moved in the negative direction.

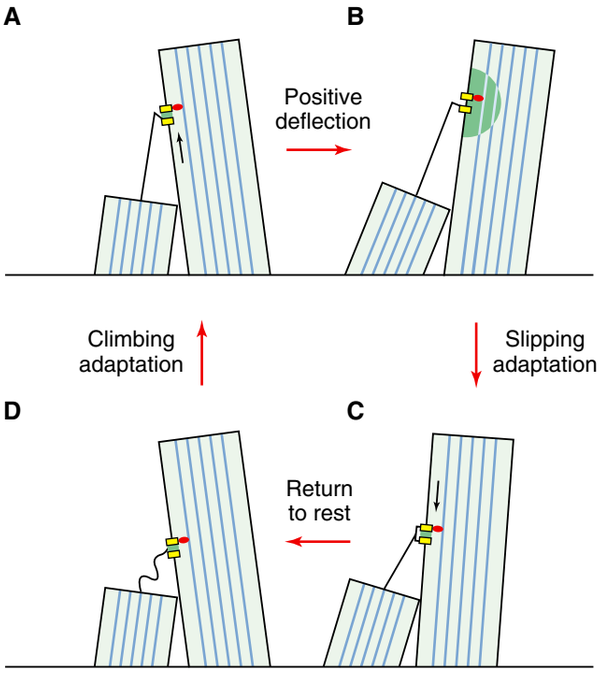

FIGURE 51-5 Slow adaptation by hair cells. (**A**) . Resting hair bundle. (**B**) An excitatory deflection lets in Ca^{2+} (*green*) and increases the tension felt by the motor (*blue*). (**C**) The adaptation motor responds to Ca^{2+} and high force by slipping towards the base of the stereocilium (*black arrow*). (**D**) When the bundle is returned to rest, gating spring tension is low and the motor can climb towards the tips of the stereocilia (*black arrow* in **A**).

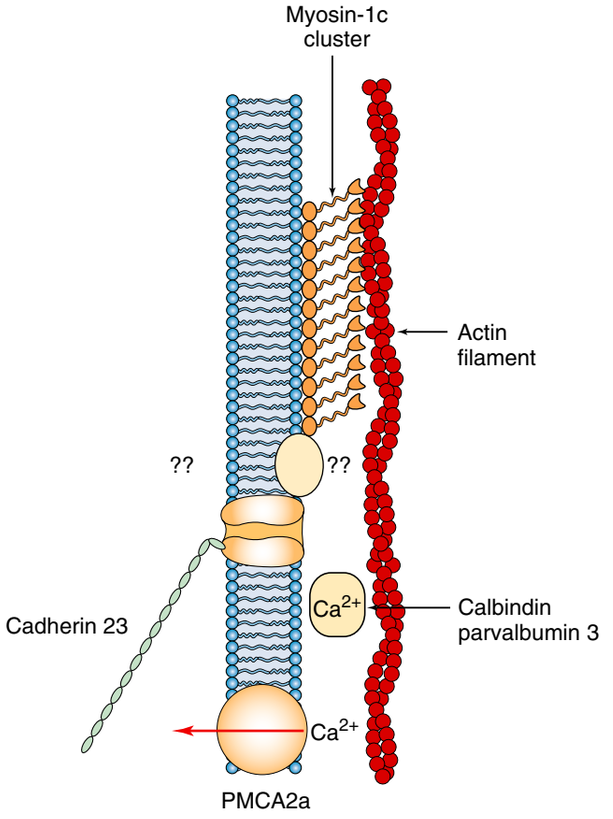

FIGURE 51-6 Molecules of the hair-cell transduction apparatus. Known molecules participating in transduction in hair cells are shown. Actin filaments, cross-linked by fimbrin and espin (not shown), are the substrate for slow adaptation. A cluster of myosin-1c molecules makes up the adaptation motor and is connected by an unknown molecule or molecules (*green oval*) to the transduction channel (*yellow*). The channel may be NompC in flies and fish but apparently is not in mammals. The channel is gated, perhaps directly, by cadherin-23, which makes up part of the tip link. Ca^{2+}, entering through open transduction channels, modulates fast and slow adaptation; it is rapidly buffered by mobile Ca^{2+} buffers including calbindin and paravalbumin 3. Ca^{2+} is eventually pumped out of the stereocilia by the Ca^{2+} pump *PMCA2a*.

Some of the molecules responsible for hair-cell transduction have been identified. A few key molecules, some already described, have been identified as part of the transduction complex (Fig. 51-6). Myosin molecules clearly play essential roles, and hair cells express a variety of myosin isoforms. Because it is located at the tips of the stereocilia, near the tip-link anchors, myosin-1c is the best candidate for the adaptation motor. Selective inhibition of a sensitized myosin-1c mutant with an ADP analog proved that this myosin participates in adaptation [14], although contribution by other myosins has yet to be ruled out. For example, mice with near-null mutations in myosin-7a have defects in auditory transduction that are consistent with alterations in the adaptation machinery, suggesting a central role for this myosin too [15].

Although mice with mutations in the myosin-6, -7a, and 15a genes are deaf, these isozymes apparently mediate essential hair-cell functions besides adaptation. Myosin-7a may play an important role in bundle integrity, as mice and fish with no functional myosin-7a protein have highly disheveled bundles.

Myosin-6 is an unusual isoform as it moves in the opposite direction along actin filaments, as compared to all other well-characterized myosins. Myosin-6 is located near the base of the hair bundle, and could play a role in anchoring the apical membrane to prevent fusion along the stereocilia shafts [16] or it may affect the forces applied to stereocilia actin filaments [17].

Myosin-15a is located at stereocilia tips and apparently controls the rate of actin filament growth [18]; stereocilia with more myosin-15a grow faster than those with lesser amounts of the myosin. Hair cells with no functional myosin-15a protein grow stereocilia that are very short and stubby, indicating that myosin-15a is important for actin filament elongation but not in the specification of the positions of the stereocilia.

Recent experiments have suggested that the tip link, a complex of two or three braided glycoprotein filaments [19], may be made in part from cadherin-23, a Ca^{2+}-dependent cell-adhesion molecule [20]. Moreover, cadherin-23 can interact directly or indirectly with myosin-1c, suggesting that these two molecules form part of the transduction complex in hair cells [20].

Although the transduction channel has attracted much attention, it too has yet to be identified. This channel passes cations, including Ca^{2+}, and can be inhibited by aminoglycoside antibiotics and amiloride derivatives. Unless controlled by tension applied through the gating spring, the channel remains shut. Most researchers in the field feel that the transduction channel belongs to the TRP channel family. TRP channels are remarkably diverse in structure and function and include many channels central for sensory function, including those involved in vision, pheromone sensing, temperature detection, and mechanical responsiveness. Indeed, TRP channels have been shown to be important in insects for mechanotransduction. In the fly, the TRP channel NompC plays a predominant role in the bristles and a lesser role in the auditory system. At least one TRP channel of the TRPV family, *nanchung*, is essential for fly hearing.

Are any of these TRP channels found in hair cells? The answer is yes for NompC, although the details are unexpected. NompC is found in zebrafish hair cells, where it has been shown to be essential for transduction [21]. Surprisingly enough, however, NompC has yet to be identified in mammalian genomes. This suggests the possibility that NompC is just one of several channel subunits required, and that other TRP channels could contribute to the transduction channel in mammals. Although several TRPV channels have been localized to hair cells in mammals, gene knockouts suggest that these channels do not play a role in transduction.

As both fast and slow adaptation mechanisms are regulated by Ca^{2+}, the stereocilia mechanisms that control the free concentration of this ion also play central roles in transduction. Entering Ca^{2+} is thought to be buffered very rapidly by the mobile buffers parvalbumin 3, calbindin, and calretinin [22, 23]. Even before bound Ca^{2+} can diffuse out of stereocilia, it is pumped back out into the endolymph by isoform 2a of the plasma-membrane Ca^{2+}-ATPase (PMCA2) [24, 25] (see also Ca^{2+} transport in Ch. 5).

DEAFNESS

About 1 in 1,000 children are born deaf, and another 1 in 1,000 develop deafness by adolescence. Although some deafness can arise during development due to external sources, much deafness in children is due to mutations in specific genes. As many forms of deafness are nonsyndromic — there is no other associated abnormality — identification of the responsible genes can lead to characterization of proteins that are essential for the auditory system (and often the vestibular system, as well).

The 'deafness gene' approach has proven fruitful for finding genes important to the auditory system. The genes responsible for ~20 recessive and ~20 dominant nonsyndromic deafness syndromes have been identified in humans, and similar numbers have been identified in mice [26]. Because some genes are associated with both recessive and dominant syndromes, and because most genes identified in humans have also been identified in mice, this tally corresponds to about 30 different genes.

The identified genes code for a variety of proteins, including transcription factors that are important for inner-ear development, cytoskeleton proteins that are responsible for the specialized architecture of hair cells, extracellular matrix molecules that make up some of the specialized acellular structures of the inner ear, and still others whose function is not yet clear.

A variety of genes have been identified, including a set of genes that apparently interact with each other in the auditory and visual systems. An example of a gene important for transduction that was identified in this manner was PMCA2. The deaf mouse *deafwaddler* was shown to have a mutation in the PMCA2 gene, which is called *Atp2b2* [27]; knockout of this gene also leads to deafness [28]. These results demonstrate that the Ca^{2+}-pumping activity of stereocilia is essential for auditory (and vestibular function), and reinforce the central role played by this ion in hair cell function.

Usher syndrome is a deafness–blindness syndrome that is caused by mutations in a number of genes [29]. Usher syndrome is divided into three classes depending on the severity of the phenotype; Usher I produces profound deafness and vestibular dysfunction, as well as adolescent blindness; in Usher II, deafness is less profound . User III is less common; in these patients, hearing and vision loss develop progressively, and vestibular dysfunction is variable.

Of the seven mapped genes underlying Usher I, five have been identified. These included cadherin-23 and myosin-7a, both described above, harmonin and SANS, both scaffold proteins, and protocadherin-15, another member of the cadherin superfamily. Biochemical evidence and phenotypic similarity suggests that these proteins may assemble into a complex [29], although conclusive evidence for such a complex is lacking.

Usher II is caused by mutations in at least four genes; only one, usherin, has been identified. Usherin is an extracellular matrix protein of unknown function; it is found in basement membranes in the eye and ear (and in other tissues as well). Similarly, at least two genes underlie Usher III, but only one (clarin-1) has been cloned. The function of clarin-1 is unknown.

The surprise is that genes clearly involved in mechanotransduction have not yet been identified by the deafness-gene approach. Clearly disruption of hair-cell function at many levels can lead to deafness, so it is expected that deafness genes include those involved in inner-ear development, in ion balance in the endolymph, and in structural integrity of hair cells. Examination of deafness genes in zebrafish has been particularly thorough, however, and

investigators using this system have several useful assays that allow them to focus only on those mutants that clearly have consequences for transduction [30].

However, the deafness-gene approach has not been successful in identifying either known (e.g. myosin-1c) or unknown (e.g. the transduction channel) components of the transduction apparatus in fish, mouse, or human. This is surprising, as the genetic approach relies on few assumptions about the nature of the systems studied. Perhaps the lesson here is that some of the important molecules for transduction play essential roles elsewhere in the organism during development; given the widespread expression of Myo1c, this presumption seems reasonable.

CONCLUSIONS

Although only a relatively small number of hair-bundle proteins have been identified, many of these clearly play important roles for transduction and bundle structure. The increasing speed of identification of important proteins suggests that a thorough accounting of the major transduction proteins for hair cells should not be too far off. The next and more interesting phase of characterization of hair-cell transduction will then ensue, determining how these molecules interact and how the hair cell assembles them during development and following normal protein turnover, in order to make a remarkably sensitive transduction apparatus.

REFERENCES

1. Sukharev, S. and Corey, D. P. Mechanosensitive channels: multiplicity of families and gating paradigms. *Sci. STKE* 2004, re4, 2004.
2. Goodman, M. B. and Schwarz, E. M. Transducing touch in *Caenorhabditis elegans. Annu. Rev. Physiol.* 65: 429–452, 2003.
3. Suzuki, H. *et al. In vivo* imaging of *C. elegans* mechanosensory neurons demonstrates a specific role for the MEC-4 channel in the process of gentle touch sensation. *Neuron* 39: 1005–1017, 2003.
4. Tobin, D. *et al.* Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in *C. elegans* neurons. *Neuron* 35: 307–318, 2002.
5. Kernan, M., Cowan, D. and Zuker, C., Genetic dissection of mechanosensory transduction: mechanoreception-defective mutations of *Drosophila. Neuron* 12: 1195–1206, 1994.
6. Walker, R. G., Willingham, A. T. and Zuker, C. S. A *Drosophila* mechanosensory transduction channel. *Science* 287: 2229–2234, 2000.
7. Eberl, D. F., Hardy, R. W. and Kernan, M. J. Genetically similar transduction mechanisms for touch and hearing in *Drosophila. J. Neurosci.* 20: 5981–5988, 2000.
8. Kim, J. *et al.* A TRPV family ion channel required for hearing in *Drosophila. Nature* 424: 81–84, 2003.
9. Chung, Y. D., Zhu, J., Han, Y. and Kernan, M. J. nompA encodes a PNS-specific, ZP domain protein required to connect mechanosensory dendrites to sensory structures. *Neuron* 29: 415–428, 2001.
10. Han, Y. G., Kwok, B. H. and Kernan, M. J. Intraflagellar transport is required in *Drosophila* to differentiate sensory cilia but not sperm. *Curr. Biol.* 13: 1679–1686, 2003.
11. Wang, V. Y., Hassan, B. A., Bellen, H. J. and Zoghbi, H. Y. *Drosophila* atonal fully rescues the phenotype of Math1 null mice: new functions evolve in new cellular contexts. *Curr. Biol.* 12: 1611–1616, 2002.
12. Fettiplace, R. and Ricci, A. J. Adaptation in auditory hair cells. *Curr. Opin. Neurobiol.* 13: 446–451, 2003.
13. Gillespie, P. G. and Cyr, J. L. Myosin-1c, the hair cell's adaptation motor. *Annu. Rev. Physiol.* 66: 521–545, 2004.
14. Holt, J. R. *et al.* A chemical–genetic strategy implicates myosin-1c in adaptation by hair cells. *Cell* 108: 371–381, 2002.
15. Kros, C. J. *et al.* Reduced climbing and increased slipping adaptation in cochlear hair cells of mice with Myo7a mutations. *Nat. Neurosci.* 5: 41–47, 2002.
16. Avraham, K. B., Hasson, T., Steel, K. P., Kingsley, D. M. and Russell, L. B. The mouse Snell's waltzer deafness gene encodes an unconventional myosin. *Nature Genetics* 11: 369–375, 1995.
17. Hasson, T. *et al.* Unconventional myosins in inner-ear sensory epithelia. *J. Cell Biol.* 137: 1287–1307, 1997.
18. Rzadzinska, A. K., Schneider, M. E., Davies, C., Riordan, G. P. and Kachar, B. An actin molecular treadmill and myosins maintain stereocilia functional architecture and self-renewal. *J. Cell Biol.* 164: 887–897, 2004.
19. Kachar, B., Parakkal, M., Kurc, M., Zhao, Y. and Gillespie, P. G. High-resolution structure of hair-cell tip links. *Proc. Natl Acad. Sci. USA* 97: 13336–13341, 2000.
20. Siemens, J. *et al.* Cadherin 23 is a component of the tip link in hair-cell stereocilia. *Nature* 428: 950–955, 2004.
21. Sidi, S., Friedrich, R. W. and Nicolson, T. NompC TRP channel required for vertebrate sensory hair cell mechanotransduction. *Science* 301: 96–99, 2003.
22. Hackney, C. M., Mahendrasingam, S., Jones, E. M. and Fettiplace, R. The distribution of calcium buffering proteins in the turtle cochlea. *J. Neurosci.* 23: 4577–4589, 2003.
23. Heller, S., Bell, A. M., Denis, C. S., Choe, Y. and Hudspeth, A. J. Parvalbumin 3 is an abundant Ca^{2+} buffer in hair cells. *J. Assoc. Res. Otolaryngol.* 3: 488–498, 2002.
24. Lumpkin, E. A. and Hudspeth, A. J. Regulation of free Ca^{2+} concentration in hair-cell stereocilia. *J. Neurosci.* 18: 6300–6318, 1998.
25. Dumont, R. A. *et al.* Plasma membrane Ca^{2+}-ATPase isoform 2a is the PMCA of hair bundles. *J. Neurosci.* 21: 5066–5078, 2001.
26. Steel, K. P. and Kros, C. J. A genetic approach to understanding auditory function. *Nat. Genet.* 27: 143–19, 2001.
27. Street, V. A., McKee-Johnson, J. W., Fonseca, R. C., Tempel, B. L. and Noben-Trauth, K. Mutations in a plasma membrane Ca^{2+}-ATPase gene cause deafness in deafwaddler mice. *Nat. Genet.* 19: 390–394, 1998.
28. Kozel, P. J. *et al.* Balance and hearing deficits in mice with a null mutation in the gene encoding plasma membrane Ca^{2+}-ATPase isoform 2. *J. Biol. Chem.* 273: 18693–18696, 1998.
29. Ahmed, Z. M., Riazuddin, S., Riazuddin, S. and Wilcox, E. R. The molecular genetics of Usher syndrome. *Clin. Genet.* 63: 431–444, 2003.
30. Nicolson, T., Rusch, A., Friedrich, R. W., Granato, M., Ruppersberg, J. P., Nusslein-Volhard, C. Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. *Neuron* 20: 271–283, 1998.

PART

–VIII–

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CHAPTER

52

Endocrine Effects on the Brain and Their Relationship to Behavior

Bruce S. McEwen

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INTRODUCTION

The brain undergoes changes in its chemistry and structure in response to changes in the environment. Circulating hormones of the adrenals, thyroid and gonads play an important role in this adaptation because the endocrine system is controlled by the brain through the pituitary gland (Fig. 52-1). This control allows environmental signals to regulate hormonal secretion. Furthermore, circulating hormones act on the brain as well as on other tissues and organs of the body to modify their structure and chemistry via two mechanisms: (i) intracellular receptors that bind to DNA and alter gene expression and (ii) cell-surface receptors that modulate ion channels and second-messenger systems.

Hormonal actions occur during sensitive periods in development, in adult life during natural endocrine cycles and in response to experience as well as during the aging process (see Ch. 30). As a result of their fundamental actions on cellular processes and genomic activity and of the control of their secretion by environmental signals, steroid and thyroid hormone actions on the brain provide unique insights into the plasticity of the brain and behavior (see also Ch. 50).

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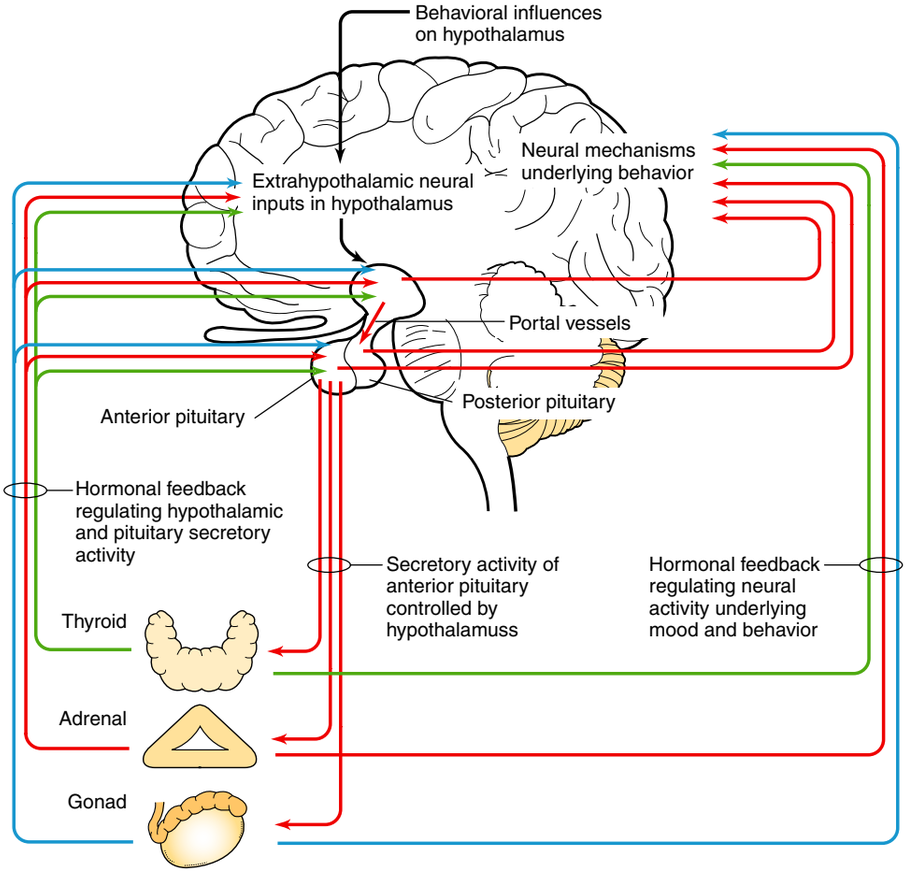


FIGURE 52-1 Schematic representation of possible and known reciprocal interactions among hypothalamic, pituitary, thyroid, adrenal and gonadal hormones.

Awareness of endocrine influences on brain function is as old as endocrinology itself. In 1849, Berthold described striking behavioral changes resulting from castration of roosters, and the reversal of these changes after testes had been transplanted into the castrated animals (see [1]). Nearly 100 years later, Beach published *Hormones and Behavior* (see [1]), which has served to instruct generations of investigators and to motivate them to explore in depth the interactions of hormones and brain. Spectacular growth of the field of neuroendocrinology (see also Ch 18) offers the present generation of neurobiologists unparalleled opportunities to explore with great sophistication the influence of neural activity on endocrine secretion, and the effect of hormones, in turn, on neural activity and behavior.

This chapter focuses on the neurochemical and molecular aspects of the influences of hormones on the nervous system and behavior, after first considering the chemical signals, behavioral events and underlying neural activity that regulate hormonal secretion.

BEHAVIORAL CONTROL OF HORMONAL SECRETION

The hypothalamic releasing factors regulate release of the anterior pituitary trophic hormones. As summarized in Figure 52-1, the releasing factors are produced in various neuronal groups within the hypothalamus and are transported to the median eminence for release into the portal circulation to the anterior pituitary. Neurons in the hypothalamus also produce the hormones oxytocin and vasopressin, which are released by the posterior pituitary into the blood. Therefore, it is not surprising that behavior and experience, which influence the hypothalamus, sometimes alter the secretion of these hypothalamic releasing factors and hormones.

Secretion of pituitary hormones is responsive to behavior and effects of experience. Consider, for example, the phenomenon of lactation, in which the sucking stimulus to the nipple triggers the release of oxytocin,

which facilitates milk ejection, and of prolactin, which helps the mammary gland to replenish the supply of milk [2]. The phenomenon of stress also illustrates the behavioral and emotional control of anterior pituitary hormone secretion. Conditions associated with tissue injury and surgical trauma, as well as the so-called psychic stresses of fear, novelty and even joy, can activate the release of adrenocorticotropic hormone (ACTH), which in turn stimulates the secretion of adrenal glucocorticoids [2]. The behavioral, emotional stimuli are mediated by neural pathways that can be modified readily by learning.

In the female rabbit, copulation activates spinal reflex pathways that stimulate the secretion of luteinizing hormone (LH), which leads to ovulation [3]. In the male rabbit, copulation also activates the secretion of LH and increases plasma testosterone [3]. Social stimuli also modify gonadotropin secretion. Olfactory cues between female mice can interrupt normal estrous cycles and lead to pseudopregnancies or to periods of prolonged diestrus, termed the Lee–Boot effect; olfactory cues from male mice can shorten the estrous cycle and either cause rapid attainment of estrus, termed the Whitten effect, or terminate pregnancy in a newly impregnated mouse, termed the Bruce effect [3]. In male rhesus monkeys, sudden, decisive defeat by other males leads to prolonged reduction in plasma testosterone, which can be reversed in the defeated male by the introduction of a female companion [3]. In men, the anticipation of sexual intercourse has been reported to increase beard growth, a process under the control of circulating androgen [3].

Hormones secreted in response to behavioral signals act in turn on the brain and on other tissues. Functional changes caused by hormones secreted in response to behavioral signals include modifications of behavior. With the sex hormones, these changes strengthen and guide the reproductive process. Thus, aggressive encounters between male birds or mammals in defense of territory during the mating period stimulate gonadotropin and testosterone secretion. Increases in these hormones further increase readiness for sexual activity by enhancing supplies of sperm and seminal fluid. This analysis was taken further by Lehrman (see [1]), who showed that among doves the behavioral sequence of courtship, mating, nest building and parenting involves complex behavioral interplay between the partners that triggers further hormonal secretion, which leads to the next phase of behavior and hormonal secretion.

Regarding the adrenal steroids, the behavioral activation of hormonal secretion in stress is part of a mechanism for restoring homeostatic balance. For example, an encounter with a predator may require rapid evasive action, in which neural activity and rapidly mobilized hormones such as epinephrine play a role. Adrenal steroid secretion is slower, reaching a peak minutes after the stressful event, and therefore is not expected to play a role in coping with the immediate situation. If the evasive action is successful and the animal survives, it will have to re-establish homeostasis; presumably, it also will learn from the experience to minimize the chances of another such encounter. Adrenal steroids facilitate such long-term adaptation; that is, they facilitate the acquisition as well as extinction of a conditioned avoidance response [4]. Suppose an animal has learned to avoid a certain place where previously it was punished; adrenals facilitate this learning in order to help 'keep it out of trouble'. Yet, if the animal later discovers that being in that place no longer results in punishment, and if that place also contains a food or water supply, it is in the best interest of the animal to extinguish the avoidance response in order to take advantage of the available food or water. Adrenal steroids have, in fact, been found to facilitate such extinction and, thus, can be said to facilitate a form of behavioral adaptation [4]. Adrenal steroids also appear to play a role in both selective attention and consolidation of a variety of learned information related to episodes or events in daily life [4]. Another aspect of adaptation in which stress-induced secretion of adrenal steroids participates, concerns the ability of the organism to cope with repeated stressful events through a variety of neurochemical changes [5].

Besides stress, adrenal steroids are secreted in varying amounts according to the time of day, and in this capacity they perform an important role in coordinating daily activity and sleep patterns with food-seeking and processing of information [5]. In nocturnally active animals, such as the rat, adrenal steroids are secreted at the end of the light period prior to onset of darkness. In humans and monkeys, adrenal steroid secretion precedes waking in the morning to begin daily activity. In both rats and primates, adrenal steroid secretion precedes the waking period, and appears to contribute, during waking, to optimal synaptic efficacy in the hippocampus for long-term potentiation, a correlate of learning. It is this aspect of adrenal steroid action that contributes to enhanced attention and improved retention of episodic memories [6] (see Ch. 50). Moreover, adrenal steroid elevation prior to waking also increases food-seeking behavior and enhances appetite for carbohydrates [5].

Cyclic changes in hormonal secretion, which are under the control of daily and seasonal light–dark rhythms, are important not only for the adrenals but for the gonads as well. Estrous cycles, menstrual cycles and seasonal breeding patterns represent adaptations of individual species to climatic conditions of the environment [1]. The feedback actions of gonadal and adrenal hormones, which are secreted in response to rhythmic output of hypothalamic and pituitary hormones, prime or activate the nervous system to perform the appropriate behavioral responses. It is important to stress that hormones themselves do not cause behaviors; rather, hormones induce chemical changes in particular sets of neurons, making certain behavioral outcomes more likely as a result of the strengthening or weakening of particular neural pathways.

CLASSIFICATION OF HORMONAL EFFECTS

Hormonal actions on target neurons are classified in terms of cellular mechanisms of action. Hormones act either via cell-surface or intracellular receptors. Peptide hormones and amino-acid derivatives, such as epinephrine, act on cell-surface receptors that do such things as open ion-channels, cause rapid electrical responses and facilitate exocytosis of hormones or neurotransmitters. Alternatively, they activate second-messenger systems at the cell membrane, such as those involving cAMP, Ca^{2+}/calmodulin or phosphoinositides (see Chs 20 and 24), which leads to phosphorylation of proteins inside various parts of the target cell (Fig. 52-2A). Steroid hormones and thyroid hormone, on the other hand, act on intracellular receptors in cell nuclei to regulate gene expression and protein synthesis (Fig. 52-2B). Steroid hormones can also affect cell-surface events via receptors at or near the cell surface.

The various modes of hormonal action summarized in Figure 52-2 may be distinguished from each other by time course. The fastest effects, in both latency and duration, are those involving direct opening of ion channels and stimulation of exocytosis. Intermediate effects involve phosphorylation of enzymes, ion channels, receptors or structural proteins, which may last for minutes or even hours. The slowest and most enduring effects are those that alter gene expression and lead to induction or repression of enzyme or receptor proteins, growth responses and even the structural remodeling of tissues.

As summarized in Figure 52-3, steroid/thyroid hormone receptors bind to other proteins as well as to DNA [7–9]. In the simplest type of action (Fig. 52-3A), the steroid/thyroid hormone receptor becomes activated after the hormone binds to it; activation results in conformational changes that include shedding of other proteins, such as heat-shock proteins, and exposing the DNA-binding domain. The receptor then binds to the specific sequence of DNA, called a 'hormone-response element' or enhancer, located on the coding strand of DNA; this enhances transcription by permitting other transcription factors as well as the RNA polymerase to bind to the promoter region [7]. A second scheme (Fig. 52-3B) is for the hormone receptor to bind with high affinity to another protein transcription factor, in this case the c-fos-c-jun complex, removing both protein complexes from binding to DNA [7]. Such a result also blocks the enhancement of transcription by either agent, although it also could reduce inhibitory effects produced by the hormone receptor through the scheme shown in Figure 52-3C. A variant on this theme, not shown in the figure, is 'squelching' in which multiple transcription factors compete with each other for a limited supply of soluble ligands that enhance their activities [7]. A third scheme, shown in Figure 52-3C, is for the steroid receptor to compete with another transcription factor for binding sites in the promoter regions. These other factors may be COUP or the cAMP-dependent transcription factors that bind to the adapter protein-1 (AP-1) response element. The result of this competition is inhibition of transcription since, in this situation, the other transcription factor enhances transcription, whereas the hormone receptor does not do so when it binds to the coding DNA strand [7].

As we have noted, second-messenger systems, through phosphorylation of nuclear proteins, can influence gene expression. There is evidence that even the classical steroid

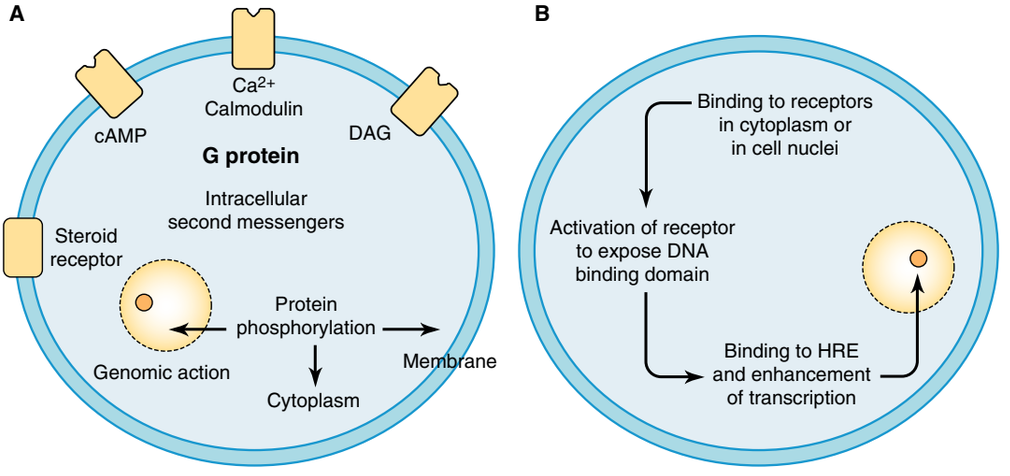


FIGURE 52-2 There are two modes of hormonal action. (**A**) Activation of cell-surface receptors and coupled second-messenger systems, with a variety of intracellular consequences. (**B**) Entry of hormone into the target cell, binding to and activation of an intracellular receptor and binding of the receptor-hormone complex to specific DNA sequences to activate or repress gene expression. *DAG*, diacylglycerol; *HRE*, hormone-response element.

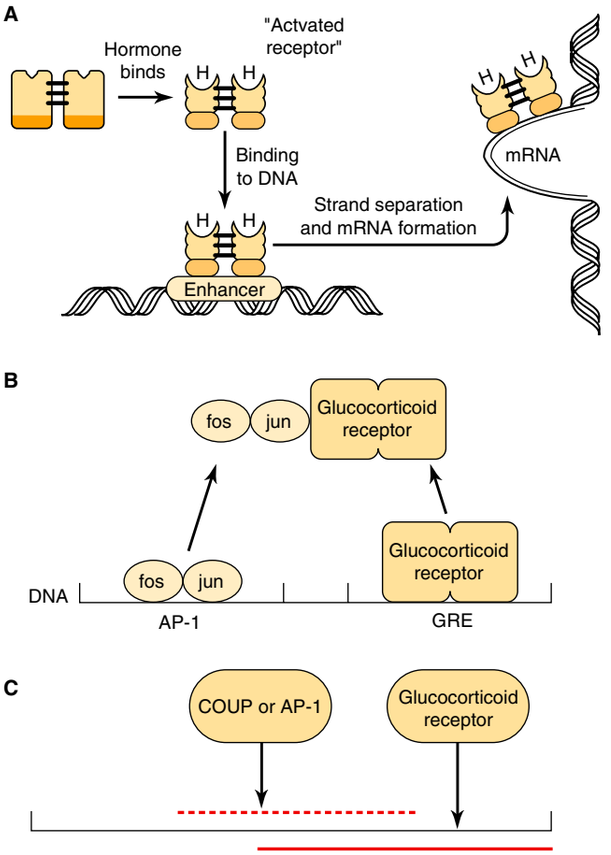


FIGURE 52-3 Intracellular receptors mediate at least three distinct types of actions on gene expression. (**A**) Binding of the hormone-receptor complex to a hormone-response element, a DNA sequence that is placed in a position where receptor binding to it can enhance unwinding of the double helix and attachment of other transcription factors. (**B**) Binding of the steroid receptor to another protein transcription factor, for example, the fos-jun complex through protein–protein interactions, removing both transcription factors from binding to DNA. *GRE*, glucocorticoid response element. (**C**) Binding of the hormone receptor to a hormone-response element located in the middle of a site for binding of another transcription factor-response element, such as the adaptor protein-1 (AP-1) or chicken ovalbumin upstream promoter-transcription factor (COUP sites), resulting in inhibition of transcription normally activated by transcription factors acting on these response elements (see [6–8]).

receptors are subject to regulation by phosphorylation and that phosphorylations promoted by a neurotransmitter such as dopamine (Ch. 12) are able to cause nuclear translocation of a steroid receptor in the absence of the steroid [7].

So far, the best understood examples of genomic regulation of neuronal function stem from the actions of gonadal and adrenal steroids and thyroid hormone, and many of these actions are involved in the plasticity of behavior that results from hormonal secretion, such as changes in aggressive and reproductive behavior and adaptation to repeated stress. In fact, hormonal actions that involve the genome are pervasive throughout the life cycle.

We can distinguish four major types of hormonal actions on the nervous system: (i) developmental actions, such as those involved in sexual differentiation and the effects of thyroid hormone and retinoic acid; (ii) reversible, and often cyclical, effects on the structure and function of neurons and glial cells that underlie corresponding cyclical changes in behavior, such as in reproduction and the daily rhythms of sleep and waking; (iii) experiential effects involving environmentally induced changes in hormonal secretion that evoke adaptive or maladaptive changes in the brain, as in stress; and (iv) effects that protect neurons or potentiate damage and lead to cell death.

It will be seen below that, in addition to genomic actions, there are nongenomic effects of steroids that modulate neurotransmitter release as well as ion traffic across the cell membrane, and do so frequently in coordination with genomic actions.

BIOCHEMISTRY OF STEROID AND THYROID HORMONE ACTIONS

Steroid hormones are divided into six classes, based on physiological effects: estrogens, androgens, progestins, glucocorticoids, mineralocorticoids and vitamin D. As shown in Figures 52-2 and 52-3, steroid hormone action on the brain and on other target tissues involves intracellular receptor sites that interact with the genome [1]. There are also important metabolic transformations of certain steroids, occurring in the nervous system, that either generate more active metabolites or result in the production of less active steroids. Such transformations are particularly important for the actions of androgens, of lesser importance for estrogens and progestins, and of practically no importance for glucocorticoids and mineralocorticoids. For vitamin D, the principal transformation to an active metabolite occurs in the kidney and liver [8]. Some metabolites, such as allopregnanolone and allotetrahydrodeoxycorticosterone, produce nongenomic effects on the $GABA_A$ receptor.

Some steroid hormones are converted in the brain to more active products that interact with receptors. The brain, like the seminal vesicles, is able to reduce testosterone to 5α-dihydrotestosterone (DHT); and, like the placenta, the brain aromatizes testosterone to estradiol (Fig. 52-4). Neither conversion occurs equally in all brain regions. The aromatization reaction is discussed below. Regional distribution of 5α-reductase activity toward testosterone in rat brain reveals that the highest activity is found in the midbrain and brainstem, intermediate activity is found in the hypothalamus and thalamus, and the lowest activity is found in the cerebral cortex [1]. The pituitary has higher 5α-reductase activity than any region of the brain, and its activity is subject to changes as

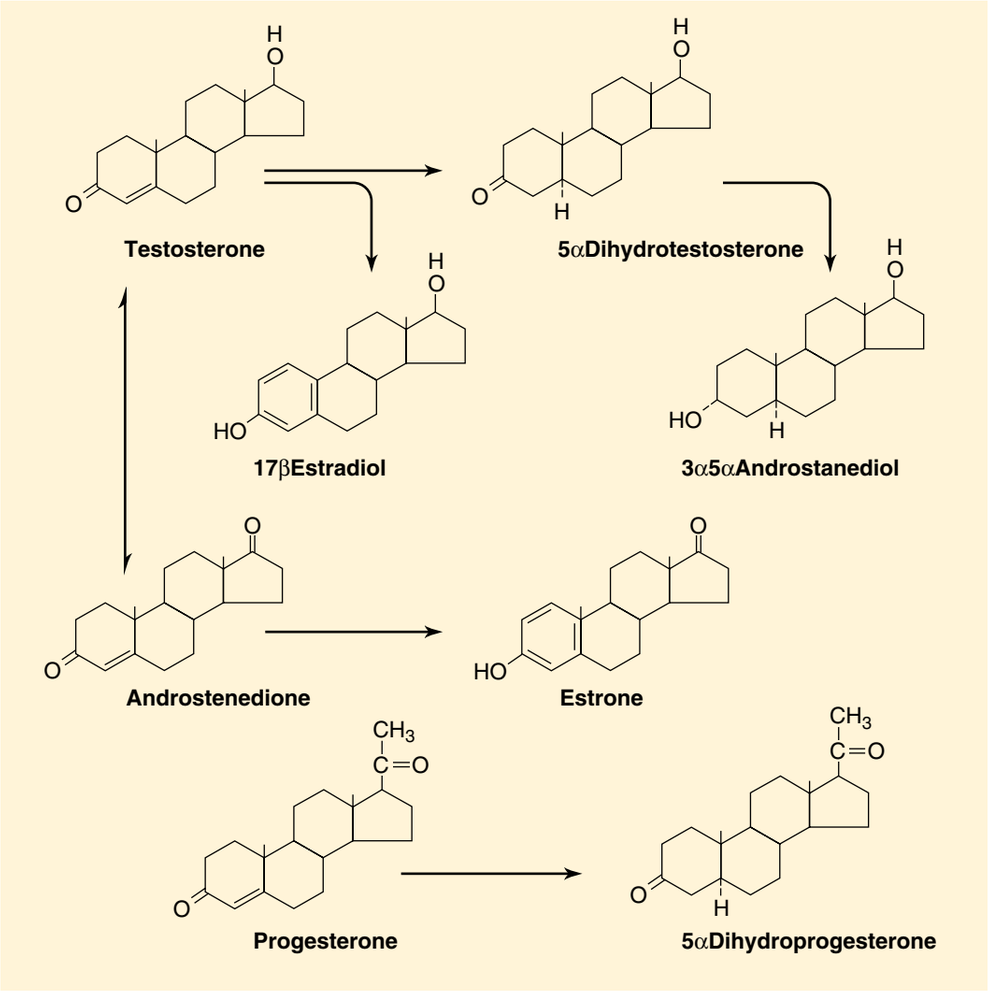


FIGURE 52-4 Some steroid transformations that are carried out by neural tissue.

a result of gonadectomy, hormone replacement and postnatal age [1]. 5α-DHT has been implicated in the hypothalamus and pituitary as a potent regulator of gonadotropin secretion, but it is relatively inactive toward male rat sexual behavior [1, 3]. Labeled metabolites with Rf values of 5α-DHT have been detected in extracts of hypothalamic and pituitary tissue after [^{3}H]-testosterone administration in both adult and newborn rats. It is interesting that progesterone inhibits 5α-reductase activity toward [^{3}H]testosterone and that [^{3}H]progesterone is converted to [^{3}H]5α-dihydroprogesterone (Fig. 52-4). Progesterone competition for 5α-reductase may explain some of the antiandrogenicity of this steroid [1]. 5β-DHT is a metabolite of testosterone formed in the avian CNS, as is 5α-DHT. 5β-DHT is inactive toward sexual behavior and is believed to represent an inactivation pathway for testosterone.

The aromatization of testosterone to form estradiol, and of androstenedione to form estrone (Fig. 52-4), has been described in brain tissue *in vitro* and *in vivo* [1, 9]. Aromatization is higher in hypothalamus and limbic structures than in cerebral cortex or pituitary gland, and, in noncastrated animals, it is higher in the male than in the female brain. Aromatization has been found in reptile and amphibian brain as well as in mammalian brain [1]. The capacity to aromatize testosterone and related androgens, therefore, may be a general property of vertebrate brains. The functional role of aromatization has been studied most extensively in the rat. Male sexual behavior is facilitated by estradiol [1], and testosterone facilitation of male sexual behavior can be blocked by a steroidal inhibitor of aromatization [1, 3]. There are indications that a similar situation exists in birds, amphibians and reptiles; that is, testosterone and estradiol can stimulate male and female heterotypical sexual behavior. Curiously, not all mammals are like the rat; for example, male sexual behavior of guinea pig and rhesus monkey is restored by the nonaromatizable androgen DHT [1, 3].

Both aromatization and 5α-reductase are regulated by gonadal steroids. In mammals such as the rat, it is principally the neural aromatase activity that is upregulated by androgens acting via neural androgen receptors [1].

In birds, both neural aromatase and 5α-reductase are induced by testosterone, and this regulation provides a way by which androgens can regulate CNS hormone sensitivity without regulating receptor number [1].

Both estrogens and glucocorticoids appear to act on brain cells without first being metabolized because both [^{3}H]estradiol and [^{3}H]corticosterone are recovered unchanged from their cell nuclear binding sites in brain [3]. However, estradiol is subject to conversion to the catechol estrogen 2-hydroxyestradiol, and this metabolite is both a moderately potent estrogen via intracellular estrogen receptors, as well as an agent capable of interacting with cell-surface receptors such as those for catecholamines, albeit at fairly high concentrations [7] (see Ch. 12). Glucocorticoids are inactivated by the enzyme 11-hydroxysteroid-dehydrogenase-2 (e.g. cortisol is converted to cortisone) and cortisone is re-activated to cortisol by the enzyme 11-hydroxysteroid-dehydrogenase-1 [10]. Organs such as the liver and brain have the ability to carry out this reactivation, which can lead to obesity via the liver and cognitive impairment via the brain [11].

Vitamin D, prior to acting in the brain, is converted to an active metabolite, 1,25-dihydroxy vitamin D_3, by enzymes in the liver and kidney [8] (see Fig. 52-5). The nervous system is also capable of cleaving the side chain from cholesterol to generate the same initial series of steroids [8] that are produced by the adrenals and gonads, namely, pregnenolone, dehydroepiandrosterone and progesterone (Fig. 52-6). In addition, neural tissue converts progesterone via reduction of the delta 4–5 double bond and reduction of the 3 keto group to 3,5α-pregnanolone, which is active on the chloride channel of the $GABA_A$ receptor [12] (Fig. 52-7). Glial cells are believed to be the primary sites of both cholesterol side-chain cleavage and generation of pregnanolone from progesterone [12]. There is also now evidence that the brain is capable of generating testosterone and estradiol from cholesterol via a process that is enhanced by excitatory neurotransmission [9].

While steroids generated in the brain have been referred to as 'neurosteroids', another useful term is 'neuroactive steroids' to refer to all steroids that affect brain function via any mechanism and irrespective of site of formation. The term neuroactive steroid also has been used to describe neuroactive steroid drugs.

Genomic receptors for steroid hormones have been clearly identified in the nervous system. The detection of intracellular, DNA-binding steroid receptors became possible with the introduction of tritium-labeled steroid hormones of high specific radioactivity: 20–25 Ci/mmol at each labeled position. The limited number of these sites had escaped detection using steroids labeled with ^{14}C at a much lower specific radioactivity. Tritium labeling also permitted histological localization of steroid receptors because the high spatial resolution of ^{3}H, 1–2 μm in light-microscopic autoradiography, allows cellular and even cell nuclear localization of the radioactivity.

Cell fractionation procedures were fundamental to the biochemical identification of steroid and thyroid hormone receptors in brain as well as in other tissues. Isolation of highly purified cell nuclei from small amounts of tissue from discrete brain regions generally is accomplished with the aid of a nonionic detergent, such as Triton X-100 [7].

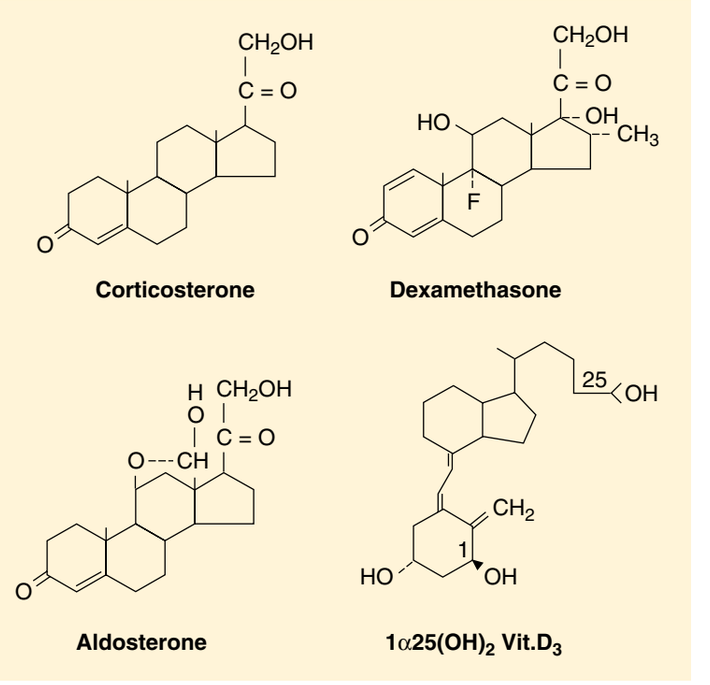


FIGURE 52-5 Formulas of four steroid hormones.

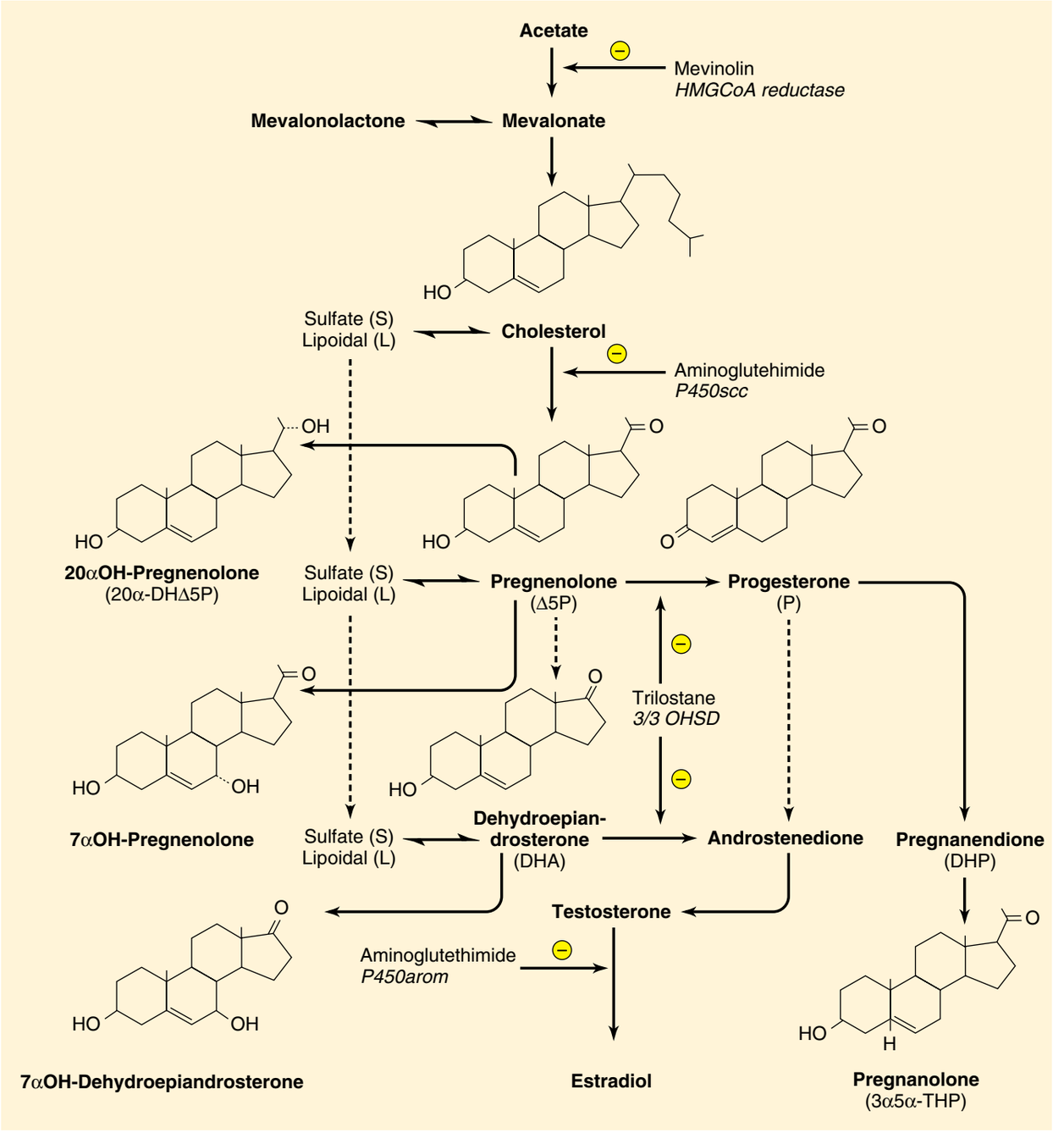


FIGURE 52-6 Biosynthesis/metabolism of steroids in the CNS. The conversion of delta5P to dehydroepiandrosterone (DHA) is postulated but not demonstrated. D5P and DHA inhibit and 3α,5α-THP potentiates $GABA_A$ receptor function, as summarized in Figure 52-7. *Solid arrows* indicate demonstrated pathways; *dotted arrows* indicate possible pathways. Metabolic inhibitors of enzymes are indicated by ⊖. (Redrawn from [12], with permission.)

Cytosolic fractions of brain tissue, prepared by centrifugation of homogenates at 105,000 g for 60 min, contain the soluble steroid-hormone-binding proteins, and a variety of methods intended to separate bound from unbound steroid have been used for measuring their binding activity [3, 7]. The most commonly employed are gel filtration chromatography and sucrose-density-gradient centrifugation. Dextran-coated charcoal or Sephadex LH20 are frequently used because they effectively absorb unbound steroid and leave intact the complexes between steroid and putative receptor. Other methods, such as gel electrophoresis and precipitation of putative receptor material with protamine sulfate, have more restricted uses.

The objective of such studies is to measure the affinity, capacity and specificity of the hormone–receptor interaction [3, 7]. Measurements of affinity and capacity are

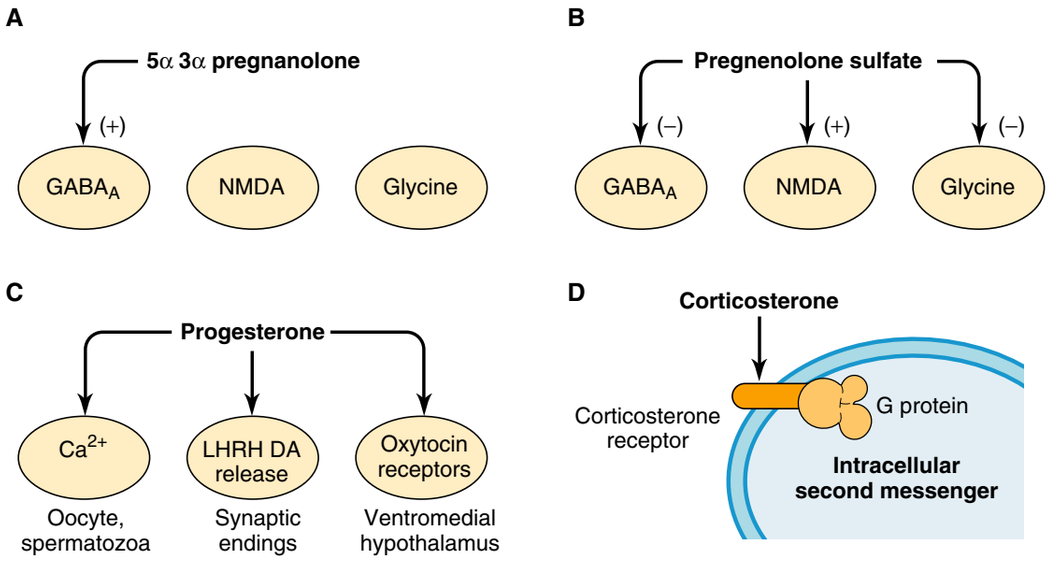


FIGURE 52-7 Schematic summary of four ways in which steroids affect cell-surface-mediated events and neuronal activity by nongenomic mechanisms. (**A**) Activation of $GABA_A$ receptor by 5α3α-pregnanolone. (**B**) Activation of NMDA receptor and inhibition of $GABA_A$ and glycine receptors by pregnenolone sulfate. (**C**) Activation of Ca^{2+} mobilization in oocytes and spermatozoa by progesterone; the same is postulated to happen in certain synaptic endings and may be involved in the rapid, direct effect of progesterone on the oxytocin receptor. (**D**) Corticosterone binds to a cell surface receptor linked to a G protein, and presumably through it to a second-messenger system, in some brain cells. *DA*, dopamine; *LHRH*, luteinizing hormone releasing hormone; *NMDA*, *N*-methyl-D-aspartate.

accomplished with equilibrium binding analysis. Specificity is based on competition between the labeled and various unlabeled ligands for binding sites.

Because the nervous system is highly heterogeneous from the standpoint of many neurochemical characteristics, including steroid and thyroid hormone receptors, the most useful techniques for mapping these receptors have been histochemical. Steroid autoradiography was the first such method. With purification of receptors and generation of antibodies, immunocytochemistry has been added as a tool. Cloning of steroid and thyroid receptors has opened the way to mapping of receptor mRNAs via *in situ* hybridization histochemistry.

Several criteria determine whether a steroid-hormone-binding site is a putative receptor. First, the steroid-hormone-binding site must be present in hormone-responsive tissues or brain regions, and absent from nonresponsive ones. Second, it should bind steroids that are either active agonists or effective antagonists of the hormone effect, and should not bind steroids that are inactive in either sense.

Understanding of the intracellular localization of steroid receptors has gone through a number of phases, beginning with the view that receptors translocated from cytoplasm to nucleus in the presence of hormone. Indeed, with the exception of thyroid hormone receptors, which are exclusively nuclear in location, cell fractionation studies have revealed that in the absence of hormone, steroid receptors are extracted in the soluble or cytosolic fraction. However, when steroid is present in the cell, many occupied receptors are retained by purified cell nuclei. Histological procedures, such as immunocytochemistry, have confirmed the largely nuclear localization of occupied receptors, but they also have revealed a nuclear localization of receptors in the absence of hormone in the tissue. This is true for estrogen, progestin and androgen receptors, but the mineralocorticoid and glucocorticoid receptors show a cytoplasmic localization in the absence of hormone. Thus, steroid receptors may exist in nuclei in a loose association that is disrupted during cell fractionation. This is not an uncommon situation for many constituents of cell nuclei [7].

At the same time, there is evidence for membrane-associated steroid receptors that are coupled to second messenger systems (see below).

INTRACELLULAR STEROID RECEPTORS: PROPERTIES AND TOPOGRAPHY

Steroid hormone receptors are phosphoproteins that have a DNA-binding domain and a steroid-binding domain. All steroid receptors have a molecular weight of 55,000–120,000. The state of phosphorylation appears to influence functional activity.

Estradiol. The first neuroactive steroid receptor type to be recognized was that for estradiol [3]. *In vivo* uptake of [^{3}H]estradiol, and binding to cell nuclei isolated from hypothalamus, pituitary and other brain regions, revealed steroid specificity closely resembling that of the uterus, where steroid receptors were first discovered [3]. Cytosolic estrogen receptors isolated from pituitary and brain tissue closely resemble those found in uterus and mammary tissue. A hallmark of the estrogen receptor is its existence

as an aggregate of subunits that dissociate during steroid-induced transformation to the DNA-binding nuclear form of the receptor. This part of the estrogen-receptor complex was cloned from human breast cancer cells and consists of a 65- to 70-kDa hormone and DNA-binding subunit [7]. The dissociation constant of estradiol binding is approximately 0.2–nM.

Estrogen receptors are found in the adult pituitary, hypothalamus, preoptic area and amygdala. They are principally in neurons, although glial cells also may express these receptors in some brain regions [13]. The developing rat brain expresses estrogen receptors in cerebral cortex and hippocampus, but these receptors largely disappear as the brain matures [13–15]. A second form of the estrogen receptor, the β-estrogen receptor, is similar to the α form in affinity and specificity but different in tissue distribution [16].

Progesterone receptors in brain were detected using the synthetic progestin R5020 (promegestrone; 17α, 21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione), which has a high affinity for the progestin receptor, with a K_d of 0.4 nM [7]. The progestin receptor, cloned from chick oviduct, consists of a steroid- and DNA-binding subunit of 108 kDa, although one 79-kDa subunit has also been described [7]. Progestin receptors with similar properties are found in pituitary, reproductive tract and most estrogen receptor-containing brain regions; these receptors are inducible by estrogen treatment [7]. There are also progestin receptor sites in brain areas lacking estrogen receptors, such as the cerebral cortex of the rat; these receptors are not induced by estradiol treatment. Nevertheless, such receptors resemble those induced by estradiol [21]. Another inducer of progestin receptors in brain is testosterone, which works through its conversion to estradiol via aromatization [7]. Progesterone acts rapidly to induce feminine sexual behavior, termed lordosis, in female rats that have been primed with estradiol to induce progestin receptors [7]. The principal site of estradiol and progesterone action is the ventromedial nucleus of the hypothalamus [17].

Androgen receptors have a steroid-binding subunit estimated to be 120 kDa [7]. The estimated K_d for active androgens is approximately 1–2 nM [3]. Androgen receptors are widely distributed in brain and pituitary tissue, although the highest concentrations are found in hypothalamus, preoptic area and limbic brain tissue [18]. Androgen receptors are deficient in the androgen-insensitivity (Tfm) mutation, and animals with this mutation show defects in sexual behavior, juvenile rough-and-tumble play behavior and certain aspects of neuroendocrine function, thus indicating the actions of testosterone that are mediated by androgen receptors, as opposed to those mediated by aromatization of testosterone to estradiol (see above) and estrogen receptors [14, 15].

Glucocorticoid. Adrenal steroid receptors have been subdivided into two categories, one of which is the classical glucocorticoid receptor [7, 19]. This receptor, cloned from human and rat sources, consists of a steroid- and DNA-binding subunit of 95 kDa [7, 19]. Such receptors, which have dissociation constants of 4–5 nM for glucocorticoids, are widely distributed across brain regions and are found in neurons and glial cells [7, 19].

Mineralocorticoid. The other type of glucocorticoid receptor is similar to the mineralocorticoid receptor originally described in the kidney [7, 19]. In the brain, receptors of this type bind the glucocorticoid corticosterone with high affinity, having a K_d of approximately 1 nM, and they are responsible for the high uptake of tracer levels of [^{3}H]corticosterone by the hippocampus [7, 19]. These corticosterone receptors, which are found in high concentrations in the hippocampus but are also widely distributed in other brain regions at lower concentrations, may be involved in mediating the effects of diurnally varying concentrations of corticosterone [5, 7, 19]. Uptake of [^{3}H]aldosterone by brain tissues reveals two types of binding sites: those in the hippocampus, which can be occupied preferentially by corticosterone, and those more diffusely distributed in the brain, which appear to retain [^{3}H] aldosterone preferentially in the presence of the normally higher concentrations of corticosterone [7]. The reasons for this selectivity of an enzyme, 11β-hydroxysteroid dehydrogenase, are that, at least in the kidney-collecting tubules, it converts corticosterone to an inactive metabolite and allows aldosterone access to the mineralocorticoid receptors [10].

Vitamin D is a steroid hormone, production of which by the body requires the action of light. Therefore, it is often necessary to provide some vitamin D in the diet [8] (see Ch. 33). Moreover, vitamin D is converted by the kidney and liver to the active metabolite 1,25-dihydroxyvitamin D3 (Fig. 52-5) [8]. Vitamin D3 receptors consist of a hormone- and DNA-binding subunit of 55 kDa [7, 18]. Receptor sites for 1,25-dihydroxyvitamin D3 are found in pituitary and brain, especially in the forebrain, hindbrain and spinal cord neurons [8]. In the brain, one site containing vitamin D3 receptors, the bed nucleus of the stria terminalis, responds to exogenous 1,25-dihydroxyvitamin D3 with an induction of choline acetyltransferase, even though the calcium-binding protein that is regulated by vitamin D3 in the intestine is not regulated by this hormone in the brain [8].

MEMBRANE STEROID RECEPTORS AND SIGNALING PATHWAYS

The known rapid effects of some steroid hormones on neuronal excitability are difficult to explain solely in terms

of genomic actions [20]. Instead, some type of membrane receptor interaction is inferred. Indeed, several types of interaction between neuroactive steroids and neural membranes have been described. Direct binding assays have revealed membrane sites for glucocorticoids and gonadal steroids and one instance of a membrane receptor coupled to a G protein (Fig. 52-7). Additionally, indirect binding assay results have implied interaction of the catechol estrogens with dopamine and with adrenergic receptors [7]. Moreover, studies with specific antibodies at the electron microscope level have revealed estrogen receptors in dendrites, presynaptic terminals and glial cell processes [21], and a membrane receptor for progesterone has been cloned from oocytes [22]. Finally, deletion of the nuclear vitamin-D receptor deprives cells of rapid membrane actions of vitamin D, indicating that this receptor may serve a dual function [23].

Progesterone produces direct membrane effects [16]. These include actions that promote maturation of spermatozoa as well as oocytes and facilitation of the release of neurotransmitters such as dopamine and LH-releasing hormone (LHRH) (Fig. 52-7). Membrane actions of progesterone also activate oxytocin receptors in the hypothalamus in a way that enables oxytocin to turn on sexual behavior in the estrogen-primed female rat [3].

Estradiol activates a variety of signaling pathways via membrane-associated receptors in many cell types [20]. In neurons, rapid actions of estradiol stimulate translation of a key protein, PSD-95, involved in synapse formation [24].

There are now indications for the interaction of progesterone metabolites with the Cl^- channel of the $GABA_A$ receptor (Fig. 52-7). The A-ring-reduced steroids, especially those with the 5α,3α configuration, are particularly active on the $GABA_A$ receptor [12]. By facilitating chloride-channel opening, these steroids produce anesthetic, anxiolytic and sedative–hypnotic effects (see Ch. 16).

Another neurosteroid (Fig. 52-6), pregnenolone sulfate (PS), produces effects that in many ways antagonize those of the steroids that open the $GABA_A$ receptor Cl^- channel [12]. PS in micromolar concentrations inhibits the $GABA_A$ receptor and the inhibitory glycine receptor, and facilitates activity of the excitatory *N*-methyl-D-aspartate (NMDA) receptor (Fig. 52-7). It is unclear, however, whether these effects are physiologically relevant, since the PS concentration needed to produce them is rather high. However, PS is produced locally in the brain and may reach sufficiently high concentrations in some compartments within the nervous system.

None of these findings undermines the importance of the intracellular genomic actions of steroids. Rather, they increase the richness of the cellular actions of steroid hormones and raise the possibility that there may be connections between genomic and nongenomic actions of steroids. For example, genomic action may induce receptors that mediate nongenomic effects. Moreover, the activation of oxytocin receptors by progesterone is dependent on the ability of estrogen priming to induce the formation of new oxytocin receptors via a genomic mechanism; these receptors are then transported along dendrites to sites where the progesterone action occurs at the membrane level [3].

BIOCHEMISTRY OF THYROID HORMONE ACTIONS ON BRAIN

Like steroid hormones, thyroid hormones interact with receptors to alter genomic activity and affect the synthesis of specific proteins during development [25–28]. As with testosterone and progesterone, metabolic transformation of thyroxine (T_4) is critical to its action [25–28]. Moreover, as with steroid hormones, thyroid hormones alter brain functions in adult life in ways that both resemble and differ from their action during development [25–28].

The initial step after cellular uptake of T_4 is metabolic transformation to 3,5,3′-tri-iodothyronine (T_3) (Fig. 52-8), which interacts with cytosolic and nuclear receptors, as well as with synaptosomal membrane binding sites of unknown function [25]. Cytosolic receptors are proteins of 70 kDa that do not appear to undergo translocation to cell nuclei, nor do they appear to be nuclear proteins that have leaked out of cell nuclei during cell rupture; nuclear receptors are proteins of 50 70 kDa that have both DNA- and hormone-binding domains [25, 26, 28].

Nuclear T_3 receptors are present in higher levels during neural development than they are in adult life [28]. In human fetal brain, nuclear T_3 receptors increase in concentration from 10 weeks of gestation to the sixteenth week, when neuroblast multiplication is high [25, 28]. Glial cells, as well as neurons, contain nuclear T_3 receptors [25, 28]. Functionally, many neurons develop prior to the appearance of significant T_3 receptor levels and, therefore, appear to be independent of large-scale thyroid influence [25–28]. Other neurons, such as those in the cortex and cerebellum, show a more profound dependence on thyroid function [25]. Although thyroid hormone affects the

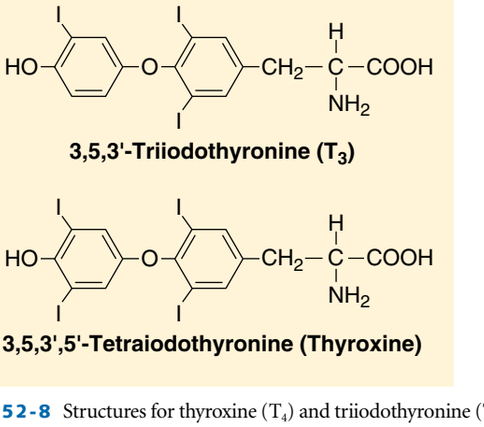


FIGURE 52-8 Structures for thyroxine (T_4) and triiodothyronine (T_3).

number of replicating cells in the external granular layer of the developing cerebellum, it is not possible to conclude that T_3 directly affects the mechanism of cell replication [25]. Rather, the most pronounced effect of hypothyroidism is a hypoplastic neuropil, with shortened dendrites and fewer spines. It has been shown that a major effect of T_3 involves development of the neuronal cytoskeleton [25–28]. Proteins, such as microtubule-associated protein (MAP2) and tau (see Ch. 27), which are polymorphic and affect microtubular assembly, are differentially affected by T_3 absence or excess [25, 28].

Developmentally, thyroid hormones interact with sex hormones such that hypothyroidism prolongs the critical period for testosterone-induced defeminization (see below) [3]; in contrast, the hyperthyroid state prematurely terminates the sensitivity to testosterone [3]. Undoubtedly, an important link in these and other effects is synapse formation. Hypothyroidism increases synaptic density, at least transiently [3]. Interesting parallels with synapse formation are reported for learning behavior in rats; neonatal hypothyroidism impairs learning ability, whereas hyperthyroidism accelerates learning initially, followed by a decline later in life [3].

The adult brain is endowed with nuclear as well as cytosolic and membrane T_3 receptors that have been visualized by autoradiography and studied biochemically [30–33]. Both neurons and neuropil are labeled by [125I]T_3, and the labeling is selective across brain regions. Functionally, one of the most prominent features of neural action of thyroid hormone in adulthood is subsensitivity to norepinephrine as a result of a hypothyroid state [27]. These changes may be reflections of loss of dendritic spines in at least some neurons of the adult brain. Clinically, thyroid hormone deficiency increases the probability of depressive illness, whereas thyroid excess increases the probability of mania (Ch. 52) in susceptible individuals [27].

DIVERSITY OF STEROID-HORMONE ACTIONS ON THE BRAIN

Steroid-hormone effects on the brain link the environment surrounding the organism with the genome of target brain cells through the process of variable genomic activity [3, 7, 29]. By this we mean that an organism experiences light, dark, heat, cold, fear and sexual arousal. These experiences influence hormonal secretion, and these hormones in turn act on the genome of receptor-containing brain cells to alter their functional state. The genome of brain cells, like that of other cells of the body, is continually active from embryonic life until death and continually responsive to intra- and extracellular signals. This activity can be seen from the high rates of RNA metabolism in neurons. The differential influence of steroid hormones on variable genomic activity is evident from studies showing rapid and brain region-specific induction of ribosomal and mRNA, as well as changes in cell nuclear diameter and chromatin and structure [1, 17]. However, variable genomic activity changes qualitatively with the state of differentiation of the target cells: embryonic neurons show growth responses that result in permanent changes in circuitry, whereas adult neurons show impermanent responses. Under other circumstances, the same hormonal signals can promote damage and even neuronal loss; under still other conditions, adult neurons can be stimulated by treatment with hormones to grow and repair the damage.

During development, steroid-hormone receptors become evident in target neurons of the brain. These receptors appear within several days of final cell division [14, 15]. Whether some receptors are also present in dividing neuronal precursors is not clear. After they have appeared, these receptors mediate a variety of developmental actions. For example, glucocorticoids direct differentiation of adrenergic/cholinergic neurons of the autonomic nervous system to develop in the adrenergic direction [7]. Glucocorticoids also increase the number of epinephrine-containing, small, intensely fluorescent cells, often referred to as SIF, in autonomic ganglia and are required for the normal postnatal ontogeny of serotonin neurons in the forebrain [7]. These effects may not all be direct but may involve hormonal modulation of growth factors produced by other cells surrounding the developing neurons.

Gonadal hormones, however, are involved in sexual differentiation of the reproductive tract and brain [1, 14, 15]. Mammals, among which animals have X and Y chromosomes, undergo sexual differentiation under the impetus of testosterone secreted by the testes during a period of perinatal life; in humans, this period is in midgestation, while in rats it is from the end of gestation into neonatal life. Key features of sexual behavior in birds are determined in the reverse manner, in keeping with the fact that the female has the chromosomal heterogeneity: females produce either estradiol or testosterone, either of which feminizes the brain, which otherwise would develop a masculine pattern in the absence of gonadal steroids [1, 15].

As for the mechanisms of sexual differentiation, we must consider the metabolism of the hormone receptor types involved and the primary-receptor-mediated events. Testosterone, as noted above, is a prohormone that is converted into either 5α-DHT or estradiol within the brain; these products exert effects on brain sexual differentiation via androgen and estrogen receptors, respectively [14, 15]. Masculinization of sexual and aggressive behavior involves either 5α-DHT alone or a combination of 5α-DHT and estradiol acting on different cells. Besides masculinization, there is in some mammals a process of defeminization, in which feminine responses that would develop in the absence of testosterone are suppressed by its presence during the critical period. Conversion to estradiol appears to be involved in this process [14, 15]. Progesterone plays no major role in brain sexual differentiation, but it does have the ability to antagonize actions at both androgen and

estrogen receptors and, thus, can moderate the degree of masculinization and defeminization.

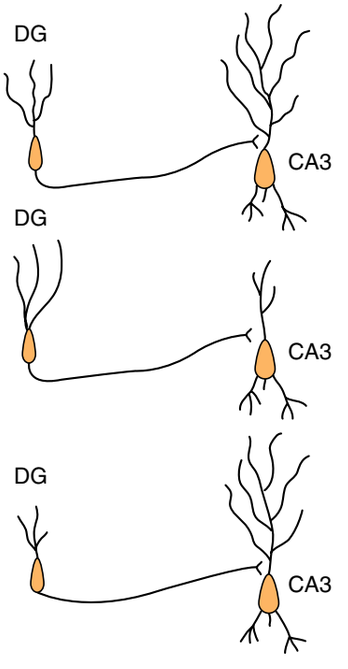
As to the primary developmental actions of testosterone, growth and differentiation appear to be involved. Testosterone or estradiol stimulates outgrowth of neurites from developing hypothalamic neurons that contain estrogen receptors [14, 15]. This is believed to be one of the principal aspects of testosterone action that increases the number and the size of neurons within specific hypothalamic nuclei in males, compared to females [1, 14, 15]. 5α-DHT may have a similar effect on androgen-sensitive neurons. Differentiation of target neurons also occurs; in adult brain tissue, hormones like estradiol can evoke responses that differ between adult male and female rats [1, 14,1 5].

Because of new research with genetic manipulations, there are other aspects of sexual differentiation that must now be incorporated into our thinking. First is that female sexual differentiation depends in part on aromatization of androgens, since knockout of the aromatase gene deprives the female of a number of normal sociosexual behavior patterns in adult life [30]. Second, is that genetic sex plays a role in brain and body sexual differentiation; more specifically, genes on the Y chromosome are believed to contribute to sex differences in the midbrain dopaminergic system, among other brain regions [31].

The response of neural tissue to damage involves some degree of structural plasticity, as in development. Collateral growth and reinnervation of vacant synaptic sites is facilitated in some cases by steroid hormones [32]. In the hypothalamus, estrogen treatment after knife cuts that destroy certain inputs promotes increases in the number of synapses. In the hippocampus, glucocorticoid treatment promotes homotypical sprouting of serotonin fibers to replace damaged serotonin input. It has also been noted that androgens enhance the regrowth of the severed hypoglossal nerve [32]. One interpretation of these steroid effects is that injury reactivates programs of steroid-responsive genomic activity that normally operate during the phase of synaptogenesis in early development [32].

Estrogens are also neuroprotective against ischemic damage [13], and aromatization of androgens to estrogens plays a role even in females, where knockout of the aromatase gene increases the vulnerability of female mice to stroke damage by a process that is prevented by estradiol administration [33].

Another aspect of neuroprotection by steroids is stabilization of neurons against death and replacement. In the dentate gyrus of both the neonate and the adult rat, neurons are born and die; rates of both birth and death are increased by adrenalectomy, and these increases are prevented by low doses of adrenal steroids acting via mineralocorticoid receptors [34] (Fig. 52-9). Regulation of the turnover rate of dentate gyrus neurons may provide the hippocampal formation of the adult with the potential to increase and decrease its volume and functional capacity, as occurs in relation to seasonal or other long-term changes in the environment. This process also occurs in the developing dentate gyrus [34].



CA3 pyramidal neurons receive the mossy fiber input from the granule neurons of the dentate gyrus. This connection is part of the 3-cell circuit of the hippocampus that is believed to be involved in learning and memory.

Chronic corticosterone treatment or repeated restraint stress promotes atrophy of apical dendrites of CA3 pyramidal neurons.

Adrenalectomy causes neurons of the dentate gyrus to die and to be replaced by new neurons, whereas pyramidal neurons are unaffected. Low levels of adrenal steroids prevent this and stabilize the dentate granule neuron population; they do this via Type I adrenal steroid receptors.

FIGURE 52-9 Summary of estrogen effects on the ventromedial nuclei related to its activation of female sexual behavior in the rat. *DG*, dentate gyrus. (From [41], with permission.)

Activation and adaptation behaviors may be mediated by hormones. Hormonal secretion by the adrenals and gonads is controlled by endogenous oscillators that can be entrained by environmental cues such as light and dark. The actions of cyclically secreted hormones on behavior and brain function are referred to as activational effects. In addition to the cyclic mode, there is another mode of secretion initiated by such experiences as stress, fear and aggressive and sexual encounters. In this case, actions of adrenal steroid hormones secreted in response to experience lead to adaptive brain responses, which help the animal cope with stressful situations [5, 19, 29]. The activational and adaptational effects are largely reversible and involve a variety of neurochemical changes, most of which appear to be initiated at the genomic level. For example, estradiol is secreted cyclically during the estrous cycle in the female rat and triggers the surge of LH, which induces a surge of progesterone from the ovary. Progesterone, in turn, stimulates sexual responsiveness to the male rat [1].

Estradiol action to promote feminine sexual behavior in the rat involves a cascade of induced protein synthesis in specific hypothalamic neurons accompanied by morphological changes indicative of increased genomic activity [1, 17, 35]. Among the induced proteins are receptors for progesterone (see above), crucial for activating sexual behavior; receptors for acetylcholine and oxytocin that are active in enabling the hypothalamic neurons to respond to afferent input; proteins that are axonally transported from the hypothalamus to the midbrain, where they may be involved in neurotransmission; and structural proteins that contribute to formation of new synapses that come and go during the estrous cycle [1, 17, 35] (Fig. 52-9).

Estradiol also induces synapses in the hippocampus and this contributes to enhanced capacity for learning and memory that is dependent on the hippocampus [13]. Estradiol exerts many other nonreproductive actions on the brain, such as fine motor coordination, seizure susceptibility, mood, protection from ischemic damage. Many of these actions occur in brain regions that show little, if any, nuclear estrogen receptors, and it seems likely that the nongenomic estrogen receptor described above may be involved [13].

Adrenal steroids secreted in the diurnal cycle are responsible for reversibly activating exploratory activity, food-seeking behavior, carbohydrate appetite and synaptic efficacy [4–6]. These appear to do so by acting on the hippocampus, where there are many mineralocorticoid and glucocorticoid receptors (see above). Adaptational effects of adrenal steroids that result from stress appear to operate via the classical glucocorticoid receptor found not only in hippocampus but also in other brain regions [19, 29]. Changes in synaptic vesicle proteins, high-affinity GABA transport, neurotransmitter-stimulated cAMP formation and central serotonin and noradrenergic sensitivity accompany repeated glucocorticoid elevations [7, 19, 29]. One view of these changes induced by glucocorticoids is that they counter-regulate some of the immediate and persistent neural effects of stress and are, therefore, part of the mechanism of adaptation [4–6, 7, 19, 29, 36]. This and other aspects of adaptation protect the organism; without adrenal secretions, the body probably would not survive many events in daily life.

The price of adaptation often includes some wear and tear and damage, called allostatic load [36]. Allostasis, meaning to achieve stability through change, is another word to describe adaptation. The wear and tear is the result of the inefficiency or overactivity of allostatic systems, including those like the adrenal cortex and autonomic nervous system, which respond to challenge and promote adaptation. This takes three forms: (i) repeated activation by many stressful events; (ii) failing to shut off after the challenge is over; and (iii) inability to be activated adequately, allowing other systems that are normally counter-regulated to become overactive, for example, inflammatory cytokines. In each of these situations, there is a cumulative as well as an immediate effect on many organs, including the heart and brain, as well as on the immune system.

Enhancement of neuronal atrophy and cell loss during aging by severe and prolonged psychosocial stress are examples of allostatic load. Repeated psychosocial stress in primates and rodents causes pathological changes in various body organs, including neuronal loss in the hippocampus [37]. Shorter exposure of rats to restraint stress, psychosocial stress or corticosterone causes atrophy of neurons in the hippocampus, particularly in the CA3 region, which receives heavy input from the dentate gyrus mossy fiber system (Fig. 52-10). The mossy fiber input is also responsible for kainic-acid- and seizure-induced damage of the CA3 region and strongly suggests the importance of excitatory amino acids [37].

Indeed, the stress-induced atrophy is blocked by NMDA receptor blockers and by an anticonvulsant drug, phenytoin. The presence of elevated glucocorticoids at the time of hypoxic damage or kainic-acid lesions (Chs 34 and 35) to the brain potentiates the necrosis produced by these treatments, especially in the hippocampus. Adrenalectomy reduces such damage and retards loss of neurons with age [37]. Thus, adrenal steroids operate in conjunction with neural excitability to produce damage, and it has been suggested that they do so by compromising the ability of the brain to obtain nutrients to support ATP generation [37].

Yet, the effect of repeated stress on the hippocampus is reversible if the stress is terminated after 3 weeks in rats, and it is best referred to as 'adaptive plasticity', since other brain areas, such as the amygdala, show a growth response to repeated stress [38].

The concept of allostatic load implies that there is a paradox in the actions of adrenal steroids: they exert protection in the short run and have the potential to cause damage in the long run if the allostatic, that is, the adaptation-promoting, responses are not managed efficiently.

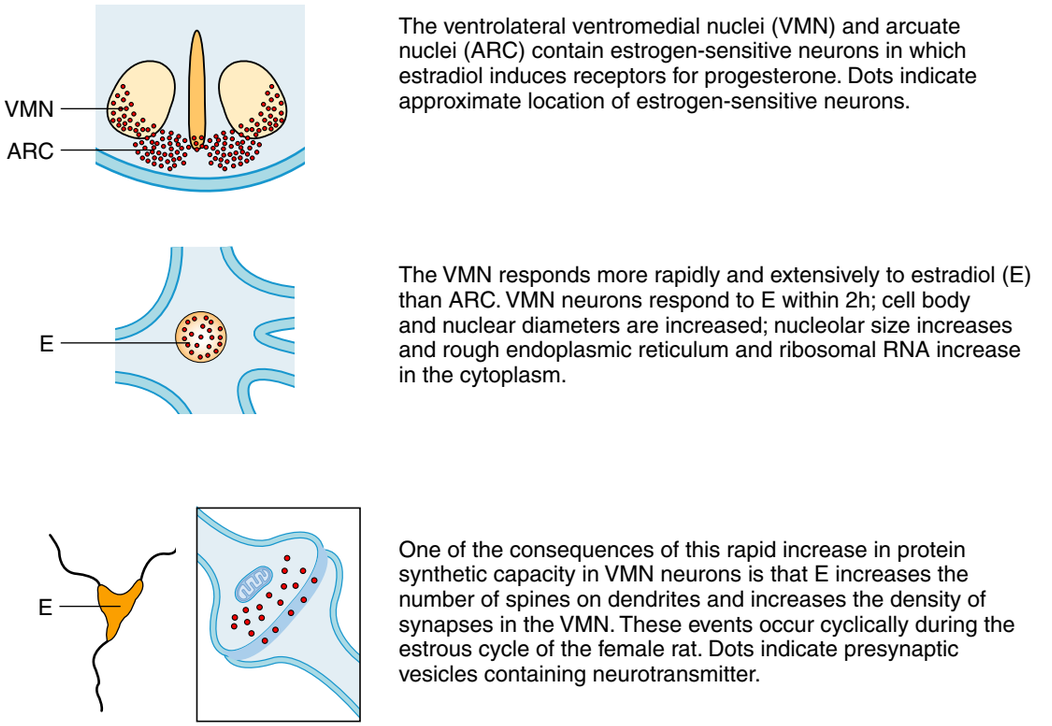


FIGURE 52-10 Summary of adrenal steroid effects on neurons of the hippocampus, illustrating their ability to protect and stabilize the population of the dentate granule neurons and to potentiate damage caused by excitatory amino-acid release upon pyramidal neurons. *E*, estrogen. (From [41], with permission.)

'Good stress' is therefore the efficient management of an allostatic response, whereas 'bad stress', or being 'stressed-out', involves the persistence or otherwise inefficient operation of these normally adaptive responses.

Stressful early life events, involving abuse or neglect, can have a life-long influence on the stress response, and lead to elevated levels of allostatic load for the lifespan. Overactivity of the stress hormone axis has been linked to prenatal stress or poor maternal care in rodent models, and this overactivity contributes to increased rates of brain and body aging [39].

There are gradients of health status across income and education (referred to as 'socioecomic status' or 'SES') that are not explained by access to health care or other simple explanations [40]. Therefore, it may be of great relevance in the future to understand the role of such factors as sense of control, helplessness, persistent fear and anxiety, diet, exercise, and the impact of the living and social (e.g. family and work) environments in regulating the allostatic systems; these factors could cause allostatic systems to operate inefficiently and lead to an acceleration of genetic predispositions towards disease.

REFERENCES

1. Becker, J. B., Breedlove, S. M., Crews, D. and McCarthy, M. M. *Behavioral Endocrinology* (2nd edn). Cambridge, MA: The MIT Press, 2002.
2. Ganong, W. *Review of Medical Physiology*. Los Altos, CA: Cange Medical Publications, 1977, notes p599.
3. McEwen, B. S. Endocrine effects on the brain and their relationship to behavior. In *Basic Neurochemistry.* Eds Siegel, G. J., Agranoff, B. W., and Katzman, R. Boston: Little Brown and Co., 1981, pp755–799.
4. Korte, S. M. Corticosteroids in relation to fear, anxiety and psychopathology. *Neurosci. Biobehav. Rev.* 25: 117–142, 2001.
5. McEwen, B. S., Sakai, R. R. and Spencer, R. L. Adrenal steroid effects on the brain: versatile hormones with good and bad effects. In *Hormonally-Induced Changes in Mind and Brain.* Ed. Schulkin, J. San Diego: Academic Press, 1993, pp157–189.
6. Lupien, S. J. and McEwen, B. S. The acute effects of corticosteroids on cognition: integration of animal and human model studies. *Brain Res. Rev.* 24: 1–27, 1997.
7. McEwen, B. S. Endocrine effects on the brain and their relationship to behavior. In *Basic Neurochemistry: Molecular, Cellular and Medical Aspects* (6th edn). Philadelphia: Lippincott-Raven Publishers, 1999, p1007.
8. Garcion, E., Wion-Barbot, N., Montero-Menei, C. N. *et al.* New clues about vitamin D functions in the nervous system. *Trends Endocrin. Metab.* 13: 100–105, 2002.
9. Hojo, Y., Hattori, T., Enami, T. *et al.* Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017α and P450 aromatase localized in neurons. *Proc. Natl Acad. Sci. USA* 101: 865–870, 2003.
10. Seckl, J. R. and Walker, B. R. Minireview: 11β-Hydroxysteroid dehydrogenase type 1 — A tissue-specific amplifier of glucocorticoid action. *Endocrinology* 142: 1371–1376, 2001.
11. Flier, J. S. The missing link with obesity? *Nature* 409: 292–293, 2001.

12. Baulieu, E. E. Neurosteroids: a function of the brain. In *Neurosteroids and Brain Function.* Eds Costa, E. and Paul, S. M. New York: Thieme, 1991, pp63–73.
13. McEwen, B. S. and Alves, S. H. Estrogen actions in the central nervous system. *Endocrine Rev.* 20: 279–307, 1999.
14. Goy, R. and McEwen, B. S. *Sexual Differentiation of the Brain.* Cambridge: MIT Press, 1980, notes p223.
15. McEwen, B. S. Gonadal steroid influences on brain development and sexual differentiation. In *Reproductive Physiology IV.* Ed Greep, R. University Park: University Park Press, 1983, pp99–145.
16. Kuiper, G. G. J. M., Shughrue, P. J., Merchenthaler, I. and Gustafsson, J.-A. The estrogen receptor β subtype: a novel mediator of estrogen action in neuroendocrine systems. *Frontiers Neuroendocrinol.* 19: 253–286, 1998.
17. McEwen, B. S., Jones, K. and Pfaff, D. Hormonal control of sexual behavior in the female rat: molecular, cellular and neurochemical studies. *Biol. Reprod.* 36: 37–45, 1987.
18. Simerly, R. B., Chang, C., Muramastsu, M. and Swanson, L. W. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. *J. Comp. Neurol.* 29: 76–95, 1990.
19. DeKloet, E. R., Vreugdenhil, E., Oitzl, M. S. and Joels, M. Brain corticosteroid receptor balance in health and disease. *Endocrine Rev.* 19: 269–301, 1998.
20. Kelly, M. J. and Levin, E. R. Rapid actions of plasma membrane estrogen receptors. *Trends Endo. Metab.* 12: 152–156, 2001.
21. Milner, T. A., McEwen, B. S., Hayashi, S *et al.* Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J. Comp. Neurol.* 429: 355–371, 2001.
22. Zhu, Y., Rice, C. D., Pang, Y. *et al.* Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc. Natl Acad. Sci. USA* 100: 2231–2236, 2003.
23. Zanello, L. P. and Norman, A. W. Rapid modulation of osteoblast ion channel responses by $1\alpha25(OH)_2$-vitamin D_3 requires the presence of a functional vitamin D nuclear receptor. *Proc. Natl Acad. Sci. USA* 101: 1589–1594, 2004.
24. Akama, K. T. and McEwen, B. S. Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway. *J. Neurosci.* 23: 2333–2339, 2003.
25. Bernal, J. Action of thyroid hormone in brain. *J. Endocrin. Investig.* 25: 268–288, 2002.
26. Konig, S., Moura Neto, V. Thyroid hormone actions on neural cells. *Cell. Mol. Neurobiol.* 22: 527–544, 2002.
27. Bauer, M., London, E. D., Silverman, D. H. *et al.* Thyroid, brain and mood modulation in affective disorders: insights from molecular research and functional brain imaging. *Pharmacopsychiatry* 36: 215–221, 2003.
28. Forrest, D., Reh, T. A. and Rusch, A. Neurodevelopmental control by thyroid hormone receptors. *Curr. Opin. Neurobiol.* 12: 49–56, 2002.
29. McEwen, B. S. Stress and hippocampal plasticity. *Annu. Rev. Neurosci.* 22: 105–122, 1999.
30. Bakker, J., Honda, S.-I., Harada, N. and Balthazart, J. The aromatase knock-out mouse provides new evidence that estradiol is required during development in the female for the expression of sociosexual behaviors in adulthood. *J. Neurosci.* 22: 9104–9112, 2002.
31. De Vries, G. J., Rissman, E. F., Simerly, R. B. *et al.* A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. *J. Neurosci.* 22: 9005–9014, 2002.
32. Matsumoto, A., Arai, Y., Urano, A. and Hyodo, S. Molecular basis of neuronal plasticity to gonadal steroids. *Functional Neurol.* 10: 59–76, 1995.
33. McCullough, L. D., Blizzard, K., Simpson, E. R. *et al.* Aromatase cytochrome P450 and extragonadal estrogen play a role in ischemic neuroprotection. *J. Neurosci.* 23: 8701–8705, 2003.
34. Cameron, H. A. and Gould, E. The control of neuronal birth and survival. In *Receptor Dynamics in Neural Development* (1st edn). Ed. Shaw, C. A. New York: CRC Press, 1996, pp141–157.
35. Pfaff, D. W. *Estrogens and Brain Function*, New York: Springer-Verlag, 1980.
36. McEwen, B. S. Protective and damaging effects of stress mediators. *New Engl. J. Med.* 338: 171–179, 1998.
37. Sapolsky, R. *Stress, the Aging Brain and the Mechanisms of Neuron Death.* Cambridge: MIT Press, 1992, Vol 1, p423.
38. McEwen, B. S. Mood disorders and allostatic load. *Biol. Psychiat.* 54: 200–207, 2003.
39. Caldji, C., Liu, D., Sharma, S. *et al.* Development of individual differences in behavioral and endocrine responses to stress: role of the postnatal environment. In *Coping with the Environment: Neural and Endocrine Mechanisms.* Ed. McEwen, B. S. New York: Oxford University Press, 2000, Vol. IV, pp271–292.
40. Adler, N. E., Marmot, M., McEwen, B. S. and Stewart, J. E. *Socioeconomic Status and Health in Industrial Nations: Social, Psychological, and Biological Pathways.* New York: The New York Academy of Sciences, Vol. 896, 1999.
41. McEwen, B. S. Our changing ideas about steroid effects on an ever-changing brain. *Semin. Neurosci.* 4: 497–507, 1991.

CHAPTER

53

Learning and Memory

Joe Z. Tsien

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BRIEF HISTORY OF MEMORY RESEARCH IN HUMANS

The ability to learn and to remember is one of the most fundamental features of the brain. Understanding how learning and memory work is important, because what we learn and what we remember determine, to a great extent, what we are and who we are. Memory, not merely facial and physical appearance, defines an individual, as everyone who has known someone with Alzheimer's disease understands all too well. Furthermore, the impact of learning and memory reaches far beyond the individual, and they form the very foundation for transmitting knowledge through generations, consequently, serving as the major forces in driving cultural and social evolution.

By definition, learning is the acquisition of new information, whereas memory is the retention of acquired information. Driven by knowledge obtained by scientists before them, various disciples of neuroscience over the course of the past 100 years have learned more about various components of learning and memory, whether at the molecular level of synapses or at the systems level of brain circuitry. The concept of memory of mind has existed since the time of Aristotle. It is only during the past 50 years or so that scientists have begun to unravel some of the anatomical and cellular bases underlying such a complex mental process.

Most neuroscientists regard the ideas and observations of Santiago Ramon Y Cajal at the end of 19th century as the beginning of the cellular exploration of just how memory is retained in the brain. Upon his original observation of synaptic conjunction between neurons, he immediately entertained the idea that the modification of these conjunctions could form the anatomical basis responsible for the persistence of memory.

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The Penfield studies were a breakthrough in human functional brain mapping. So where should we look for such changes? The answer to this seemingly simple question lies at the core of modern neuroscience. Scientists must first find out where memories reside in the brain. A breakthrough was made in the mid 1950s by Wilder Penfield who had the opportunity to stimulate the cortical surface of over 1,000 of epileptic patients in the course of neurosurgery for removing epileptic tissue [1]. Since the brain does not have pain receptors, the operation only needs local anesthesia on the scalp and the patients remained fully conscious during surgery. Some patients reported that they were hearing voices and music, or seeing images and having other mental perceptions uniquely when electrical stimulation was delivered to a brain area known as the temporal lobe (Fig. 53-1). Those electrical stimulation-triggered mental experiences often had dream-like qualities, which suggested that they reflected flashbacks to past experiences.

Further studies have shown that electrical activation of specific limbic structures within the temporal lobe system, such as the hippocampus and amygdala (Fig. 53-1), are capable of generating experiential responses [2]. These fascinating reports were the first indications that the temporal lobe system may play a crucial role in representing memories and thoughts. In fact, we now know that memories are processed in many regions of the brain, far beyond the temporal lobe system [3]. Many studies suggest that memory is both distributed and localized. In other words, no single memory center exists in the brain, but rather memory is encoded along many pathways in the brain by a set of specific circuits.

Amnesia patients showed the role of temporal lobe in memory. Almost at the same time of Penfield's studies, Brenda Milner of the Montreal Neurological Institute examined a patient, known by his initials as H. M., who had undergone bilateral surgical removal of the temporal lobe (medial temporal cortex, amygdala, and two-thirds of the hippocampus). The surgery was apparently a success in terms of relieving his severe epilepsy, but left him with a devastating loss of ability to form memories [4]. For example, although H. M. recognized his childhood pictures and remembered well childhood events, he had trouble recalling major personal and social events that had taken place a couple of years before his operation. This inability to remember things that happened several years preceding the surgery is called *retrograde amnesia.*

More strikingly, in H. M.'s case, the surgery also produced severe *anterograde amnesia* — the inability to form new memories about events, places and people he encountered after the operation. For example, H.M would not recognize Dr Milner following the surgery, even though she had been examining him very frequently for more than 40 years. His anterograde amnesia was so severe that H. M. could not even recognize current photos of himself, despite the fact that he viewed himself in a mirror every morning.

Interestingly, H. M. preserved his ability to form short-term memory. For example, with rehearsal and as long as he was not interrupted, he could remember for several minutes a string of six-digit numbers. His intact short-term memory suggests that the temporal lobe is crucial for the conversion of new memory into long-term memory, a process termed *memory consolidation.* Even more amazingly, H. M. exhibited normal learning and memory for perceptual–motor tasks such as mirror-drawing (to learn to draw pictures by looking at his hand in a mirror) despite his inability to remember the repeated daily learning sessions. Thus, it seemed clear that H. M.'s ability to learn and retain new motor skills and procedural operations remained intact.

H. M. was the first human case in which specific amnesia could be linked to selective regions of the brain. Since then, many patients have been identified as having selective lesions to the temporal lobe system, especially within the hippocampus. They have exhibited amnesias similar to H. M.'s. For example, *amnesic patient*, R. B, who had a specific lesion in the CA1 region of hippocampus, showed profound loss of ability to form new memories of people, places, and events [5]. R. B. also lost memories regarding public and personal events that he had experienced two

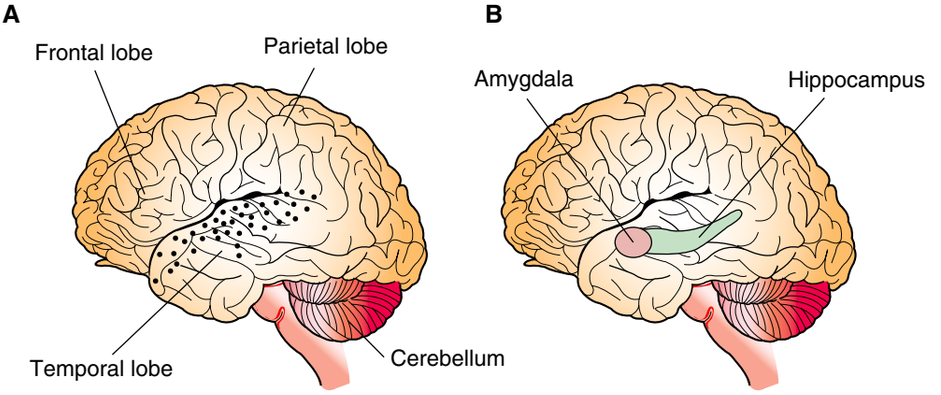

FIGURE 53-1 Illustrative drawing of the temporal lobe system in the human brain. (**A**) Anatomical sites, marked by *black dots*, within the temporal lobe where electrical stimulation evoked experiential responses in Penfield's patients. (**B**) The location of the hippocampus and amygdala inside the temporal lobe.

years before his CA1 lesion. Such clinical observations have established the view that the hippocampus system is critically involved in memory processes.

DIVISIONS OF MEMORY

Declarative memory vs. procedural memory. Based on the types of memory selectively affected in such amnesic patients, memory can be divided into two major classes: declarative memory and procedural memory.

Declarative memory, also termed *explicit memory*, is memory of places, events, facts and people, and is dependent on the temporal lobe system. Retrieval of these memories requires conscious recollection. This type of memory tends to form easily and be forgotten easily.

Declarative memory can be divided further into two subclasses: episodic memory and semantic memory. *Episodic memory* refers to memory of episodic events that contain what, where and when information. This is the major type of memory encoded in our daily life. *Semantic memory* refers to memory of facts and knowledge that are no longer ascribable to any particular occasion in life (without necessarily remembering where and when the person acquires it). Thus, semantic memory, created through either single or repeated experience, represents a more abstract generalization of experience that may give rise to concepts and categorization. Lesions in the temporal lobe, such as the hippocampus, are known to greatly impair patients' ability to learn new facts, concepts, vocabulary and knowledge about the world.

Procedural memory, also termed *nondeclarative memory* or *implicit memory,* is the counterpart of declarative memory and encompasses a variety of perceptual–motor learning skills and mental operations. This type of memory does not depend on the structural integrity of the temporal lobe system. For example, H. M. showed good ability to reduce the time required for completing a ten-choice tactile maze after 80 trials, despite the fact that he never remembered the correct sequence of turns during the maze test. Actually, amnesic patients with temporal lobe lesions retain other learning capacities such as classical conditioning (such as eye-blink conditioning), habituation, sensitization, priming (the facilitation of performance by prior exposure to words) and mental operations such as how to put together a jigsaw puzzle and use of numbers. It should be mentioned that priming is a short-lived phenomenon, whereas learned motor skills (such as how to ride a bike) can endure for a long time. Currently, the brain regions involved in encoding procedural memory are not clear, at least in humans. In rodents, lesion studies suggest that the striatum may be engaged.

Short-term memory vs. long-term memory. The classification of memories into declarative memory and nondeclarative memory is based on the anatomy of the temporal lobe system. However, memory is often classified temporally: *short-term memory* has a time course of the order of seconds to hours, whereas *long-term memory* has a time course of weeks, months and years. The basis for temporal division of the memory process is revealed by observations that newly formed memories (short-term memories) are more vulnerable to interferences and disruptions. For example, if someone involved in a car accident lapses into a coma from head trauma, he or she very likely will not remember the events leading up to and including the accident on regaining consciousness,. Similarly, someone who receives electroconvulsive treatment will lose memories of events immediately preceding the convulsion. However, these same treatments have no effects on old memories which were generated long ago (long-term memories). These phenomena have led to the notion that memories are initially created in a much more labile status, and are gradually converted into a more stable state (*memory consolidation*). As we recall from the cases of H. M. and R. B., those patients exhibited severe impairment in the formation of new long-term memories while preserving old long-term memories as well as intact short-term memories. These clinical studies, together with lesions in laboratory animals, clearly suggest that the hippocampal system is crucial for helping the brain to convert short-term memory into long-term memory.

MOLECULAR MECHANISMS OF LEARNING

Hebb's rule, long-term potentiation (LTP) and experimental models of synaptic plasticity. By knowing the neurobiological classifications of memory as well as identifying some of the brain regions crucial for the memory process, you may want to ask the next question: 'How is memory formed and stored in those brain regions?' As mentioned in the introduction, the idea that changes in synaptic efficacy are important can be traced back to Cajal 100 years ago, when he showed that networks of neurons are linked with each other at the specialized junctions that Sherrington called synapses. Cajal was the first to hypothesize that modification of the physical structures of individual signaling units, i.e. synapses, may account for the cellular event of memory storage.

The foundations for exploring the molecular and cellular mechanisms of learning and memory were laid in 1949, when Canadian psychologist Donald O. Hebb came up with a simple yet profound idea to explain how memory is represented and stored in the brain. According to Hebb's postulate [6]:

> 'When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.'

These 49 words have formed what now is known as *Hebb's Learning Rule*. Although some minor modifications have been required over the years, the essence of Hebb's rule remains unchanged: a memory is produced by coincident neural activity; when two connected nerve cells are active simultaneously, the strength of their synaptic connection increases; this confers a basis for the persistence of memory.

To understand whether the brain actually uses 'coincidence-detection' logic to modify synaptic efficacy and store information, one needs to know where to look for such synaptic changes. As we recall from the pioneering studies made by Dr Brenda Milner, the hippocampus and its nearby structures are crucial for the formation of memories about facts, people, and events. In the 1970s, Timothy Bliss and Terji Lomo observed that the perforant-dendate gyrus pathway in the anesthetized rabbit hippocampus exhibits increased synaptic responses after a train of high-frequency stimulation. The increase in synaptic transmission, as often measured by either the amplitude or slope of the EPSP, can last for hours *in vitro* and days and weeks *in vivo*. This phenomenon is called *long-term potentiation* (LTP) (see also relation to glutamate receptors in Ch. 15) [7]. Later studies by many scientists have shown that high-frequency stimulation of every pathway within the hippocampus, including the CA3–CA1 Schaffer-collateral pathway, can produce LTP (Fig. 53-2). In fact, we now know that LTP exists in many animal species and in many brain regions including the neocortex, amygdale, and striatum. Moreover, LTP occurs in several distinct forms.

A series of investigations suggests that LTP generally possesses four basic characteristics: (1) temporal specificity; (2) cooperativity, (3) associativity, and (4) input-specificity. *Temporal specificity* means that the strengthening of synaptic efficacy requires the presynaptic cell(s) to fire *before* the postsynaptic cell. This temporal requirement resembles the temporal feature of associative Pavlov conditioning in which the conditioned stimulus (e.g. a bell-ring) must precede the unconditioned stimulus (e.g. food) if the conditioning is to be successful. *Cooperativity* refers to the fact that many synapses are required to produce enough depolarization to induce LTP: activation of a single synapse can rarely trigger a spike in the brain. Because of variations in the internal states of many synapses, LTP induction thresholds are not fixed, and often require different intensities and patterns of tetanic stimulation. *Associativity* refers to the special scenario in which strong activation of one set of synapses can facilitate LTP induction at a set of recently and weakly activated adjacent synapses of the same postsynaptic cell. Finally, *input-specificity* of LTP means that potentiation is only induced at the synapses receiving stimulation, and not at unstimulated synapses on the same cell. This property is crucial since it guarantees the specificity of altered connections, and may also increase the capacity of individual neurons to process and store information.

The NMDA receptor in the hippocampus induces LTP. What molecular machinery controls these forms of synaptic plasticity? One of the most studied brain regions is the CA1 region of the hippocampus: the CA1 region is not only crucial for memory formation (profound amnesia in patient R. B with selective CA1 lesion), but also exhibits a well-organized laminar structure ideal for electrophysiological recording.

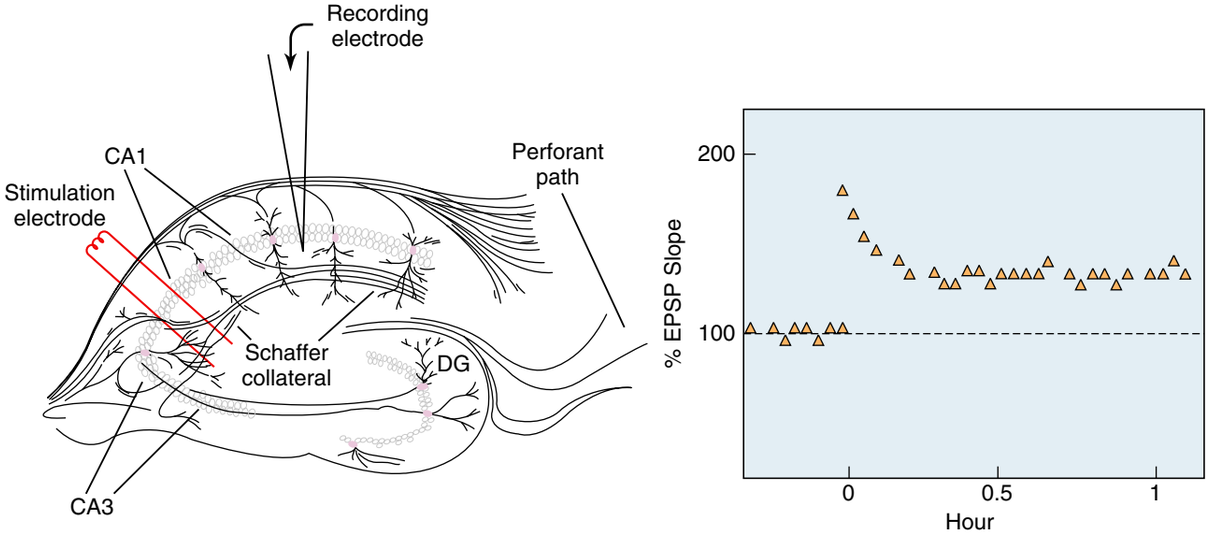

FIGURE 53-2 A drawing of the major pathways and synaptic plasticity of the hippocampus. The hippocampus is made of three major synaptic pathways (the left panel). The first one is called the *perforant*-pathway which transfers information from the entorhinal cortex to the granule cells of the dentate gyrus. The second relay is from the dentate gyrus to the CA3 pyramidal cell, and is called the *mossy-fiber* pathway. The third pathway is from the CA3 cells to CA1 pyramidal cells, termed the *Schaffer collateral* pathway. This pathway is one of the most studied systems *in vitro*. Long-term potentiation, as measured by the increase in EPSPs of CA1 cells in response to stimulation of the CA3 axon bundle, is induced by the high-frequency stimulation in the Schaffer-collateral pathway (the right panel). This increased potentiation can last for several hours *in vitro* and days and weeks *in vivo*.

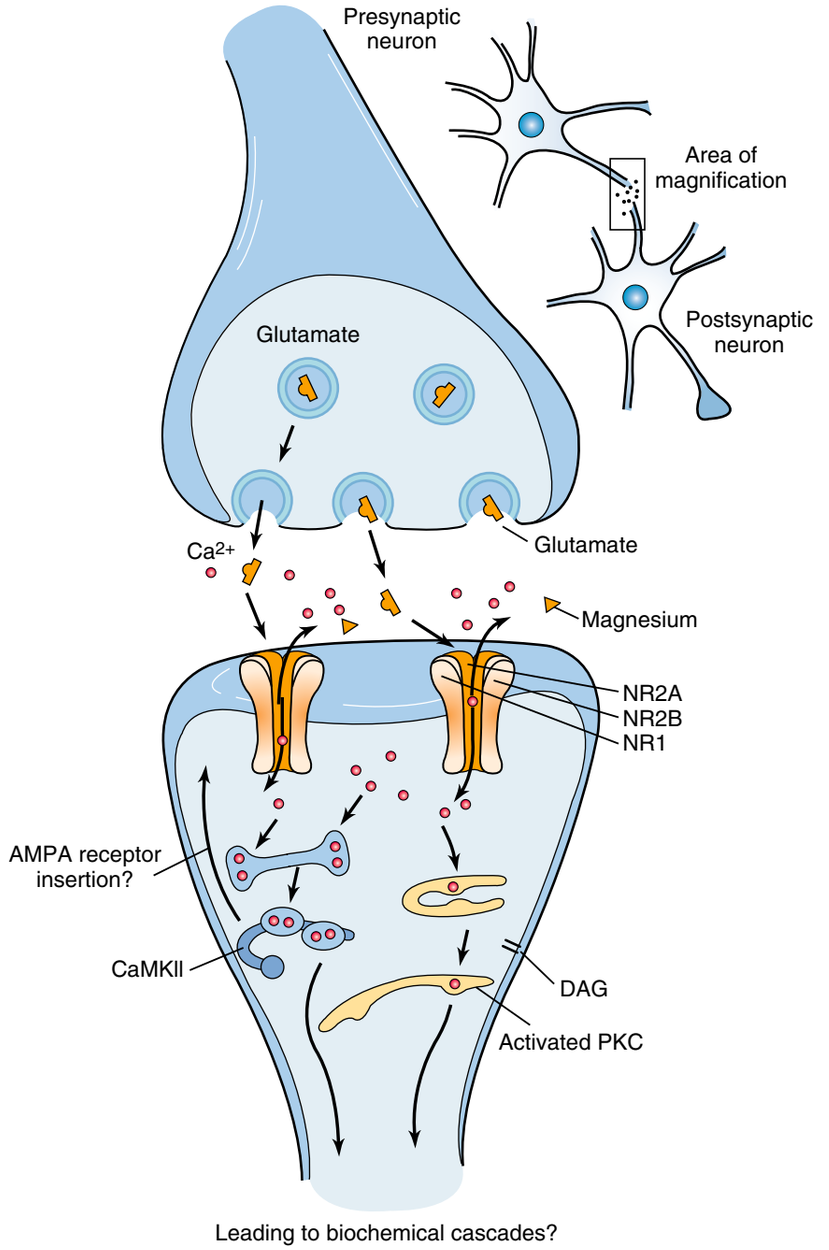

FIGURE 53-3 An illustration of a synapse between the presynaptic and postsynaptic neurons. The glutamate released from presynaptic terminals activates both AMPA and NMDA receptors. While the AMPA receptor is responsible for basal synaptic transmission, the NMDA receptor acts like the volume controller regulating the efficacy of synaptic transmission. Synaptic transmission is enhanced if the NMDA receptor detects the co-activity of the presynaptic (release and binding of glutamate) and postsynaptic neuron (enough depolarization to expel Mg^{2+} from the channel pore). When such a coincidence event occurs, the NMDA receptor is activated, which opens the channel pore and allows Na^{+} and Ca^{2+} to rush in and K^{+} to rush out. The influx of Ca^{2+} then activates biochemical cascades that eventually strengthen the synapse. It is believed that some of these kinases bind directly to the *C*-terminus of the NR2B subunit, allowing efficient signal detection and amplification (see also Ch. 15).

It is well-established that the *induction mechanism of LTP* at the CA3–CA1 synapse of the Schaffer collateral pathway requires postsynaptic activation of the NMDA receptors (Ch. 15). The NMDA receptor seems to be a perfect cellular device to detect the synaptic coincidence between pre-synaptic and post-synaptic neurons, and to associate two events at the cellular level [8].

What is the molecular underpinning for the coincidence-detector property of the NMDA receptor? The NMDA receptor is a channel protein that sits in the postsynaptic membrane (Fig. 53-3). The electrical stimulation of a pre-synaptic cell releases glutamate which binds to the post-synaptic AMPA and NMDA receptors. Glutamate binding to the NMDA receptor alone is not sufficient to activate

the channel because, at the usual resting membrane potential, the Mg^{2+} blocks the pore of the NMDA receptor, thereby preventing the channel from opening. The relief of the Mg^{2+} blockade comes when the postsynaptic cell is sufficiently depolarized by the repetitive activations of the AMPA receptor, which cause Na^+ influx and consequently increase EPSP. Thus, the opening of the NMDA receptors is both ligand-dependent (release and binding of glutamate) and voltage-dependent (the depolarization of postsynaptic cell). Because the NMDA receptor is permeable not only to Na^+ and K^+ but also to Ca^{2+}, calcium influx into a dendritic spine triggers activation of protein kinases which initiate a cascade of biochemical events (Fig. 53-3). These biochemical cascades are believed to modify synaptic strength. Thus, the NMDA receptor is a gating switch for the induction of synaptic plasticity.

Thus, the mechanistic properties of the NMDA receptor can help account for the properties of temporal specificity, cooperativity, and associativity of LTP. They can also explain why both high-frequency stimulation (100 Hz) and pairing low-frequency stimulation with postsynaptic depolarization can induce LTP. The occurrence of presynaptic activity followed by postsynaptic activity determines a temporal sequence and specificity. To generate sufficient depolarization in the postsynaptic cell to expel Mg^{2+} from NMDAR channels usually requires cooperative depolarization at many synapses. Moreover, the requirement of postsynaptic depolarization also underlies associativity since the depolarization caused by the strongly activated synapses can relieve the Mg^{2+} blockade of the NMDA receptors on weakly activated synapses.

Molecular mechanisms underlying the early- and late-phase expressions of LTP. While opening the NMDA receptors is crucial for coincidence-detection, the sensitivity and robustness of coincidence-detection is not solely determined by the opening time-window. It is dependent on at least three other features: summation of the opening duration, the peak amplitude and proper intracellular signal transduction.

Among many signaling molecules involved in the relevant biochemical cascades, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays a key role in mediating the early-phase expression of LTP [9]. Ca^{2+} entry through NMDA receptors promotes binding of calcium/calmodulin to CaMKII, which causes physical translocation of α-CaMKII to post-synaptic density zones (PSD) by binding to the *C*-terminus of the NMDA receptor NR2B subunits at synapses (Fig. 53-3). Autophosphorylation at Thr^{286} of α-CaMKII further enhances the Ca^{2+}/CaM binding affinity with the CaMKII and prolongs the association of the CaMKII holoenzyme at PSDs. It is believed that the activated CaMKII at the PSD zone is responsible for potentiating synapses, probably by causing synaptic insertion of AMPA receptors and/or increasing their single channel conductance. Several other kinases, such as PKC and MAP kinase, may also be involved in the expression of LTP. This phosphorylation-dependent modification of synaptic potentiation is believed to be capable of supporting LTP for 1–3 h. This period is termed *the early phase of LTP*.

For maintaining synaptic potentiation beyond the initial three hours, protein kinase A(PKA) and ERK pathways may be involved. This is termed *late-phase LTP* and appears to require gene transcription and protein synthesis [10]. At the level of transcription, it has been further hypothesized [11] that the phosphorylation of the nuclear transcription factor, CREB (*c*AMP *R*esponse *E*lement-*B*inding protein), is the key regulator for turning on gene expression. Initial analyses indicated that the CREB manipulations seemed to be correlated with changes in long-term memory. Since then, the role of CREB as a central switch for the long-term plasticity and memory formation has been promoted as a central dogma in neuroscience.

However, a series of recent crucial experiments have begun to question the scientific validity of this view. For example, the original 'transcription requirement for the late phase LTP' experiments could not be reproduced. Also, the transgenic fly overexpressing the dCREB2-a transgene originally reported to produce enhanced long-term memory (touted as 'photographic memories') actually carries a mutation that produces a translational reading-frame shift with the consequent formation of a stop codon at predicted amino-acid position 79. Overexpression of a *corrected* dCREB2-a transgene failed to show any enhancement of LTM [12]. Furthermore, it turns out that the original CREB global knockout mice did not delete all forms of CREB isoforms and their performance of memory tasks may be due to developmental abnormalities. Moreover, once crossed to different genetic background their memory deficits disappeared or were greatly alleviated.

Most importantly, later genetic experiments, using conditional knockout technique to delete all forms of CREB in either the entire forebrain or hippocampus, showed that these conditional knockout mice have normal hippocampal early- and late-phase LTP, and normal performance in hippocampal-dependent memory tasks [13]. Therefore, those newer findings cast significant doubt on the role of CREB as a central switch for long-term plasticity.

While gene transcription may still provide a mechanism for laying down long-term synaptic plasticity, the complex morphological specialization and large number of synapses would require that newly synthesized proteins are selectively transported to the activated synapses without altering the function of the other synapses in the neuron. One hypothesis to deal with this issue is that synaptic plasticity may be partially mediated via local production of new proteins only at specific subsets of synapses or individual spines (translational control) [14]. Another hypothesis is that the activated synapses may create some types of 'synaptic tagging' signals by which the newly synthesized proteins can find their ways to the supposed sites [15]. So far, the molecular identities of 'synaptic tagging' remain unclear (but see ref. [16]).

Although the exact molecular basis underlying long-term plasticity is not clear, the general consensus has been that structural plasticity is a fundamental property of

neurons, and that it is a component of mechanisms for storing long-term memories (both Ramon y Cajal (in 1893) and Taniz (in 1893) put forward the idea that changes in dendritic spine number and morphology may provide a cellular basis for memory storage). The search for relationships between structural changes and LTP is complicated by the difficulty in identifying and tracking changes at synapses which have been activated by electrode stimulation to undergo LTP. In order to increase the chance of detecting any learning-induced subtle structural change, researchers have employed a behavioral model known as *the enriched environment paradigm*. In this model, animals are raised in, or allowed to explore daily, a large space containing a variety of toys, exercise wheels, small houses and tunnels. This paradigm has yielded consistent improvements in learning and memory functions as well as increases in dendritic spines and synapses.

Other forms of synaptic plasticity include long-term depression (LTD) and NMDA-receptor-independent LTP. In addition to its ability to produce LTP, a synapse also possesses the ability to decrease its synaptic efficacy. For instance, it has been shown that low-frequency (~1 Hz) stimulation of the hippocampal Schaffer-collateral pathway for 15 min. produces decreased ESPS responses at CA3–CA1 synapses. This type of synaptic plasticity can last at least one hour, and is called *long-term depression* (LTD) (see glutamate transmission in Ch. 15). Similar to the typical form of LTP at the CA1 region, the induction of LTD also requires NMDA receptor activation [17]. Pharmacological studies suggest that, in contrast to the involvement of α-CaMKII in the expression of LTP, Ca^{2+}/calmodulin-dependent phosphatase (see Ch. 23) plays a major role in the expression of LTD. It is interesting to note that although both LTP and LTD induction are dependent on the activation of the NMDA receptor and Ca^{2+} influx, experiments indicate that differential levels of Ca^{2+} influx distinguish which of these signaling pathways will be activated. High Ca^{2+} influx, produced by tetanic stimulation, leads to activation of CaMKII-mediated cascades, whereas lower Ca^{2+} elevation, produced by low-frequency stimulation, favors activation of protein phosphatase cascades (see Ch. 22).

It is now known that synapses in other regions can produce the NMDA-receptor-independent LTD following the same low-frequency stimulation. This, in fact, was true of the first reported case of LTD: the original observation of synaptic weakening after stimulation was made by Masao Ito and his colleagues who recorded in the cerebellum. In the cerebellar cortex Purkinje cells receive input from two major pathways, namely, climbing fibers from the inferior olive nuclei of the brain stem, and the parallel fiber of the cerebellar granule cells. Interestingly, a single climbing fiber makes hundreds of excitatory synapses on a Purkinje cell, whereas a single parallel fiber makes only one synapse (but a single Purkinje cell receives as many as 100,000 passing parallel fibers). Ito and his colleagues found that the parallel-fiber–Pukinje synapses become weaker after the low-frequency pairing of climbing fiber stimulation with stimulation of the parallel fibers. It is now known that induction of this *cerebellar LTD* requires activation of metabotrophic glutamate receptors (mGluR). This type of glutamate receptor is coupled to G proteins that activate phospholipase C. The activation of this enzyme leads to the production of a second messenger, diacylglycerol (DAG) which in turn activates protein kinase C (Ch. 15).

Like NMDA-receptor-independent LTD, at least two types of LTP can occur independently of NMDA receptor activation. First, in the CA1 Schaffer-collateral pathway and some pathways in the visual cortex, extremely-high-frequency stimulation (200–250 Hz) can lead to slow development, over 20–30 min. after stimulation, of synaptic potentiation that is dependent on the activation of voltage-gated calcium channels (VGCC) ([18] and Ch. 6). Thus, this form of plasticity is also known as *the voltage-gated calcium-channel-dependent LTP*, or VGCC–LTP. Another interesting feature of VGCC–LTP is that in contrast to the synaptic localization of the NMDA receptors, voltage-gated calcium channels are situated at the base, or around the base, of dendritic spines. This suggests that this type of LTP may be able to spread to nearby synapses.

A second type of NMDA-receptor-independent LTP exists in the mossy-fiber pathway at the dentate granule cell-to-CA3 pyramidal cell synapse [19]. This form of LTP, termed *mossy fiber-CA3 LTP*, is believed to involve PKA activation in the presynaptic cell which leads to increased neurotransmitter release. However, the exact induction mechanism is not yet clear.

Despite the various exceptions described above, NMDA-receptor-dependent forms (both LTP and LTD) are the most prevalent CNS forms of synaptic plasticity. The pivotal role of the NMDA receptors in synaptic plasticity is also highlighted by the peculiar fact that the effect of NMDA receptor blockade on the induction of LTP and LTD is one of few things that most LTP laboratories can agree on. This contrasts with more variable results of blocking downstream molecules. In a sense, this is not entirely surprising since even a slight variation in experimental protocols (e.g. short tetanus, long tetanus, repeated tetanus, theta-burst, slice incubation temperature, etc) can often result in LTP with different magnitudes, decay and shapes, reflecting activation or recruitment of different molecular downstream pathways.

Initial evidence linking Hebb's coincidence detection rule to learning and memory. As the unique receptor in the brain with the coincidence-detection property, the NMDA receptor is an ideal candidate to gate the formation of memory at the synaptic level. Early observations demonstrated that infusion of NMDA receptor blockers into brain ventricles resulted in animals' poor performance in the hidden-platform water maze. At first, this seemed to provide evidence for the role of hippocampal LTP in memory formation. Unfortunately, careful analyses revealed that poor performances in the water maze tests

were probably due to drug-induced side-effects such as sensorimotor disturbances.

The first crucial experimental test for the role of Hebb's rule in learning and memory came in mid 1990s as a result of development of *conditional gene knockout technology* [20]. This region-specific gene knockout technique allows the NR1 gene, which encodes the core subunit of the NMDA receptor, to be selectively deleted in excitatory pyramidal neurons in the CA1 region [21] (Fig. 53-4). This second-generation knockout technique is based on the Cre/loxP recombination system, a trick employed for millions of years by a virus that infects bacteria. This new method overcomes many shortcomings of the first-generation gene knockout, which deletes the gene of interest in every tissue and during embryonic development. Therefore, the conditional gene knockout can allow researchers to avoid developmental abnormalities and the tissue nonspecificity associated with global gene knockout. Using this brain-region-specific knockout method, mutant mice were engineered, in which NMDA receptors were selectively and post-developmentally absent in the CA1 region of the hippocampus. These mice lacked NMDA-receptor-dependent forms of LTP and LTD at the CA1–CA3 synapse, and did not learn and remember well in a variety of spatial and nonspatial tasks that were dependent on the hippocampus [21, 22]. This series of experiments strongly suggest the important role of CA1 NMDA receptors in controlling the formation of hippocampal-dependent learning and memory. Intriguingly, daily exposures of these CA1-specific NR1 mice to enriched environments can significantly alleviate some of those learning and memory deficits [22]. Later studies showed that daily enrichment produces significant changes in the expression of several hundred genes, as well as an increase in dendritic spines in the brains of those mutant mice. This suggests that environmental enrichment is capable of compensating the hippocampal deficits in the CA1-specific knockout mice. However, it is interesting to note that similar enrichment seems to be incapable of rescuing the memory deficits in the forebrain-specific NMDA-receptor knockout, suggesting that the enrichment may exert its effects through a cortical NMDA-receptor-mediated mechanism.

While the conditional gene knockout experiments are supportive of a role for the NMDA receptors in memory, they are less than fully conclusive in linking the synaptic coincidence-detection feature of the NMDA receptor to memory formation. Like all 'loss-of-function' studies, CA1-specific gene-knockout experiments could, in theory, produce memory impairment via a mechanism independent of the coincidence-detection function of the NMDA receptor. For example, one may argue that the physical absence of the NMDA receptor channels may cause subtle structural reconfiguration at the synapse, thereby altering normal synaptic transmission. Therefore, the 'memory impairment' in CA1-specific NR1 knockout mice does not allow a firm conclusion that the coincidence-detection function of NMDA receptors controls learning and memory processes at the cellular level.

Genetic engineering of 'smart mice' is a more stringent test of Hebb's rule. In seeking a more stringent test of Hebb's rule for learning and memory, a somewhat unconventional design was devised: if Hebb's prediction were correct, his rule implies that enhancing the coincidence-detection feature of the NMDA receptor might serve as a theoretical basis to genetically engineer '*smart mice*' with superior learning and memory functions. If such a 'memory enhancement' experiment worked, it would offer far more convincing evidence that the NMDA receptor is Hebb's gating switch for memory formation. Among several advantages, this is a 'gain-of-function' approach that relies on fine-tuning a particular channel property of the NMDA receptor, rather than the earlier sledgehammer approach of knocking out the entire receptor function. In 1999, researchers reported just such 'gain-of-function' experiments [23].

To do this, they focused on the molecular composition and channel biophysics of the NMDA receptor. At the molecular level, NMDA receptors are believed to be tetrameric complexes consisting of the NR1 subunit and NR2

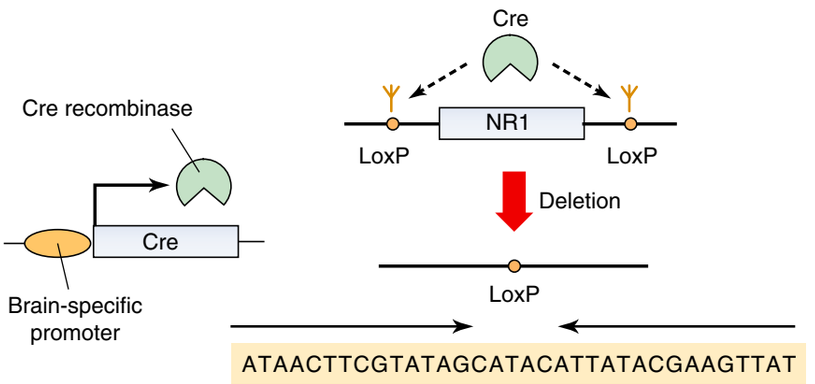

FIGURE 53-4 The strategy for achieving brain-region-specific gene knockout. This second-generation genetic technique employs a trick used by bateriophage to infect host cells: the Cre/loxP recombination system. The Cre recombinase acts like a DNA scissor which cuts specifically at the loxP sites. By expressing the Cre gene in a specific region of the brain, any gene flanked by loxP sites, which are inserted by embryonic cell homologous recombination, can be deleted. The bottom picture illustrates the efficient deletion restricted to the CA1 region of the mouse hippocampus.

or NR3 subunits (see Fig. 53-3 and Ch.15). The core component is the NR1 subunit, deletion of which leads to complete loss of the NMDA receptor function, as in the memory-deficient CA1-specific NR1 knockout mice. But the NR1 subunit needs to associate with different partners, because these other subunits determine and regulate many of the coincidence-detection features such as channel gating and Mg^{2+} dependency.

Among NR2 subunits, NR2A and NR2B are particularly important because they are the main subunits available for association with NR1 to form channels in cortex and hippocampus, the primary regions for learning and memory. Coincidently, these two subunits impart strong Mg^{2+} dependency, a feature ideal for coincidence-detection. Interestingly, the NR2C and NR2D, which exhibit greatly reduced Mg^{2+} dependency (and are thereby less suitable for coincidence function), are mainly expressed in the cerebellar granule cells and interneurons in the midbrain regions, respectively. In addition, the NMDAR complex contains many other modulation sites for fine-tuning the channel properties. For example, some neurosteroids can selectively potentiate NR2A- and NR2B-containing NMDA receptors, whereas they inhibit NR2C- or NR2D-containing NMDA receptors [24].

Integration of this knowledge with two other phenomena contributed to the design of this test:

1. In species ranging from birds to rodents to primates, the NMDA receptor almost invariably stays open longer in young animals than in adults;
2. The molecular composition of NMDA receptors in brains exhibits dynamic changes as a function of age.

During the transition from juvenile to adult, there is a gradual switch from NR2B to NR2A as the preferred partner for NR1 in the cortex and hippocampus. Moreover, it is known from *in vitro* studies that NR2B prolongs the duration of channel opening, whereas NR2A shortens it [25].

Therefore, researchers reasoned that the natural switch with age of NR1's partner could explain why the NMDA receptor in adult brain has a much narrower window of time for cellular association to occur, and it might explain why adult animals find it harder to learn and register new information [26]. So a copy of the NR2B gene was linked to a forebrain-specific promoter that increases NR2B's expression in the adult mouse forebrain (Fig. 53-5A), thereby counteracting the natural decline of the NR2B expression in the adult.

As a result of making more NR2B as a partner for NR1 in the adult cortex and hippocampus, the NMDA receptor underwent a subtle but significant change: instead of staying open for about 130 ms, as does the NR2A-containing NMDA receptor in the normal adult brain, these transgenic NMDA channels now opened for about 230 ms, in form of NR2B-containing receptor. Thus the transgenic channels retain some juvenile receptor properties which make them better at detecting synaptic coincidence and forming stronger synaptic connections between co-activated cells. The performance of these genetically modified mice was tested in a variety of situations that addressed different aspects of learning and memory. They found that these NR2B transgenic animals (nicknamed *Doogie*) learn faster, remember longer, and outperform wild-type littermates in at least six different behavioral tests [23] (Fig. 53-5B).

Recently, other research groups have independently confirmed these findings [27], and further shown that upregulation of NR2B expression at synapses can be obtained by over-expressing KIF-17, a kinesin motor protein for transporting NR2B protein from soma to dendrites. This procedure also enhances learning and memory in mice [28]. Together, these 'smart mice' experiments substantially validate Hebb's theory, by clearly demonstrating that NMDA-receptor-mediated coincidence-detection serves as a common cellular mechanism underlying learning and memory in brain. These experiments also confirm that the change in the molecular ratio of NR2A:NR2B at the onset of sexual maturity is a genetic basis for the gradual cognitive declines in adulthood. Perhaps Hamlet had a different meaning in mind when he declaimed '2B or not 2B that is the question'.

Experimentally induced LTP may be disassociated from memory function. While the evidence for the coincidence-detector function of the NMDA receptor in learning and memory is consistent, correlations between experimental LTP and specific learning and memory functions remain controversial. Although many gene knockout studies have correlated deficiency in hippocampal LTP with decreases in learning and memory, there are major exceptions to this simplistic relationship between LTP and memory. For example, in mutant mice lacking the PSD-95 gene, enhanced LTP is associated with impaired performance in the water maze [29]; in contrast, glutamate-receptor-type-A (GluR-A or GluR1) knockout mice have an impaired LTP (induced by 100 Hz ×1 s tetanus), but perform normally in the hidden-platform water maze [30]. Thus, analyses of correlations between LTP and memory formation have not given researchers a simple 'Yes-or-No' answer. This may not be entirely surprising, since evaluations of LTP mostly depend on artificial-induction in brain slices. Not only are such measurements prone to variations of experimental protocols and incubation conditions, but also LTP, as a form of an artificially induced phenomenon, is unlikely to capture all hallmark features of actual synaptic mechanisms elicited by natural learning conditions.

MOLECULAR MECHANISMS OF MEMORY CONSOLIDATION AND STORAGE

Retrograde amnesia and post-learning consolidation occur in the hippocampus. Researchers have sought to understand the biological mechanisms underlying the formation of long-term memory since Muller and

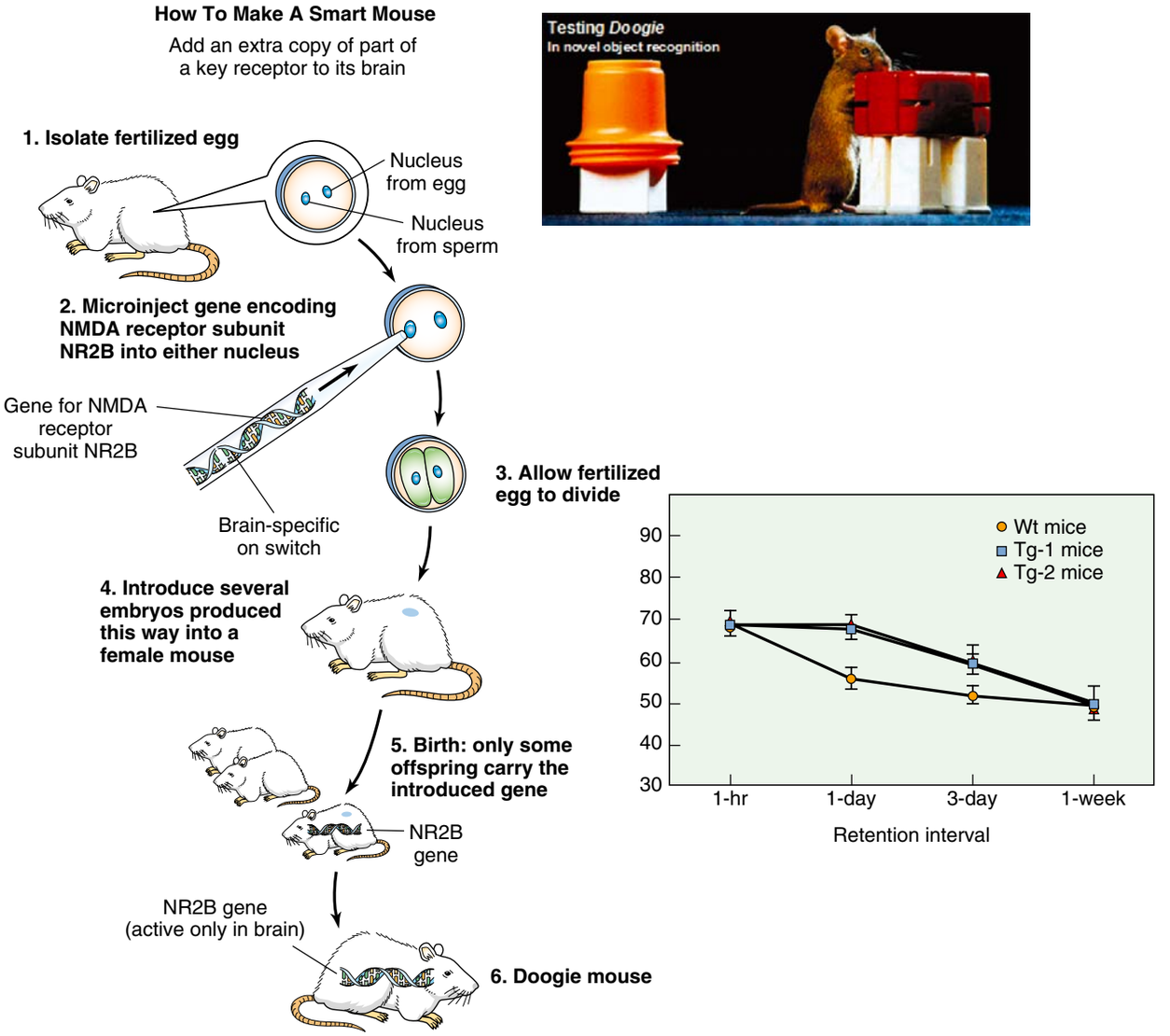

FIGURE 53-5 The procedure to make 'smart mice' through the transgenic microinjection technique. A genetically engineered smart mouse is performing the novel object recognition task. This task allows researchers to measure the amount of time the animal spent on exploring either the old toy (in *orange* on the left) or new one (the *red* one on the right). If the mouse remembers the old toy, he tends to spend more time playing with the new one. The graph shows that transgenic NR2B mice are capable of remembering for at least three days, whereas the wild-type littermates retain the memory for only one day. This task assesses only one type of memory. In other tests, the transgenic mice also showed greater learning ability (modified from Tsien, J. Z. *Scientific American,* 282: 62–68, 2000).

Pilzecker at the turn of 20th century [31]. Recall from the previous sections, that studies of amnesiac patients and experimental animals have revealed two effects of hippocampal lesion: *anterograde amnesia* (lost ability to form new memories) and *retrograde amnesia* (loss of recently formed memories). The later effect suggests that the hippocampus is involved in the conversion of short-term memory into long-lasting memory even long after learning has occurred. This post-learning process is termed *memory consolidation.* Memory consolidation requires the hippocampus to continue its engagement for an additional period of time after learning — estimated to be a couple of weeks in rodents, and up to one or two years in humans. Those observations have led to the general notion that the memory process is not an instant and unitary, but rather a gradual and continuous process which can be divided into four distinct temporal stages: acquisition (learning), consolidation, storage, and retrieval. However, analysis of molecular mechanisms underlying each memory stage has not been an easy task, since precise temporal control of molecular functions has been lacking.

A dominant view is that long-term memory is ultimately expressed in the form of changes in synaptic structure, resulting from a learning-triggered molecular cascade consisting of receptor activation, transient changes in protein phosphorylation levels, new protein synthesis, and new gene expression. This '*single cascade hypothesis*' has guided many experimental designs as well as conceptual thinking in the past decades [11]. However, emerging evidence suggests that the single cascade hypothesis is insufficient to account for the formation and consolidation of long-term memory in the mammalian brain. First, the time scales for a molecular cascade and memory consolidation do not match each other. A single molecular cascade typically operates on the timescale of minutes to hours, whereas the hippocampus-mediated consolidation of long-term memories operates on the timescale of week(s) in rodents and years in humans [5, 32, 33]. Second, although protein synthesis inhibitors seem to produce long-term memory deficits (often tested within a day or days of training), spontaneous recovery or reminder-induced recovery of memory over the period of a week or weeks has been reported in animals initially thought to be amnesic [34]. Finally, individual synaptic receptor and structural protein molecules have relatively short lifetimes, in brain as in other organs. For example, a recent study shows that synaptic NMDA receptors are degraded within 5 days in brains of freely behaving mice. Such turnover rates of synaptic receptors and their associated signaling and structural proteins would inevitably lead to deleterious drifts in synaptic efficacy, thereby destabilizing the stored memory traces, in the absence of restorative mechanisms. Thus, the observed metabolic turnovers of synaptic proteins raise a fundamental question: how can the structural changes at synapses, as envisioned by the Hebb's biochemical cascade, sustain memories over long periods of time in spite of such dynamic turnover?

Synaptic re-entry reinforcement (SRR) is a candidate cellular process for consolidating and storing memory traces. To address this question, the third-generation knockout technique has been developed to allow manipulation of gene function in an inducible, reversible, and region specific manner (Fig. 53-6). This technique allows one to genetically engineer a strain of mice in which the NMDA receptors can be selectively switched on and off in the CA1 region during various stages of the memory process in freely behaving mice. This has been employed to demonstrate that NMDAR are required not only during learning, but also during the initial post-training week(s). This reveals that memory consolidation is a continuous process and dependent on multiple rounds of NMDAR reactivations because those synaptic changes (or synaptic memory traces) created by initial learning can be repeatedly reinforced (Fig. 53-7A). This molecular reactivation process, known as *synaptic re-entry reinforcement (SRR)*, may form the crucial mechanism underlying consolidation of recently acquired memories [35].

Computational analysis has been further illustrated by repeatedly reinforcing synaptic traces via SRR mechanism within the relevant hippocampal circuits; coherent reactivations of hippocampal neurons may also act as a '*coincidence-regenerator*' to provide coordinated input that drives the collective reactivations of cortical neurons previously corresponding to different sensory modules (Fig. 53-7B). Such collective reactivations of cortical neurons consequently permit the SRR-process-based synaptic consolidation of cortical memories. Indeed, experiments demonstrate that activation of the hippocampus triggers the re-entrance of activity specifically into the deep layer of the entorhinal cortex [36]. Such re-entrance activations would result in the progressive strengthening of cortical memory traces through reactivation of cortical NMDA receptors. Once these cortical traces are fully consolidated and stabilized via cortical SRR into remote memory status, the hippocampus itself becomes dispensable for the retrieval of these cortically stored remote memories. In the absence of SRR, hippocampal memory traces could not remain stable in face of synaptic turnovers. As a result, it would be difficult for the hippocampus to maintain its ability to provide coherent output to drive the cortical neurons to reactivate together, thereby preventing the consolidation and binding of cortical memories spanning multiple sensory modules.

Consistent with the requirement of NMDA-receptor-reactivation for memory consolidation, it has been found that memory consolidation is also dependent on reactivations of CaMKII, the key intracellular mediator for the NMDA receptor activation, during the first post-learning week (see CaMKII in Chs. 15, 23). Using *inducible protein knockout technique* (Fig. 53-8), it was shown that mice with either the normal or higher level of CaMKII activity can learn and consolidate fear memories equally well. However, switching back-and-forth between two different levels of CaMKII activity within the first post-learning week severely disrupts synaptic consolidation of 1-month long-term fear memories. In other words, memory consolidation needs to occur at the same level of CaMKII activity as the one used during learning. Interestingly, the similar alterations in the CaMKII expression level during the second, third and fourth post-training weeks were no longer capable of disrupting memory consolidation. This shows that memory consolidation has a critical time-period during which precise reactivation of NMDA-receptor-mediated cascades is required. Additional experiments suggest that the SRR process appears not to be restricted to the consolidation of hippocampal-dependent memory, but is also required for the consolidation of hippocampal-independent memory. Therefore, synaptic re-entry reinforcement may present a common off-line mechanism for memory consolidation at both synaptic and systems levels in the brain.

Mechanistically speaking, SRR-mediated off-line strengthening of synaptic connections requires only pair-wise reactivation between two activated neurons. Thus, this synaptic reactivation feature is different from the reactivation of memory at the cognitive level. Although the SRR

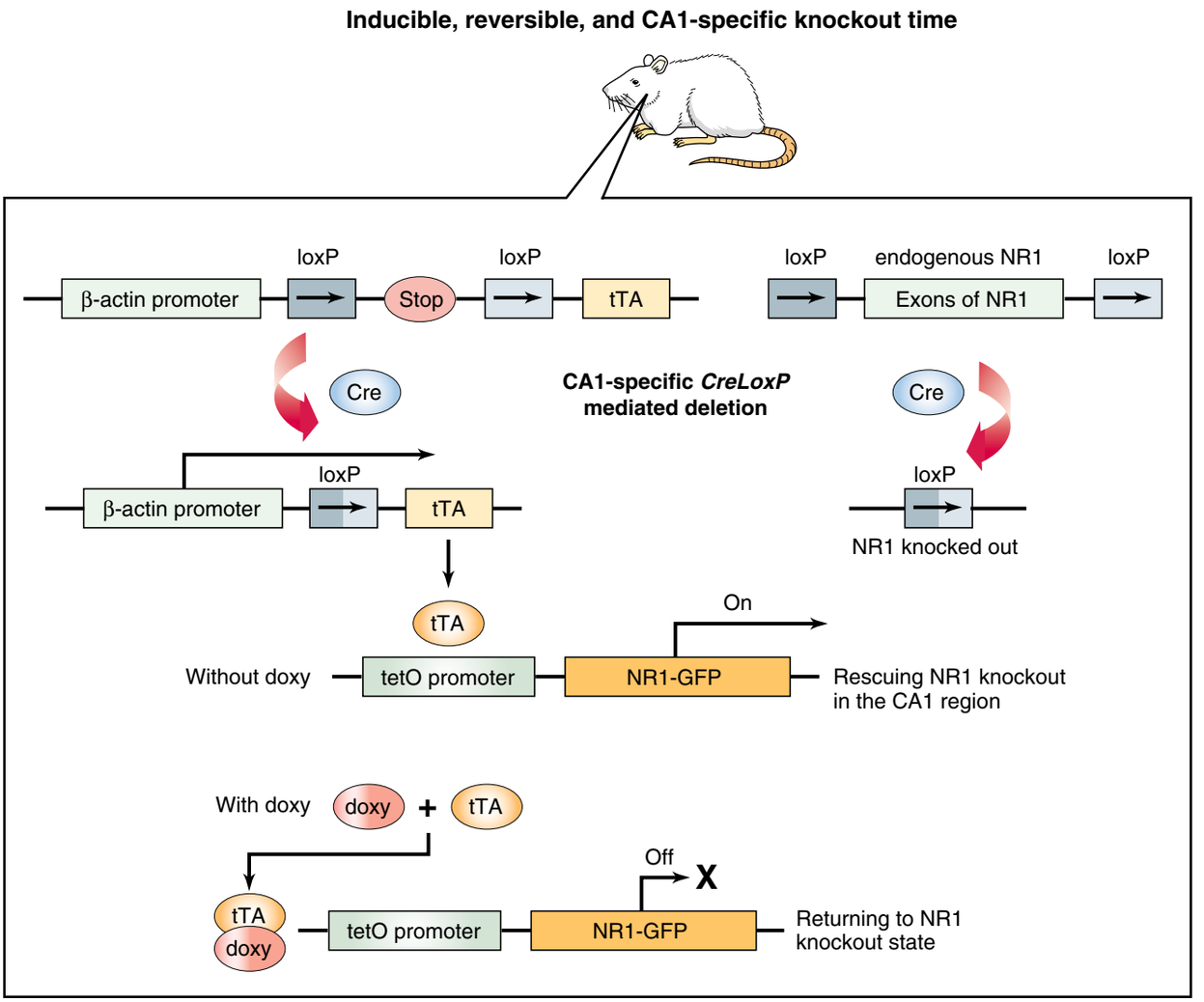

FIGURE 53-6 The strategy for making the inducible, reversible, and region-specific gene knockout. Through a series of genetic engineering techniques, any gene can be knocked out in inducible, reversible and region-specific manner. This third-generation knockout method employs both the tetracycline-based transactivator (tTA) gene expression system and the Cre/loxP recombination system. In the inducible knockout mice, expression of the tTA transgene in the CA1 neurons is achieved by the Cre/loxP-mediated deletion of the 'STOP' sequence, which then allows the expression of the NR1-green fluorescence protein (*NR1-GFP*) fusion protein in these neurons, thereby rescuing the knockout of floxed endogenous NR1 gene in CA1. Feeding the mice with doxy, a tetracycline analog compound pulling tTA off the tetO promoter, will switch off NR1-GFP transgene expression, and return the CA1 cells to the NMDA receptor knockout state. On the other hand, the withdrawal of doxy from their food or drinking water will restore NR1-GFP expression, thereby reversing NMDA receptor function (modified from Shimizu, E. *et al. Science* 290: 1170–1174, 2000).

process was initially proposed to describe the consolidation of long-term memory traces, most recent evidence shows that a similar process also seems to be required for long-term storage of remote memories in the brain [37]. Furthermore, the time necessary for detrimental manifestation of synaptic drift, in the absence of the NMDA receptors, has been estimated to be about one month. This suggests that the brain may use the SRR process to counteract cumulative synaptic drift caused by metabolic turnovers of synaptic proteins, and thereby to dynamically preserve brain stability.

When and how does the SRR process occur — a role for sleep? Currently, it is not clear what triggers the SRR process and when SRR usually takes place. We can only entertain a couple of ideas at this stage. One conceivable triggering mechanism could be that conscious recall can initiate the SRR process. This is consistent with common experience, where the more frequently a particular event is recalled, the better and longer it will be remembered. Another triggering mechanism could be the subconscious reactivation of the brain network. This could most likely be achieved during sleep, because various sleep states are

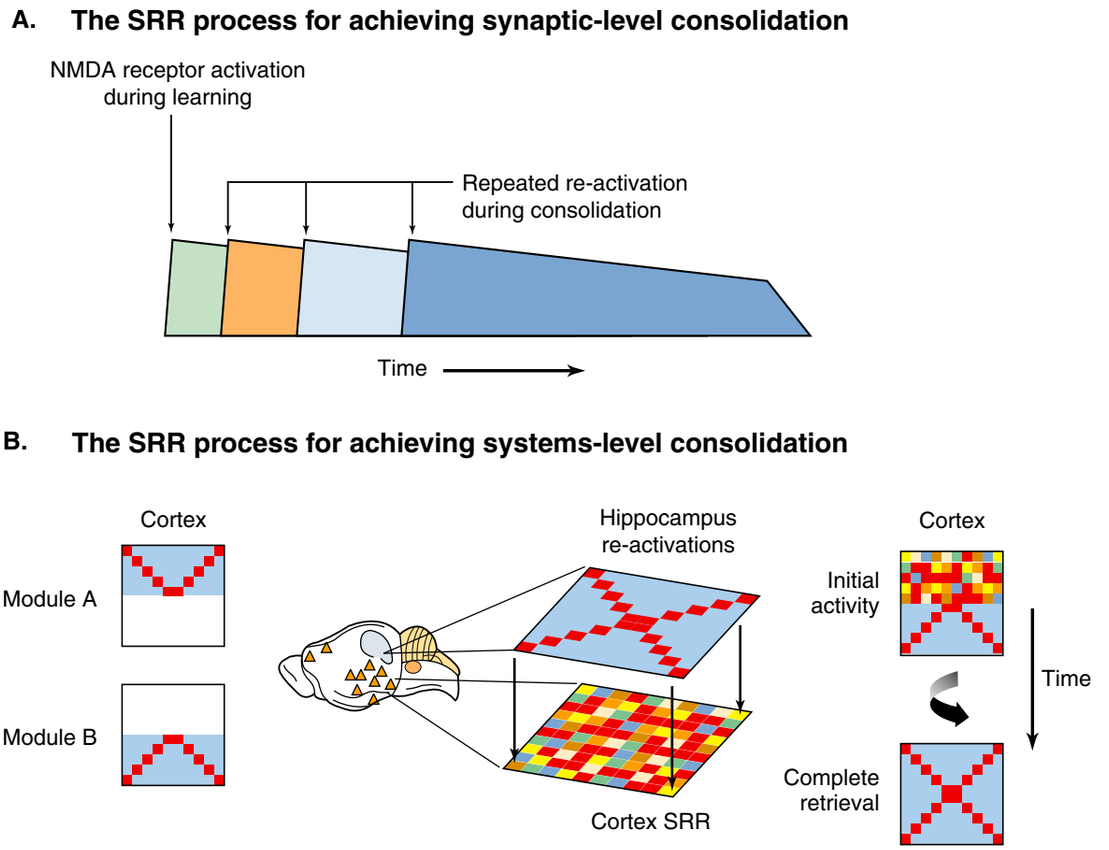

FIGURE 53-7 The synaptic re-entry reinforcement model for the consolidation of long-term memory. (**A**) Re-activations of the NMDA receptor during the consolidation period trigger the repeated reinforcements of hippocampal synaptic modifications initiated by original learning. The molecular reactivations strengthen the synaptic connections between neurons involved in the learning. (**B**) Re-activations of the hippocampus also serve as the coincidence-regenerator for co-activating the cortical neurons belonging to different sensory modules, resulting in the SRR-based strengthening of cortical synaptic efficacies, primarily between cortical modules *A* and *B*, as well as within each module. Once fully consolidated, the cortically stored memories across all modules can be retrieved by partial cues.

known to produce high levels of synchronized neuronal activity across many brain regions. For instance, recent studies show that learning-induced correlations in the firing of hippocampal place cells reappear during sleep [38]. Such a coordinated reactivation of these neurons suggests the existence of a natural condition in which NMDA receptors can be reactivated, thus reinforcing the synaptic connections between them. It is important to point out that the cellular re-activation producing SRR during subconscious consolidation is not the same as the conscious re-activation of memory traces — in the sense that it does not require sequential activation of coding assemblies at the circuitry level. In theory, consolidating synapses only require pair-wise coreactivation between the connected neurons to maintain their existing synaptic efficacy.

Clearance of outdated memory traces in the hippocampus — a role for adult neurogenesis? What happens to memory traces left behind in the hippocampus after long-term memories have been stored in the cortex? It has been estimated that the hippocampus may have limited storage capacity. For instance, there are only roughly 200,000–300,000 CA3 and 300,000–400,000 CA1 pyramidal cells and 700,000–1,000,000 granule cells in rodent hippocampus. It is conceivable that continued accumulation of outdated memory traces in the hippocampus could overload the system over time, eventually disabling the ability of hippocampal functioning in memory consolidation. So how might the hippocampus deal with this overloading problem?

A possible answer comes unexpectedly from experiments originally aimed at investigating the role of the presenilin-1 gene, whose mutations lead to familial early-onset Alzheimer's disease. This type of Alzheimer's disease is known to be the most aggressive form that can cause severe memory loss and dementia in patients as early as in their 30s. Recent studies have shown that forebrain-specific

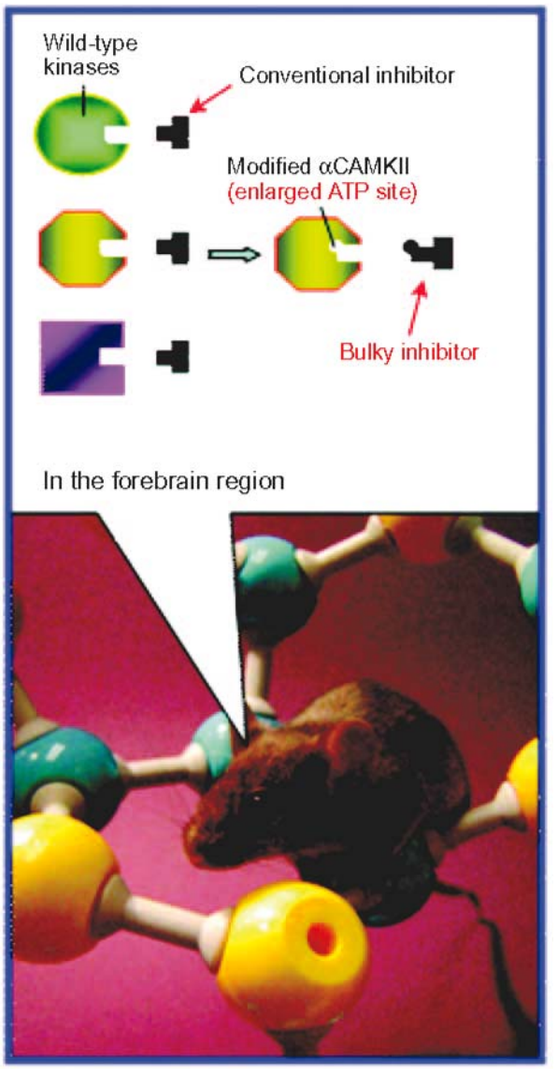

FIGURE 53-8 Inducible protein knockout strategy. This fourth-generation genetic knockout technique is based on the 'bump-and-hole' strategy (Wang, H. *et al. Proc. Natl Acad. Sci. USA*, 100: 4287–4292, 2003). The ATP-binding pocket of a protein kinase (e.g. aCaMKII) is enlarged such that a bulky inhibitor can be rationally designed to fit only the enlarged pocket and not the unmodified pocket of all other wild-type kinases. This protein knockout method offers superior temporal resolution that allows the knockout of protein function *in vivo* within several minutes, in comparison to the relatively slow inducible gene knockout, which intrinsically relies on the complete turnovers of the existing protein before phenotypic manifestation.

knockout of the presenilin-1 gene results in a pronounced deficiency in enrichment-induced adult neurogenesis in the dentate gyrus [39]. Deficient neurogenesis in the dentate gyrus is associated with impaired clearance of outdated memory traces from the hippocampus. This suggests that adult neurogenesis in the dentate gyrus plays a role in periodic clearance of outdated hippocampal memory traces after cortical memory consolidation, thereby ensuring that the hippocampus is continuously able to process new memories.

Adult neurogenesis refers to the phenomenon of continued proliferation of some progenitor cells in the olfactory bulb and the dentate gyrus of the hippocampus into neurons throughout adulthood. This capability is one of the mysteries of neuroscience, since nearly all neurons in the brain stop dividing shortly after birth. But some cells in these two regions retain this property even in adulthood. Moreover, although this phenomenon is preserved across many mammalian species, ranging from rodents to monkeys to humans, nobody knows the actual function of adult neurogenesis.

The proposed '*neurogenesis-memory clearance hypothesis*' is attractive because addition and removal of adult-born neurons in local network architecture could gradually destabilize the stored memory traces. Also, adult-generated neurons within the dentate gyrus, the upstream location in the hippocampus, potentially can amplify the 'destabilization' effects. Coincidently, these newborn neurons are short-lived, typically with a life-span of three weeks in rodents [40], which seems to correlate well with the duration of hippocampal dependence of declarative memories.

FUTURE DIRECTIONS AND CHALLENGES

As we can see from the above, over the past century scientists have made tremendous progress in understanding the molecular and cellular bases of learning and memory. Through a series of genetically engineered mice, some of the central molecular components of learning and memory have been assembled. Now we know that the NMDA receptor is a molecule with major responsibility for memory acquisition, consolidation, and storage. This memory switch is activated during learning and then needs to be reactivated for consolidating newly formed memories as well as for chronic storage of remote memories. However, many fundamental questions still remain. What is a memory trace? What is the organizing principle that the brain uses to achieve its real-time encoding of memory? How is memory retrieved? Can the stored memory be selectively erased through experimental manipulation? With new and exciting technologies emerging at the horizon, it is safe to predict that many new discoveries will be made in the coming years and decades.

From the history of the advance of sciences, it is often the case that our human quest to understand nature unexpectedly creates knowledge and technology that will have profound impact, and potentially unpredictable consequences for society and civilization. Neuroscience is no exception to this rule. For example, the genetic experiments, which were designed to test the predictions of Hebb's rule, have resulted in the creation of smart '*Doogie*' mice. While there is clearly a giant, perhaps even an impossible, gap in leaping from mice to human beings, it has already triggered widespread discussion of many issues: 'Where do all those revolutionary genetic technologies lead us?'

'Should people use genetic manipulations in human embryos to correct gene mutations which would otherwise lead to Alzheimer's disease or learning disability?' 'If so, is it ethical to use the same procedure to improve cognitive function in persons who are healthy but with low intellectual ability?' 'How should society define *normal*?' 'Where should we draw a line between moral compassion and unethical obsession?'

Although some may think these questions are premature; unfortunately, avoiding or ignoring these issues would not be a wise or lasting strategy. History has shown that scientists bear a particular burden and responsibility for directly engaging in public education and policy, because the scientific ignorance of citizens and policy-makers will not be in the long-term interest of either the scientific community or society in general.

REFERENCES

1. Penfield, W. W. and Jasper, H. *Epilepsy and the Functional Anatomy of the Human Brain*. Boston, MA: Brown, 1954.
2. Halgren, E. Walter, R. D., Cherlow A. G. and Crandall, P. H. Mental phenomena evoked by electrical stimulation of the human hippocampal formation and amygdale. *Brain* 101: 83–117, 1978.
3. Fuster, J. M. Memory in the Cerebral Cortex. *An Empirical Approach to Neural Networks in the Human and Nonhuman Primate*. Cambridge, MA: The MIT Press, 1994.
4. Scoville, W. B. and Milner, B. Loss of recent memory after bilateral hippocampal lesions. *J. Neurol. Neurosurg. Psychiatry* 20: 11–21, 1957.
5. Zola-Morgan, S, Squire, L. R. and Amaral, D. Human amnesia and the medial temporal region: Enduring memory impairment following a bilateral lesion limited to the CA1 field of the hippocampus. *J. Neuroscience* 6: 2950–2967, 1986.
6. Hebb, D. O. *The Organization of Behavior*. New York: Wiley, 1949.
7. Bliss, T. V. and Collingridge, G. L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361: 31–39, 1993.
8. Wigstrom, H. and Gustafsson, B. On long-lasting potentiation in the hippocampus: a proposed mechanism for its dependence on coincidence pre- and post-synaptic activity. *Acta Physiol Scand.* 123: 519–522, 1985.
9. Nicoll, R. A. and Malenka, R. C. Expression mechanisms underlying NMDA receptor-dependent long-term potentiation. *Ann. NY Acad. Sci.* 868: 515–525, 1999.
10. Nguyen, P. V., Abel, T. and Kandel, E. R. Requirement of a critical period of transcription for induction of a late phase of LTP. *Science* 265: 1104–1107, 1994.
11. Kandel, E. R. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 294: 1030–1038, 2001.
12. Perazzona B, Isabel G, Preat T and Davis R. L. The role of cAMP response element-binding protein in Drosophila long-term memory. *J. Neurosci.* 24: 8823–8828, 2004.
13. Balschun, D., Wolfer D. P., Gass, P. *et al.* Does cAMP response element-binding protein have a pivotal role in hippocampal synaptic plasticity and hippocampal-dependent memory? *J. Neuroscience* 23: 6304–6314, 2003.
14. Steward, O. and Schuman, E. M. Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci.* 24: 299–325, 2001.
15. Frey U. and Morris R. G. Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. *Trends Neurosci.* 21: 181–188, 1998.
16. Si K., Giustetto M. *et al.* A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. *Cell* 115: 993–904, 2003.
17. Bear, M. and Abraham, W. C. Long-term depression in hippocampus. *Annu. Rev. Neurosci.* 19: 437–462, 1996.
18. Teyler, T. J. Cavus, I., Coussens, C. *et al.* Multideterminant role of calcium in hippocampal synaptic plasticity. *Hippocampus*, 4: 623–634, 1994.
19. Zalutsky R. A. and Nicoll R. A. Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* 248: 1619–1624, 1990.
20. Tsien J. Z. *et al.* Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87: 1317–1326, 1996.
21. Tsien, J. Z., Herta, P. T. and Tonegawa, S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* 87: 1327–1338, 1996.
22. Rampon, C., Tang, Y., Goodhouse, J. *et al.* Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. *Nature Neurosci.* 3: 238–244, 2000.
23. Tang, Y., Shimizu, E., Dube, G. R. *et al.* Genetic enhancement of learning and memory in mice. *Nature* 401: 63–69, 1999.
24. Monyer, H., Burnashev, N., Laurie, D. J. *et al.* Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12: 529–540, 1994
25. Malayev A, Gibbs, T. T. and Farb, D. H. Inhibition of the NMDA responses by pregnenolone sulphate reveals subtype selective modulation of NMDA receptors by sulphated steroids. *Brit. J. Pharmacol.* 135: 901–909, 2002.
26. Tsien, J. Z. Building a brainier mouse. *Scientific American* 282: 62–68, 2000.
27. White, T. L. and Youngentob, S. L. The effect of NMDA-NR2B receptor subunit over-expression on olfactory memory task performance in the mouse. *Brain Res.* 1021, 1–7, 2004.
28. Wong, R. W., Setou, M., Teng, J. *et al.* Overexpression of motor protein KIF17 enhances spatial and working memory in transgenic mice. *Proc. Natl Acad. Sci. USA* 99: 14500–14505, 2002.
29. Migaud, M., Charlesworth, P., Dempster, M. *et al.* Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 396: 433–439, 1998.
30. Zamanillo, D. Sprengel, R., Hvalby, O. *et al.* Importance of AMPA receptor for hippocampal synaptic plasticity but not for spatial learning. *Science* 284: 1805–1811, 1999.
31. Muller G. E. and Pilzecker A. *Psychol.* 1: 1–288, 1900.
32. Kim, J. and Fanselow, M .S. Modality specific retrograde amnesia of fear. *Science* 256: 675–677, 1992.
33. Bontempi, B., Luanrant-Demir, C., Destrade, C. and Jaffard, R. Time-dependent reorganization of brain-circuitry underlying long-term memory storage. *Nature* 400: 671–675, 1999.
34. Miller, R. R. and Springer, A. D. Implications of recovery from experimental amnesia. *Psychol. Review* 81: 470–473, 1974.

35. Wittenberg, G. M. and Tsien, J. Z. An emerging molecular and cellular framework for memory processing by the hippocampus. *Trends Neurosci.* 25: 501–505, 2002.
36. Kloosterman, F., Haeften, T. V., Witter, M. and Lopes da Silva, F. H. Electrophysiological characterization of interlaminar entorhinal connections: an essential link for re-entrance in the hippocampal–entorhinal system. *Eur. J. Neurosci.* 18: 3037–3052, 2003.
37. Cui, Z. Z., Wang, H., Tan, Y. *et al.* Inducible and reversible NR1 knockout reveals crucial role of the NMDA receptor in preserving remote memories in the brain. *Neuron* 41: 781–793, 2004.
38. Wilson, M. A. and McNaughton, B. L. Reactivation of hippocampal ensemble memories during sleep. *Science* 265: 676–679, 1994.
39. Feng, R. *et al.* Deficient neurogenesis in forebrain-specific presenilin-1 knockout mice is associated with reduced clearance of hippocampal memory traces. *Neuron* 32: 911–926, 2001.
40. Cameron, H. A., Woolley, C. S., McEwen, B. S. and Gould, E. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 56: 337–344, 1993.

CHAPTER

54

The Neurochemistry of Schizophrenia

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CLINICAL ASPECTS OF SCHIZOPHRENIA

Schizophrenia is a severe, chronic disabling mental disorder. Schizophrenia affects approximately 1% of the population worldwide. Symptomatic onset occurs in late adolescence and early adulthood in males and somewhat later in females, who tend to be somewhat less severely affected. It is estimated to be the seventh most costly medical illness to society in terms of cost of care and loss of productivity, because less than 30% of affected individuals experience complete remission after its symptomatic onset at the beginning of their productive lives.

The conceptualization of schizophrenia as a specific disorder occurred at the turn of the last century. The German psychiatrist, Amil Kraepelin, identified a group of psychotic patients characterized by the onset of symptoms in early adulthood, impaired cognition and poor outcomes, which he labeled *dementia praecox*. The term *schizophrenia* was introduced a decade later by Eugen Bleuler, who characterized the splitting ('schizo') of affect (i.e. emotional tone) from cognition. He further identified four characteristic symptoms: autism, ambivalence, blunted affect and disturbances of volition. While these early clinicians readily accepted that schizophrenia was a brain disorder with heritable vulnerabilities, this biological conceptualization of the disorder was eclipsed by the rise of psychoanalysis in the 1930s, and the dominance for the next half century of its theoretical conceptualization that intra-psychic conflicts are the cause of psychiatric disorders. Thus, until recently, many clinicians viewed schizophrenia as a consequence of pathologic maternal child interactions and deviant family communication.

This chapter will review the compelling evidence that schizophrenia is a brain disorder with a high degree of heritability. It will draw upon four major research strategies that have transformed our understanding of this disorder: neuropsychopharmacology, brain imaging, genetics and postmortem studies of the brains of individuals affected with schizophrenia. Over the last decade, the convergence of findings from these different approaches is pointing to plausible, final common pathways accounting for the pathophysiology of schizophrenia.

Schizophrenia is characterized by three partially independent symptom clusters. These symptom clusters are designated: *positive symptoms, negative symptoms and cognitive impairments* [1]. The positive symptoms are the most dramatic and are manifestations of psychosis.

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They include hallucinations, typically auditory in nature wherein the individual hears voices commenting on their thoughts or actions, and delusions, which often are of a paranoid nature. The patients feel that they are being persecuted and neutral life-events take on special meaning to support this belief. More bizarre delusional experiences are common, such as the feeling that their mind or body is being controlled by someone else or that thoughts are being inserted or withdrawn from their mind. Cognitive processes can become quite disorganized, resulting in loose associations with tangential thinking, illogical reasoning and emotional states inconsistent with thought content.

The positive symptoms are the most responsive to antipsychotic medications, such as chlorpromazine or haloperidol. Initially, these drugs were thought to be specific for schizophrenia. However, psychosis is not unique to schizophrenia, and frequently occurs in bipolar disorder and in severe major depressive disorder in which paranoid delusions and auditory hallucinations are not uncommon (see Ch. 55). Furthermore, in spite of early hopes based on the efficacy of antipsychotic drugs in treating the positive symptoms, few patients are restored to their previous level of function with the typical antipsychotic medications [2].

The more enduring and disabling components of the disorder that remain after effective treatment of the positive symptoms are the negative symptoms and cognitive impairments. The term 'negative' refers to a loss or deficit, and includes the blunting of affect, social incompetence and the loss of initiative. The cognitive impairments, which are associated with a modest reduction in IQ, disrupt problem-solving abilities, verbal declarative memory, delayed word recall and verbal fluency. While clearly disabling, the cognitive deficits do not become as severe as those associated with dementia or delirium. The degree of cognitive impairment correlates with the severity of negative symptoms. Neither correlates well with positive symptoms.

Retrospective and prospective studies of children who are at genetic high-risk for schizophrenia indicate that subtle abnormalities in cognitive function and attention, social oddness and motor clumsiness are often present years before the onset of psychosis [3]. These observations on the developmental aspects of schizophrenia, as well as the enduring nature of the negative symptoms and cognitive impairments over the lifetime of the schizophrenic patient, has led to the conceptualization of these symptoms as the *endophenotype* of schizophrenia — i.e. the core symptomatic features — with psychosis viewed as a secondary phenomenon. The variation in symptomatic features of schizophrenia across individuals, and the common nature of the disorder, strongly suggest that there will probably be multiple causes that result in the phenotype.

Schizophrenia is a disorder of complex genetics. Adoption, twin and family studies carried out over the last 40 years have provided compelling evidence for the heritability of schizophrenia [4]. Meta-analysis of the twin studies indicates a concordance rate of approximately 50% in identical twins if one twin is affected, which is 5-fold greater than the concordance rate in fraternal twins or siblings. The absence of complete concordance in identical twins points to additional epigenetic factors, which can transform genotype to phenotype. Perinatal insults such as second trimester viral infection and anoxia at birth figure prominently as environmental factors influencing the risk of schizophrenia. Findings from family studies support the role of complex genetics. The risk of developing schizophrenia in first-degree relatives of a proband is approximately 10%, which is 10-fold greater than the risk in the general population. In second-degree relatives the risk falls to 3%. Based upon a multigroup model, evidence supports substantial additive genetic effects, indicating that the heritable liability to develop schizophrenia is approximately 80%. The pattern of distribution of schizophrenic symptoms within affected families, with a rapid falling off of concordance from that in identical twins to first-degree relatives to second-degree relatives, is consistent with complex genetics in which many genes of small effect interact to contribute to the phenotype.

The family environment, such as communicational deviancy, is unlikely to contribute to risk, because adoption studies indicate that the risk for schizophrenia in offspring of schizophrenics (i.e. first degree relatives) is unchanged by being adopted into and raised in a family without schizophrenia. Nevertheless, adverse family environments characterized by high levels of expressed emotions negatively affect the course of schizophrenia for those who are already symptomatic.

Meta-analysis of the 20 genome-wide scans for linkage to schizophrenia has identified loci on 16 of the autosomes at which susceptibility genes are likely located[5]. Thus far, there is no evidence for autosomal dominant/recessive Mendelian-like genes that would account for schizophrenia. Potential risk genes will be discussed in the section **Cellular and Molecular Studies**.

Current treatment of schizophrenia relies on atypical antipsychotic drugs. The antipsychotic efficacy of chlorpromazine for the treatment of schizophrenia was discovered serendipitously by Laborit, Delay and Denicker in France in 1953, following the observation of the peculiar calming and poikilothermic effects of chlorpromazine in experimental animals[6]. In the early 1960s, placebo-controlled double-blind studies demonstrated that chlorpromazine was superior to placebo and the sedative phenobarbital in reducing psychosis in schizophrenic patients. A number of other effective antipsychotic drugs were subsequently discovered over the next 30 years on the basis of animal behavioral screens. These screens, we now know, selected for the drug's ability to block dopamine D2 receptors, as shown by their ability to prevent apomorphine-induced vomiting in dogs and amphetamine-induced hyperactivity in rodents (dopamine

receptors are discussed in detail in Ch. 12). In 1963 Arvid Carlsson was the first to propose that antipsychotic drugs exert their therapeutic effects by blocking dopamine receptors, based on the observations that these drugs cause Parkinsonian side-effects, which were known to be associated with loss of striatal dopamine, and that these drugs also increased the turnover of dopamine in the striatum[7] (see also Ch. 46).

Fifteen years later with the development of radio-ligand binding assays, the dopamine D2 receptor was identified by the specific, high-affinity binding of [^{3}H]haloperidol to brain membranes that displayed the requisite regional and pharmacologic characteristics for a dopamine receptor distinct from the dopamine receptor-associated adenylyl cyclase activity (D1 receptor, see Chs 12 and 21). Furthermore, a compelling correlation between the affinity of antipsychotic drugs, regardless of structural class, for the D2 receptor, and their clinical potency was demonstrated. Abuse of stimulants like amphetamine, which releases dopamine, was known to cause psychotic symptoms, especially paranoid delusions, similar to those observed in schizophrenia. Given the antipsychotic effects of these D2 blocking drugs in schizophrenia, the *dopamine hypothesis* was articulated, in which schizophrenia was proposed to be caused by excessive dopaminergic neurotransmission [9].

The typical antipsychotic drugs, which for 50 years have been the mainstay of treatment of schizophrenia, as well as of psychosis that occurs secondary to bipolar disorder and major depressive disorder, affect primarily the positive symptoms[10]. The behavioral symptoms, such as agitation or profound withdrawal, that accompany psychosis, respond to the antipsychotic drugs within a period of hours to days after the initiation of treatment. The cognitive aspects of psychosis, such as the delusions and hallucinations, however, tend to resolve more slowly. In fact, for many patients the hallucinations and delusions may persist but lose their emotional salience and intrusiveness. The positive symptoms tend to wax and wane over time, are exacerbated by stress, and generally become less prominent as the patient becomes older.

Among the commonly used typical antipsychotic drugs are chlorpromazine, haloperidol, fluphenazine, molindone and thioridazine [10]. In spite of their structural heterogeneity, there is no evidence that they differ in efficacy in the treatment of psychosis, although they do differ in their clinical potency and in their side-effect profiles. A major limitation of the typical antipsychotic drugs is the high risk of extrapyramidal side-effects due to a blockade of striatal dopamine D2 receptors. Manifestations of these include extrapyramidal or Parkinson's-disease-like syndrome, characterized by bradykinesia, tremor and rigidity. The associated mask-like face and 'zombie-like' gait are stigmatizing for patients (see also Ch. 46). Younger patients are particularly prone to the development of dystonic reactions, with cramping of tongue, neck or back muscles causing grotesque posturing. *Akathisia* causes a sense of inner tension that is relieved only by movement (i.e. pacing). As akathisia is quite uncomfortable, and it is a major cause of noncompliance in taking antipsychotics. Finally, with long-term use of the antipsychotics, a relatively irreversible neurologic syndrome can occur known as *tardive dyskinesia,* which is characterized by writhing movements of the tongue and choreiform movements of the extremities. These movements persist long after the discontinuation of the antipsychotic drugs.

Additional side-effects, reflecting the various degrees of muscarinic receptor antagonist actions of the antipsychotic drugs, result in dry mouth and constipation. α-Adrenergic receptor antagonism is associated with hypotension, tachycardia and impotence. Histamine H1 antagonism contributes to sedation. Needless to say, this panoply of side-effects has been associated with real problems in compliance with medication, resulting in discontinuation of therapy and recurrence of psychosis.

Because of the problems with compliance and stigma, neuropharmacologists have long sought antipsychotic drugs that are devoid of neurologic side-effects. The first such agent identified was clozapine[11]. It does not produce catalepsy in rodents, nor does it cause acute extrapyramidal side-effects or tardive dyskinesia in humans. Furthermore, low doses of clozapine have been used effectively in Parkinson's disease to treat psychotic symptoms induced by dopamine replacement therapy without exacerbating the underlying neurologic symptoms (Ch. 46). The clinical use of clozapine has been limited because of a 1% risk of a fatal hematologic complication known as *agranulocytosis.* To avoid this complication, patients receiving clozapine are subject to regular hematologic tests to identify those at risk. Clozapine has been found to be particularly effective in treating a subgroup of schizophrenic patients who respond poorly to typical antipsychotic drugs, in whom it reduces negative symptoms, improves cognition and is associated with a marked reduction in suicide (the lifetime risk for suicide in schizophrenia is 10%).

Deciphering the mechanism of action of clozapine has been difficult because clozapine and its metabolites interact with several neurotransmitter receptors aside from the D2 receptor, including the dopamine D4 receptor that is predominantly expressed in cortico–limbic regions, muscarinic receptors, α-adrenergic receptors, histamine receptors and the serotonin 5-HT_{2A} receptor (see also Chs 11–14). Since blockade of the 5-HT_{2A} receptor appears to mitigate against extrapyramidal symptoms resulting from D2 receptor blockade, a number of effective antipsychotic drugs have been developed which have dual D2 and 5-HT_{2A} antagonism; these include risperidone, olanzapine, quetiapine and ziprasidone. Since these newer agents have a lower propensity for causing extrapyramidal symptoms, they have been designated 'atypical antipsychotic drugs'. Because of their low risk for neurologic side-effects and their possibly greater efficacy than typical antipsychotic drugs, the second generation of atypical antipsychotic drugs have largely replaced the typical antipsychotic drugs in the management of schizophrenia.

Nevertheless, some atypical antipsychotic drugs, such as clozapine and olanzapine, have been linked to substantial weight gain, hyperlipidemia and type II diabetes, a new range of medically serious side-effects.

Extrapyramidal side-effects generally appear with blockade of dopamine D2 receptors in excess of 80%, whereas clinical efficacy in treating psychosis is associated with 60–70% D2 receptor blockade [12]. Recently, a partial agonist for the D2 receptor known as aripiprazole has been developed, which results in approximately 70% antagonism/30% agonism at the D2 receptor. It is an effective antipsychotic, has low risk for extrapyramidal symptoms, and does not cause elevated levels of prolactin as do the full antagonists at D2 receptors.

BRAIN IMAGING

Brain imaging studies provide unequivocal evidence that schizophrenia is a disease of brain. Brain imaging exploits three different strategies:

- Morphometric analysis utilizing computer assisted tomography (CAT) or magnetic resonance imaging (MRI) to reveal brain structure;
- Functional brain imaging, which monitors blood flow or glucose utilization through Positron Emission Tomography (PET) or functional (f) MRI;
- Measurement of brain neurochemical features by means of PET ligand binding studies or MRI spectroscopy (MRS), which can quantify chemicals of high concentration in the brain.

These methodologies are discussed in Chapter 58.

The first report of cortical volume loss in schizophrenia must be credited to Alois Alzheimer, who is most noteworthy for his clinical and neuropathologic description of the primary dementia that bears his name (Ch. 47). However, the unequivocal proof of his initial observations awaited the application of CAT scanning and quantitative morphometric MRI nearly 100 years later. Initial findings to support this inference came from CAT scans documenting an increase in the size of the lateral ventricles in schizophrenia. MRI studies that exploit sophisticated statistical analyses demonstrate unequivocally reduced volume of the cerebral cortex that is unevenly distributed and affects predominantly the frontal cortex and temporal lobe [13]. Segmentation studies indicate that these effects are borne predominantly by the gray matter, although some reductions in white matter volume have been demonstrated. Magnetic tensor imaging studies also indicate disruption of axon terminals in the cerebral cortex in schizophrenia.

The reduction in cortical volume observed in schizophrenia is substantially less than that found in the adult onset primary dementias such as Alzheimer's disease and Huntington's disease. A subject of controversy has been whether the reduction in cortical volume in schizophrenia is the result of developmental cortical hypoplasia or of a progressive cortical atrophic process after development. Studies of patients in the earliest stages of schizophrenia do indicate cortical volume reduction as well as functional abnormalities. Nevertheless, prospective studies have revealed increases in ventricular volume, progressive loss in cortical volume and progressive impairments in cortical function in a substantial portion of, but not all, subjects with schizophrenia [14]. Notably, the degree of cortical volume reduction correlates with the severity of cognitive impairments and negative symptoms in patients suffering from chronic schizophrenia.

Magnetic resonance spectroscopy has provided compelling evidence of significant persistent neurochemical abnormalities in the brains of individuals with schizophrenia. *N*-acetyl-aspartate (NAA), an amino acid of poorly understood function, is concentrated in neurons, including their somata, axons and dendrites, and appears to be a marker of neuronal functional status. A number of studies have demonstrated small but highly significant reductions in the levels of NAA in the prefrontal cortex, temporal lobe and hippocampal formation in schizophrenia. Levels of NAA in prefrontal cortex correlate negatively with symptom severity [15]. Furthermore, NAA deficits in the hippocampal formation and in the dorsolateral prefrontal cortex correlate with working memory impairments. In contrast, the level of glutamine, a precursor to the neurotransmitter glutamate, has been reported to be elevated in the anterior cingulate cortex and in the thalamus in never-treated patients with schizophrenia. ^{31}Phosphorus MRS studies have consistently shown membrane phospholipid abnormalities in individuals with schizophrenia [16]. Thus, glycerophospho-ethanolamine has been found to be decreased in the anterior cingulate, prefrontal cortex and thalamus in schizophrenia. There have been consistent reports of a decrease in phosphomonoester and an increase in phosphodiester in prefrontal cortex, These membrane lipids are discussed in Chapters 2 and 3.

Functional imaging studies have consistently shown cortico–limbic abnormalities in schizophrenia. Two non-invasive brain imaging techniques have been used to understand neural function in schizophrenia: surface electroencephalographic recordings, such as the event-related potentials (ERP); and brain hemodynamic activity, as measured by ^{15}O regional cerebral blood flow by positron emission tomography (PET) or blood oxygen level desaturation (BOLD) measured with fMRI (Ch. 58).

Electroencephalography allows real-time measurement of neural activity with millisecond temporal resolution. When coupled with the repeated presentation of a stimulus, the electroencephalograms can be averaged to produce an event-related potential (ERP), whose components develop and resolve within tens or hundreds of milliseconds. These potentials are designated by the millisecond lag between presentation of the stimulus and the appearance of the ERP, with the P50 (i.e. potential at 50 ms after

the stimulus) and P300 being the subject of most study in schizophrenia. The P300 ERP is smaller in amplitude and longer in latency in patients with schizophrenia as compared with controls [17]. The latency is increased with the duration of disease, although the P300 amplitude is unaffected by the severity of symptoms or antipsychotic medications. The P50 is a measure of sensory gating. The subjects receive two brief auditory stimuli separated by 400 ms. In normal subjects, a negative wave at 50 ms after the second stimulus is suppressed, whereas this is not the case in subjects with schizophrenia. An allelic variant in the promoter region of the α7 nicotinic receptor gene (CHRNA7), which has been linked to schizophrenia, is associated with abnormal P50 potential [18]. Notably, the atypical antipsychotic, clozapine, corrects this abnormality in schizophrenic patients, whereas other typical and atypical antipsychotic drugs do not. In a related task, *pre-pulse inhibition* (PPI), there is a warning auditory stimulus followed by a loud noise, causing a startle response. After a few experiences, normal subjects recognize the low volume warning stimulus and suppress the startle response, whereas schizophrenics fail to suppress the startle response.

Early functional imaging studies performed in subjects at rest suggested that blood flow was reduced somewhat in the frontal cortex in patients with schizophrenia. However, when subjects with schizophrenia are compared with normal subjects while performing cognitive tasks that require the engagement of the frontal cortex, such as the Wisconsin Card Sort Task, robust differences between controls and the patients are observed [19]. Similarly, when patients with schizophrenia are compared with controls using a difficult memory-recall task, patients perform more poorly than the controls and also exhibit little increase in blood flow to the hippocampus [20]. Measurement of the absolute blood flow to the hippocampus indicated a significantly elevated blood flow at rest in the schizophrenic subjects, suggesting that the failure to activate reflected a 'ceiling effect'. Functional imaging during the performance of cognitive tasks, such as word recognition, demonstrates regions of reduced blood-flow (such as the dorsolateral prefrontal cortex and paralimbic regions) in schizophrenic subjects as compared with controls, but also areas of increased blood flow (such as the anterior prefrontal cortices) in comparison with controls, indicating that schizophrenic subjects engage alternative neuronal systems in their attempts to perform such tasks.

One of the most prominent positive symptoms in schizophrenia is the auditory hallucinations that are perceived as distinct voices emanating from outside the individual. Regional cerebral-blood-flow studies in patients experiencing auditory hallucinations reveal activation of the associational auditory cortex during the episodes of hallucinations, but not in their absence. One theory holds that auditory hallucinations occur as a consequence of the inability of individuals with schizophrenia to monitor effectively their inner speech. fMRI studies suggest that this monitoring function requires activation of the temporal, parietal and parahippocampal cortices. Schizophrenic subjects exhibit much lower activation of these regions when generating inner speech.

PET studies are helpful in measuring trace markers, such as receptors and transporters in the brains of living subjects. Because antipsychotic drugs all block dopamine D2 receptors, the status of forebrain dopaminergic systems has been of particular interest with regard to schizophrenia research. Quantitating dopamine transporters (DAT), a marker thought to be an index of dopaminergic terminal density, with [^{123} I]β-CIT has revealed no apparent difference in DAT density in schizophrenic subjects as compared with controls. Most evidence indicates no difference in the density of D2 receptors in the striatum in schizophrenic subjects as compared with controls in PET scanning studies. Investigators have exploited the PET ligand [^{11}C]raclopride, which has high specificity but low affinity for the D2 receptor, to monitor endogenous dopamine release through its displacement of the ligand from the D2 receptor [21]. Challenge with D-amphetamine, which releases endogenous dopamine, causes a greater displacement of [^{11}C]raclopride in schizophrenic subjects than controls. Consistent with this finding, several studies using [^{18}F] or [^{11}C]DOPA have documented an increased incorporation of the dopamine precursor in the striatum in schizophrenic subjects as compared with controls, suggesting an increased turnover of dopamine, indicating increased dopamine release in schizophrenic subjects. Hypofunction of NMDA subtype of glutamate receptors has also been hypothesized as a core feature of schizophrenia (see NMDA receptors in Ch. 15). Studies of striatal D2 receptor occupancy with [11C]raclopride show that normal subjects receiving the NMDA receptor antagonist ketamine exhibit a more robust release of dopamine than subjects naive to ketamine, replicating the findings from schizophrenic subjects.

A modification of the dopamine hypothesis proposes that dopaminergic neurotransmission is impaired in the prefrontal cortex, although elevated in subcortical regions [22]. Studies with ligands for the dopamine D1 receptor have revealed an increase in the density of these receptors in the frontal subcortical region, which correlates with the severity of negative symptoms. This finding comports with the modified dopamine hypothesis that dopaminergic function is reduced in the frontal cortex, which would provoke D1 upregulation in subcortical regions.

In summary, brain imaging studies have provided compelling evidence of structural, neurochemical and functional abnormalities in the cerebral cortex, limbic system and thalamus in patients suffering from schizophrenia. The structural abnormalities, primarily loss of gray matter in discrete cortical regions and in the thalamus, correlate with the impairments in the cognitive functions associated with these regions. Finally, these cognitive impairments correlate with regionally specific abnormalities in neural processing. These findings provide a solid

foundation for inferring neurochemical abnormalities in the brains of individuals with schizophrenia.

CELLULAR AND MOLECULAR STUDIES

The dopamine hypothesis has dominated schizophrenia research for over 40 years. Given the current evidence that schizophrenia is a highly heritable disorder with complex genetics involving multiple genes of small or modest effects interacting to produce the phenotype, it is now clear that dysfunction of a single neural system will be unlikely to account for the pathophysiology of the disorder. Rather, the emerging evidence from postmortem studies, in the context of the accumulating genetic findings, is that there are a number of neural systems that can be affected in schizophrenia but that none appear to be affected in all cases. Postmortem tissue studies have become much more comprehensive with the introduction of DNA-micro-array analyses capable of measuring up to 30,000 transcripts. Cellular specificity has also been markedly improved, with the use of laser-capture single-cell analysis as well as *in situ* hybridization. The extensive array of antibodies against specific proteins augurs well for proteomic strategies to measure proteins, as well as their posttranslational modifications such as their phosphorylation state. Finally, informatics methodology identifies interrelationships among proteins that reveal families of proteins involved in common functional or structural roles. Freeware, such as Expression Analysis Systemic Explorer (EASE), available through the NIH Database for Annotation, Visualization and Integrated Discovery (DAVID), facilitates such analyses. This strategy assists in linking seemingly isolated abnormalities in gene or protein expression that may be involved in related functions such as myelinization or mitochondrial oxidative metabolism [23].

Despite the ability to measure dopamine, its metabolite homovanillic acid (HVA) and other presynaptic markers for dopaminergic neurons in the striatum and cortico–limbic regions for 40 years, most postmortem findings of dopaminergic markers in schizophrenia have been inconsistent and largely negative. The only reproducible finding is that antipsychotic drugs increase cerebrospinal fluid (CSF) and plasma HVA, consistent with preclinical studies demonstrating that D2 blockade causes an increase in dopamine turnover.

The dopamine receptors, so designated by the sequence of their discovery, consist of DA1, which is positively coupled to adenylyl cyclase, and DA2, which is negatively coupled to adenylyl cyclase[8] (Ch. 12). Cloning strategies yielded three additional DA receptors: D3 and D4, which are D2-like, and D5, which is D1-like. Given the remarkable correlation of D2 receptor affinity and clinical potency of antipsychotic drugs, perhaps it is surprising to note that over a generation of studies have revealed no consistent evidence of alterations in D2 receptor as measured by ligand binding, receptor autoradiography and *in situ* hybridization in brains from schizophrenic patients, who were untreated or not recently treated with antipsychotic drugs (to avoid the confounding effects of D2 antagonists on D2 expression). D3 receptors are concentrated in the ventral striatum and their expression is regulated by brain-derived neurotrophic factor (BDNF). Studies to determine alterations of expression of D3 receptors in schizophrenia have been largely inconclusive. However, results of studies analyzing the serine-9-glycine polymorphism of the D3 receptor suggest that the glycine-9 allele may confer susceptibility to tardive dyskinesia.

The D4 receptor differs from D2 and D3 receptors by exhibiting a disproportionately high affinity for the atypical antipsychotic clozapine, as well as for some other antipsychotic drugs. The D4 receptor is relatively enriched in cortico–limbic regions including the hippocampus in humans, but is expressed at very low levels in the striatum. While receptor autoradiographic techniques that measured indirectly D4 receptor density suggested an increase in the striatum in schizophrenia, PCR and *in situ* hybridization studies have not revealed consistent alterations in D4 receptors in striatum or in most areas of cortex in schizophrenia. The D1 receptors, for which antipsychotic drugs have a highly variable affinity, are highly expressed in frontal cortex and they play an important role in cognitive functions.

Since the ventral-tegmental-area (VTA) cortical-dopaminergic fibers express negligible amounts of dopamine transporter (DAT), the inactivation of extracellular dopamine occurs primarily through catabolism by catechol-*O*-methyltransferase (COMT) (see Ch. 12). In this regard, a common allelic variant of COMT, valine-158-methionine, is associated with substantial differences in COMT activity, as the valine allele is much more temperature sensitive, resulting in lower enzymatic activity than the methionine allele [24]. Cognitive testing in normal subjects matched for IQ demonstrates that those with the least active COMT genotype (valine–valine), which would enhance cortical dopaminergic neurotransmission, perform best on a task that requires prefrontal cortical function as compared with those with the methionine allele. Schizophrenics with higher activity genotypes for COMT exhibit greater impairments in cognitive functioning, suggesting that this may be a risk gene for the disorder. These genotype differences have been confirmed in postmortem studies.

Hypofunction of NMDA receptors may contribute to the endophenotype of schizophrenia. The hypothesis that hypofunction of a subpopulation of NMDA receptors contributes to the pathophysiology of schizophrenia has gained considerable support over the last decade (**see Fig. 54-1**). The dissociative anesthetics including phencyclidine (PCP) and ketamine when introduced clinically 40 years ago were noted to produce a syndrome that was difficult to distinguish from schizophrenia. These agents act as non-competitive open-channel blockers of the NMDA receptor.

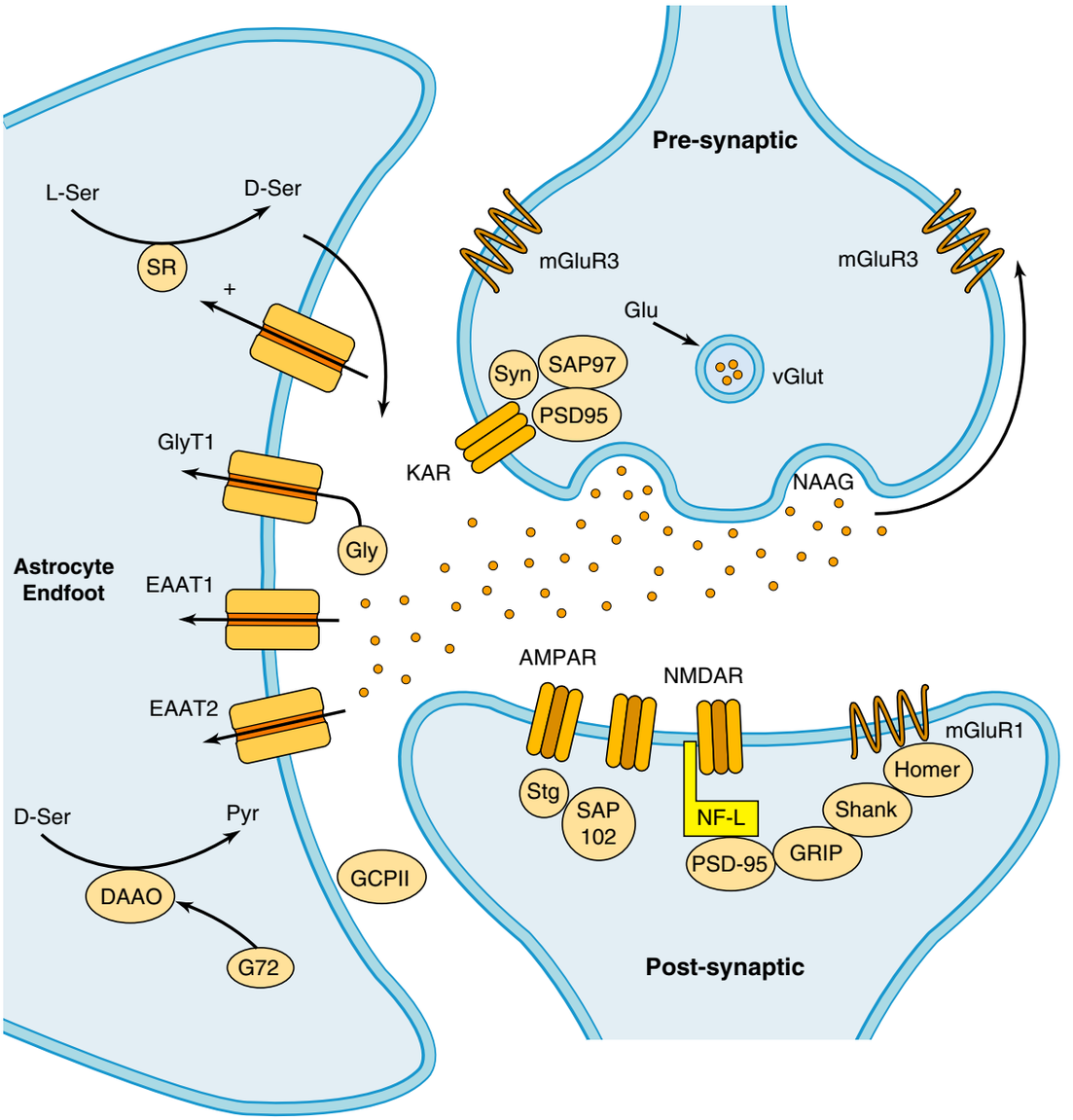

FIGURE 54-1 Schematic relationship of the tripartite glutamatergic synapse. This synapse consists of a presynaptic glutamatergic bouton, the postsynaptic spine and the astrocytic endfoot. Presynaptic mGluR3 receptors inhibit the release of glutamate and are selectively activated by *N*-acetyl aspartyl glutamate (*NAAG*). Presynaptic kainic acid receptors (*KAR*) regulate glutamate release. Postsynaptic AMPA receptors (*AMPAR*) and NMDA receptors (*NMDAR*) generate the excitatory postsynaptic currents when activated by glutamate. The astrocyte expresses two types of Na^+-dependent glutamate transporters (*EAAT1* and *EAAT 2*), a glycine transporter (*GlyT1*), which maintains subsaturating concentrations of glycine at the synaptic NMDA receptor, Serine Racemase (*SR*), which synthesizes D-serine (*ser*), Glutamate Carboxypeptidase II (*GCPII*), which hydolyzes NAAG, and D-Amino-Acid Oxidase (*DAAO*), which degrades D-serine. The receptors are associated with a variety of structural proteins that anchor them in the synapse including *PSD-95* (Post-Synaptic Density, kDa 95), Stargazin (*STG*), *SAP-97* and *SAP -102* (Synapse Associated Protein, kDa 97 and 102), Syntenin (*SYN*), Glutamate Receptor Interacting Protein (*GRIP*) and Neurofilament Light protein (*NF-L*). Adapted from Coyle *et al.* [26] and Meador-Woodruff *et al.* [29].

Controlled clinical investigations with careful titration of doses in normal subjects demonstrate that ketamine produces negative symptoms, such as withdrawal and the subtle cognitive impairments associated with schizophrenia [25]. As is the case for schizophrenia, these symptoms occur without clouding of consciousness or frank dementia. Positive symptoms with auditory hallucinations and fully formed delusions do not typically occur with acute administration of ketamine to normal subjects, but do occur with chronic abuse. Furthermore, low-dose ketamine produces several physiologic abnormalities associated with schizophrenia, such as abnormal eye-tracking, enhanced subcortical-dopamine-release, impaired prepulse inhibition, hypofrontality and abnormal event-related potentials.

In vivo dialysis studies indicate that acute blockade of the NMDA receptor in experimental animals results in a disinhibition of forebrain dopamine-release, suggesting that dopamine-mediated psychosis could be secondary to a more primary defect in cortical glutamatergic signaling.

Postmortem studies combined with genetic findings have generated compelling evidence that disruption in the modulation of subtypes of NMDA receptors contribute to psychopathology in schizophrenia [26]. Glutamate carboxy peptidase II (GCPII) degrades the neuropeptide *N*-acetyl-aspartyl-glutamate (NAAG), which is co-localized and released by glutamatergic neurons as well as other neuronal systems (cholinergic motor neurons, noradrenergic locus coeruleus neurons). NAAG is an agonist at the mGluR3 receptor, which reduces glutamate release, and a reversible glycine antagonist at hippocampal NMDA receptors (see Ch. 15). Postmortem studies with cohorts from three different brain banks have documented significant reductions in its enzyme activity, and in its mRNA levels in frontal and temporal cortex and in hippocampus. A translocation associated with schizophrenia affects a locus on chromosome 11q13 in close proximity to the gene encoding GCP II.

D-serine is a full agonist at the glycine modulatory site on the NMDA receptor and its levels are determined by the catabolic enzyme D-amino acid oxidase (DAAO) since the levels of the former are inversely correlated with the activity of the latter. A mutation in a protein designated *G72*, which results in robust activation of DAAO, has been described in two genetic studies of schizophrenia [27]. Single-nucleotide polymorphisms (SNPs) of DAAO have been associated with increased risk for schizophrenia. The levels of D-serine in plasma, which appears to come primarily from the CNS, are decreased in schizophrenics. Kynurenic acid is another endogenous antagonist of the glycine modulatory site on the NMDA receptor. The levels of kynurenic acid are significantly elevated in prefrontal cortex but not motor cortex in schizophrenic patients as compared with controls, which appears to be unrelated to antipsychotic drug exposure. Tryptophan-2,3-dioxygenase, an upstream enzyme in kynurenic acid synthesis, is also up-regulated in frontal cortex in schizophrenia [28]. Notably, double-blind placebo-controlled clinical trials of agents that directly or indirectly enhance NMDA receptor function at the glycine modulatory site show significantly reduced negative symptoms and enhanced cognitive function in patients with chronic schizophrenia.

NMDA receptors are anchored in the postsynaptic density (PSD95, 95 kDa), a complex with which over 80 proteins have been associated (see Ch. 15). Postmortem studies have examined the expression of the subunits of the NMDA receptors as well as components of the PSD [29]. In one study in the thalamus, the NR1 and NR2B subunits were decreased in schizophrenia and PSD95, SAP102 (Synapse Associated Protein kDa 102) and NF-L (Neurofilament-Light), components of the PSD, were also significantly reduced with the latter reduction also found in bipolar disorder. Similarly, other studies have shown significant reductions in the expression of SAP97 (Synapse Associated Protein, kDa 97), SAP102 and PSD95 in the prefrontal cortex in schizophrenia [30]. Decreased phosphorylation of NR1 at serine 97, which impairs NMDA receptor function, has been found in prefrontal cortex in two cohorts of schizophrenic subjects. That the postsynaptic density complex may be an important target for the pathophysiology of schizophrenia is reinforced by the highly significant association of SNPs for distobrevin and dysbinden, two proteins in the PSD complex, with the risk for schizophrenia. Notably, dysbinden expression is reduced in prefrontal cortex.

Nitric oxide (NO) synthesis in brain is, in part, driven by NMDA receptor activation through Ca^{2+}/calmodulin-dependent activation of NO-synthase [23]. Several studies suggest that NO interacting with NMDA receptor thiols causes inhibition of the receptor. In this regard, it is noteworthy that arginine, the substrate for NO-synthase, is elevated threefold, and the expression of endothelial and inducible NO-synthase are significantly elevated in the frontal cortex in schizophrenia. While these alterations would be expected to associate with oxidative stress, which promotes DNA damage and apoptosis, DNA fragmentation is found to be markedly reduced in prefrontal cortex in schizophrenic subjects as compared with both controls and bipolar subjects.

It is important to note that other components of the glutamatergic signaling system are also affected. For example, the glutamate transporters, Excitatory Acid Amino Acid Transporters 1 and 2 are elevated in the thalamus, which should further compromise glutamatergic neurotransmission [29] (see also Chs. 5 and 15). Consistent reductions in kainic acid receptor have been documented by ligand binding, *in situ* hybridization of subunits and DNA microarray studies in the prefrontal cortex and hippocampus [31]. SNPs for the mGluR3 receptor gene, which down-regulates glutamate release, have been linked to the risk for schizophrenia in three different studies, although the effects on its expression or function remain unclear at present.

Neuregulins are cell–cell signaling proteins that are ligands for the tyrosine kinase receptor of the Erb family [32]. At least three separate studies have associated allelic variants of neuregulin 1 and the risk for schizophrenia. Neuregulin has complex effects on glial differentiation, astrocyte function and synapse stabilization (Ch. 25). Postmortem studies have revealed reductions in neuregulin expression in cortex in schizophrenia. Mice homozygous for a null mutation of neuregulin or its ErbB4 receptor display hyperactivity that responds to clozapine and exhibit reduced numbers of NMDA receptors. Individually and in concert, these alterations would attenuate NMDA receptor function in schizophrenia.

GABAergic neurons are also implicated in schizophrenia. Early postmortem neurochemical studies suggested reductions in presynaptic markers for the GABAergic neurons, such as glutamatic acid decarboxalase (GAD) activity, in prefrontal cortex in schizophrenic subjects

[33]. However, the validity of these findings was undermined by the finding that agonal events, such as anoxia and a slow death, to which schizophrenics in particular are prone, can markedly reduce the activity of GAD independent of diagnosis. Later studies profitably took advantage of more discrete anatomical analyses of GABAergic interneurons in the frontal cortex and hippocampal formation. Disruption in the migration of these neurons in frontal cortex, a process occurring during the second trimester, has been noted in a subpopulation of patients with schizophrenia. Several studies have shown reduced expression of GAD67, and immunohistochemical studies have shown reduced GABA transporter (GAT) staining on the GABAergic 'cartridges' from Chandelier neurons synapsing on pyramidal cell dendrites. Consistent with this is the finding of decreased GAT mRNA in these intermediate level GABAergic interneurons in prefrontal cortex. In support of a discrete reduction in GABAergic innervation, increased ligand binding for the GABA-A receptor has also been noted in the same sector, consistent with denervation supersensitivity. GABAergic neurons that co-express calbindin appear to be more affected. Most of these findings have been confirmed in different laboratories using different sources for brain material (see Ch. 16 for further discussion of GABA transmission and transporters).

Whether the concerted reduction in the function and the number of a subpopulation of cortico–limbic GABAergic neurons is primary or secondary to some other process in schizophrenia remains to be determined, although thus far no putative candidate gene, with the exception of reelin, has been directly associated with GABAergic neurons [34]. Several groups have demonstrated a significant reduction in the levels of reelin in schizophrenia, a high-affinity peptide ligand for integrin receptors. The reelin–integrin system modulates early cortical development, especially neuronal migration. Notably, reelin is expressed by virtually all GABAergic neurons throughout life.

There are data that suggest a link between the hypothesized NMDA receptor hypofunction and the cortical GABAergic abnormalities. Electrophysiologic evidence suggests that limbic GABAergic interneurons may be differentially more sensitive to NMDA receptor antagonists than those located on pyramidal neurons. The expression of NMDA NR2 B-subunit is reduced on GAD67, expressing GABAergic interneurons in prefrontal cortex in schizophrenia. Furthermore, chronic treatment with the noncompetitive NMDA receptor antagonist dizocilpine (MK801) results in down-regulation in the expression of GAD67 and GAT and up-regulation of GABA-A receptors in rat cortex, mirroring the alterations in these parameters observed in the cortex in schizophrenic subjects.

The cholinergic system has also been implicated in schizophrenia. Cigarette smoking is remarkably prevalent among patients suffering from schizophrenia. Speculating that this may reflect a form of self-medication, Freedman and colleagues demonstrated that nicotine improved the sensory gating abnormalities that occurs in the P50 potential in schizophrenia [18]. Further studies also revealed similar P50 abnormalities in many first-degree relatives of schizophrenic probands, suggesting that the gating abnormality may be a heritable risk factor for schizophrenia. Subsequent linkage studies identified a locus on 15q13 that was associated with increased risk for schizophrenia. This region contains the gene for the α7 nicotinic receptor (CHRNA7), a subtype of nicotinic receptor with intriguing parallels to the NMDA receptor because of its calcium ion conductance, sensitivity to kynurenic acid inhibition and involvement in neuroplasticity (see also Ch.12). An allelic variant in the promoter region of CHRNA7 is associated with P50 abnormalities and results in reduced expression of α7 receptors. This allele has been documented in some patients with schizophrenia.

Some intracellular signal transduction molecules are reduced in schizophrenia. The release of neurotransmitters is regulated by a family of proteins that coordinate vesicular trafficking (see Ch. 9). Of these, the expression of complexin I and II appears to be decreased in prefrontal cortex and subfields of the hippocampal formation, and the ratio of complexin I to complexin II is elevated in the hippocampus [35]. SNAP-25 (Synaptosomal Associated Protein, kDa 25) has inconsistently been found to be down-regulated in both these regions. Synapsin expression is also reduced, but more robust decrements have been observed in bipolar disorder (Ch. 55).

Regulators of G-protein signaling (RGS) comprise a large family of modulators of synaptic neurotransmission. RGS4 has been implicated in schizophrenia in part because its gene is located at 1q21-22, a locus associated with high risk for the disorder [4]. The expression of RGS4 has been shown to be consistently down-regulated in prefrontal cortex in schizophrenia. The AKT (phosphatidylinositide 3-kinase, PI3K) and glycogen synthase kinase-3β (GSK3β) are downstream components of the neuregulin and wingless (Wnt) signaling pathways. GSK3β is an important kinase for tau phosphorylation in brain, thereby regulating tau function. GSK3β is itself regulated via the Wnt proteins and several other kinases that phosphorylate it on the ser-9 residue, which inhibits its kinase activity (see Fig. 55-1 and legend for further details). Association studies have implicated the neuregulin gene at 8p12 as a risk gene for schizophrenia. Postmortem studies have revealed decreased levels of AKT1 protein and reduced phosphorylation of serine 9 on GSK3β as well as decreased GSK3β expression in frontal cortex and hippocampus in schizophrenia [36].

Proteins involved in the fundamental structure and function of neurons are decreased in schizophrenia. Several postmortem studies have identified consistent reductions in the expression of mitochondrial associated genes involved in oxidative metabolism, such as cytochrome oxidase and cytochrome C reductase [23]. Reduced oxidative metabolism is consistent with evidence of increased brain lactate,

documented with MRS and postmortem measures, as well as decreased brain pH observed in postmortem studies in schizophrenia Morphologic studies have also shown a decreased number of mitochondria in both frontal cortex and striatum.

Robust decreases in the expression of the various proteasome subunits and ubiquitin-conjugating enzymes have been described in prefrontal cortex in schizophrenia. Neuronal ubiquitin and proteasomes play an important role in the assembly, function and plasticity of the synapse. Structural proteins including tubulin and α-spectrin also show decreased expression in prefrontal cortex.

Glia may play a role in schizophrenia. Disruption in myelin is a novel pathophysiologic mechanism recently identified in schizophrenia [37]. The results of imaging studies strongly suggest abnormalities in myelin tracks, with reduction in the total white-matter volume in whole brain, significant decreases in myelin or axonal membrane integrity as seen with magnetization transfer ratio, and decreased anisotropy in cortical regions indicative of myelin disruption, as documented by the diffusion tension imaging. DNA microarray studies have repeatedly shown reduced levels of mRNA encoding proteins associated with oligodendroglia and myelinization [38]. In several cases, these findings have been confirmed using quantitative RT-PCR. Among these are mRNAs for *myelin associated glycoprotein* (MAG), *myelin lymphocyte protein* (MAL), the neuregulin receptor *ErbB3* and *transferrin*. See Myelin Formation, Structure and Biochemistry, Chapter 4. The genes encoding these proteins are located at loci on the human genome that have been associated with heritable risk for schizophrenia. It should be noted that down-regulation of the expression of several of these genes has also been observed in postmortem cortex from individuals with bipolar disorder. This suggests that these myelin abnormalities may account for features such as psychosis that are shared between schizophrenia and bipolar disorder. Nevertheless, the unexpected implication of myelin dysfunction in schizophrenia speaks to the power of microarray technology for surveying efficiently an extraordinary large number of gene transcripts in tissue.

Although the apparent number of glial fibrillary acidic protein (GFAP) positive astrocytes are unchanged in schizophrenia, the expression of GFAP is reduced in prefrontal cortex. While reduction in glial numbers has been associated primarily with affective disorders, reduced numbers of astrocytes have been reported for the anterior cingulate cortex in schizophrenia [39]. Both the activities and the protein levels of glutamine synthetase and glutamate dehydrogenase, astrocyte-associated enzymes, are altered in prefrontal cortex in schizophrenia.

CONCLUSION

Our understanding of the pathophysiology of schizophrenia has advanced remarkably over the last decade through the combined use of *in vivo* imaging techniques, genetic studies and neurochemical (in the broadest sense) analyses of postmortem brain. Interdigitating the findings from these different methods of analysis indicates that the disorder is highly heritable and involves interactions of multiple genes, each producing a small effect, which in total probably affect several functional domains. Aside from dopamine, which may be central to psychosis, the glutamatergic, GABAergic and cholinergic neurotransmitter systems are credible participants. As several of the affected proteins modulating NMDA receptor function are expressed in astrocytes and multiple candidate genes, and their products are involved in myelinization, non-neuronal cell types may also be important participants in the pathophysiology of schizophrenia. A major challenge for the future will be disentangling those alterations in gene expression that are primary from those that are secondary, and relating these alterations in a meaningful way to the symptoms of schizophrenia. These advances will provide the hope for developing more effective treatments that will probably be unrelated to the dopaminergic system, which has dominated pharmacotherapy of schizophrenia for the last 50 years.

REFERENCES

1. Liddle, P. F., Barnes, T. R., Morris, D. and Haque, S. Three syndromes in chronic schizophrenia. *Br. J. Psychiatry* Suppl. 7: 119–122, 1989.
2. Lewis, D. A. and Liebnerman, J. A. Catching up on schizophrenia: natural history and neurobiology. *Neuron* 28: 325–334, 2002.
3. Cornblatt, B. A., Lencz, T., Smith, C. W. *et al.* The schizophrenia prodrome revisited: a neurodevelopmental perspective. *Schizophr. Bull.* 29: 633–651, 2003.
4. Harrison, P. J. and Owen, M. J. Genes for schizophrenia? Recent findings and their pathophysiological implications. *The Lancet* 361: 417–419, 2003.
5. Lewis, C. M., Levinson, D. F., Wise, L. H. *et al.* Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am. J. Hum. Genet.* 73: 34–48, 2003.
6. Deniker, P. Impact of neuroleptic chemotherapies on schizophrenic psychoses. *Am. J. Psychiatry* 135: 923–927, 1978.
7. Carlsson, A. A paradigm shift in brain research. *Science* 294: 1021–1024, 2001.
8. Seeman, P. and Van Tol, H. H. Dopamine receptor pharmacology. *Trends Pharmacol. Sci.* 15: 264–270, 1994.
9. Snyder, S. H. The dopamine hypothesis of schizophrenia: focus on the dopamine receptor. *Am. J. Psychiatry* 133: 197–202, 1976.
10. Coyle, J. T. The clinical use of antipsychotic medications. *Med. Clin. North Am.* 66: 993–1009, 1982.
11. Meltzer, H. Y. What's atypical about atypical antipsychotic drugs? *Curr. Opin. Pharmacol.* 4: 53–57, 2004.
12. Tamminga, C. A. and Carlsson, A. Partial dopamine agonists and dopaminergic stabilizers, in the treatment of psychosis. *Curr. Drug Targets CNS Neurol. Disord.* 2: 141–147, 2002.

13. Kuperberg, G. R., Broome, M. R., McGuire, P. K. *et al.* Regionally localized thinning of the cerebral cortex in schizophrenia. *Arch. Gen. Psychiatry* 60: 878–888, 2003.
14. Sporn, A. L., Greenstein, D. K., Gogtay, N. *et al.* Progressive brain volume loss during adolescence in childhood-onset schizophrenia. *Am. J. Psychiatry* 160: 2181–2189, 2003.
15. Callicott, J. H., Bertolino, A., Egan, M. F. *et al.* Selective relationship between prefrontal *N*-acetylaspartate measures and negative symptoms in schizophrenia. *Am. J. Psychiatry* 157: 1646–1651, 2000.
16. Jensen, J. E., Al-Semaan, Y. M., Williamson, P. C. *et al.* Region-specific changes in phospholipids metabolism in chronic, medicated schizophrenia: (31)P-MRS study at 4.0 Tesla. *Br. J. Psychiatry* 180: 39–44, 2002.
17. Winterer, G., Egan, M. F., Raedler, T. *et al.* P300 and genetic risk for schizophrenia. *Arch. Gen. Psychiatry* 60: 1158–1167, 2003.
18. Leonard, S., Gault, J., Hopkins, J. *et al.* Association of promoter variants in the alpha7 nicotinic acetylcholine receptor subunit gene with an inhibitory deficit found in schizophrenia. *Arch. Gen. Psychiatry* 59: 1085–1096, 2002.
19. Weinberger, D. R., Berman, K. F. and Zec, R. F. Physiologic dysfunction of dorsolateral prefrontal cortex in schizophrenia. I. Regional cerebral blood flow evidence. *Arch. Gen. Psychiatry* 43: 114–124, 1986.
20. Heckers, S. Neuroimaging studies of the hippocampus in schizophrenia. *Hippocampus* 11: 520–528, 2001.
21. Laruelle, M., Kegeles,. L. S. and Abi-Dargham, A. Glutamate, dopamine and schizophrenia from pathophysiology to treatment. *Ann. N. Y. Acad. Sci.* 1003: 138–158, 2003.
22. Davis, K. L., Kahn, R. S., Ko, G. and Davidson, M. Dopamine in schizophrenia: a review and reconceptualization. *Am. J. Psychiatry* 148: 1474–1486, 1991.
23. Prabakaran, S., Swatton, J. E., Ryan, M. M. *et al.* Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. *Mol. Psychiatry*, 9: 684–697, 2004.
24. Akil, M., Kolachana, B. S., Rothmond, D. A. *et al.* Catechol-*O*-methyltransferase genotype and dopamine regulation in the human brain. *J. Neurosci.* 23: 2008–2013, 2003.
25. Krystal, J. H., D'Souza, D. C., Mathalon, D. *et al.* NMDA receptor antagonist effects, cortical glutamatergic function and schizophrenia: toward a paradigm shift in medication development. *Psychopharmacology* 169: 215–233, 2003.
26. Coyle, J. T., Tsai, G. and Goff, D. Converging evidence of NMDA receptor hypofunction in the pathophysiology of schizophrenia. *Ann. N. Y. Acad. Sci.* 1003: 318–327, 2003.
27. Chumakov, I., Blumenfeld, M., Guerassimenko, O. *et al.* Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc. Nat. Acad. Sci. USA* 99: 13675–13680, 2002.
28. Miller, C. L., Llenos, I. C., Dulay, J. R. *et al.* Expression of the kynurenine pathway enzyme tryptophan 2,3-dioxygenase is increased in the frontal cortex of individuals with schizophrenia. *Neurobiol. Dis.* 15: 618–629, 2004.
29. Meador-Woodruff, J. H., Clinton, S. M., Beneyto, M. and McCullumsmith, R. E. Molecular abnormalities of the glutamate synapse in the thalamus in schizophrenia. *Ann. N. Y. Acad. Sci.,* 1003: 75–93, 2003.
30. Toyooka, K., Iritani, S., Makifuchi, T. *et al.* Selective reduction of a PDZ protein, SAP-97, in the prefrontal cortex of patients with chronic schizophrenia. *J. Neurochem.* 83: 797–806, 2002.
31. Harrison, P. J., Law, A. J. and Eastwood, S. L. Glutamate receptors and transporters in the hippocampus in schizophrenia. *Ann. N. Y. Acad. Sci.* 1003: 94—101, 2003.
32. Falls, D. L. Neuregulins: functions, forms and signaling strategies. *Experimental Cell Research* 284: 14–30, 2003.
33. Lewis, D. A., Volk, D. W. and Hashimoto, T. Selective alterations in prefrontal cortical GABA neurotransmission in schizophrenia: a novel target for the treatment of working memory dysfunction. *Psychopharmacology* 174: 143–150, 2004.
34. Costa, E., Chen, Y., Davis, J. *et al.* Reelin and schizophrenia: a disease at the interface of the genome and the epigenome. *Molecular Interventions* 2: 47– 57, 2002.
35. Sawada, K., Young, C. E., Barr, A. M. *et al.* Altered immunoreactivity of complexin protein in prefrontal cortex in severe mental illness. *Mol. Psychiatry* 7: 484–492, 2002.
36. Emamian, E. S., Hall, D., Birnbaum, M. J. *et al.* Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia. *Nature Genetics* 36: 131–137, 2004.
37. Davis, K. L., Stewart, D. G., Friedman, J. I. *et al.* White matter changes in schizophrenia: evidence for myelin-related dysfunction. *Arch. Gen. Psychiatry* 60: 443–456, 2003.
38. Tkachev, D., Mimmack, M. L., Ryan, M. M. *et al.* Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *The Lancet* 362: 798–805, 2003.
39. Stark, A. K., Uylings, H. B., Sanz-Arigita, E. and Pakkenberg, B. Glial cell loss in the anterior cingulated cortex, a subregion of the prefrontal cortex, in subjects with schizophrenia. *Am. J. Psychiatry* 161: 882–888, 2004.

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CHAPTER

55

Neurobiology of Severe Mood and Anxiety Disorders

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Basic Neurochemistry: Molecular, Cellular and Medical Aspects
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Mood and anxiety disorders are common, severe, chronic, and often life-threatening illnesses. There is a growing appreciation that, far from being diseases with purely psychological manifestations, severe mood and anxiety disorders are systemic diseases with deleterious effects on multiple organ systems. The emerging evidence suggests that the combination of genetics, and various stressors ultimately determines individual responsiveness to mood and anxiety disorders. In this chapter, we review emerging evidence for the involvement of critical neurotransmitter and cellular plasticity cascades, and discuss their implications, not only for changing existing conceptualizations regarding the pathophysiology of these disorders, but also for the strategic development of improved therapeutics.

MOOD DISORDERS

Three common diagnoses comprise the major mood disorders. The most common mood disorder diagnoses are:

1. major depressive disorder, also known as unipolar depression,
2. dysthymia, a less severe but more chronic form of depression akin to a mood trait, and
3. bipolar disorders, or manic-depression, characterized by both depressive episodes and episodes of mania and hypomania.

Epidemiological surveys report yearly mood disorder prevalence rates in the U.S.A. of 5.1–11.1% of the population. By subtype, yearly prevalence rates are 4.5–10.1% for a major depressive episode, 4–8.9% for major depressive disorder, 0.5–1.3 % for bipolar I, and 0.2% for bipolar II disorder [1]. Women have higher rates of major depressive disorder, approximately twice that of men, whereas bipolar disorder is equally prevalent in men and women [1]. Globally, major depression is the leading cause of disease burden among females aged 5 years and above. Individuals with a mood disorder have greater overall mortality risk, including higher risk of death by unnatural causes. Much of the excess mortality amongst individuals with mood disorders is attributable to higher rates of suicide. Approximately 60% of all suicides occur in relation to mood disorders [2]. Lifetime mortality due to suicide in discharged hospital populations is approximately 20% in bipolar disorder, and 15% in major depressive disorder. A diagnosis of mood disorder also carries an elevated risk for nonfatal suicide attempts, with epidemiological studies documenting lifetime rates of suicide attempt of 29.2% in bipolar disorder, 15.9% in major depressive disorder, and 4.2% in all other DSM III Axis I disorders [3].

Mood disorders are recurrent in about 80% of cases. The reason most likely lies in genetic and developmental effects having created a neurobiological diathesis for recurrent episodes of major depression or mania. It is of note that the clinical picture of major depression varies greatly from episode to episode within the same patient [4]. The pleomorphic nature of recurrent mood disorders implies that there is a super-family of mood disorders with a potentially common set of causal genes and environmental causes. The specific manifestation of an episode of a mood disorder must therefore be sensitive to state-dependent factors, and pathophysiology should reflect both a common trait-dependent component and a more variable state-dependent component. An example of the former is the serotonin system abnormality observed during and between episodes, brought on by acute tryptophan depletion or fenfluramine challenge. An example of the latter is dysregulation of the hypothalamic–pituitary–adrenal axis (see Ch. 52), or regional alterations in brain glucose metabolism as seen on positron emission tomography [5] (see also Ch. 58).

Both genetic and nongenetic factors play roles in the transmission of mood disorders. The familial nature of mood disorders is well established. Studies over the past 20 years have consistently documented higher rates of mood disorder in the relatives of individuals with major depression and bipolar disorder than in relatives of healthy controls [6, 7]. The familial aggregation of mood disorders is the outcome of both genetic and environmental factors.

The heritability of bipolar and major depressive disorders has been demonstrated in twin and adoption studies. In twin studies, monozygotic twins have two- to three-fold higher concordance for major depressive disorder than dizygotic twins [7], and four- to eight-fold higher concordance in bipolar disorder [6]. A higher concordance rate indicates that genetic factors play a role over and above shared environmental factors. Shared environment may be greater for monozygotic twins than for dizygotic twins, and the confound of shared environment can be avoided by adoption studies. In studies of depressed adoptees, higher rates of depression are observed in biological parents than adoptive parents, indicating a genetic component underlying the familial transmission of mood disorders. Genetic factors are estimated to account for 37– 75% of the liability for major depressive disorder [8] and 60–85% of liability for bipolar disorder [6].

Major depressive and bipolar disorders arise from the interactions between the susceptibility genes and environmental factors. Parental psychopathology contributes to the transmission of mood disorders to offspring both by transmission of susceptibility genes and through environmental effects. Parental mood disorder might result in adverse childrearing practices and environments, with greater exposure to stressors such as childhood abuse, marital discord in parents, impoverished parenting due to parental illness, substance misuse, poverty, and early separation from a parent due to loss or departure [9]. Deficient parenting by depressed parents, particularly maternal interaction with children, has also been associated with mood disorders in children (see Beardslee *et al.* [9] for a review). Such adverse child-rearing environments can

precipitate onset of depressive disorders in children and adolescents with a genetic susceptibility [10], or may interfere with treatment response.

Genetic studies have examined a number of candidate genes in association and linkage studies with inconclusive, but suggestive, results. In bipolar disorder, genetic studies have tentatively implicated chromosomes 18, 11 and X (for a review see Johansson *et al.* [11]). There have been fewer studies of major depressive disorder and results remain inconclusive. A less-well-defined clinical phenotype may be one reason. Linkage analyses of families with major depression have not observed a significant linkage to any of the genetic markers examined, with the possible exception of gamma-aminobutyric acid (GABA) A receptor $\alpha 5$ subunit gene [12]. With respect to candidate genes, association studies of the promoter regions of the norepinephrine transporter gene, the dopamine D2 receptor gene, and the serotonin transporter gene have not found consistent differences between individuals with major depression and healthy controls. One large postmortem study of the 5-HTTLPR polymorphism of the serotonin transporter found more heterozygotes in major depression; however, this relationship did not explain the lower transporter binding which has been observed in the prefrontal cortex, anterior cingulate, and brainstem raphe nuclei [13]. It is possible such an effect is present but obscured by regulatory effects at nerve terminals, such as rate of transporter internalization, which is influenced by the intrasynaptic level of serotonin.

A major obstacle faced by studies in psychiatric genetics is uncertainty over the degree of the biological heterogeneity encompassed within any particular psychiatric diagnoses. For example, we have reported that the pattern of symptoms and signs of major depressive episodes can vary considerably from episode to episode [4]. This implies that, although within an individual patient with a constant genetic makeup, successive episodes of major depression may vary in terms of the most prominent clinical features, the fact they all occur within a single patient suggests they are part of a single, super-family of major depression [4]. Thus, although the clinical phenotype may vary, the genetic underpinnings must be the same.

The interaction of environmental factors with genetic susceptibility in the development of mood disorders also complicates genetic studies. For example, Caspi *et al.* [10], found that childhood maltreatment favored the onset of major depressive episodes and responses to life events in individuals harboring the short allele of the promoter region of the serotonin transporter gene; while those individuals carrying the long allele of this gene, which is more highly expressed, were more resistant to the development of major depressive episodes in the context of a past history of adverse childhood experiences or in response to recent life events in adulthood. Replication of these potentially important findings is required because, in general, replication of results in genetic studies has proven difficult. This is due to factors such as environment–gene interaction, the likelihood that the same clinical phenotypes are informed by multiple genes and gene combinations, and the low power of sample sizes in most studies to date.

NEUROTRANSMITTER AND NEUROPEPTIDE SYSTEMS ARE IMPLICATED IN THE PATHOPHYSIOLOGY OF BIPOLAR AND MAJOR DEPRESSIVE DISORDERS

The serotonergic system has long been thought to play a role in major depression and bipolar disorders, although the exact mechanisms are yet to be determined.

In 1969, the indoleamine hypothesis of depression was proposed [14], wherein the vulnerability to either depression or mania was related to low serotonergic activity, attributable to either less serotonin release, or fewer serotonin receptors, or impaired serotonin receptor-mediated signal transduction. Prange *et al.* [15] formulated a permissive hypothesis of serotonin function in bipolar disorder, in which a deficit in central serotonergic neurotransmission permits the expression of bipolar disorder, and both the manic and depressive phases of bipolar disorder are characterized by low central serotonergic neurotransmission. Studies of the serotonergic system over the last 30 years, have reinforced its role in mood disorders, and have identified additional associations with suicidal behavior, impulsivity, aggression, eating disorders, obsessive–compulsive disorder, anxiety disorders, personality disorders, seasonal changes in mood and behavior and alcohol misuse disorders (see also Addiction, Ch. 56). The serotonergic system also plays a role in the regulation of a range of basic biological functions, including sleep, appetite, circadian rhythm, and cognition. These functions are often disrupted in episodes of major depression.

Serotonergic function has been investigated by using multiple methods. Assaying the major metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid (CSF) has been widely used (Ch. 13). This method assumes that CSF 5-HIAA is related to brain serotonin activity. This premise is supported by the rostral–caudal concentration gradient of CSF 5-HIAA and the observation in postmortem studies that CSF 5-HIAA correlates with levels of 5-HIAA in prefrontal cortex [16], both of which suggest that CSF 5-HIAA is a reasonable index of prefrontal serotonin turnover .

Some, but not all, studies observe low CSF 5-HIAA in major depressive episodes. Numerous studies, though not all, have also reported no difference between patients with mania or depression in CSF 5-HIAA levels, consistent with both Prange *et al.*'s [15] permissive hypothesis for bipolar disorders and the indoleamine hypothesis.

This implies that CSF 5-HIAA is low in both manic and depressive states and thus is not a mood-state-dependent effect. Moreover, the level of CSF 5-HIAA generally does not correlate with severity of depression. There is an independent association between lower levels of CSF 5-HIAA and serious suicidal behavior in depressed patients [17]. A history of serious suicide attempts rather than severity of depression distinguishes depressed patients with lower CSF 5-HIAA. Low CSF 5-HIAA is also related to lifetime aggressive behavior, and has been shown to predict both future suicide and pathological aggression. This may be due to low serotonin input into the ventral prefrontal cortex and anterior cingulate gyrus, resulting in less behavioral inhibition and thus a greater likelihood of suicide or violence.

Platelets are more accessible for study than CSF, and share many properties with central serotonin neurons, including uptake, storage, and release of serotonin, some serotonin receptors, and serotonin transporter binding sites. Platelets have been used as a peripheral model for serotonin neurons in studies of serotonin function in mood disorders. Ligands such as [^{3}H]imipramine and [^{3}H]paroxetine bind to the serotonin transporter on the presynaptic nerve terminal and in the platelet. Over 20 years ago, lower platelet [^{3}H]imipramine and [^{3}H]paroxetine binding were first observed in depressed patients [18]; however, no difference in platelet [^{3}H]imipramine binding has been observed in manic patients compared with healthy controls [19]. Suicidal behavior has been associated with higher platelet 5-HT_{2A} binding [20], and aggressive behaviors related to higher platelet serotonin content [21]. Serotonin receptors are discussed in detail in Ch. 13.

Hormonal response to the serotonin releasing-agent/uptake-inhibitor fenfluramine, and other related direct and indirect serotonin agonists, have also been examined in mood disorder research as an indicator of central serotonin system function. A release of serotonin from raphe nuclei projections to the hypothalamus causes the release of prolactin, cortisol and adrenocorticotropic hormone (ACTH, see Ch. 52). Thus, prolactin response is an index of central serotonin responsivity. Using this method one can measure net serotonin transmission, including elements of presynaptic and postsynaptic serotonergic functioning, which is not possible in cerebrospinal fluid and platelet studies. A blunted prolactin response to fenfluramine, indicating less serotonin release and/or serotonin 5-HT_{1A} or 5-HT_{2A} receptor signal transduction, in depressed patients has been reported [22] but not in all studies. Discrepancies between studies may be due to differences in the composition of study populations; for example, the proportion of patients with suicidal behavior or certain personality traits in study samples [22, 23]. Depressed patients who have made high lethality suicide attempts show lower prolactin response to fenfluramine than those who have made low-lethality suicide attempts [23]. Depressed patients who experience anger attacks have significantly blunted prolactin response to fenfluramine compared to patients without anger attacks [24], and in personality-disorder patients reduced prolactin response to fenfluramine correlated with ratings of impulsive aggression [22].

Another method of studying the serotonergic system has been through depletion of serotonin by either inhibition of tryptophan hydroxylase (TPH), the rate-limiting biosynthetic enzyme for serotonin, with parachlorophenylalanine (PCPA), or acute tryptophan depletion. These studies have demonstrated that, in long-term-remitted medication-free depressed patients, depression recurs after serotonin or tryptophan depletion. An elevated rate of induction of depression by acute tryptophan depletion has also been observed in the relatives of mood-disorder patients, consistent with familial serotonin hypofunction. Similarly, a transient return of depressed symptoms following acute serotonin or tryptophan depletion occurs in patients who have responded to selective serotonin reuptake inhibitors (SSRIs), indicating that the antidepressant effect of this class of medications is dependent on a continuous enhancement of serotonergic function.

Medications, such as SSRIs, that block the serotonin transporter site and selectively inhibit reuptake of serotonin, have proven to be effective antidepressants. Some antidepressant drugs act specifically at one or another of the serotonin receptor subtypes, for example buspirone and gepirone are 5-HT_{1A} receptor agonists. Fewer 5-HT_{1A} receptors have been implicated in the pathophysiological mechanism of depression and anxiety. In positron emission tomography (PET) studies of the regional 5-HT_{1A} receptor binding potential, that compare depressed subjects with primary, recurrent, familial mood disorders with healthy controls, mean 5-HT_{1A} receptor binding potential has been reported as lower in the midbrain raphe and mesial hippocampus [25]. Single positron emission tomographic study [SPECT) studies have also found less brainstem serotonin transporter binding sites in depressed patients [26]. Postmortem studies have confirmed this effect in the brainstem, and have extended it to the prefrontal cortex [13].

The role of 5-HT_{2A} receptors has been examined in postmortem and imaging studies. In postmortem studies of suicide victims, both with and without depressive diagnoses, there is evidence that 5-HT_{2A} receptors are up-regulated in the dorsal prefrontal cortex (areas 8 and 9) in suicide, but remain unchanged in the rostral pole of the prefrontal cortex (area 10) (see [27] for a review). Imaging studies of 5-HT_{2A} receptor binding in depressed subjects have reported conflicting results, with some observing increased 5-HT_{2A} receptor binding in the prefrontal cortex, others reporting a decrease in binding potential in the cingulate, insular, and inferior frontal cortex, and others observing no alterations [27]. The variation in results is possibly explained by the effects of antidepressant medications which have been shown to downregulate 5-HT_{2A} receptor binding across widespread areas of the

prefrontal cortex [28]. There is disagreement about 5-HT_{2A} receptor binding in medication-free depressed subjects in imaging [29], and postmortem studies [30]. The cause of higher 5-HT_{2A} receptor binding in depression is unclear, but animal studies show that stress can up-regulate 5-HT_{2A} receptor binding.

Given the heritability of major depressive disorders, a genetic contribution to serotonergic dysfunction seems likely. Animal studies have demonstrated that CSF 5-HIAA levels are partly under genetic control [31]. As noted above, linkage and association studies of candidate genes related to the serotonin system are numerous. In one such study, Mann *et al.* [13] assayed prefrontal cortical (PFC) serotonin transporter binding in postmortem brain from major depression and suicide victims and examined the relationship to the functional 5-HTTLPR allele in the promoter region. Lower binding to the serotonin transporter (5-HTT) throughout the PFC of subjects with a history of a major depressive episode was observed, and the 5-HTTLPR genotype was associated with major depression, but not with suicide or 5-HTT binding. The authors consider that a diffuse reduction of transporter binding in the PFC of individuals with major depression may be indicative of a widespread impairment of serotonergic function. It was unrelated to genotype at the level of the cortex, although Heinz *et al.* [32] reported a relationship in the brainstem using SPECT.

Studies of serotonin function in major depression suggest both hypofunction and accompanying compensatory alterations to increase serotonergic activity. A role for serotonergic system underactivity in the pathogenesis of depression is suggested by findings such as lower serotonin and 5-HIAA levels in postmortem brainstem, relapse of depression with acute depletion of tryptophan, fewer serotonin transporter sites in prefrontal cortex, fewer postsynaptic 5-HT_{1A} receptors, and the antidepressant properties of medications that enhance serotonergic transmission. Conversely, reports of more 5-HT_{2A} receptor binding in the frontal cortex of depressed individuals who committed suicide, fewer brainstem 5-HT_{1A} autoreceptors, and fewer serotonin transporters in the cortex, would be consistent with homeostatic changes designed to increase deficient serotonergic transmission in major depression. Future investigations of serotonergic activity in mood disorders will need to further differentiate primary pathogenesis from such compensatory changes.

There is evidence for the contribution of serotonin dysfunction to mania, and in the mechanism of action of mood stabilizers [19], however, specific data on the serotonergic system and mania are fewer and variable. Moreover, altered functioning of other neurotransmitters in mania such as norepinephrine, dopamine, acetylcholine, and GABA, and their interaction with serotonin, are also likely to be involved in the pathogenesis of mood disorders. Differences in these neurotransmitter systems possibly underlie differences in the pathogenesis of depressive and manic episodes.

The catecholamine hypothesis of depression proposed that major symptoms of the disorder are explained by a deficiency in noradrenalin or its receptors. Early evidence that the noradrenergic system is crucial in the pathophysiology of depression appeared in the mid-1950s, when a number of patients became profoundly depressed when treated for hypertension with tetrabenazine and reserpine, which deplete cathecholamines in the central and peripheral nervous system [33]. More recently, evidence of the role of the noradrenergic system in depressive disorders is seen in the effect of α-methyl-*p*-tyrosine (AMPT), which induces more biochemically specific catecholamine depletion by inhibiting tyrosine hydroxylase, the rate-limiting step in catecholamine synthesis (details of synthesis are given in Ch. 12). AMPT has negligible effects on mood in healthy subjects, but produces a return of depressive symptoms in recovered depression patients treated with noradrenalin reuptake inhibitors.

Multiple lines of investigation provide evidence for a role of the noradrenergic system in depressive disorder, although considerable variability in findings indicate that much remains to be learned regarding specific mechanisms. The noradrenalin metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), has been investigated in plasma, urine and CSF studies. Early studies of urinary MHPG reported lower levels in depressed patients compared with controls and higher MHPG levels in manic states in bipolar patients. However, subsequent studies have shown considerable variability in MHPG levels in patients with depression [34]. These inconsistencies may in part be due to the low contribution of CNS catecholamines to urinary metabolite levels.

Investigators have sought more accurate measures of brain noradrenalin activity by measuring CSF metabolite levels. Again, significant heterogeneity of results appears in studies of major depressive disorder. For example, of six studies of CSF noradrenalin metabolites, three found higher levels in depression, one found lower levels, and two reported no difference [34]. Studies of bipolar disorder have yielded somewhat more consistency, with bipolar type I patients generally showing lower levels of noradrenalin metabolites in the depressed state; however, results also vary [35]. While there is some contribution from the spinal cord, most MHPG comes from the brain. The observation in rodents that lower CNS noradrenergic activity is associated with greater sympathetic nervous system (SNS) activity suggests that mood disorders may be characterized by lower CNS noradrenergic activity and greater SNS activity. Thus, CSF MHPG levels in mood disorders may reflect the balance of changes in brain and SNS noradrenergic activity.

Noradrenalin turnover is decreased with antidepressant treatment. This is a compensatory effect of elevated intrasynaptic noradrenalin causing feedback inhibition of tyrosine hydroxylase. This effect has been consistently observed with noradrenergic-specific antidepressants, as expected, but has also been reported with serotonergic-specific

agents, and with ECT [36]. The former effect may be due to the action of the raphe serotonergic neurons on activity of locus coeruleus noradrenergic neurons.

Receptor studies also provide evidence of noradrenergic anomalies in mood disorders. No consistent changes in α_1-adrenergic receptor numbers have been observed in unmedicated depressed patients; however, downregulation and hyposensitivity of β-adrenergic, and possibly α_2-adrenergic, receptors have been reported [37]. Antidepressant treatment decreased the number of α_2 and β_1-adrenergic receptors and increased the density of α_1-adrenergic receptors in animal studies [37]. α_2-adrenergic receptors can also be studied indirectly through challenge with the α_2-receptor agonist clonidine, which induces growth hormone secretion, primarily through an action on postsynaptic receptors. Following clonidine challenge, attenuated growth hormone secretion, indicating decreased responsiveness of postsynaptic α_2-adrenergic receptors, has been observed in depressed patients [38]. Elevated plasma cortisol levels have been observed in depressed patients following administration of yohimbine, an α_2-adrenergic receptor agonist, likewise indicating subsensitivity of postsynaptic α_2-adrenergic receptors [39].

Postmortem brain studies offer further evidence of noradrenergic abnormality in depression, finding greater cortical norepinephrine, and less high-affinity β_1-adrenergic receptor binding. More tyrosine hydroxylase in the locus coeruleus (LC) may result from depletion of stores of NE, and that may thereby trigger a compensatory increase in NE synthesis. There are fewer noradrenergic neurons reported in depressed suicides [40], which may indicate a lower functional reserve of the noradrenergic system in suicide and/or major depression. Thus, there is greater likelihood of noradrenergic depletion in the face of severe or prolonged stress, such as the stress of a depressive illness. Noradrenergic response to stress in adulthood appears to be greater in those with an adverse experience in childhood, potentially putting them at greater risk in adulthood for norepinephrine depletion because of fewer noradrenergic neurons, and therefore for major depression [41].

There is ample support for the hypothesis of noradrenergic system dysfunction in depression; however, the inconsistencies in findings rule out any simple model of increased or decreased noradrenergic activity. It is important to determine which noradrenergic system abnormalities relate specifically to the pathogenesis of mood disorders, and which are related to nonspecific effects of stress, homeostatic mechanisms, or comorbid psychopathology. More work is needed on the mood-state-dependence of noradrenergic function.

Altered dopaminergic system function has been implicated in major depression. Patients with major depression have been reported to have lower CSF levels of the dopamine metabolite homovanillic acid (HVA) compared to nondepressed controls, with more severely depressed patients having still lower CSF HVA levels (see [42] for a summary). By increasing the sensitivity of CSF HVA as a measure of CNS dopamine turnover through using probenecid to block the transport of HVA from the CSF, researchers found lower CSF HVA in patients with depression, particularly in a subgroup of patients with psychomotor retardation [43]. However, not all studies have observed lower CSF HVA in patients with depression. In other measures of dopaminergic activity, lower serum dopamine β-hydroxylase activity, higher plasma dopamine and HVA concentrations have been observed in psychotically depressed patients compared with nonpsychotic depressed patients [44]. Adding support to the hypothesis that the dopaminergic system is implicated in depressive disorders are the antidepressant properties of dopamine-releasing stimulants methylphenidate and dextroamphetamine, which are useful in the treatment of depression.

There might be an increased ratio of cholinergic to adrenergic activity in depression, and the reverse in mania. This concept was proposed by Janowsky *et al.* [45]. More recently, Poland *et al.* [46] suggested that cholinergic hyperactivity could contribute to depression. Further support for over-activity of the cholinergic system in the pathogenesis of depression is offered by such findings as:

- Cholinergic input reduces rapid eye movement sleep (REM) latency, and decreased REM latency is seen in depression;
- The anticholinergic properties of some antidepressants;
- In some cases mania is reduced and depression induced by lecithin, an acetylcholine precursor;
- Following abrupt withdrawal of anticholinergic medications cholinergic rebound can cause a relapse of depression [37].

The antidepressant effects reported for some drugs that antagonize NMDA receptors suggest a role for the glutamatergic system in depressive disorders. Lower glucose uptake, as indicated by PET imaging studies of the prefrontal cortex in depression using the glucose analogue [^{18}F]-FDG, could reflect lower glutamate–glutamine turnover as a result of lower glutamatergic activity in the cortex [47]. Morphometric studies of pyramidal cells in neocortex and magnetic resonance spectroscopic analysis of glutamate will help clarify the state of the glutamatergic system in mood disorders.

Reduced GABAergic activity may play a role in depression. In depressed patients, magnetic resonance spectroscopy studies have observed lower GABA levels in occipital cortex, and lower CSF and plasma concentrations of GABA have also been reported [48]. We have found that low CSF GABA may be inversely proportional to severity of anxiety and depression in major depression. Lamotrigine enhances GABAergic activity, and has demonstrated antidepressant prophylactic effects, preventing recurrent

depressions in bipolar disorders [49]. Anticonvulsant mood-stabilizers acting by enhancing GABAergic function suggest GABA plays a role in mood stability, and a GABA deficiency may favor mood swings. These pathophysiological contributions of GABA and therapeutic effects of GABAergic medications in mood disorders may also be mediated via effects on other neurotransmitter systems [37].

The cortical–hypothalamic–pituitary–adrenal axis has been implicated in major depression. Multiple methods of measuring cortical–hypothalamic–pituitary–adrenal axis activity have provided evidence of altered function in patients with major depression, including: elevated corticotropin-releasing factor (CRF) concentrations in CSF ; blunted cortical–adrenocorticotropic hormone (ACTH) and β-endorphin responses after intravenous CRF administration; lower CRF binding in frontal cortex; pituitary gland enlargement; adrenal gland enlargement; hypercortisolemia and elevated CSF cortisol concentrations; blunted plasma glucocorticoid, ACTH, and β-endorphin nonsuppression after dexamethasone administration; higher urinary free cortisol concentrations; elevated 5-hydroxytryptophan-induced cortisol secretion; higher ACTH-induced cortisol secretion; and higher ACTH and cortisol responses to CRF after dexamethasone pretreatment.

In depressed patients, cortical–hypothalamic–pituitary–adrenal axis hyperactivity can be explained by the hypersecretion of CRF, and secondary pituitary and adrenal gland hypertrophy. Impaired negative feedback at various CNS sites, including the hippocampus and pituitary are also likely to contribute. Downregulation of hippocampal mineralocorticoid receptors and expression is reported in depressed suicides [50]. In bipolar disorder, hyperactivity of the cortical–hypothalamic–pituitary–adrenal axis has been observed [51]. This increase in cortical–hypothalamic–pituitary–adrenal axis activity has also been observed in mixed mood states, mania and in depression in rapid-cycling patients. Partial reversal of HPA overactivity is associated with treatment and recovery from depression.

Thyroid dysfunction may produce depression. Approximately 5–10% of individuals evaluated for depression have previously undetected or subclinical thyroid dysfunction [52]. Jackson [53] suggests that most patients with depression, although generally viewed as chemically euthyroid, have altered thyroid function, including slight elevation of the serum thyroxine (T4), loss of the nocturnal TSH rise, and blunted thyrotropin (TSH) response to thyrotropin-releasing-hormone (TRH) stimulation. A down-regulation of TRH receptors in the pituitary in response to the increased levels of TRH secreted into the hypophyseal-portal circulation might explain the blunting of the TSH response to TRH challenge.

Bipolar disorder patients have shown abnormalities of the hypothalamic–pituitary-thyroid axis, demonstrated by an exaggerated TSH response to TRH and elevated basal plasma concentrations of TSH [54]. In contrast, a blunted TSH response to TRH, the presence of antithyroid microsomal or antithyroglobulin antibodies [55], and a blunted or absent nocturnal surge in levels of plasma TSH [56], have also been observed in patients with bipolar disorder.

Depression has been associated with disorders of various neuropeptides.

Growth hormone. Mood disorders have been related to alterations in the activity of the growth-hormone axis. A blunted growth-hormone response to clonidine, an α_2 receptor agonist, has been consistently found in depression. Increased growth-hormone secretion during the day and decreased nocturnal growth-hormone secretion have also been observed in depressed patients. Depressed patients have lower CSF concentrations of somatostatin, compared to those with schizophrenia and normal controls. While lower CSF somatostatin is a state-dependent marker of depression, it occurs in a number of unrelated nonpsychiatric conditions, and thus appears to be relatively nonspecific.

Brain-derived neurotrophic factor. Brain growth and development is affected by multiple neurochemical factors, including thyroid hormones, somatostatin, growth hormone, and brain-derived neurotrophic factor (BDNF). BDNF, a major neurotrophic factor in the brain, is critical to the survival and guidance of neurons during development, and is also required for the survival and function of neurons in the adult brain [57]. For example, it has been shown to play a critical role in long-term potentiation, a cellular model of learning and memory, thus influencing plasticity [58].

Antidepressant treatment has, in recent studies, been shown to upregulate the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) cascade and expression of BDNF [59]. This upregulation of CREB and BDNF raises the possibility that antidepressant treatment could oppose the cell death pathway, possibly via increased expression of the oncogene *Bcl-2*. Studies are necessary to determine if antidepressant treatment increases *Bcl-2* expression. Increased expression of *Bcl-2* in brain and cultured cells, and inhibition of apoptosis of cultured cerebellar granule neurons have been reported with lithium treatment [57]. Mice lacking the BDNF TrkB receptor fail to show behavioral and neurogenic responses to antidepressants.

Substance P, an undecapeptide, is abundant both in the periphery and in the central nervous system. It is usually co-localized with one of the classical neurotransmitters, most commonly serotonin. Substance P is thought to have a role in the regulation of pain, asthma, psoriasis, inflammatory bowel disease and, in the CNS, emesis, migraine, schizophrenia, depression and anxiety. The substance-P-preferring receptor neurokinin-1 has been focused on most intensively in drug development, and existing

preclinical and clinical literature is suggestive, but not conclusive, of a role for substance P and neurokinin-1 receptors in the pathophysiology of depression and/or anxiety disorders. Originally studied as potential analgesic compounds, recent evidence suggests that neurokinin-1 receptor antagonists may possess antidepressant and anxiolytic properties. If confirmed by further controlled clinical studies, this will represent a mechanism of action distinct from all existing antidepressant agents. Evidence of impaired substance-P-mediated neurotransmission in depression is lacking, and CSF studies of substance P in depressed subjects are not in agreement.

ABNORMALITIES OF SLEEP RHYTHMS IN MOOD DISORDERS

Disturbances of sleep are typical of mood disorders, and belong to the core symptoms of major depression. More than 90% of depressed patients complain of impaired sleep quality [60]. Typically, patients suffer from difficulties in falling asleep, frequent nocturnal awakenings, and early morning awakening. Not only is insomnia a typical symptom of depression but, studies suggest, conversely, insomnia may be an independent risk factor for depression. In bipolar disorders sleep loss may also be a risk factor for the development of mania. Hypersomnia is less typical for depression [61] and, in contrast to insomnia, may be related to certain subtypes of depression, such as seasonal affective disorder (SAD).

Polysomnographic sleep research has demonstrated that besides disturbances of sleep continuity in depression, sleep disturbance is also characterized by a reduction of slow-wave sleep and a disinhibition of REM sleep, with a shortening of REM latency, a prolongation of the first REM period, and increased REM density [62]. Most effective antidepressant agents suppress REM sleep , and depressive symptoms are at least transiently alleviated by manipulations of the sleep–wake cycle, such as sleep deprivation or a phase advance of the sleep period [63]. Thus, there appears to be a bidirectional relationship between sleep, sleep alterations and mood.

NEUROANATOMICAL AND NEUROPATHOLOGICAL CORRELATES OF MOOD DISORDERS

Neuroimaging technology provides opportunities for elucidating the anatomic correlates of mood disorders. Neuroimaging studies of major depression have identified structural and functional abnormalities in multiple areas of the orbital and medial prefrontal cortex, hippocampus, amygdala, and related parts of the striatum and thalamus [25, 64]. Neuromorphology and neuromorphometry in primary mood disorders, and localization of pathology in major depressive episodes arising secondary to cerebral lesions, have been assessed with structural magnetic resonance imaging (MRI). See Hastings *et al.* [65] and Lochhead *et al.* [66] for reviews on structural imaging findings in major depressive disorder and bipolar disorders, respectively. Briefly, although the specific structure affected may vary between studies and between patients, there is a convergence of findings from structural imaging studies implicating a circuit involving the anterior cingulate, ventral striatum, pallidum, thalamus and hippocampus. There has been no clear evidence of global atrophy in mood disorders, although there are reports that patients with major depression have smaller basal ganglia, cerebellum, and possibly frontal lobe, perhaps indicating local atrophy. Bipolar disorder is associated with smaller cerebellum, possibly smaller temporal lobe, and changes in the hippocampus. Bipolar and elderly major depression patients both show increased subcortical white matter and periventricular hyperintensities. Lesions in these selected brain regions caused by injuries, such as anterior tumors or stroke, can be involved in the pathogenesis of mood disorders. The aging process may also contribute to the emergence of depression by causing lesions in these areas.

The emergence of functional neuroimaging methods permits the study of patients across the course of their illness. Functional imaging tools such as positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and single photon emission computed tomography (SPECT), allow *in vivo* characterization of neurophysiologic and/or receptor pharmacologic correlates of normal and pathologic emotional states, chronic or recurrent illness, and treatment response and resistance (see detailed discussion in Ch. 58).

Functional neuroimaging findings have consistently observed prefrontal lobe dysfunction, indicated by lower blood flow and glucose metabolism, in mood disorders. There is evidence of abnormalities in basal ganglia, temporal lobe and related limbic structures, that accompany or are independent of structural changes. Major depressive disorder is primarily associated with dysfunction in the prefrontal cortex and basal ganglia, while bipolar depression appears to be associated with dysfunction in the temporal lobe, in addition to these other areas (see Blumberg *et al.* [67], Soares and Mann. [64] for reviews). Some of these functional abnormalities appear mood-state-dependent and are located in regions where cerebral blood flow increases during normal and some pathologic emotional states. Such neurophysiologic differences between depressives and control subjects may thus indicate areas where physiologic activity changes to mediate, or respond to, the emotional, behavioral and cognitive manifestations of major depressive episodes.

Other abnormalities are more trait-like, and persist following symptom remission. They are found in orbital and medial prefrontal cortex areas where postmortem studies have also documented reductions in cortex volume and histopathologic changes in primary mood disorders [68]. Evidence from brain mapping, lesion analysis and electrophysiologic studies of humans and experimental

animals, suggests that these areas appear to modulate emotional behavior and stress responses. Thus, it is hypothesized that dysfunction involving these regions plays a role in the pathogenesis of depressive symptoms. Taken together, these findings implicate interconnected neural circuits in which pathologic patterns of neurotransmission may result in the emotional, cognitive, motivational and behavioral manifestations of primary and secondary mood disorders.

Well-characterized postmortem human brain tissue banks permit study of the brain changes in mood disorders, and such findings can then be correlated with those of functional neuroimaging studies [68]. *In vivo* neuroimaging data, delimiting areas where gray matter volume is abnormal and characterizing the clinical conditions under which such abnormalities are evident, are beginning to also guide postmortem studies of mood disorders.

The development of selective ligands for neuroreceptor imaging provides expanding capabilities for noninvasive quantitation of *in vivo* receptor binding and neurotransmitter function. For example, PET and SPECT studies have yielded important information regarding the role of serotonergic receptors in the pathogenesis of mood disorders (see above). Such studies will facilitate a more complete characterization of the neurotransmitter abnormalities suggested by studies of postmortem tissue, body fluids, and neuroendocrine function. Combining PET and SPECT technology with genotyping functional polymorphisms in serotonin receptor genes, allows for more precise characterization of potentially important biological intermediate endophenotypes that are more sensitive indices of the pathology of mood disorders. Repeated studies in different mood states, and examination of familial and developmental effects using such methods, will help understand the pathophysiology of mood disorders; and will perhaps provide useful biomarkers for measuring and monitoring treatment effects.

STRESS, GLUCOCORTICOIDS AND NEUROPLASTICITY IN THE PATHOLOGY OF MOOD DISORDERS

The potential hyperactivation of the HPA axis in mood disorders has been revisited in recent years, in large part due to the growing recognition of the specific brain areas in which atrophy (loss), a neuroplastic event, may be present in many patients. To what extent these findings of atrophy represent the sequelae of the biochemical changes (for example, in glucocorticoid levels) accompanying repeated affective episodes, remains to be fully elucidated.

This suggestion receives support from the observation that chronic stress or glucocorticoid administration has been demonstrated to produce atrophy and death of vulnerable hippocampal neurons in rodents and primates. Furthermore, MRI studies have revealed reduced hippocampal volumes in patients with Cushing's disease and post-traumatic stress disorder (PTSD), conditions associated with hypercortisolemia. One of the most consistent effects of stress on cellular morphology is atrophy of hippocampal neurons. This atrophy is observed in the CA3 pyramidal neurons, but not in other hippocampal cell groups [69, 70] (i.e. CA1 pyramidal and dentate gyrus granule neurons). Atrophy of CA3 pyramidal neurons also occurs upon exposure to high levels of glucocorticoids, suggesting that activation of the HPA axis probably plays a major role in mediating the stress-induced atrophy. In addition, long-term exposure to stress (i.e. for several months) has also been associated with loss of hippocampal neurons in the CA3 pyramidal cell layer [69, 70].

Thus, it is possible that recurrent mood disorders, as in the case of bipolar disorder, may lower the threshold for cell death and/or atrophy in response to a variety of other physiological (e.g. normal aging) and pathological (e.g. ischemic) events, and thereby contribute to a variety of deleterious health-related effects.

INTRACELLULAR SIGNALING PATHWAYS INTEGRATE ENVIRONMENTAL AND GENETIC CUES INVOLVED IN MOOD

Recent neurobiological research into the pathophysiology and treatment of mood disorders has moved from a focus on neurotransmitters and cell surface receptors to intracellular signaling cascades. Multicomponent cellular signaling pathways interact at various levels, thereby forming complex signaling networks which allow the cell to: (a) receive internal and external cues, (b) process these cues, and (c) respond to information [71–73]. These signaling networks facilitate the integration of signals across multiple timescales, assist in the generation of distinct outputs depending on input strength and duration, and regulate intricate feed-forward and feedback loops [71–73]. Given their widespread and crucial role in the integration and fine-tuning of physiologic processes, it is not surprising that abnormalities in signaling pathways have now been identified in a variety of human diseases. Furthermore, signaling pathways represent major targets for a number of hormones, including glucocorticoids, thyroid hormones and gonadal steroids. The biochemical effects of these signaling pathways may play a role in mediating certain clinical manifestations of altered hormonal levels in mood-disorder subjects, e.g. the frequent onset of bipolar disorder in puberty, triggering of episodes in the postpartum period, association of depression and potentially rapid cycling with hypothyroidism, and triggering of mood episodes in response to exogenous glucocorticoids.

Complex signaling networks may be especially important in the CNS, where they 'weigh' and integrate diverse neuronal signals and then transmit them to effectors, thereby forming the basis of a complex information processing network. See Figure 55-1 for details of these

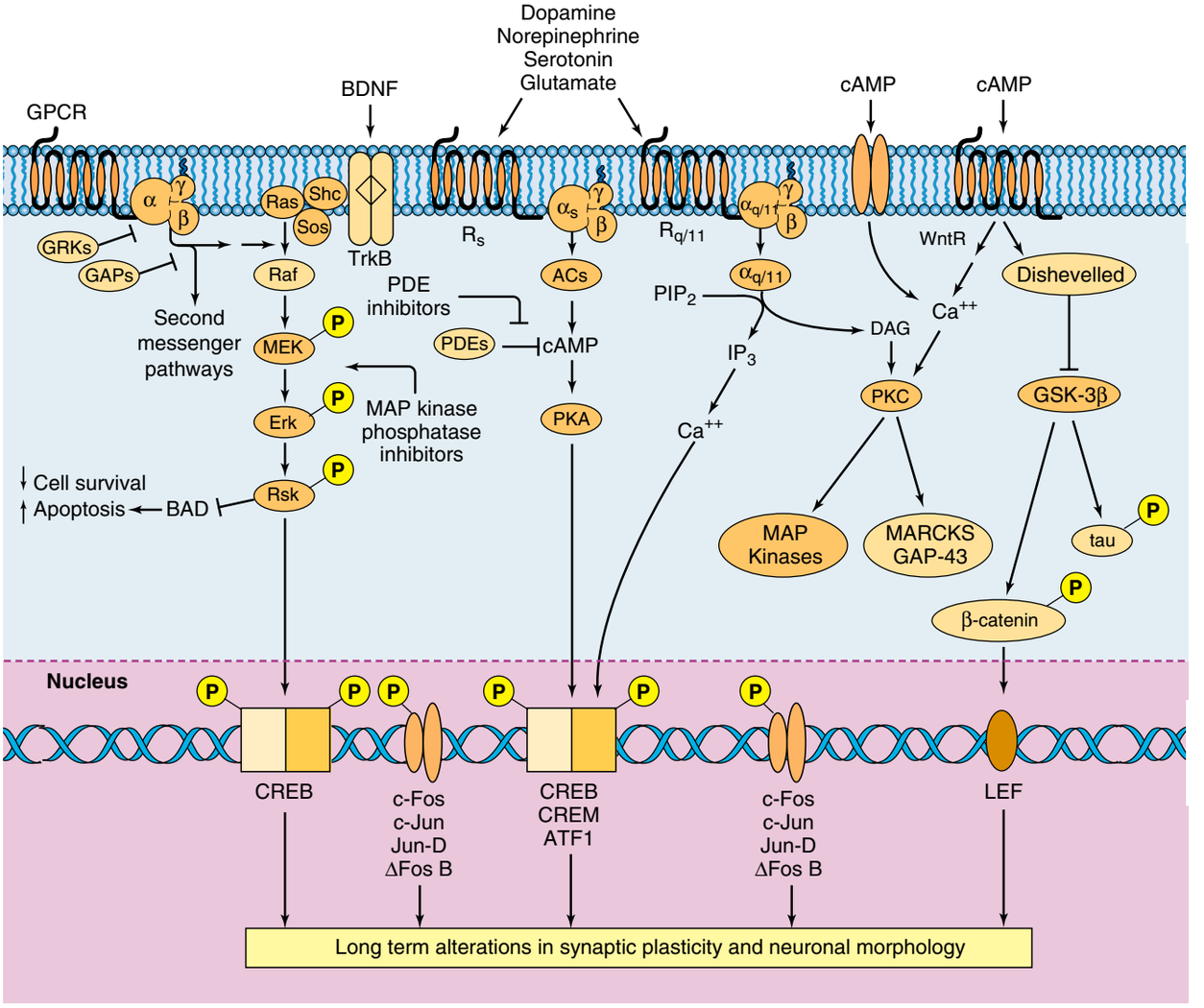

FIGURE 55-1 Major intracellular signaling pathways involved in neural and behavioral plasticity. The figure depicts some of the major intracellular signaling pathways involved in neural and behavioral plasticity. Cell-surface receptors transduce extracellular signals such as neurotransmitters and neuropeptides into the interior of the cell. Most neurotransmitters and neuropeptides communicate with other cells by activating seven transmembrane spanning G-protein-coupled receptors (*GPCRs*). As their name implies, GPCRs activate selected G proteins, which are composed of α and βγ subunits (Ch. 19). Two families of proteins turn off the GPCR signal, and may therefore represent attractive targets for new medication development. G-protein-coupled receptor kinases (*GRKs*) phosphorylate GPCRs and thereby uncouple them from their respective G proteins. GTPase activating proteins (*GAPs*, also called RGS or regulators of G-protein-signaling proteins) accelerate the G-protein turn-off reaction (an intrinsic GTPase activity). Two major signaling cascades activated by GPCRs are the *cAMP* generating second messenger system (Ch. 21), and the phosphoinositide (*PI*) system (Ch. 20). cAMP activates protein kinase A (*PKA*), a pathway which has been implicated in the therapeutic effects of antidepressants. Among the potential targets for the development of new antidepressants are certain phosphodiesterases (*PDEs*). PDEs catalyze the breakdown of cAMP; thus PDE inhibitors would be expected to sustain the cAMP signal, and may represent an antidepressant augmenting strategy. Activation of receptors coupled to PI hydrolysis results in the breakdown of phosphoinositide 4,5-biphosphate (PIP_2) into two second messengers — inositol 4,5-trisphosphate (IP_3) and diacylglycerol (*DAG*). IP_3 mobilizes Ca^{2+} from intracellular stores (Ch. 22), whereas DAG is an endogenous activator of protein kinase C (*PKC*), which is also directly activated by Ca^{2+}. PKC, PKA and other Ca^{2+}-dependent kinases directly or indirectly activate several important transcription factors, including *CREB*, *CREM*, *ATF-1*, *c-Fos*, *c-Jun*, *Jun-D*, and *ΔFos B* (Ch. 26). Endogenous growth factors such as brain-derived neurotrophic factor (*BDNF*) utilize different types of signaling pathways (Ch. 27). BDNF binds to and activates its tyrosine kinase receptor (*TrkB*); this facilitates the recruitment of other proteins (*SHC*, *SOS*), which results in the activation of the ERK-MAP kinase cascade (via sequential activation of *Ras*, *Raf*, *MEK*, *Erk*, and *Rsk*). In addition to regulating several transcription factors, the ERK-MAP kinase casade, via Rsk, down-regulates BAD, a pro-apoptotic protein. Enhancement of the ERK-MAP kinase cascade may have effects similar to those of endogenous neurotrophic factors; one potential strategy is to utilize inhibitors of MAP kinase phosphatases (which would inhibit the turn-off reaction) as potential drugs with neurotrophic properties. In addition to utilizing GPCRs, many neurotransmitters (e.g. glutamate and GABA) produce their responses via ligand-gated ion channels (see detailed discussions in Chs. 15 and 16). Although these responses are very rapid, they also bring about more stable changes via regulation of gene transcription. One pathway gaining increasing recent attention in adult mammalian neurobiology is the *Wnt* signaling pathway. Wnts are a group of glycoproteins active in development, but now known to play important roles in the mature brain.

Continued

potential pathways. The high degree of complexity generated by these signaling networks may be one mechanism by which neurons acquire the flexibility for generating the wide range of responses observed in the nervous system. These pathways are thus undoubtedly involved in regulating such diverse vegetative functions as mood, appetite and wakefulness, and are therefore likely to be involved in the pathophysiology of mood disorder.

Abnormalities in G protein subunits/cAMP signaling pathway have been observed in bipolar disorder. Postmortem brain studies in bipolar patients show increased levels of the stimulatory G protein ($G\forall_s$) accompanied by increases in postreceptor-stimulated adenylyl cyclase (AC) activity in bipolar disorder. These observations are further supported by a study showing increased agonist-activated [^{35}S]GTP(S binding to G protein $\forall_s$ subunits in the frontal cortical membranes of bipolar disorder patients [74]. Several studies have also found elevated $G\forall_s$ protein levels and mRNA levels in peripheral circulating cells in bipolar disorder, although the dependency of this finding on clinical state remains unclear. However, there is at present no evidence to suggest that the alterations in the levels of $G\forall_s$ are due to a mutation in the $G\forall_s$ gene itself. There are numerous transcriptional and post-transcriptional mechanisms which regulate the levels of G protein subunits (see Ch. 26), and the elevated levels of $G\forall_s$ could potentially represent the indirect sequelae of alterations in any one of these other biochemical pathways.

There is growing consensus that the ability of a 'simple' monovalent cation like lithium to treat multiple aspects of an illness as complex as bipolar disorder arises from its major effects on intracellular signaling pathways, rather than on any single neurotransmitter system *per se*. Although it appears that the lithium ion (at therapeutic concentrations) does not directly affect G protein function, there is considerable evidence that chronic lithium administration affects G protein function [75, 76]. It might be postulated that these G-protein effects of lithium, which would theoretically attenuate excessive signaling through multiple pathways, probably contribute to lithium's long term prophylactic efficacy in protecting susceptible individuals from spontaneous cyclic affective episodes induced by stress and drugs (e.g. antidepressants and stimulants).

Protein kinase C (PKC) exists as a family of closely related kinase subspecies with a heterogeneous distribution in the brain. These kinases have particularly high levels in presynaptic nerve terminals and, together with other kinases, appear to play a crucial role in the regulation of synaptic plasticity and various forms of learning and memory. PKC is one of the major intracellular mediators of signals generated upon external stimulation of cells via a variety of neurotransmitter receptors that induce the hydrolysis of various membrane phospholipids; these neurotransmitter receptors include muscarinic M1, M3, and M5 receptors, noradrenergic $\forall_1$ receptors, metabotropic glutamatergic receptors and serotonergic $5HT_{2A}$ receptors.

To date, there have only been a limited number of studies directly examining PKC in bipolar disorders [77]. Although undoubtedly an oversimplification, particulate (membrane) PKC is sometimes viewed as the more active form of PKC, and thus an examination of the subcellular partitioning of this enzyme can be used as an index of the degree of activation. Friedman *et al.* [78] investigated PKC activity and PKC translocation in response to serotonin in platelets obtained from bipolar-disorder patients before and during lithium treatment. They reported that the ratios of platelet-membrane-bound to cytosolic PKC activities were elevated in the manic patients. In addition, serotonin-elicited platelet PKC translocation was found to be enhanced in those patients. With respect to brain tissue, Wang and Friedman [74] measured PKC isozyme levels, activity and translocation in postmortem brain tissue from patients with bipolar disorder, and reported increased PKC activity and translocation in the brains of bipolar patients compared with controls, effects which were accompanied by elevated levels of selected PKC isozymes in cortices of bipolar disorder patients.

Evidence accumulating from various laboratories has clearly demonstrated that lithium, at therapeutically relevant concentrations, exerts significant effects on the PKC signaling cascade. Current data suggest that chronic lithium attenuates PKC activity, and down-regulates the expression of PKC isozymes $\forall$ in the frontal cortex and hippocampus [79, 80]. Chronic lithium has also been demonstrated to dramatically reduce the hippocampal levels of a major PKC substrate, myristoylated-alanine-rich C kinase substrate (MARCKS), which has been implicated in regulating long-term neuroplastic events.

Although these effects of lithium on PKC isozymes and MARCKS are striking, a major problem inherent in neuropharmacologic research is the difficulty in attributing therapeutic relevance to any observed biochemical finding. It is thus noteworthy that the structurally dissimilar

FIGURE 55-1—cont'd Binding of Wnts to the Wnt receptor (*WntR*) activates an intermediary protein, Disheveled, which regulates a glycogen synthase kinase (*GSK*-3β). GSK-3βexerts many cellular effects; it regulates cytoskeletal proteins, including *tau*, and plays an important role in determining cell survival/cell death decisions. GSK-3β has recently been identified as a target for Li^+'s actions. GSK-3β also regulates phosphorylation of β-*catenin*, a protein that when dephosphorylated acts as a transcription factor at LEF (lymphoid enhancer factor) sites. *CREB*, cAMP response element binding protein; R_q and R_o, extracellular GPCRs coupled to stimulation or inhibition of adenylyl cyclases (ACs), respectively. *Rq/11*, GPCR coupled to activation of phospholipase C (PLC), *MARCKS*, myristoylated-alanine-rich C kinase substrate, a protein associated with several neuroplastic events. Modified and reproduced, with permission from Payne, J. L. *et al.* Cellular biology of bipolar disorder. In *Neurobiology of Mental Illness*, Charney, D. S. and Nestler E. J. (eds). Oxford University Press, 2004.

antimanic agent valproate (VPA) produces effects very similar to those of lithium on PKC ∀ and on isozymes and MARCKS protein [79, 80]. Interestingly, lithium and VPA appear to bring about their effects on the PKC signaling pathway by distinct mechanisms. These biochemical observations are consistent with the clinical observations that some patients show preferential response to one or the other of the agents, and that one often observes additive therapeutic effects in patients when the two agents are co-administered.

In view of the pivotal role of the PKC signaling pathway in the regulation of neuronal excitability, neurotransmitter release, and long-term synaptic events, it was postulated that the attenuation of PKC activity may play a role in the antimanic effects of lithium and VPA. In a pilot study it was found that tamoxifen, a nonsteroidal anti-estrogen drug known to be a PKC inhibitor at higher concentrations, may possess antimanic efficacy [81]. Clearly, these results have to be considered preliminary, due to the small sample size thus far. However, in view of the preliminary data suggesting the involvement of the PKC signaling system in the pathophysiology of bipolar disorder, these results suggest that PKC inhibitors may be very useful agents in the treatment of mania. Larger double-blind, placebo-controlled studies of tamoxifen and of novel selective PKC inhibitors in the treatment of mania are warranted.

A crucial kinase that functions as an intermediary in numerous intracellular signaling pathways is the enzyme glycogen synthase kinase-3 (GSK-3). Given the evidence noted thus far of the importance of intracellular signaling pathways in bipolar disorders, GSK-3 may therefore play an important role in bipolar disorder research [82, 83]. GSK-3, a highly conserved enzyme in evolution, is found in two nearly identical isoforms (variations) in mammals, α and β. This enzyme was first discovered (and named) based on its ability to phosphorylate, and thereby inactivate, the enzyme glycogen synthase, an action that leads to a decrease in the synthesis of glycogen. GSK-3 is unique among kinases in that it is generally constitutively active, and thus most intracellular signals to GSK-3 inactivate this enzyme by phosphorylating it on its serine-9 residue, or, possibly, by inhibiting a putative phosphatase. Signals deactivating GSK-3 arise from insulin stimulation, numerous growth factors (e.g. phosphoinisitide [PI] 3-kinase), and developmental signals. A number of endogenous growth factors (e.g. nerve-growth factor and brain-derived neurotrophic factor [BDNF]) use the PI-3-kinase signaling cascade as a major effector system. Thus, growth factors may bring about many of their neurotrophic and/or neuroprotective effects, at least in part, by GSK-3 inhibition. GSK-3 phosphorylates — and thereby inactivates — many transcription factors, and modulates the function of cytoskeletal proteins such as the Alzheimer's disease protein tau (another name for GSK-3 is tau kinase). Inhibition of GSK-3 thus results in the release of this inhibition, and activation of multiple cellular targets.

Rapidly increasing evidence suggests that GSK-3 plays an important role in regulating neuroplasticity and cellular resilience. Studies have suggested that changes in GSK-3 mediated MAP-1B (a cytoskeletal protein) phosphorylation are associated with the loss and/or unbundling of stable axonal microtubules. Furthermore, GSK-3 inhibition results in the accumulation of synapsin I, a protein involved in synaptic vesicle docking and release of growth-cone-like areas.

In addition to its putative role in regulating synapse formation and axonal growth, there is considerable excitement regarding the role of GSK-3 in regulating cell death (apoptosis) in mature neuronal tissue, and the development of GSK-3 inhibitors as novel therapeutic agents for bipolar disorder and classical neurodegenerative diseases. Although it was initially reported that GSK-3 activity was required for β-amyloid-induced neurotoxicity in primary hippocampal neurons, this observation was not followed-up until very recently. More-current studies have demonstrated that GSK-3 may regulate cell death beyond its role in β-amyloid-induced toxicity. For example, GSK-3 overexpression induces apoptosis in cultured cells, which is prevented by dominant negative mutants. Furthermore, the expression of FRAT-1, a protein that inhibits GSK-3, also rescues primary sympathetic neurons from cell death induced by PI-3-kinase inhibition.

Activation of the HPA axis appears to play a critical role in mediating hippocampal atrophy. In addition to directly causing neuronal atrophy, stress and glucocorticoids also appear to reduce cellular resilience, thereby making certain neurons more vulnerable to other insults, such as ischemia, hypoglycemia, and excitatory amino-acid toxicity (see Ch. 32). The reduction in the resilience of hippocampal neurons may also reflect the propensity for various stressors to decrease the expression of BDNF in this region [84]. BDNF and other neurotrophic factors are necessary for the survival and function of neurons, implying that a sustained reduction of these factors could affect neuronal viability. Increasing evidence suggests that neurotrophic factors inhibit cell death cascades by (in large part) activating the mitogen activated protein (MAP) kinase signaling cascade, and up-regulating major cell survival proteins such as bcl-2 [85].

Bcl-2 is now recognized as a major neuroprotective protein, since bcl-2 overexpression protects neurons against diverse insults, including ischemia, the neurotoxic agent methyl-phenyl-tetrahydropyridine (MPTP), β-amyloid, free radicals, excessive glutamate and growth factor deprivation [86]. Accumulating data suggest that bcl-2 is not only neuroprotective, but also exerts neurotrophic effects and promotes neurite sprouting, neurite outgrowth and axonal regeneration [86]. If enhanced bcl-2 expression appears to be capable of offsetting the potentially deleterious consequences of stress-induced neuronal endangerment, then pharmacologically induced upregulation of bcl-2 may have considerable utility. Overall, it is clear

that the neurotrophic factor/MAP kinase/bcl-2 signaling cascade plays a critical role in cell survival in the CNS, and that there is a fine balance maintained between the levels and activities of cell survival and cell death factors. Modest changes in this signaling cascade or in the levels of the bcl-2 family of proteins (potentially due to genetic, illness, or insult-related factors) may therefore profoundly affect cellular viability.

Interestingly, pretreatment with pramipexole, a dopaminergic agonist agent, increased bcl-2 and inhibited methylphenylpyridinium-induced apoptosis in human neuroblastoma SH-SY5Y cells, and treatment of rats for 4 days with pramipexole markedly increased bcl-2 immunoreactivity in neuronal dendritic processes in both cerebral cortex and hippocampus. Our group recently demonstrated the antidepressant efficacy of this agent in a double-blind placebo-controlled study, in bipolar II depressed patients on therapeutic levels of lithium or valproate [87].

INTRACELLULAR CALCIUM SIGNALING STUDIES IN BIPOLAR DISORDER

Calcium ions play a critical role in regulating the synthesis and release of neurotransmitters, in neuronal excitability, and in long-term neuroplastic events, and it is thus not surprising that a number of studies have investigated intracellular Ca^{2+} in peripheral cells, particularly in bipolar disorder.

Impaired regulation of Ca^{2+} cascades has been found as the most reproducible biological measure abnormality described in bipolar disorder research. For this reason, mechanisms involved in Ca^{2+} regulation have been postulated to underlie aspects of the pathophysiology of bipolar disorder. To date, approximately 15 studies have consistently revealed elevations in basal intracellular Ca^{2+} levels in platelets, lymphocytes or neutrophils of patients with bipolar disorder. By contrast, there are only four negative studies. Although this may represent (in part) the phenomenon of publication bias, the elevation in basal Ca^{2+} represents one of the most replicated findings in bipolar disorder research. Higher platelet intracellular Ca^{2+} elevations have also been found in patients with bipolar disorder in response to stimulation with thrombin, platelet activator factor (PAF), serotonin, dopamine and thapsigargin. In lymphocytes, the same higher elevations were observed when the cells were stimulated with phytohemaglutinin, concavalin A, thrombin and (as in platelets) with thapsigargin and serotonin.

However, a few caveats to these positive studies should be noted. There is considerable evidence that a variety of circulatory factors may influence the activity of blood cells and elements, and bipolar disorder patients are known to have numerous neurohormonal abnormalities (such as catecholamine and cortisol level abnormalities). Furthermore, many of these studies did not employ an extensive medication washout period, raising the possibility that the elevations of Ca^{2+} in circulating cells are simply secondary manifestations. Yet, in an elegant series of studies, Epstein–Barr-virus-immortalized B lymphoblasts (BLCL) were grown in culture (away from the patients' confounding circulatory environment) for weeks. These studies found that even in the immortalized lymphoblasts, patients with bipolar disorder showed elevated basal Ca^{2+} concentrations compared with healthy subjects or patients with other psychiatric disorders. In an extension of these studies, these investigators examined the components of the storage-operated Ca^{2+} entry (SOCE), and found a reduction in the mRNA expression of the TRPC7 (TRPM2) gene (whose gene product is implicated in SOCE functioning), in BLCLs from a subgroup of bipolar disorder I patients [88].

The regulation of free intracellular Ca^{2+} is a complex, multi-faceted process (see Chs. 5 and 22), and the abnormalities observed in bipolar disorder could arise from abnormalities at a variety of levels. Ongoing studies should serve to delineate the specific regulatory sites at which the impairment occurs in bipolar disorder.

ANXIETY DISORDERS

The development of mild forms of anxiety and neurovegetative and/or cognitive responses to stress may represent an adaptive evolutionary step against environmentally (external) or self-triggered (internal) threats, but maladaptive reactions have also emerged in human evolution. Thus, anxiety disorders are maladaptive conditions in which disproportionate responses to stress, or even self-evoked responses, are displayed. Anxiety disorders are one of the most frequent psychiatric illnesses, and have a lifetime prevalence of 15– 20% [1, 89]. The most common presentations are generalized anxiety disorder, with a lifetime prevalence rate of close to 5% [1, 89]; social anxiety disorder, with very variable lifetime prevalence rates ranging from 2 to 14% [90]; panic disorder, with rates from 2 to 4% [1, 89]; and post-traumatic stress disorder (PTSD), with a prevalence rate close to 8%. Specific phobias, acute stress and obsessive–compulsive behavior are other clinical presentations of anxiety disorders.

Animal models of fear and anxiety have primarily used the rat, the mouse and, to a lesser extent, nonhuman primates. It is not particularly difficult to evoke or measure anxiety in these species. However, difficulties arise when one attempts to define exactly how a stimulus and resultant behavioral response are related to human behavior, i.e. when a mouse exhibits freezing behavior to an unfamiliar and threatening cue, what is the human equivalent? Or, similarly, what stimulus could one present to a rat to best model the anxiety-inducing-experience of

public speaking? The unique set of symptoms and physiological markers associated with each anxiety disorder makes the development of truly specific models difficult. There is also the extremely important distinction between models which simply measure the anxious response of normal animals exposed to pharmacological or other manipulations, as might be used to screen drugs or genes, and those which attempt to create a pathological model of abnormal behavior.

There are three generally accepted criteria for validating animal models for human psychiatric disorders: face validity, construct validity, and predictive validity. Face validity refers to the outward appearance of the model, i.e. does the animal's behavior adequately reflect the human behavior being modeled? In this dimension, anxiety models have a clear advantage over other psychiatric models; it is usually quite apparent if an animal is frightened, whereas it is a much more difficult to assess whether an animal is displaying psychotic-like or depressive-like behavior, for example.

Construct validity refers to the fundamental causality and etiology for the behavior. Again, animal models of anxiety perform relatively well in this regard; the purpose and underlying anatomical and neurochemical substrates of anxiety are well conserved. However, although the general modeling of fear and anxiety has both face and construct validity, models of specific disorders are much more meager in these respects.

Predictive validity is the ability of a model to predict the effect that pharmacological or other manipulations will have on the condition being modeled. This criterion can present a real difficulty, in that drug development is often dictated by animal models. For example, if a given model only detects a subset of effective compounds (i.e. those belonging to a specific chemical class), then useful candidates will be discarded long before clinical trials, and the flaw in the model's predictive validity will not be discovered. Thus, the possibility that a model will yield false negatives cannot be ruled out.

Currently, a wide variety of methods are employed to measure animal anxiety. In rodents, the most commonly used measures employ these animals' natural fear of brightly lit, open spaces. Measures may include exploratory behavior and social behaviors (in the social interaction test), with the expectation that such behaviors will decrease as anxiety increases. Advantages of these methods, including light–dark exploration and open-field tests, lie primarily in their ease of use. These models have had great predictive validity for benzodiazepines and barbiturates, but have been only marginally successful in detecting the serotonergic and noradrenergic anxiolytics (anti-anxiety drugs). These methods are also currently enjoying great popularity in the behavioral testing of transgenic and knockout mice (mice with genes added or removed, respectively).

In operant conflict models, animals are trained to respond for a reward, such as food. The reward is subsequently paired with a punishment (e.g. foot shock), causing a reduction in response frequency. The degree to which the animal ceases to respond is thought to reflect its anxiety about receiving punishment. The predictive validity of this method is somewhat better than the elevated-plus-maze and social interaction measures, in that chronic administration of antidepressants or serotonin $(5\text{-HT})_{1A}$ partial agonists results in an anxiolytic effect, at least in rats. Furthermore, the requirement for chronic treatment with these drugs appears to be preserved in this model. However, the long training periods and specialized equipment required for this technique makes it impractical for many laboratories.

Ultrasonic vocalizations are emitted by rat pups (under the age of 14 days) when they are isolated from their mother, and are thought to reflect anxiety. This measure has proven sensitive to both anxiolytic and anxiogenic manipulation of GABA neurotransmission. However, the early developmental window used is problematic, in that chronic drug administration probably results in a variety of compensatory changes not seen in adulthood, and may alter development of relevant brain systems. Indeed, in contrast to the clinical situation, the antidepressant clomipramine has acute, but not chronic, anxiolytic efficacy in this model.

Nonhuman primates offer another, although clearly much more difficult, avenue to investigate fear and anxiety [91]. The interspecies variation in neurobiology and behavior is substantially reduced compared with rodents. A variety of measures to test anxiety have been developed, using stressors such as social isolation, conspecific (of the same species) intruders, extraspecific (human or predator) threat, and pharmacological anxiogenesis. These models have demonstrated validity, and may be especially valuable in the later stages of preclinical drug development.

Animal models thus allow us to study the biology of anxiety behavior, and the manipulation of that behavior through environmental, pharmacological and genetic mechanisms. However, the ability of these models to adequately replicate the character and diversity of human anxiety disorders is far from clear. Given the rapid advancement of techniques for probing human brain function *in vivo*, we will increasingly be able to discern disorder-specific biology, and thereby develop more valid animal models.

Transgenic and knockout mice are employed to investigate the genetics of anxiety. The recent ability to manipulate the genome of mice — either through adding a gene (transgenic) or removing a gene (knockout) — has enabled an entirely novel type of study: relating a particular gene directly with a particular behavior. Using these genomic manipulation strategies in animals, a large number of genes have been implicated, to some degree, in the pathophysiology of anxiety in humans.

In principle, the knowledge that can be gained by genome manipulation is impressive. Given sufficient knowledge

about both the gene and the behavior(s) in question, it is possible to simultaneously investigate in a 'top-down' and 'bottom-up' fashion; in other words, one may proceed upward from the gene function level to the cell biology and physiology level (bottom-up), or proceed downward from the behavioral to systems and circuit-level neuroscience (top-down). In this way, one may aggressively approach from two different directions the complex problem that is the relationship between genes and behavior (see also Learning and Memory, Ch. 53).

However, genes and behaviors cannot be associated on a simplistic, one-to-one basis; the true relationship between a gene and a behavior is probably closer to chaos theory's 'sensitive dependence on initial conditions'. For example, there is presumably no gene for language; there are a number of genes which pattern the embryonic brain in such a way as to facilitate, and allow the physiological processes necessary for, language acquisition. In a similar regard, no single gene has been found to code for a human psychiatric condition. It is more likely that susceptibility genes interact with developmental factors, both day-to-day and profound environmental events, epigenetic DNA modifications, and possibly entirely stochastic mechanisms, eventually leading to the development of normal and abnormal human behaviors.

Although many difficulties exist in the interpretation of knockout and transgenic studies, one of the most troubling is the relative role of immediate versus developmental and adaptive effects. Many knockout gene studies have been interpreted in much the same way as a pharmacological manipulation, assuming that the only difference between the wild versus the experimental animal is the presence or absence of a specific protein. In truth, the actual effect of a gene knockout can be far-reaching and highly complex. Given the universality of homeostatic regulation in biology, from the intracellular to the organism level, it is almost inevitable that adaptations will occur in the chronic absence of a particular gene. Furthermore, the consequences of developmental absence are almost certainly even more profound. The recent use of *conditional knockouts*, in which gene expression is only altered in particular anatomical regions and/or during particular times, has illustrated this point quite clearly and has simultaneously offered a solution. Perhaps the most relevant example to the field of anxiety comes from genetic studies of the 5-HT_{1A} receptor.

The 5-HT_{1A} receptor, as a target of many useful anxiolytic agonists, has been studied more than any other gene in the context of anxiety research. As might be expected, most knockout studies have reported increased anxiety-like behavior in the mutant animals. Seemingly, these data support a very simple, pharmacological view-that 5-HT_{1A} activation counteracts anxiety. However, the recent work of Gross and colleagues has placed this interpretation in doubt [122]. Using a knockout/rescue approach with regional and temporal specificity, it was demonstrated that the anxiety-related effect of the 5-HT_{1A} receptor knockout was actually developmental. Specifically, 5-HT_{1A} receptor expression limited to the hippocampus and cortex during early postnatal development was sufficient to counteract the anxious phenotype of the mutant, even though the receptor was still absent in adulthood. Thus, pharmacological, genetic, and developmental effects of the 5-HT_{1A} receptor on anxiety appear to be distinguishable. In conclusion, although there is no debating the usefulness of genetic manipulation, the interpretation of behavioral genetics studies must be thoughtful and cautious.

There are undoubtedly many developmental insults, genetic and otherwise, that can alter the expression of anxiety-related behaviors. The dissection of a gene's role in anxiety must include the acute, adaptive, compensatory, and developmental effects. For further discussion of the genetic approaches to animal anxiety, the reader is referred to several recent reviews [92–94].

Neurotransmitters and neuropeptides have been implicated in fear and anxiety. Much of our knowledge of the human neural substrates of fear and anxiety is derived from pioneering work using cat and rodent models. As techniques have advanced, our understanding of the anatomy, neurochemistry and physiology of these responses has progressed. In particular, the development of functional imaging techniques has allowed us to confirm that observations made in a number of animal species may also apply to humans.

The fear response necessarily begins with perception. Most sensory information is relayed via the thalamus to the sensory cortices, which are responsible for recognition and cognitive appraisal of a threat. Two exceptions to this pathway are olfactory input, which may reach the amygdala and entorhinal cortex directly, and visceral organ input, which proceeds from nuclei in the brainstem to the locus ceruleus (LC). The LC appears to have several roles that are important in the regulation of anxiety. On one level, noradrenergic efferents appear to have a key role in regulating the peripheral sympathetic response; LC firing appears necessary for the generation of a physiological response to anxiogenic stimuli. Additionally, the LC, perhaps as a mediator of sensory information in the cortex-to-thalamus pathway, also plays a role in directing attention towards salient, threatening stimuli. Notably, there is evidence that the release of corticotrophin releasing hormone (CRH) in the LC is necessary for the enhanced firing seen in high-anxiety conditions.

The amygdala is perhaps the best-studied, and most strongly implicated, brain structure in anxiety and fear. Electrical stimulation of the amygdala produces fear-like behavioral and physiological responses in animals, and increases the suggestive experience of fear in human subjects. Additionally, amygdala stimulation leads to corticosterone secretion and HPA-axis activation in animals, probably via outputs to the hypothalamus and the bed nucleus of the stria terminalis. It has been suggested

that the amygdala may represent a sort of 'master switch' of fear, which projects to a variety of areas. This unitary-mediator hypothesis explains the constellation of behavioral and physiological responses that concur so consistently.

Multiple studies suggest that particular pharmacological agents often provoke symptoms of anxiety in susceptible individuals. These agents can be generally classified into two categories: (a) those that reduce available oxygen, and appear to act on peripheral areas, and (b) those that directly manipulate neurotransmitter systems. A number of pharmacological agents have been noted to increase anxiety (generally measured as panic symptoms in patients with panic disorder) in susceptible individuals.

In one sense, these findings provide important clues in understanding the underlying neurobiology of anxiety disorders. However, the broad spectrum of agents that can produce anxiety symptoms makes it difficult to define one system, or pathway, in the brain most responsible for anxiety. It is possible, however, to broadly define the targets of compounds that may have general mechanisms of action. In this regard, carbon dioxide, sodium lactate and bicarbonate fall into the first category — all act peripherally, resulting in increased respiration, heart rate, and other signs of sympathetic nervous system activity. These mechanisms of action helped form a hypothesis in which patients with anxiety disorders may interpret physiological changes (such as increased heart rate, increased respiration, etc) as more serious than they really are, and thus the patients respond with increasing anxiety.

It may be that any peripherally adverse stimulus — especially one that stimulates sympathetic activity — thus has the potential to activate brain areas of prime importance in the formation of anxiety symptoms. As a result of pharmacological challenge studies, biochemical assays, neuroimaging and studies of animal models, a number of centrally acting neurotransmitters, and their relevant neural circuits, are implicated in anxiety. These neurotransmitters include norepinephrine, serotonin, GABA, neuropeptide Y, cholecystokin and substance P.

Noradrenergic systems. Preclinical studies using pharmacological manipulation and electrical stimulation have suggested the involvement of norepinephrine and the LC in anxiety- and fear-like behaviors. The strongest human evidence comes from studies of the anxiogenic and anxiolytic properties of centrally acting selective noradrenergic drugs, some of which exert these actions by increasing LC activity. The selective β-agonist isoproterenol induces anxiety and panic attacks in some patients. However, directly attributing the effects of isoproterenol to enhanced central β-adrenergic receptor throughput is problematic, because this drug does not appear to cross the blood–brain barrier. NE receptors are discussed in Ch. 12.

More direct evidence of noradrenergic effects comes from studies using the α2 antagonist, yohimbine. α2 adrenergic receptors are known to be present on the cell bodies and terminals of norepinephrine neurons, where they regulate the firing rate and amount of norepinephrine released per nerve impulse respectively. Thus, blockade of α2 receptors has been clearly established to increase the firing on LC norepinephrine neurons, as well as norepinephrine release. In patients with panic disorder, yohimbine increases anxiety and the frequency of panic attacks compared with controls, effects which have been attributed to enhanced LC firing and norepinephrine release [95, 96]. Consistent with the behavioral data are neurobiological data showing that yohimbine increases cardiovascular responses and plasma MHPG and cortisol in panic patients relative to control subjects. Yohimbine has also been reported to produce a decrease in frontal blood flow in this patient population, effects which were not seen in control subjects.

Additional pharmacologic support for the role of the LC norepinephrine system in mediating anxiety symptoms comes from studies using clonidine, a centrally active α2 receptor agonist, which decreases LC firing and norepinephrine release. Thus, clonidine has been shown to have some efficacy in the treatment of anxiety and in both spontaneous and induced panic disorder. Interestingly, the beneficial effects of clonidine subside over time, perhaps due to desensitization of the α2 receptor with continued stimulation by a direct agonist. Furthermore, clonidine administration results in a greater degree of hypotension and larger reductions in plasma MHPG in patients with panic disorder relative to control subjects, raising the possibility of altered α2 receptor sensitivity in panic disorder. Consistent with such a contention, norepinephrine and its metabolites measured in urine, blood and CSF are elevated in panic disorder, PTSD, specific phobias, social anxiety and GAD, suggesting a dysregulation of the central and peripheral norepinephrine systems.

It has been postulated that the inhibitory inputs from the frontal cortex to limbic regions may be disrupted in anxiety disorders, resulting in unrestrained amygdala activity, which results in an increase in anxiety. In this context, yohimbine decreases metabolism in the cortical brain areas in PTSD patients, whereas in control subjects cerebral metabolism is increased. Additionally, as mentioned, functional studies in panic disorder suggest an abnormal blood flow following administration of agents that result in panic symptoms. In addition to yohimbine [97], these studies have also been performed with lactate infusion. These findings in the cortex are consistent with the model by which cortical input to the amygdala provides a 'top down' inhibition that is lacking in patients with anxiety disorders — and functions as a contributor to anxiety in patients. Also consistent with these data is the general finding that while low levels of anxiety increase cortical metabolism in humans, high levels of anxiety have been shown to decrease it [98, 99]. Thus, abnormal cerebral metabolism in anxiety may allow for abnormal amygdala activity. In this regard, recent preclinical work in rats has shown that stimulating specific areas of the medial PFC can increase the extinction of fear responses [100].

Serotonergic systems. Evidence on several levels implicates the serotonergic system in the treatment of anxiety, although not conclusively. The raphe nuclei presumably play an important role in the serotonergic aspects of fear and anxiety. An excitatory projection from the LC to the dorsal raphe may be important in the serotonin release observed in the PFC, amygdala, and hypothalamus in response to anxiogenic stimuli. Additionally, projections from the dorsal raphe also extend to and inhibit the LC, suggesting a possible negative-feedback mechanism. Chronic, but not acute, SSRI administration suppresses LC firing in rats. The medial raphe may also have some opposing effects; there is some evidence that output from this nucleus to the dorsal hippocampus acts to increase stress resistance [101], whereas LC projections to the medial raphe suppress its firing.

A number of medications used in the treatment of anxiety have effects on serotonin neurotransmission (Ch. 13). These medications include tricyclic antidepressant medications, SSRIs, and monoamine oxidase inhibitors (MAOIs). However, because these medications take weeks to exert their full anxiolytic effects, it is unlikely that blocking the reuptake (and thus increasing synaptic levels) of either serotonin or norepinephrine selectively is responsible for their anxiolytic properties — rather it is suspected that the therapeutic effects are due to changes in gene expression, protein levels, and eventually changes in synaptic connections between neurons.

In spite of the large number of medications that target serotonin neurotransmission, consistent evidence implicating serotonin neurotransmission in the pathophysiology of anxiety is lacking. As reviewed in refs [102] and [103], markers of the density and/or activity of serotonin reuptake transporters has been found to be increased, unchanged or decreased in panic disorders, depending on the study and experimental conditions. Furthermore, the application of pharmacological challenges to patients with anxiety disorders has been almost equally ambiguous. Thus, responses to serotonin precursors (such as L-tryptophan and 5-hydroxytryptophan) do not appear to be different between control subjects and those with panic disorder, or to be anxiogenic. Challenges with other more direct serotonin agonists are also inconclusive. In this regard, the 5-HT-releasing agent fenfluramine has been reported to be anxiogenic and produce greater increases in plasma prolactin and cortisol in patients with panic disorder compared with control subjects. The 5-HT agonist m-chlorophenyl-piperazine (mCPP) has been shown to increase anxiety and plasma cortisol in some studies of panic-disorder patients compared with control subjects. Tryptophan depletion in anxiety disorders has also not been generally informative. Indirect evidence also implicates serotonergic dysfunction in PTSD, but more work needs to be done [102, 103].

GABA. The GABA system is the primary target for the acute treatment of anxiety. The benzodiazepines (BDZs) function by binding to a potentiator site on the GABA-A receptor, increasing the amplitude and duration of inhibitory postsynaptic currents in response to GABA binding (see Ch. 16). Unfortunately, the BDZs have significant negative side-effects, including sedation, cognitive impairment and addictive liability. Our growing understanding of the GABA system may enable us to design more tolerable medications.

The GABA-A receptor displays enormous heterogeneity; it is composed of a combination of five subunits, of which there are at least 18 subtypes. The various receptors display variation in functional pharmacology, hinting at the multiple, finely tuned roles that inhibitory neurotransmission plays in brain function. The majority of GABA-A receptors in the brain are targets of diazepam and other BDZs. For this reason, there has been considerable interest in determining if the desirable and undesirable effects of BDZs might be based on the differing receptor subtypes.

Much work has been focused in this direction, particularly using gene knockout technology. For instance, mutation of the BDZ-binding site of the GABA-A ∀-1 subunit in mice blocks the sedative, anticonvulsive, and amnesic, but not the anxiolytic, effects of diazepam. In contrast, the ∀-2 subunit (expressed highly in the cortex and hippocampus) is necessary for diazepam anxiolysis and myorelaxation. Thus, there is now optimism that an ∀-2 selective ligand will soon provide effective, acute treatment of anxiety disorders without the unfavorable side-effects profile of current BDZs. A compound with this preferential affinity has already been demonstrated to exert fewer sedative and depressant effects than diazepam in rat behavioral studies.

It is believed that novel medications under development will specifically target subtypes of GABA receptors, resulting in medications that are more specific for anxiety, and that have fewer side-effects. Current GABAergic drugs exert their actions almost immediately upon initiation of treatment, and there is optimism that novel medications of this class will also have immediate effects, thereby circumventing one of the primary problems of many current anxiolytic drugs, namely the delay in onset of action. For more on the pharmacology of benzodiazepines, see [104].

CRH and stress axes. The HPA axis is a major pathway by which stress exerts its effects on the brain and the rest of the body. The HPA axis is also believed to be relevant to development of anxiety and anxiety disorders [105]. In the HPA axis pathway, the lateral ventricular nucleus of the hypothalamus releases CRH, which stimulates the production of ACTH by the pituitary gland. This hormone (ACTH) stimulatesthe production of cortisol by the adrenal gland. Cortisol is considered a primary stress hormone of the body, having varied effects on metabolism, neurovegetative behaviors of organisms, and on functions of neurons and neuronal systems. A primary receptor for cortisol — the glucocorticoid receptor — is localized to many brain regions important in the stress response, including a high density of receptors in the hippocampus.

CRH and its receptors have generated increasing interest as a target for medications. In addition to the pituitary, CRH receptors have been localized to the cortex, the nuclei of the amygdala, the LC, and regions of the hypothalamus. It has also been reported that CRH increases LC activity, and that local injection of CRH into the LC increases behavioral responses consistent with increased anxiety [105]. Furthermore, CRH antagonists have been repeatedly shown to have anxiolytic effects in animal models.

Supporting the notion of an overactivation of the HPA axis in anxiety, are a number of clinical studies suggesting that patients with PTSD have a smaller hippocampal volume than matched control subjects, a finding that is consistent with what has been observed in animal models of stress. To date, no quantitative neuro-imaging studies have been performed in panic disorder, phobic disorder or generalized anxiety disorder — but a single study does suggest the presence of abnormalities in the temporal lobe in panic disorder [103, 106]. Studies also suggest an increase in cortisol release in response to stress in PTSD and panic disorder. Furthermore, a generally consistent finding is a chronic increase of CRH in patients with anxiety. Additional studies suggest changes in other mediators of the HPA axis, but these are generally less consistent.

A great deal of effort has gone into the development of antagonists to the type 1 and 2 CRH receptors, because CRH agonism has proven anxiogenic in a variety of animal models, and CRH antagonists have demonstrated anxiolytic properties in rodent and primate studies, and even in a preliminary clinical study for depression [107]. Drugs targeting the CRH family of ligands and receptors are perhaps the most promising new class of anxiolytics and antidepressants. The orally active CRH antagonist, antalarmin, significantly reduces fear and anxiety responses in nonhuman primates and may be a candidate for further development. Several pharmaceutical companies have developed substituted-pyrimidine small-molecule CRH antagonists [108]. Clinical trials are underway to examine the efficacy of these treatments in depression, and it is likely that complementary trials for the treatment of anxiety will also be undertaken. Targeting glucocorticoid receptors may also represent a possible mechanism to prevent stress-mediated neuronal plasticity changes.

Thus, the evidence that stress, and the stress hormone corticosterone, may cause changes in hippocampal structure, hippocampal connections, and changes in gene and protein expression, suggest that viable targets of anxiolytic agents are cellular signaling pathways involved in neuroplasticity and the maintenance of cellular resilience.

Neuropeptide Y (NPY) and its receptors may also be important in the regulation of anxiety and stress. NPY is synthesized in the arcuate nucleus, which receives LC input. In a number of rodent models, NPY administration has anxiolytic and, at somewhat higher doses, sedative effects. Likewise, NPY antagonizes CRH-induced stress responses, and suppresses LC firing when injected into the brainstem. There is some evidence to suggest that NPY is low under unstressed conditions, but is stimulated as a counteradaptation to stress. Additionally, NPY projections to the central amygdala, the nucleus accumbens, septum, periaqueductal gray (PAG), hippocampus and other regions, may also be involved in NPY anxiolysis. As our understanding of the differing roles played by the various NPY receptors improves, NPY receptor agonists are likely to become a goal of anxiolytic development [109].

Cholecytokinin (CCK) is another neuropeptide with importance to anxiety disorder research. CCK-B receptor agonists reportedly have an anxiogenic effect in animals, and are anxio- and panico-genic in normal subjects and panic disorder patients (patients are more sensitive). Likewise, suppression of CCK-B receptor expression or pharmacological antagonism blocks the acquisition of conditioned-fear response. However, CCK antagonists have yet to succeed in clinical trials [110]. Nevertheless, the CCK system remains an attractive target for drug development, particularly for panic disorders.

Substance P. The neuropeptide substance P (Sub P) binds to the neurokinin-1 receptor (NK-1; also known as tachykinin-1 receptor). Although originally targeted as a nociceptive pathway, antagonists of this receptor may exert anxiolytic and antidepressant effects. There is also evidence for an anxiolytic effect of Sub P when it is injected into the cholinergic nucleus basalis magnocellularis, suggesting region-specific effects. Furthermore, NK-1 activation in the hypothalamus inhibits CRH secretion. Several NK-1 antagonists appear anxiolytic in animal studies, and in a preliminary clinical trial, the antagonist MK-869 was found to be as effective as paroxetine in treating anxiety and depression. For additional discussion about the potential usefulness of NK-1 antagonists, see [111].

INTRACELLULAR TARGETS FOR ANXIETY DISORDERS

Whereas genetic and pharmacological studies have focused predominantly on the receptors, transporters and metabolic enzymes of neurotransmitters already implicated in anxiety, there also exists a more diverse group of gene knockouts with anxiety-related phenotypes. A number of animal studies have investigated anxiety-like behavior in knockouts of key intracellular signaling enzymes, including calcium/calmodulin-dependent protein kinase-II (CaMK-II) α, adenylate cyclase type VIII, and PKC ε and γ. These studies raise the possibility that pharmacological manipulation of signaling cascades within cells might be exploited in the development of new anxiolytics.

Studies of PKC and anxiety provide a particularly good example. The knockout of either the ε or γ isoform confers resistance to anxiety and potentiates the action of benzodiazepines. Furthermore, PKC inhibition may have

anxiolytic effects in humans; in a study of psychosocial functioning in women on long-term tamoxifen, which possesses both PKC- and estrogen-inhibiting properties, there was a statistical trend towards reduced anxiety in the treatment group. Likewise, valproic acid, an anticonvulsant and mood stabilizer that reduces expression of several PKC isoforms, may have efficacy in the treatment of panic disorder [112] and PTSD [113]. The specific roles of PKC isozymes in anxiety behavior require study, but specific inhibitors may someday serve as anxiolytics or anxiolytic-potentiators.

Inositol, the building block of the phosphoinositide intracellular signaling pathway, has been examined as a potential anxiolytic. In clinical trials, inositol has reported to be effective in both panic disorder [114, 115] and depression [116], and animal data are also favorable. Because of inositol's status as a dietary supplement, there is little financial backing for studies of its efficacy and safety. The mechanism of action of inositol is not entirely clear, but it is believed to facilitate phosphoinositide-signaling-coupled neurotransmission.

FUTURE DIRECTIONS AND THE DEVELOPMENT OF NOVEL THERAPEUTICS

This chapter describes neurobiological findings that are generating considerable scientific excitement about the development of novel agents for the treatment of mood and anxiety disorders. Most notably, there is a considerable body of evidence — both conceptually and experimentally — suggesting that impairments in neuroplasticity and cellular resilience are demonstrable in certain mood and anxiety disorders. Thus, in addition to a variety of neurochemical changes, many patients also have pronounced structural alterations (e.g. reduced spine density, neurite retraction, overall neuropil reductions, and/or volumetric changes on neuroimaging measures) in critical neuronal circuits. Therefore, for these disorders, optimal treatment may only be attained by providing both trophic and neurochemical support. The trophic support would enhance and maintain normal synaptic connectivity, thereby allowing the chemical signal to reinstate the optimal functioning of critical circuits necessary for normal affective functioning. It is thus noteworthy that many novel strategies currently being investigated for these disorders — including CRH antagonists, GR antagonists, glutamatergic agents, and phosphodiesterase inhibitors — can be conceptualized as 'plasticity enhancers', which would be expected to exert trophic effects in addition to their effects on specific neurochemical systems.

It is hoped that our ever-expanding knowledge of the neurobiology of anxiety and anxiety disorders will yield entirely new pharmacological approaches to the treatment of these disorders. Despite some concerns with their validity, animal models continue to provide a useful screening mechanism for the development of novel anxiolytics. There are presently a number of promising new targets for anxiety disorders, and a new generation of drugs directed at some of these may come to market in the coming years. Traditionally, the neurochemical systems targeted in the treatment of anxiety disorders have been GABA, serotonin, and norepinephrine. Continuing the development of drugs modulating these transmitters will probably yield modestly more effective and/or better-tolerated medications. However, the results achieved with more innovative targets mentioned above promise both markedly better drugs and a more sophisticated understanding of pathological anxiety. Nongenetic mechanisms of gene regulation, termed epigenetic, probably play a role in the formation of cellular memory and the modulation of neural circuitry. The interplay of transcription factors interacting with covalent DNA modifications is probably involved in modulating how previous experience may regulate subsequent behavioral responses. In conclusion, emerging results from a variety of clinical and preclinical experimental and naturalistic paradigms suggest that a reconceptualization about the pathophysiology, course and optimal long-term treatment of severe mood and anxiety disorders may be warranted. An increasing number of strategies are being investigated to develop small-molecule agents to enhance cellular plasticity; this progress holds much promise for the development of novel therapeutics for the long-term treatment of these devastating disorders.

REFERENCES

1. Kessler, R. C., McGonagle, K. A., Zhao, S. *et al.* Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. *Arch. Gen. Psych.* 51: 8–19, 1994.
2. Isometsa, E., Henriksson, M., Marttunen, M. *et al.* Mental disorders in young and middle aged men who commit suicide. *BMJ* 310: 1366–1367, 1995.
3. Chen, Y. W. and Dilsaver, S. C. Lifetime rates of suicide attempts among subjects with bipolar and unipolar disorders relative to subjects with other Axis I disorders. *Biol. Psych.* 39: 896–899, 1996.
4. Oquendo, M. A., Barrera, A., Ellis, S. P. *et al.* Instability of symptoms in recurrent major depression: a prospective study. *Am. J. Psychiatry* 61: 255–261, 2004.
5. Milak, M. S., Parsey, R. V., Keilp, J. *et al.* Neuroanatomical correlates of psychopathological components of major depressive disorder. *Arch. Gen. Psych.* 62: 397–408, 2005.
6. Smoller, J. W. and Finn, C. T. Family, twin, and adoption studies of bipolar disorder. *Am. J. Med. Genet. C Semin. Med. Genet.* 123C: 48–58, 2003.
7. Tsuang, M. T. and Faraone, S. V. The inheritance of mood disorders. In *Genetics and Mental Illness. Evolving Issues for Research and Society*. Ed. Hall L. L. New York: Plenum Press, 1996, pp 79–109.
8. McGuffin, P., Katz, R., Watkins, S. and Rutherford, J. A hospital-based twin register of the heritability of DSM-IV unipolar depression. *Arch Gen. Psych.* 53: 129–136, 1996.

9. Beardslee, W. R., Versage, E. M. and Gladstone, T. R. Children of affectively ill parents: a review of the past 10 years. *J. Am. Acad. Child Adolesc. Psychiatry* 37: 1134–1141, 1998.
10. Caspi, A., Sugden, K., Moffitt, T. E. *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science J. –Sci.* 301: 386–389, 2003.
11. Johansson, C., Jansson, M., Linner, L. *et al.* Genetics of affective disorders. *European Neuropsychopharm.* 11: 385–394, 2001.
12. Oruc, L., Verheyen, G. R., Furac, I. *et al.* Positive association between the GABRA5 gene and unipolar recurrent major depression. *Neuropsychobiol.* 36: 62–64, 1997.
13. Mann, J. J., Huang, Y., Underwood, M. D. *et al.* A serotonin transporter gene promoter polymorphism (5-HTTLPR) and prefrontal cortical binding in major depression and suicide. *Arch. Gen. Psych.* 57: 729–738, 2000.
14. Lapin, I. P. and Oxenkrug, G. F. Intensification of the central serotoninergic processes as a possible determinant of the thymoleptic effect. *Lancet* 132–136, 1969.
15. Prange, A. J., Jr, Wilson, I. C., Alltop, L. B. and Stikeleather, R. A. L-Tryptophan in mania: contributions to a permissive hypothesis of affective disorders. *Arch. Gen. Psych.* 30: 56–62, 1974.
16. Stanley, M., Traskman-Bendz, L. and Dorovini-Zis, K. Correlations between aminergic metabolites simultaneously obtained from human CSF and brain. *Life Sciences* 37: 1279–1286, 1985.
17. Mann, J. J., Malone, K. M., Sweeney, J. A. *et al.* Attempted suicide characteristics and cerebrospinal fluid amine metabolites in depressed inpatients. *Neuropsychopharm.* 15: 576–586, 1996.
18. Mann, J. J. Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. *Neuropsychopharm.* 21: 99S–105S, 1999.
19. Shiah, I. S. and Yatham, L. N. Serotonin in mania and in the mechanism of action of mood stabilizers: a review of clinical studies. *Bipolar Disord.* 2: 77–92, 2000.
20. Pandey, G. N., Pandey, S. C., Dwivedi, Y. *et al.* Platelet serotonin-2A receptors: a potential biological marker for suicidal behavior. *Am. J. Psych.* 152: 850–855, 1995.
21. McBride, P. A., Brown, R. P., DeMeo, M. *et al.* The relationship of platelet 5-HT2 receptor indices to major depressive disorder, personality traits, and suicidal behavior. *Biol. Psych.* 35: 295–308, 1994.
22. Coccaro, E. F., Siever, L. J., Klar, H. M. *et al.* Serotonergic studies in patients with affective and personality disorders. Correlates with suicidal and impulsive aggressive behavior. *Arch. Gen. Psych.* 46: 587–599, 1989.
23. Malone, K. M., Corbitt, E. M., Li, S. and Mann, J. J. Prolactin response to fenfluramine and suicide attempt lethality in major depression. *Brit. J. Psych.* 168: 324–329, 1996.
24. Fava, M., Vuolo, R. D., Wright, E. C. *et al.* Fenfluramine challenge in unipolar depression with and without anger attacks. *Psych. Res.* 94: 9–18, 2000.
25. Drevets, W. C., Frank, E., Price, J. C. *et al.* PET imaging of serotonin 1A receptor binding in depression. *Biol. Psych.* 46: 1375–1387, 1999.
26. Malison, R. T., Price, L. H., Berman, R. *et al.* Reduced brain serotonin transporter availability in major depression as measured by [123I]-2β-carbomethoxy-3β-(4-iodophenyl)-tropane and single photon emission computer tomography. *Biol. Psych.* 44: 1090–1098, 1998.
27. Stockmeier, C. A. Involvement of serotonin in depression: evidence from postmortem and imaging studies of serotonin receptors and the serotonin transporter. *J. Psychiatr. Res.* 37: 357–373, 2003.
28. Yatham, L. N., Liddle, P. F., Dennie, J. *et al.* Decrease in brain serotonin 2 receptor binding in patients with major depression following desipramine treatment: a positron emission tomography study with fluorine-18-labeled setoperone. *Arch. Gen. Psych.* 56: 705–711, 1999.
29. Mintun, M. A., Sheline, Y. I., Moerlein, S. M. *et al.* Decreased hippocampal 5–HT2A receptor binding in major depressive disorder: *in vivo* measurement with [18F]altanserin positron emission tomography. *Biol. Psych.* 55: 217–224, 2004.
30. Rosel, P., Arranz, B., San, L. *et al.* Altered 5-HT2A binding sites and second messenger inositol trisphosphate (IP3) levels in hippocampus but not in frontal cortex from depressed suicide victims. *Psych. Res. Neuroimag.* 99: 173–181, 2000.
31. Higley, J. D., Thompson, W. W., Champoux, M. *et al.* Paternal and maternal genetic and environmental contributions to cerebrospinal fluid monoamine metabolites in Rhesus monkeys (*Macaca mulatta*). *Arch. Gen. Psych.* 50: 615–623, 1993.
32. Heinz, A., Ragan, P., Jones, D. W. *et al.* Reduced central serotonin transporters in alcoholism. *Am. J. Psych.* 155: 1544–1549, 1998.
33. Schildkraut, J. J. The catecholamine hypothesis of affective disorders: a review of supportive evidence. *Am. J. Psych.* 122: 509–522, 1965.
34. Potter, W., Grossman, G. and Rudorfer, M. Noradrenergic function in depressive disorders. In *Biology of Depressive Disorder, Part A: A System Perspective.* Ed. Kupfer, D. J. New York: Plenum Press, 1993, pp 1–27.
35. Maas, J. W., Kocsis, J. H., Bowden, C. L. *et al.* Pre-treatment neurotransmitter metabolites and response to imipramine or amitriptyline treatment. *Psych. Med.* 12: 37–43, 1982.
36. Owens, M. J., Morgan, W. N., Plott, S. J. and Nemeroff, C. B. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J. Pharm. Exp. Ther.* 283: 1305–1322, 1997.
37. Dubovsky, S. L. and Ruzan, R. Mood disorders. In *The American Psychiatric Press Textbook of Psychiatry*. Ed. Talbott, J. A. Washington, DC: Am. Psych. Press, 1999, pp 479–565.
38. Siever, L. J., Trestman, R. L., Coccaro, E. F. *et al.* The growth hormone response to clonidine in acute and remitted depressed male patients. *Neuropsychopharm.* 6: 165–177, 1992.
39. Price, L. H., Charney, D. S., Rubin, A. L. and Heninger, G. R. Alpha 2-adrenergic receptor function in depression. The cortisol response to Yohimbine. *Arch. Gen. Psych.* 43: 849–858, 1986.
40. Underwood, M. D., Mann, J. J. and Arango, V. Serotonergic and noradrenergic neurobiology of alcoholic suicide. *Alc. Clin. Exp. Res.* 28: 57S–69S, 2004.
41. Heim, C. and Nemeroff, C. B. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol. Psych.* 49: 1023–1039, 2001.
42. Kapur, S. and Mann, J. J. Role of the dopaminergic system in depression. *Biol. Psych.* 32: 1–17, 1992.

43. Goodwin, F. K., Post, R. M., Dunner, D. L. and Gordon, E. K. Cerebrospinal fluid amine metabolites in affective illness: the probenecid technique. *Am. J. Psychiatry* 130: 73–79, 1973.
44. Devanand, D. P., Bowers, M. B., Jr, Hoffman, F. J., Jr. and Nelson, J. C. Elevated plasma homovanillic acid in depressed females with melancholia and psychosis. *Psych. Res.* 15: 1–4, 1985.
45. Janowsky, D. S., el Yousef, M. K., Davis, J. M. and Sekerke, H. J. A cholinergic–adrenergic hypothesis of mania and depression. *Lancet* 2: 632–635, 1972.
46. Poland, R. E., McCracken, J. T., Lutchmansingh, P. *et al.* Differential response of rapid eye movement sleep to cholinergic blockade by scopolamine in currently depressed, remitted, and normal control subjects. *Biol. Psych.* 41: 929–938, 1997.
47. Kegeles, L. S., Malone, K. M., Slifstein, M. *et al.* Response of cortical metabolic deficits to serotonergic challenge in familial mood disorders. *Am. J. Psych.* 160: 76–82, 2003.
48. Petty, F. and Schlesser, M. A. Plasma GABA in affective illness. A preliminary investigation. *J. Affect. Disord.* 3: 339–343, 1981.
49. Bowden, C. L. Novel treatments for bipolar disorder. *Expert Opin. Investig. Drugs* 10: 661–671, 2001.
50. Lopez, J. F., Chalmers, D. T., Little, K, Y. and Watson, S. J. A. E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. *Biol. Psych.* 43: 547–573, 1998.
51. Kiriike, N., Izumiya, Y., Nishiwaki, S. *et al.* TRH test and DST in schizoaffective mania, mania, and schizophrenia. *Biol. Psych.* 24: 415–422, 1988.
52. Thase, M. E. Mood disorders: neurobiology. In *Kaplan & Sadock's Comprehensive Textbook of Psych*iatry. Ed. Sadock, V. A. Philadelphia: Lippincott Williams & Wilkins, 2000, pp1318–1328.
53. Jackson, I. M. The thyroid axis and depression. *Thyroid* 8: 951–956, 1998.
54. Haggerty,J. J., Jr. ., Simon, J. S., Evans, D. L. and Nemeroff, C. B. Relationship of serum TSH concentration and antithyroid antibodies to diagnosis and DST response in psychiatric inpatients. *Am. J. Psychiatry* 144: 1491–1493, 1987.
55. Lazarus, J. H., McGregor, A. M., Ludgate, M. *et al.* Effect of lithium carbonate therapy on thyroid immune status in manic depressive patients: a prospective study. *J. Affect. Disord.* 11: 155–160, 1986.
56. Souetre, E., Salvati, E., Wehr, T. A. *et al.* Twenty-four-hour profiles of body temperature and plasma TSH in bipolar patients during depression and during remission and in normal control subjects. *Am. J. Psychiatry* 145: 1133–1137, 1988.
57. Duman, R. S., Malberg, J., Nakagawa, S. and D'Sa, C. Neuronal plasticity and survival in mood disorders. Biol. Psych. 2000; 48: 732–739
58. Figurov A, Pozzo-Miller LD, Olafsson P *et al.* Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. *Nature* 381: 706–709, 1996.
59. Duman, R. S., Malberg, J. and Thome, J. Neural plasticity to stress and antidepressant treatment. *Biol. Psych.* 46: 1181–1191, 1999.
60. Mendelson, W. B., Gillin, J. C. and Wyatt, R. D. *Human Sleep and its Disorders.* New York: Plenum Press, 1977.
61. Hawkins, D. R., Taub, J. M. and Van de Castle, R. L. Extended sleep (hypersomnia) in young depressed patients. *Am. J. Psychiatry* 142: 905–910, 1985.
62. Riemann, D., Berger, M. and Voderholzer, U. Sleep and depression—results from psychobiological studies: an overview. *Biol. Psychol.* 57: 67–103, 2001.
63. Wu, J. C. and Bunney, W. E. The biological basis of an antidepressant response to sleep deprivation and relapse: review and hypothesis. *Am. J. Psychiatry* 147: 14–21, 1990.
64. Soares, J. C. and Mann, J. J. The anatomy of mood disorders — review of structural neuroimaging studies. *Biol. Psych.* 41: 86–106, 1997.
65. Hastings, R. S., Parsey, R. V., Oquendo, M. A. *et al.* Volumetric analysis of the prefrontal cortex, amygdala, and hippocampus in major depression. *Neuropsychopharmacol* 29: 952–959, 2004.
66. Lochhead, R. A., Oquendo, M. A., Mann, J. J. and Parsey, R. V. Regional brain gray matter volume differences in bipolar disorder patients as assessed by optimized voxel-based morphometry. *Biol. Psych.* 55: 1154–1162, 2004.
67. Blumberg, H. P., Charney, D. S. and Krystal, J. H. Frontotemporal neural systems in bipolar disorder. *Semin. Clin. Neuropsychiatry* 7: 243–254, 2002.
68. Mann, J. J. and Arango, V. Abnormalities of brain structure and function in mood disorders. In *Neurobiology of Mental Illness.* Ed. Bunney, B. S. New York: Oxford University Press, 1999, pp385–393.
69. Sapolsky, R. M. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch. Gen. Psych.* 57: 925–935, 2000.
70. McEwen, B. S. Stress and hippocampal plasticity. *Annu. Rev. Neurosci.* 22: 105–122, 1999.
71. Bourne, H. R. and Nicoll, R. Molecular machines integrate coincident synaptic signals. *Cell* 72(Suppl): 65–75, 1993.
72. Bhalla, U. S. and Iyengar, R. Emergent properties of networks of biological signaling pathways. *Science* 283: 381–387, 1999.
73. Weng, G., Bhalla, U. S. and Iyengar, R. Complexity in biological signaling systems. *Science* 284: 92–96, 1999.
74. Wang, H. Y. and Friedman, E. Enhanced protein kinase C activity and translocation in bipolar affective disorder brains. *Biol. Psych.* 40: 568–575, 1996.
75. Jope, R. S. Anti-bipolar therapy: mechanism of action of lithium. *Mol. Psych.* 4: 117–128, 1999.
76. Manji, H. K. and Lenox, R. H. The nature of bipolar disorder. *J. Clin. Psych.* 61(Supp 13): 42–57, 2000.
77. Hahn, C. G. and Friedman, E. Abnormalities in protein kinase C signaling and the pathophysiology of bipolar disorder. *Bipolar Disord.* 1: 81–86, 1999.
78. Friedman, E., Hoau Yan, W., Levinson, D. *et al.* Altered platelet protein kinase C activity in bipolar affective disorder, manic episode. *Biol Psych.* 33: 520–525, 1993.
79. Manji, H. K. and Lenox, R. H. Ziskind–Somerfeld Research Award. Protein kinase C signaling in the brain: molecular transduction of mood stabilization in the treatment of manic-depressive illness. *Biol. Psych.* 46: 1328–1351, 1999.
80. Manji, H. K. and Lenox, R. H. Signaling: cellular insights into the pathophysiology of bipolar disorder. *Biol. Psych.* 48: 518–530, 2000.
81. Bebchuk, J. M., Arfken, C. L., Dolan-Manji, S. *et al.* A preliminary investigation of a protein kinase C inhibitor in the treatment of acute mania. *Arch. Gen. Psych.* 57: 95–97, 2000.

82. Chen, G., Huang, L. D., Jiang, Y. M. and Manji, H. K. The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* 72: 1327–1330, 1999.
83. Gould, T. D. and Manji, H. K. Signaling networks in the pathophysiology and treatment of mood disorders. *J. Psychosom. Res.* 53: 687–697, 2002.
84. Duman, R. S. Synaptic plasticity and mood disorders. *Mol. Psych.* 7(Suppl 1): S29–34, 2002.
85. Manji, H. K. and Chen, G. PKC, MAP kinases and the bcl-2 family of proteins as long-term targets for mood stabilizers. *Mol Psych.* 7(Suppl 1): S46–56, 2002.
86. Manji, H. K., Moore, G. J. and Chen, G. Lithium up-regulates the cytoprotective protein Bcl-2 in the CNS *in vivo*: a role for neurotrophic and neuroprotective effects in manic depressive illness. *J. Clin. Psych.* 61(Suppl 9): 82–96, 2000.
87. Zarate, C. A., Payne, J. L., Singh, J. *et al.* Pramipexole for bipolar II depression: a placebo-controlled proof of concept study. *Biol Psych.* 56: 54–60, 2004.
88. Yoon, I. S., Li, P. P., Siu, K. P., Kennedy, J. L. *et al.* Altered TRPC7 gene expression in bipolar-I disorder. *Biol. Psych.* 50: 620–626, 2001.
89. Kringlen, E., Torgersen, S. and Cramer, V. A Norwegian psychiatric epidemiological study. *Am. J. Psych.* 158: 1091–1098, 2001.
90. Stein, M. B. Attending to anxiety disorders in primary care. *J. Clin. Psych.* 64(Suppl 15): 35–39, 2003.
91. Barros, M. and Tomaz, C. Non-human primate models for investigating fear and anxiety. *Neurosci. Biobehav. Rev.* 26: 187–201, 2002.
92. Belzung, C. The genetic basis of the pharmacological effects of anxiolytics: a review based on rodent models. *Behav. Pharmacol.* 12: 451–460, 2001.
93. Clement, Y., Calatayud, F. and Belzung, C. Genetic basis of anxiety-like behaviour: a critical review. *Brain Res. Bull.* 57: 57–71, 2002.
94. Bilbo, S. D. and Nelson, R. J. Behavioral phenotyping of transgenic and knockout animals: a cautionary tale. *Lab. Anim. (NY)* 30: 24–29, 2001.
95. Charney, D. S., Heninger, G. R. and Breier, A. Noradrenergic function in panic anxiety. Effects of yohimbine in healthy subjects and patients with agoraphobia and panic disorder. *Arch. Gen. Psych.* 41: 751–763, 1984.
96. Uhde, T. W., Boulenger, J. P., Post, R. M. *et al.* Fear and anxiety: relationship to noradrenergic function *Psychopathol.* 17(Suppl 3): 8–23, 1984.
97. Woods, S. W., Koster, K., Krystal, J. K. *et al.* Yohimbine alters regional cerebral blood flow in panic disorder. *Lancet* 2: 678, 1988.
98. Gur, R. C., Gur, R. E., Resnick, S. M. *et al.* The effect of anxiety on cortical cerebral blood flow and metabolism. *J. Cereb. Blood Flow Metab.* 7: 173–137, 1987.
99. Rodriguez, G., Cogorno, P., Gris, A. *et al.* Regional cerebral blood flow and anxiety: a correlation study in neurologically normal patients. *J. Cereb. Blood Flow Metab,* 9: 410–416, 1989.
100. Milad, M. R. and Quirk, G. J. Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature* 420: 70–74, 2002.
101. Graeff, F. G., Guimaraes, F. S., De Andrade, T. G. and Deakin, J. F. Role of 5-HT in stress, anxiety, and depression. *Pharmacol. Biochem. Behav.* 54: 129–141, 1996.
102. Charney, D. S. and Drevets, W. C. The neurobiological basis of anxiety disorders. In *Neuropsychopharmacology: the Fifth Generation of Progress.* Ed. Nemeroff, C. Philadelphia: Lippincott Williams & Wilkins, 2002, pp901–930.
103. Charney, D. S. and Bremner, J. D. The neurobiology of anxiety disorders. In *Neurobiology of Mental Illness.* Ed Bunney, B. S. New York: Oxford University Press, 1999, pp494–517.
104. Mohler, H., Fritschy, J. M. and Rudolph, U. A new benzodiazepine pharmacology. *J. Pharmacol. Exp. Ther.* 300: 2–8, 2002.
105. Bremner, J. D. and Charney, D. S. Neural circuits in fear and anxiety. In *Textbook of Anxiety Disorders.* Ed. Washington, H. E. Arlington: American Psychiatric Publishing, Inc., 2002, pp43–56.
106. Ontiveros, A., Fontaine, R., Breton, G. *et al.* Correlation of severity of panic disorder and neuroanatomical changes on magnetic resonance imaging. *J. Neuropsychiatry Clin. Neurosci.* 1: 404–408, 1989.
107. Zobel, A. W., Nickel, T., Kunzel, H. E. *et al.* Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. *J. Psychiatr. Res.* 34: 171–181, 2000.
108. Arborelius, L., Owens, M. J., Plotsky, P. M. and Nemeroff, C. B. The role of corticotropin-releasing factor in depression and anxiety disorders. *J. Endocrinol.* 160: 1–12, 1999.
109. Kask, A., Harro, J., von Horsten, S. *et al.* The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. *Neurosci. Biobehav. Rev.* 26: 259–283, 2002.
110. Pande, A. C., Greiner, M., Adams, J. B. *et al.* Placebo-controlled trial of the CCK-B antagonist, CI-988, in panic disorder. *Biol. Psych.* 46: 860–862, 1999.
111. Stout, S. C., Owens, M. J. and Nemeroff, C. B/ Neurokinin(1) receptor antagonists as potential antidepressants. *Annu. Rev. Pharmacol. Toxicol.* 41: 877–906, 2001.
112. Baetz, M., Bowen, R. C. Efficacy of divalproex sodium in patients with panic disorder and mood instability who have not responded to conventional therapy. *Can. J. Psych.* 43: 73–77, 1998.
113. Clark, R. D., Canive, J. M., Calais, L. A. *et al.* Divalproex in posttraumatic stress disorder: an open-label clinical trial. *J. Trauma Stress* 12: 395–401, 1999.
114. Benjamin, J., Levine, J., Fux, M. *et al.* Double-blind, placebo-controlled, crossover trial of inositol treatment for panic disorder. *Am. J. Psych.* 152: 1084–1086, 1995.
115. Palatnik, A., Frolov, K., Fux, M., Benjamin, J. Double-blind, controlled, crossover trial of inositol versus fluvoxamine for the treatment of panic disorder. *J. Clin. Psychopharmacol.* 21: 335–339, 2001.
116. Levine, J., Barak, Y., Gonzalves, M. *et al.* Double-blind, controlled trial of inositol treatment of depression. *Am. J. Psych.* 152: 792–794, 1995.
117. Sheline, Y. I., Sanghavi, M., Mintun, M. A. and Gado, M. H. Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. *J. Neurosci.* 19: 5034–5043, 1999.
118. Strakowski, S. M., Wilson, D. R., Tohen, M. *et al.* Structural brain abnormalities in first-episode mania. *Biol Psych.* 33: 602–609, 1993.
119. Hirayasu, Y., Shenton, M. E., Salisbury, D. F. *et al.* Subgenual cingulate cortex volume in first-episode psychosis. *Am. J. Psych.* 156: 1091–1093, 1999.

120. Graham, Y. P., Heim, C., Goodman, S. H. *et al.* The effects of neonatal stress on brain development: implications for psychopathology. *Dev. Psychopathol.* 11: 545–565, 1999.
121. Francis, D., Diorio, J., Liu, D., Meaney, M. J. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* 286: 1155–1158, 1999.
122. Gross, C., Zhuang, X., Stark, K. *et al.* Serotonin 1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* 416: 396–400, 2002.

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CHAPTER

56

Addiction

Marina E. Wolf

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GENERAL PRINCIPLES

Addiction is characterized by compulsive drug use, despite severe negative consequences. It follows a chronic course, with periods of abstinence followed by relapse. Vulnerability to relapse can persist even after years of abstinence, suggesting that long-lasting and perhaps permanent neurobiological changes underlie addiction. There are tremendous individual differences in vulnerability, reflecting both genetic and environmental influences, and many people experiment with potentially addictive drugs without progressing to compulsive use. Nevertheless, substance abuse accounts for more deaths, illnesses and disabilities than any other preventable health condition. Approximately one in four of all deaths in the U.S.A. are attributable to the use of alcohol, nicotine or illicit drugs [1].

Long-term drug exposure produces many physiological and behavioral changes that contribute to addiction. *Tolerance* is the need for increasing doses of a drug to

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achieve the same effect. *Sensitization* refers to the enhancement of drug responses as a result of repeated drug exposure. For a given drug, it is possible for certain effects to show tolerance and others to show sensitization. *Dependence* is an adapted physiological state of cells or systems that develops to compensate for excessive stimulation by a drug. When drug intake stops, unmasking of this adapted state leads to a *withdrawal syndrome* that may have somatic (physical) components as well as affective and motivational components.

Many forces may drive compulsive drug use. The incentive-sensitization theory of addiction proposes that sensitization occurs in the neural systems that attribute incentive salience to drugs and drug-associated cues [2]. Drug 'wanting' sensitizes, even though drug 'liking' typically shows tolerance. Alternatively, addiction has been characterized as a spiraling cycle of hedonic dysregulation driven by many factors but with an important role ascribed to negative affective states that occur during periods of drug abstinence [3]. Other theories emphasize the role of learning in addiction, based on the ability of drugs to facilitate some forms of learning and trigger neuroadaptations also seen in learning. Abnormally strong forms of learning are proposed to underlie habitual behaviors associated with addiction [4–6]. Finally, drugs may produce dysregulation of cortical systems that normally exert inhibitory control over behavior, leading to poor decision-making and impulsivity, which in turn drive compulsive pursuit of drugs [7]. These theories are not mutually exclusive. Different factors may contribute at different times in the cycle of addiction.

NEURONAL CIRCUITRY OF ADDICTION

Natural reinforcers and drugs of abuse use similar circuits. Drugs of abuse — like food, drink and sex — are reinforcing. That is, they 'stamp in' or 'reinforce' learned associations, such that behaviors associated with obtaining the reinforcer tend to be repeated. Different drug classes have different initial targets in brain (Fig. 56-1). However, a common mechanism underlying the reinforcing actions of most addictive drugs — and those of natural reinforcers — is activation of mesocorticolimbic dopamine neurons (Ch. 12). These neurons originate in the ventral tegmental area (VTA) of the midbrain and project to cortical and limbic target regions (Fig. 56-2). Elevation of dopamine levels in one of these target regions, the nucleus accumbens, is particularly important for reinforcement. The nucleus accumbens serves as an interface between limbic and cortical regions important for motivation, and motor circuits responsible for execution of motivated behaviors. Dopamine neurons in the substantia nigra, which project primarily to dorsal striatum, are also important, particularly in the learning and performance of habitual behaviors associated with addiction.

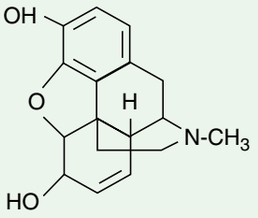
The exact role of dopamine in reinforcement remains an active area of research [8–10]. According to the incentive-sensitization theory of addiction (above), dopamine signals the incentive salience attributed to drugs and drug associated cues, causing them to be 'wanted' [2]. Another influential theory holds that activation of dopamine neurons is important in learning and predicting the likelihood of reward when an animal is presented with reward-related stimuli [11]. Thus, a normal function of dopamine may be to enable reward-related stimuli to shape behavior, and so promote the learning of adaptive behaviors that enable survival.

While dopamine neurons are activated to a modest degree by natural rewards, drugs of abuse produce a much stronger elevation in dopamine levels that may not be subject to normal regulatory mechanisms. For example, dopamine released by natural rewards will be removed from the synapse by the dopamine transporter. However, cocaine works by blocking this transporter, so cocaine produces larger and more prolonged increases in dopamine levels. Other drugs activate dopamine neurons through indirect mechanisms depicted in Figure 56-3. The unregulated release of dopamine initiates compensatory changes in dopamine-receptive neurons that lead to a chain reaction of molecular and cellular adaptations throughout the circuits depicted in Figure 56-2. A major challenge is to understand the relationship between these adaptations and the behavioral changes that characterize addiction. One reason for the difficulty is the sheer number of adaptations that have been described, encompassing signal transduction, gene expression and structural changes. Further complicating the picture, some adaptations reduce drug responsiveness, favoring homeostasis, while others increase drug responsiveness in a feed-forward manner. The molecular and cellular adaptations that underlie addiction are discussed in more detail in subsequent sections of this chapter.

The neuronal circuitry underlying drug craving has been intensely studied to develop strategies for preventing relapse. Drug craving in human addicts, and the reinstatement of drug-seeking behavior in animal models of relapse, can be triggered by three types of stimuli: cues associated with prior drug use (such as drug paraphernalia), re-exposure to a low dose of drug and exposure to stress. Animal studies have shown that these stimuli trigger reinstatement of drug-seeking through distinct but overlapping neuronal circuits [12]. For example, the basolateral amygdala is critical for the ability of conditioned cues to maintain and reinstate drug-seeking behavior [13, 14]. Cocaine-primed reinstatement requires activation of glutamate projections from the prefrontal cortex to the nucleus accumbens [12]. Stress-induced reinstatement involves brain corticotrophin-releasing factor (CRF) and norepinephrine systems [15]. Stress also contributes to other aspects of addiction. For example, stress increases the rate at which rats will learn to

A. Opiates

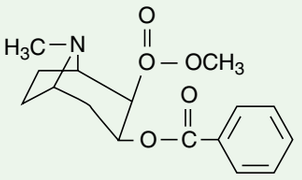
Morphine



Primary target: μ opioid receptor

B. Psychostimulants

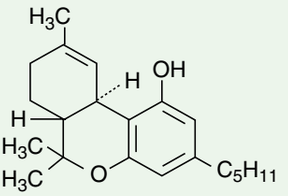
Cocaine



Primary target:
Monoamine transporters

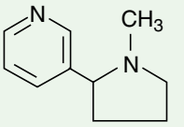
C. Cannabinoids

Δ^9-tetrahydrocannabinol



Primary target:
Cannabinoid CB1 receptor

D. Nicotine



Primary target:
Nicotinic acetylcholine receptor

E. Alcohol

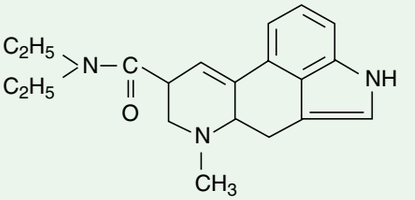
Ethanol

$H_3C - CH_2 - OH$

Primary target:
Ligand gated and voltage gated ion channels

F. Hallucinogens

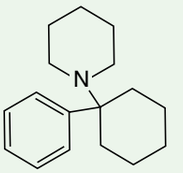
Lysergic acid diethylamine



Primary target:
Serotonin receptors

G. Dissociative Drugs

Phencyclidine



Primary target:
NMDA receptor

FIGURE 56-1 Major drug classes, structures of prototypical agonists and 'primary targets' implicated in drug class reward. See Figure 56-4 for structures of amphetamines.

self-administer addictive drugs [16]. After chronic administration of many drugs of abuse, including heroin, cocaine and ethanol, there are profound adaptations in brain, hypothalamic and pituitary stress systems. These adaptations contribute importantly to dependence and withdrawal [17].

Functional imaging studies (Ch. 58) in human addicts have found that cue-elicited craving is associated with activation of the amygdala, the anterior cingulate cortex, the orbitofrontal cortex, and the dorsolateral prefrontal cortex [18]. Activation of other regions has been reported less consistently. The amygdala is critical for associative

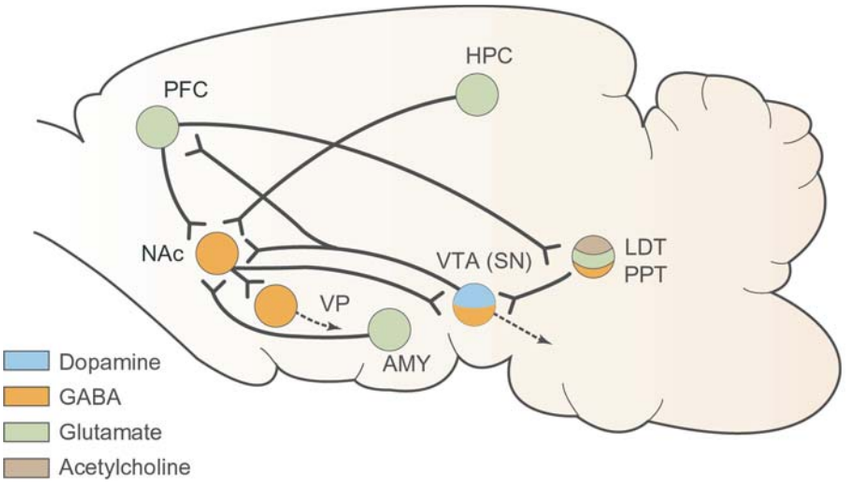


FIGURE 56-2 The mesocorticolimbic dopamine system and associated circuits. The ventral tegmental area (*VTA*) contains both dopamine and GABA neurons that innervate the nucleus accumbens (*NAc*), prefrontal cortex (*PFC*) and other forebrain targets not shown. Nucleus accumbens neurons, which use GABA as their transmitter, receive glutamate inputs from the PFC, amygdala (*AMY*), hippocampus (*HPC*), and thalamus (not shown). These glutamate inputs convey information important for goal-directed behaviors. NAc neurons integrate this information, and then transmit it to brain regions important for execution of these behaviors, including the ventral pallidum (*VP*), VTA and substantia nigra (*SN*), as well as other motor regions (*dashed arrows*). The prefrontal cortex influences this circuitry at many levels by sending descending glutamate projections to many targets, including the NAc, VTA, SN, pedunculopontine tegmental nucleus (*PPT*) and laterodorsal tegmental nucleus (*LDT*). The PPT and LDT send mixed cholinergic, glutamate and GABA projections to the VTA and SN, that exert an important regulatory influence on dopamine and GABA cells in the latter regions.

learning related to reward, while the three cortical areas are important for decision-making based on integration of cognitive and motivationally relevant information. Disruption of these circuits in addicts may be associated with alterations in dopamine transmission [10].

Overall, the results of functional imaging studies are consistent with results obtained using animal models, and both underscore the importance of conditioned responses and cognitive dysfunction in drug craving. Compulsive drug use may reflect a combination of an increase in motivation to take a drug, driven by enhanced responsiveness of memory and motivational circuits to drugs or drug-associated cues, and a decrease in the ability to exert inhibitory control and exercise appropriate judgment, due to alterations in executive function.

OPIATES

Opiates are drugs derived from opium, including morphine and heroin. Opium, extracted from poppy plants, has been used for recreational and medicinal purposes for thousands of years. Morphine was identified as the active pharmacological ingredient of opium in the early 1800s. Heroin was synthesized from morphine in the late 1800s in an attempt to develop a nonaddicting cough suppressant. Opioid is a more inclusive term that includes opiates, as well as endogenous, or naturally occurring, opioid peptides. These include the enkephalins, endorphins, dynorphins and endomorphins (see Ch. 18).

Acutely, opiates produce analgesia, autonomic inhibition and an intense 'high'. Prolonged use leads to tolerance and dependence, and to craving and withdrawal symptoms when drug use is terminated. Withdrawal symptoms include anxiety, nausea, insomnia, hot and cold flashes, muscle aches, perspiration, and diarrhea. Opiate actions in spinal cord and brain stem are important for analgesic effects, autonomic effects, and physical withdrawal symptoms. Opiate effects on the mesocorticolimbic system are important for acute rewarding effects, some psychological withdrawal symptoms, and craving.

There are three classical opioid receptor types, μ, δ, and κ. The μ-opioid receptor is responsible for the reinforcing effects of heroin and morphine. Morphine is relatively selective for μ receptors. Endorphins and enkephalins bind to μ and δ receptors, while dynorphin binds selectively to κ-opioid receptors. Endomorphins are another class of potential endogenous opioid peptide with high affinity and selectivity for μ receptors. The N/OFQ receptor, cloned in 1994, has high structural homology with classical opioid receptors, but very low affinity for conventional opioid ligands. Orphanin (nociceptin) is the endogenous ligand for the N/OFQ receptor, and may modulate opioid effects.

Opioid receptors generally mediate neuronal inhibition. They couple to G_i or G_o, and produce inhibition of Ca^{2+} channels and opening of K^+ channels. They also inhibit adenylyl cyclase. Through this and other downstream signaling pathways, opioid receptors modulate

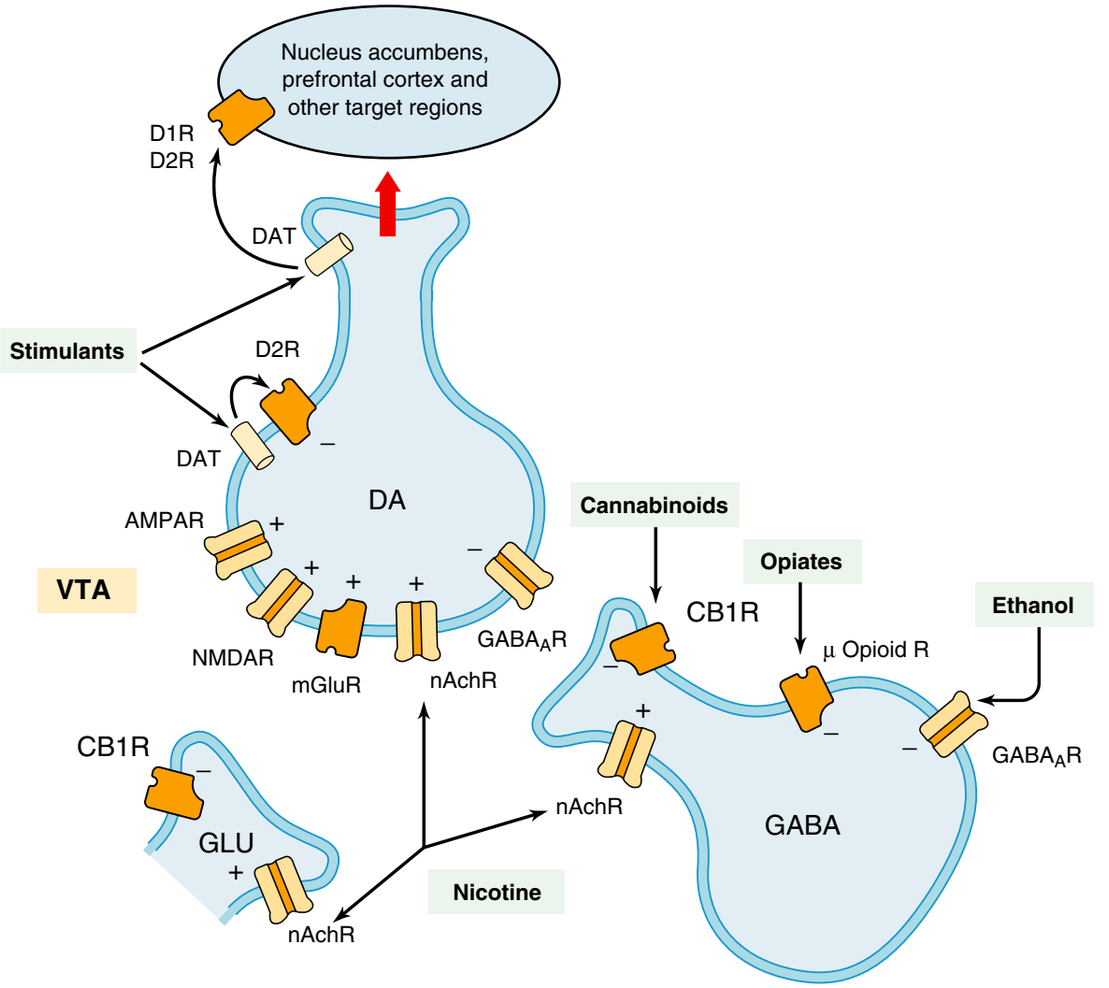


FIGURE 56-3 All reinforcing drugs increase dopamine transmission in the mesocorticolimbic dopamine system, but they use different mechanisms. Opiates, ethanol and cannabinoids decrease GABA transmission in the ventral tegmental area (*VTA*), thereby disinhibiting dopamine neurons. Psychomotor stimulants interact with the DA transporter (*DAT*) to elevate extracellular dopamine levels. Nicotine excites dopamine cells directly and promotes glutamate release from glutamate nerve terminals in the VTA.

synaptic plasticity and gene expression [19]. Recent work has shown that opioid receptors interact to form homodimers and hetero-dimers. This generates signaling units with different pharmacology and signaling ability compared to the cloned receptor subtypes [20]. The significance of this finding is only beginning to be explored, but it may contribute to the pharmacological diversity of opioid receptors, and has exciting implications for drug design.

The reinforcing effects of opiate drugs involve a number of neuronal pathways [21]. In the VTA, opiates stimulate μ-opioid receptors on GABA neurons that synapse on dopamine neurons. This inhibits the GABA neurons, leading to disinhibition of the dopamine neurons and enhanced dopamine release in the nucleus accumbens and other target areas (Fig. 56-3). Opiates also exert dopamine-independent effects in the nucleus accumbens by activating μ-opioid receptors on nucleus accumbens neurons themselves. The relative contribution of these two mechanisms to the reinforcing effects of opiates remains unclear. There is increasing evidence that endogenous cannabinoid systems are also important in mediating the rewarding effects of opiates, and vice versa.

Chronic opiate treatment results in complex adaptations in opioid receptor signaling. Much has been learned from studies on mechanisms of tolerance to the analgesic effects of opiates. This is a major clinical problem, as it means that ever-escalating doses are required for the treatment of chronic pain. The classic view was that tolerance reflects a decrease in functional opioid receptors via desensitization and internalization. Desensitization occurs when receptors are uncoupled from G proteins as a result of phosphorylation by G-protein-coupled receptor

kinases (GRKs) and arrestin binding (Ch. 19). These events also lead to internalization of opioid receptors by promoting their association with clathrin-coated pits. However, the paradoxical observation that morphine produces tolerance but does not promote efficient receptor internalization has led to a re-evaluation of this view and prompted exciting research on opioid receptor regulation [22]. Although much remains to be learned, one possibility is that lack of internalization promotes tolerance because it is associated with abnormally sustained signaling and resultant adaptations in the target cell and downstream circuits. A key element of this theory is that different agonists direct opioid receptors towards different signaling and trafficking pathways. Understanding the rules governing the function of ligand–receptor complexes may help in the development of opiates that are effective analgesics but have reduced liability to cause tolerance and dependence.

Upregulation of the cAMP second-messenger pathway is a well-established molecular adaptation. It occurs in many brain regions after chronic administration of opiates and other drugs of abuse. The locus coeruleus (LC) has been a useful model system for studying upregulation of the cAMP pathway [23]. Located in the brain stem, the LC is the largest cluster of norepinephrine-containing neurons in the brain and normally participates in regulation of autonomic function and attentional states. Acutely, opiates suppress the activity of LC neurons via G_i-coupled receptors that inhibit adenylyl cyclase activity (Chs 19 and 21). Chronic opiate administration leads to compensatory upregulation of specific subtypes of adenylyl cyclase and specific subunits of protein kinase A. The transcription factor CREB plays a critical role in this adaptive response. Upregulation of the cAMP pathway increases the excitability of LC neurons, enabling them to fire at normal rates despite the continued presence of opiates. When opiates are withdrawn, upregulation of the cAMP pathway is no longer opposed by inhibitory effects of opiates, leading to a dramatic rebound activation of LC neurons that contributes to somatic withdrawal symptoms. Of course, upregulation of the cAMP pathway is only one of many cellular mechanisms contributing to adaptive changes in the LC and other regions after chronic opiate administration [19].

The nucleus accumbens is another brain region in which the cAMP pathway is upregulated by chronic administration of opiates. This leads to increased transcription of CREB-regulated proteins, including dynorphin, the endogenous ligand for the κ-opioid receptor. Dynorphin stimulates presynaptic κ-opioid receptors on dopamine nerve terminals, inhibiting dopamine release. In the presence of opiates, this effect of dynorphin may serve as a homeostatic adaptation that diminishes drug responsiveness by reducing activation of the dopaminergic system. However, once opiates are no longer present, decreased dopamine release may contribute to the anhedonia and dysphoria that characterize the early phase of opiate withdrawal.

There are two main treatments for the opiate withdrawal syndrome. One is replacement therapy with methadone or other μ agonists that have a longer half-life than heroin or morphine, and produce mild stimulation rather than euphoria. They also produce cross-tolerance to heroin, lessening heroin's effect if patients relapse. Withdrawal is also treated with the α_2 agonist clonidine, which inhibits LC neurons, thus counteracting autonomic effects of opiate withdrawal — such as nausea, vomiting, cramps, sweating, tachycardia and hypertension — that are due in part to loss of opiate inhibition of LC neurons.

Endogenous opioid systems are an integral part of the reward circuitry. As such, they contribute to adaptations that underlie addiction to many drug classes. For example, interactions of endorphins and enkephalins with μ and δ receptors contribute to reinforcing effects of cocaine and ethanol through mechanisms involving activation of mesolimbic dopamine transmission. Dynorphin is upregulated by other drugs of abuse, e.g. cocaine, and its interaction with κ-opioid receptors produces aversive states that oppose reinforcement by decreasing mesolimbic dopamine transmission (see above). Naltrexone, a nonspecific opioid receptor antagonist, is approved in the U.S.A. for the treatment of individuals with alcohol dependence. It appears to block some of the reinforcing properties of alcohol, and reduces rates of relapse to alcohol drinking.

PSYCHOMOTOR STIMULANTS

This drug class includes cocaine and amphetamine derivatives. Low-to-moderate doses lead to increased activity, talkativeness and feelings of euphoria and general well-being, along with decreases in fatigue and in food intake. Repetitive motor activity (stereotyped behavior) is produced by higher doses, and very high doses can produce convulsions, hyperthermia, coma and death. Stimulants have some therapeutic uses. For example, amphetamine is used to treat narcolepsy, and methylphenidate (Ritalin) is used in the treatment of children with attention deficit hyperactivity disorder. Because repeated administration of stimulants can produce sensitization of their reinforcing effects, there is concern that long-term childhood exposure to methylphenidate may increase vulnerability to drugs of abuse later in life. However, some studies suggest that methylphenidate may have protective effects [24].

Transporters for dopamine (DAT), serotonin (SERT) and norepinephrine (NET) are the initial targets for psychomotor stimulants. By interacting with these transporters (Chs 12 and 13), psychomotor stimulants increase extracellular levels of monoamine neurotransmitters. Cocaine is a monoamine uptake inhibitor. The reinforcing effects of cocaine correlate best with its binding potency at the DAT. However, experiments with monoamine transporter-deficient mice suggest that cocaine actions at

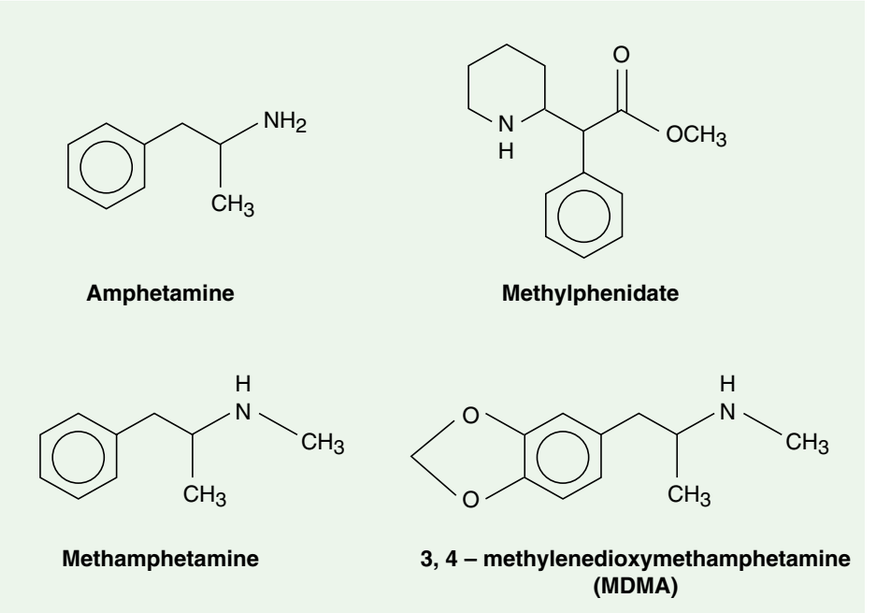


FIGURE 56-4 Amphetamine and other important stimulants.

the SERT and NET are also important for its reinforcing effects [25]. Amphetamine and other important stimulants are shown in Figure 56-4. These drugs interact with DAT, SERT and NET with varying relative affinities. Some, like methylphenidate, appear to block uptake in a manner similar to cocaine, while others, including amphetamine itself, are transported substrates. Through the latter mechanism they promote monoamine efflux by reverse transport via a process known as exchange diffusion [26]. The amphetamines are also lipophilic weak bases and likely transported substrates of the synaptic vesicle monoamine transporters (VMAT). Once inside the vesicles, they collapse the vesicular pH gradient that drives dopamine uptake into synaptic vesicles (Chs 5, 12). This promotes redistribution of dopamine from vesicles to the cytosol, making more dopamine available for reverse transport [26]. In addition, amphetamine elevates cytosolic monoamine levels by inhibiting monoamine oxidase and stimulating tyrosine hydroxylase. Recent studies suggest that amphetamine and cocaine can also cause DAT internalization, a novel mechanism for reducing DAT function.

In experimental animals, repeated exposure to high doses of some psychomotor stimulants produces long-term decreases in markers for the integrity of dopamine and serotonin nerve terminals [27]. This involves a neurodegenerative process mediated by free radicals and oxidative stress [28]. In rats, high-dose amphetamine preferentially damages dopamine terminals, methamphetamine damages serotonin and dopamine terminals, and 3,4-methylenedioxymethamphetamine (MDMA) preferentially damages serotonin neurons. MDMA is the major constituent of the street drug ecstasy. The important and controversial questions are whether similar neurotoxic changes occur in the human brain at the lower doses used by humans, whether these doses produce functional impairment (detected in behavioral or brain imaging studies), whether functional changes are attributable to neurotoxicity versus other mechanisms, and whether any observed changes are reversible. The answers depend on the stimulant in question. There is emerging evidence that methamphetamine has long-term effects on motor, cognitive and motivational function that are associated with reductions in DAT levels and brain metabolism; some effects recover and others persist, even after 12–17 months of abstinence [29]. In MDMA users, there is some evidence for mild impairments of memory and cognition [30], but no strong evidence linking these impairments to neurotoxicity.

Cocaine and amphetamines produce neuronal adaptations by repeatedly elevating monoamine levels. The resulting overstimulation of monoamine receptors, located on target neurons postsynaptic to monoamine nerve terminals, leads to a complex cascade of downstream changes that involves many brain regions and transmitter systems. Many adaptations have been characterized that are associated with behavioral sensitization, an animal model of addiction [2]. During repeated drug administration, behavioral responses to psychomotor stimulants gradually intensify or 'sensitize', including responses that provide an index of motivation for drug-taking. For example, a sensitized rat will work harder to obtain drugs in a self-administration experiment [31]. Sensitization persists for months after discontinuing drug exposure, reminiscent of the persistence of vulnerability to relapse in recovering addicts. Although sensitization is

best characterized for psychomotor stimulants, most drugs of abuse produce sensitization and cross-sensitization occurs across drug classes.

The dopamine system itself undergoes complex adaptations during behavioral sensitization to psychomotor stimulants [32, 33]. During the first few days after discontinuing drug administration, the firing rate of dopamine neurons increases due to transient changes in regulatory mechanisms in the VTA, including the development of LTP at excitatory synapses onto dopamine neurons. Adaptations in the VTA dissipate with longer withdrawal times, giving way to more persistent adaptations in the nucleus accumbens and other forebrain regions, some of which increase the responsiveness of the dopamine system to subsequent drug exposure. For example, there are alterations in Ca^{2+} signaling within dopamine nerve terminals that allow more dopamine to be released by depolarizing stimuli and psychomotor stimulants. There are also alterations in postsynaptic dopamine receptor signaling (see next section).

Stimulant-induced changes in glutamate transmission have received considerable attention in recent years [32, 33]. In part, this is due to glutamate's central role in plasticity and the recognition that addiction is a form of plasticity, a topic considered in the last section of this chapter. However, the focus on glutamate also reflects the fact that postsynaptic neurons in most dopamine-innervated brain regions are co-regulated by convergent dopamine and glutamate inputs, with dopamine serving a neuromodulatory role. Glutamate transmission in the nucleus accumbens is the critical mediator of cocaine-seeking behavior in rats [12].

Dopamine receptor signaling through the PKA pathway mediates many effects of psychomotor stimulants. Dopamine receptors can be divided into two major families, based on pharmacology and signal transduction mechanisms, the D1-like receptors (D1 and D5) and the D2-like receptors (D2, D3 and D4) (Ch. 12). This section will focus on D1 and D2 receptors. D1 receptors are positively coupled to adenylyl cyclase, while D2 receptors are negatively coupled to adenylyl cyclase. In addition, both receptors influence other signaling mechanisms. Both serve as postsynaptic receptors in the nucleus accumbens, dorsal striatum, amygdala, prefrontal cortex and other dopamine innervated regions. D2 receptors also serve as presynaptic autoreceptors. Both D1 and D2 receptors mediate psychomotor stimulant actions, but D1 receptor signaling through the cAMP-dependent protein kinase (PKA) pathway may be more important for persistent drug effects (Fig. 56-5).

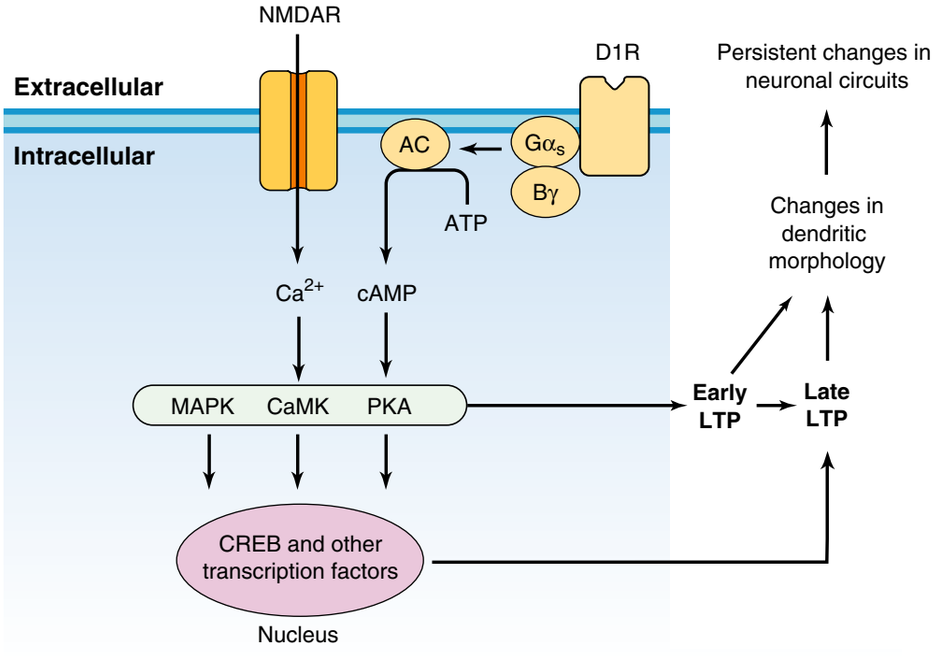


FIGURE 56-5 The D1 receptor–PKA signaling pathway influences neuronal excitability by regulating ion channels and receptors in the membrane (see text) and influences gene expression by activating transcription factors such as CREB. Note that some effects of this pathway are mediated indirectly by DARPP-32, a potent inhibitor of protein phosphatase-1 that is involved in many aspects of addiction (see Ch. 23). There is considerable cross-talk between the D1 receptor–PKA signaling pathway and other receptors located in dopamine-receptive neurons, for example, NMDA, AMPA, mGluR, D2 dopamine, serotonin, adenosine, opiate and $GABA_A$ receptors. The NMDA receptor plays an important role in activating Ca^{2+}-dependent signaling pathways. Convergent activation of cAMP and Ca^{2+} signaling pathways is necessary for some responses, e.g. CREB activation. The same cascades are critical for activity-dependent forms of plasticity such as LTP. During the early component of LTP, which requires activation of several protein kinases, synaptic strength is increased by mechanisms that include the synaptic insertion of new glutamate receptors. This is followed by a later more persistent component of LTP that requires protein synthesis. Over time, morphological changes occur in the postsynaptic density, perhaps related to the insertion of new glutamate receptors. Ultimately, dendritic spines and even presynaptic terminals undergo complex remodeling, leading to persistent changes in the activity of neuronal circuits. The ability of addictive drugs to influence the same signaling pathways that mediate LTP may explain their ability to produce persistent structural and functional changes in neuronal pathways related to motivation and reward (see Fig. 56-7).

Through this pathway, dopamine regulates the phosphorylation state of many important proteins that influence neuronal excitability on different time scales. For example, D1 receptors can exert rapid effects on neuronal excitability via PKA phosphorylation of ligand- and voltage-gated ion channels, including NMDA receptors, AMPA receptors, Na^+ channels and Ca^{2+} channels. D1 receptors exert longer lasting effects by regulating transcription factors such as CREB (Ch. 26).

Repeated cocaine administration, like repeated morphine administration, leads to upregulation of the cAMP–CREB pathway in the nucleus accumbens [23]. The resulting changes in gene expression and cellular biochemistry have yet to be fully characterized, but one consequence is CREB induction of dynorphin, which serves a homeostatic function by decreasing dopamine release (see Opiates above). It is important to keep in mind that repeated stimulant exposure produces adaptations in dopamine receptive neurons in addition to those related to D1 receptor–PKA signaling, and that convergent activation of PKA and Ca^{2+} signaling may be necessary for the recruitment of mechanisms that enable synaptic and structural plasticity (Fig. 56-5). Finally, although this section has focused on the role of dopamine receptors, serotonin and norepinephrine receptors are also important mediators of stimulant effects in both naïve and drug-experienced animals.

CANNABINOIDS (MARIJUANA)

Marijuana and hashish are derivatives of the cannabis sativa plant. Although cannabinoids have been used for centuries for recreational and therapeutic purposes, dramatic advances in cannabinoid neurobiology have occurred since 1990 [34–37]. This is attributable to the cloning of cannabinoid receptors and the discovery of endogenous cannabinoids, termed endocannabinoids.

Marijuana's major effect in humans is a mildly euphoric and relaxing 'high'. Other effects include increased appetite, and apparently reversible cognitive impairments related to attention and memory. Less common effects are anxiety, paranoia and panic. The psychoactive component of cannabis is Δ^9-tetrahydrocannabinol (THC). Tolerance develops to most effects of marijuana, but it disappears rapidly. Very few people seek treatment for marijuana addiction, and withdrawal symptoms are rare. However, a mild withdrawal syndrome has been observed in users who suddenly stop after heavy, daily marijuana use and in experimental animals treated chronically with THC and then administered a cannabinoid receptor antagonist (precipitated withdrawal). Cannabinoid withdrawal, like opioid withdrawal, is associated with upregulation of the cAMP pathway. But this occurs mainly in the cerebellum, accounting in part for much milder symptoms. Cannabinoids also have therapeutic potential. Marijuana is used for the treatment of nausea produced by cancer chemotherapy, and wasting syndrome caused by AIDS. Cannabinoid agonists and antagonists are being considered for use in a number of conditions, including movement disorders, eating disorders, and even for drug and alcohol addiction (see below).

Cannabinoid effects in the CNS are mediated by the CB1 receptor. This G_i/G_o-protein-coupled receptor was cloned in 1990. Its counterpart in the periphery, CB2, was cloned three years later. The brain may contain other undiscovered cannabinoid receptors. CB1 is by far the most abundant G-protein-coupled receptor in the mammalian brain. It is highly expressed in the basal ganglia, cerebellum, hippocampus, cortex and brain stem. Accordingly, cannabinoids modulate motor activity, motivation, learning, memory, and pain processing. Many CB1 receptors are located presynaptically on GABA and glutamate nerve terminals, where they decrease GABA or glutamate release by inhibiting Ca^{2+} currents and altering K^+ channel gating. Presynaptic CB1 receptors inhibit the release of other transmitters as well. CB1 receptors are also coupled to inhibition of adenylyl cyclase and activation of the MAP kinase/ERK pathway, as well as other protein kinase signaling cascades that regulate gene expression (see Part 3, Intracellular Signaling). Like other GPCRs, CB1 receptors undergo agonist-induced desensitization involving G-protein uncoupling in response to phosphorylation by GRKs and receptor internalization.

Cannabinoids share with other drugs of abuse the ability to increase the firing rate of VTA dopamine neurons and increase dopamine release in the nucleus accumbens. One mechanism involves stimulation of presynaptic CB1 receptors on GABA nerve terminals in the VTA, leading to decreased GABA release and disinhibition of dopamine neurons (Fig. 56-3). CB1 receptors in other locations, including the nucleus accumbens, may also contribute to reinforcing effects of cannabinoids. Endogenous opioid systems play a role in cannabinoid effects on dopamine release and, more generally, in the reinforcing effects of cannabinoids, as opioid antagonists block cannabinoid self-administration in animals.

Endocannabinoids are endogenous ligands for the CB1 receptor. The best established are anandamide (*N*-arachidonoylethanolamine) and 2-AG (2-arachidonoylglycerol). Others may also exist. Pathways involved in the formation and inactivation of anandamide and 2-AG are shown in Figure 56-6. Some steps in their formation are Ca^{2+}-dependent. This explains the ability of neuronal depolarization, which increases postsynaptic intracellular Ca^{2+} levels, to stimulate endocannabinoid formation and release. Some neurotransmitter receptors (e.g. the D2 dopamine receptor) also stimulate endocannabinoid formation, probably by modulating postsynaptic Ca^{2+} levels or signaling pathways (e.g. PLC) that regulate endocannabinoid formation.

Endocannabinoids are derived from lipids, making them different from classical and peptide transmitters in several important respects. The latter are stored in vesicles after their synthesis and released by exocytosis in response

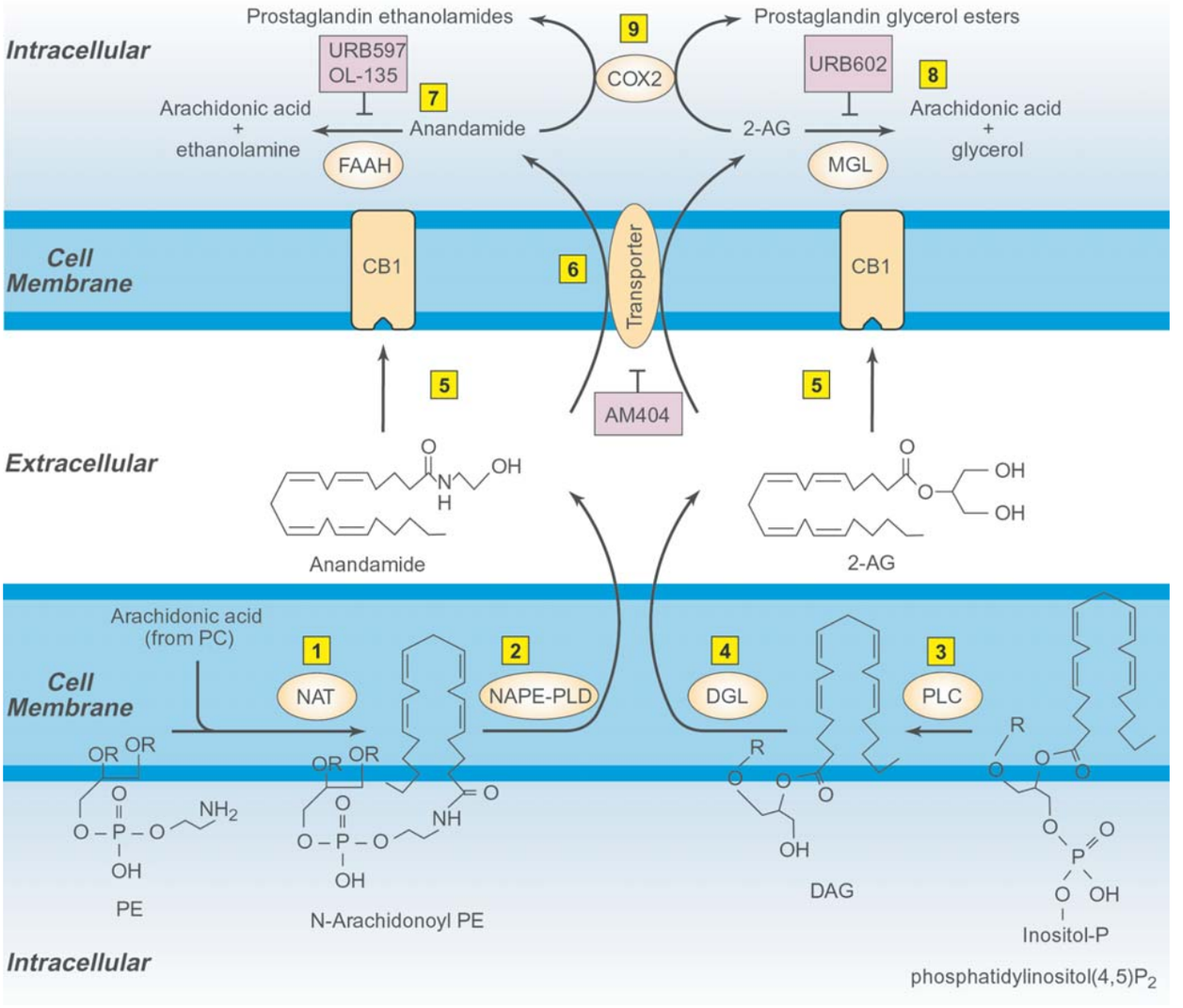


FIGURE 56-6 Formation and inactivation of the endocannabinoids anandamide and 2-AG. (**1**) *N*-arachidonoyl phosphatidylethanolamine (*N*-arachidonoyl PE), required for synthesis of anandamide, may be formed by *N*-acyl transferase (*NAT*), which transfers an arachidonate moiety, derived from the sn-1 position of phospholipids such as phosphatidylcholine (*PC*), to the primary amino group of PE. (**2**) Anandamide is generated from the hydrolysis of *N*-arachidonoyl PE, catalyzed by *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (*NAPE-PLD*). (**3**) Phospholipase C (*PLC*) catalyzes the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) to diacylglycerol (*DAG*) and inositol (1,4,5)-triphosphate. (**4**) DAG lipase (*DGL*) catalyzes the formation of 2-AG from DAG. An alternative pathway for 2-AG formation involves the formation of a 2-arachidonoyl-lysophospholipid such as lyso-PI (catalyzed by phospholipase A1) followed by its hydrolysis to 2-AG (catalyzed by lyso-PLC) (see Piomelli [36]). (**5**) Anandamide and 2-AG are agonists at the CB1 receptor. (**6**) Anandamide and 2-AG are removed from the extracellular space by carrier-mediated transport, which is inhibited by AM404. (**7**) Anandamide is hydrolyzed by a membrane-bound fatty acid amidohydrolase (*FAAH*), which is inhibited by URB597 and OL-135. (**8**) 2-AG is hydrolyzed by monoacylglycerol lipase (*MGL*). MGL is inhibited by URB602. (**9**) Anandamide and 2-AG are converted to prostaglandin ethanolamides and glycerol esters, respectively, by cyclooxygenase-2 (COX-2). Abbreviations: *AA*, arachidonic acid; *R*, fatty acid group.

to action potential invasion of the nerve terminal. In contrast, endocannabinoids are produced 'on demand' when neuronal activity or occupation of membrane receptors leads to cleavage of membrane lipid precursors. Cannabinoid release is poorly understood, but it is not vesicular. Their hydrophobic nature raises questions about how they cross the extracellular space. It is possible that this is facilitated by extracellular lipid-binding 'carrier' proteins.

Endocannabinoids serve as retrograde messengers. They are released by postsynaptic neurons and act on presynaptic CB1 receptors on neighboring nerve terminals. Retrograde signaling by endocannabinoids is essential for many forms of synaptic plasticity that are initiated by postsynaptic depolarization and increased postsynaptic intracellular Ca^{2+}, but expressed as a presynaptic decrease in the probability of transmitter release. Examples include some forms of long-term depression (LTD; see Ch. 53) at GABA

synapses in the hippocampus and the amygdala, and at glutamate synapses in the striatum and nucleus accumbens. Marijuana may alter normal endocannabinoid-mediated synaptic effects, perhaps leading to abnormal synaptic plasticity. This may be related to the short-term disruption of memory and learning associated with marijuana use, and to motivational and rewarding effects of marijuana.

There are many similarities between endogenous opioid and cannabinoid systems. Both CB1 and μ-opioid receptors are G_i/G_o-coupled receptors that share some signaling mechanisms and cellular effects, such as presynaptic inhibition. Both opioids and cannabinoids produce analgesic and rewarding effects. Finally, both systems are integral components of the reward circuitry and thus participate in responses to other drug classes. For example, in animals, blockade of endocannabinoid transmission attenuates reinstatement of cocaine- and heroin-seeking behavior and decreases motivation for alcohol consumption. It is likely that the mechanism involves CB1 receptor-mediated modulation of synaptic transmission and synaptic plasticity in reward-related brain regions such as the VTA, nucleus accumbens, dorsal striatum and amygdala. It is possible that drugs targeting endocannabinoid transmission may be useful in treating some aspects of addiction.

NICOTINE

Nicotine is responsible for the highly addictive properties of tobacco products. Addiction occurs in 30% of those who experiment with tobacco products, and more than 80% of those who attempt to quit smoking will relapse within a year. Withdrawal from nicotine produces a syndrome characterized by nicotine craving as well as dysphoria, anxiety, irritability, restlessness and increased appetite. It is treated with nicotine replacement therapies, such as nicotine gum and patches, and/or with buproprion, a drug that is classified as an antidepressant but has multiple and complex effects in brain. Buproprion reduces craving in some smokers. Nicotine addiction has been reviewed recently at cellular and systems levels [38–41].

Nicotine is an agonist at the nicotinic acetylcholine receptor (nAChR). Activation of this receptor depolarizes target cells (see Ch. 11). nAChRs are composed of five subunits surrounding a central ion-channel pore. Twelve different nicotinic receptor subunits are expressed in the nervous system (α2–α10 and β2–β4). Of these, a subset is expressed in the VTA (α3–α7 and β2–β4). It is thought that α7 receptors form homomeric receptors; α3, α4 and α6 form heteromeric channels with β2 or β4; and α5 and β3 can associate with other α/β pairs. Studies in knockout mice implicate several subunits in the ability of nicotine to modulate dopamine neurons (α4, α6, α7, β2, β3) but suggest that β2-containing receptors play a critical role in reinforcement, as β2-subunit knockout mice do not self-administer nicotine.

nAChRs exist in three basic conformational states: closed, open and desensitized. There are both short- and longer-lasting desensitized states. The extent to which desensitization occurs depends on the subunit composition of the nAChR, as well as the concentration and duration of agonist exposure. For example, prolonged exposure to low agonist concentrations, such as occurs during smoking, can induce receptor desensitization. Cycles of receptor activation and desensitization contribute to smoking behavior. For example, the first cigarette of the day may be perceived as the most pleasurable because high-affinity nAChRs are still available for activation. These include the β2-containing receptors on dopamine and GABA cells of the VTA, which are readily desensitized during smoking (see below). With long-term nicotine exposure, the number of nAChRs is upregulated, probably as a homeostatic response to nAChR desensitization. This may contribute to agitation and withdrawal symptoms through increased nAChR transmission. Additional cigarettes may 'medicate' the smoker by desensitizing these excess receptors.

The ventral tegmental area (VTA) is a critical site for nicotine action. Intra-VTA nicotine is sufficient to produce behavioral effects, whereas injections outside the VTA are not effective. Activation of dopamine transmission by nicotine is important for its reinforcing effects in drug-experienced animals. For example, nicotine self-administration is reduced by blocking dopamine transmission in the nucleus accumbens. Complicating the picture, however, is evidence implicating dopamine activation in aversive effects of intra-VTA nicotine in naïve animals. Brain regions connected to the VTA also play important roles in nicotine's actions, notably the pedunculopontine tegmental nucleus and the adjacent laterodorsal tegmental nucleus (see Fig. 56-2). These regions send cholinergic (as well as glutamate and GABA) projections to the VTA that are important regulators of dopamine cell activity. Thus, the acetylcholine system, along with endogenous opioid and cannabinoid systems, can be considered part of the natural reward circuitry. In this context, it is not surprising that nAChR transmission is implicated in the reinforcing effects of other drug classes, including psychomotor stimulants and ethanol.

Within the VTA, nicotine exerts its effects via nAChRs located on dopamine neurons, GABA neurons and glutamate nerve terminals. Dopamine and GABA neurons of the VTA express β2-containing receptors, which include α4 as well as other subunits. α7-containing receptors are expressed by glutamate nerve terminals in the VTA, and by a subset of dopamine and GABA neurons. Differential desensitization of nAChRs is important for nicotine's action in the VTA. While β2 receptors on the dopamine cells and GABA cells desensitize readily in response to a

smoker's nicotine levels, the α7 receptors on glutamate terminals desensitize to a lesser extent and continue to promote glutamate release, which now excites the dopamine cells even more effectively due to the decrease in inhibitory GABA synaptic inputs. This enables sustained activation of dopamine transmission. Furthermore, by enhancing glutamate release, presynaptic α7 receptors in the VTA can produce LTP at excitatory synapses onto dopamine neurons. This further enhances the firing of dopamine neurons. nAChRs also influence synaptic plasticity in other regions, including the striatum and hippocampus.

ETHANOL, SEDATIVES AND ANXIOLYTICS

Alcoholism is a chronic relapsing disorder. The risk of alcoholism depends on interactions between genetic, environmental and neurobiological factors. Historically, ethanol's actions were attributed to nonspecific disruption of the lipid bilayer of neurons. It is now recognized that ethanol has specific targets, and that effects of long-term ethanol exposure are due to neuroadaptations as well as neurotoxicity [42–45].

The symptoms of mild ethanol intoxication vary among individuals, ranging from stimulation to sleepiness. At higher doses, sedating effects increase. Intoxicating doses impair memory, judgment, reaction time and self-control. After repeated exposure, tolerance can be substantial. Alcoholics may attain very high blood alcohol levels without appearing grossly sedated. Heavy use inevitably leads to physical dependence. The severity of the ethanol withdrawal syndrome depends on the degree of dependence and the number of prior withdrawal episodes. Symptoms can include tachycardia, sweating, tremor, hypertension, anxiety and agitation, and can progress to include perceptual changes, confusion, seizures and, in some cases, other life-threatening complications. The severity of these symptoms is one factor that contributes to the resumption of alcohol consumption early in withdrawal. However, the major problem in the treatment of alcohol dependence in humans is continued vulnerability to relapse even after prolonged withdrawal periods.

Ethanol interacts directly with ligand-gated and voltage-gated ion channels. This is important both for acute effects and long-term adaptations. Ethanol interacts with a wide range of ion channels, including $GABA_A$, NMDA, glycine, nACh and 5-HT3 receptors and voltage-gated calcium channels. Most attention has focused on ethanol's ability to act as a positive allosteric modulator of $GABA_A$ receptors and a negative allosteric modulator of NMDA receptors. Ethanol inhibits ion flux through the NMDA receptor channel by binding to a hydrophobic pocket distinct from other modulatory sites of the NMDA receptor. During long-term ethanol exposure, there are compensatory changes in the expression of ethanol-responsive ion channels and receptors. NMDA receptors are upregulated, while $GABA_A$ receptors are downregulated. This results in increased neuronal excitability during acute withdrawal, when ethanol is no longer present to decrease NMDA receptor function and enhance GABA transmission. The increase in glutamate tone may contribute to withdrawal-related seizures and neurotoxicity. Different mechanisms may come into play during protracted ethanol withdrawal.

Multiple neuronal systems contribute to the reinforcing effects of ethanol. Activation of dopamine neurons is most important for reinforcing effects associated with ethanol's mild stimulant actions. In the VTA, $GABA_A$ receptors are predominantly located on GABA neurons. By enhancing $GABA_A$ receptor transmission in VTA, ethanol inhibits these GABA neurons, thus disinhibiting the dopamine neurons (Fig. 56-3). Ethanol also directly excites dopamine neurons by decreasing a potassium conductance. After chronic ethanol exposure, adaptations develop in the mesolimbic dopamine system to offset excitatory effects of ethanol, such that withdrawal from chronic ethanol leads to decreased dopamine cell activity and extracellular dopamine levels in the nucleus accumbens. This may contribute to dysphoria. There is also an important role for endogenous opioid systems in ethanol addiction. Antagonists of μ and δ opioid receptors decrease ethanol consumption and reinforcement. One locus for opioid effects is the nucleus accumbens. Opioids influence ethanol reinforcement through both dopamine-dependent and dopamine-independent actions in the nucleus accumbens. Other transmitters (e.g. endocannabinoids and neuropeptide Y) and other brain regions (e.g. the extended amygdala) may also contribute to ethanol action.

There is a need for improved therapies for alcoholism. Disulfiram is used to help prevent relapse, but compliance is often low. Disulfiram inhibits aldehyde dehydrogenase, an enzyme involved in alcohol metabolism, resulting in the buildup of acetaldehyde when alcohol is consumed. This produces unpleasant effects such as tachycardia, nausea and anxiety. The opioid antagonist naltrexone is used to decrease alcohol consumption and craving, based on the role of opioid receptors in the reinforcing effects of ethanol (above). Acamprosate, a newer drug used currently in Europe to maintain abstinence, may produce therapeutic effects via modulation of NMDA receptor function, although $GABA_B$ receptors and voltage-dependent Ca^{2+} channels may also be involved.

Barbiturates and benzodiazepines are used to treat anxiety. Sedatives such as the barbiturates were commonly

used until the introduction of benzodiazepines in the early 1960s. Both of these drug classes have abuse liability, although less than stimulants and opiates. There is cross-tolerance and cross-dependence among ethanol, barbiturates and benzodiazepines, as all are positive allosteric modulators of $GABA_A$ receptors, and produce downregulation of $GABA_A$ receptors after chronic treatment. Accordingly, benzodiazepines are commonly used, both in the clinic and on the street, to treat symptoms of alcohol withdrawal.

HALLUCINOGENS AND DISSOCIATIVE DRUGS

Hallucinogens produce an altered state of consciousness. Users may experience perceptual distortions and sometimes hallucinations, mood changes (ranging from elation to depression or paranoia) and intense arousal. High doses can elicit a 'mystical–religious' experience, accounting for the important role of naturally occurring hallucinogens, such as mescaline (the psychoactive component of peyote), in religion and philosophy in early cultures. Hallucinogen use tends to be episodic and limited, not chronic or compulsive, so these drugs are not associated with drug dependence or addiction. Nevertheless, there can be serious adverse consequences, including accidents due to impaired judgment and 'bad trips' characterized by severe anxiety. Psychotic reactions have been reported in a small proportion of users. In addition, flashbacks which resemble the original LSD experience occur in a small proportion of former users. This is termed hallucinogen persisting perception disorder (HPPD).

Two categories of hallucinogens can be defined based on chemical structure: the tryptamines, which include LSD, and the phenethylamines, which include mescaline and MDMA. MDMA is discussed in the section on Psychomotor Stimulants above. This section will focus on LSD, the most-potent hallucinogenic drug [46]. Based on structural similarity between tryptamines and serotonin, interactions between LSD and serotonin systems have been suspected since the 1950s. The hallucinogenic properties of LSD are now attributed mainly to its partial agonist effects at $5\text{-}HT2_A$ receptors. However, LSD's mechanism still remains puzzling, because its high *in vivo* potency is not predicted from its receptor affinity or intrinsic activity at known signaling pathways. A possible explanation is related to the realization that different agonists can activate different signaling pathways through the same receptor (agonist-directed signaling). Perhaps LSD produces a unique pattern of activation of 5-HT2 receptor signaling pathways. It is also possible that other monoamine receptors contribute to LSD's actions. At the neuronal systems level, frontal cortex and thalamus are important sites of LSD action. Modulation of glutamate transmission in these regions probably underlies distortions in perceptual and cognitive function. Effects of LSD on neurons of the locus coeruleus, an important region for gating of sensory inputs, may contribute to enhanced sensory experiences after LSD.

Phencyclidine (PCP) is a dissociative drug. PCP and ketamine were developed in the 1950s as general anesthetics for surgery. They were discontinued because patients sometimes became delusional during recovery from anesthesia. PCP became a drug of abuse in the 1970s. It is classified as a dissociative drug because it distorts perception and produces feelings of dissociation from reality. Its effects can be powerfully euphoric as well as dysphoric. Notably, PCP mimics both negative symptoms (e.g. apathy and social withdrawal) and positive symptoms (e.g. delusions, paranoia and hallucinations) of schizophrenia. Tolerance and withdrawal have not been well studied in humans. Long-term users of PCP report problems with memory, thinking and mood. PCP overdose is associated with dangerous physical complications.

PCP is a noncompetitive antagonist of NMDA-type glutamate receptors. It binds to a site within the open NMDA receptor ion channel (Ch. 15). Because PCP mimics schizophrenic symptoms, the discovery that it blocks glutamate transmission was a major impetus for the development of glutamate-based theories of schizophrenia (see Ch. 54). Although PCP is a weak dopamine-uptake inhibitor, its reinforcing effects in rat self-administration models reflect NMDA receptor blockade, not an increase in dopamine transmission. However, PCP can activate dopamine neurons indirectly, by blocking NMDA receptors that influence dopamine neurons. Similarly, other transmitter systems are influenced by PCP via pathways 'downstream' of NMDA receptor blockade. Thus, the pharmacology of PCP action in animal models of addiction is complex. Very high doses of PCP and other noncompetitive NMDA receptor antagonists produce neurodegeneration in animal models.

ADDICTION AND NEURONAL PLASTICITY SHARE COMMON CELLULAR MECHANISMS

Addiction may result from inappropriate neuronal plasticity. As discussed in earlier sections of this chapter, drugs of abuse activate the same neuronal pathways as natural reinforcers. However, they do so in a strong and unregulated manner that is hypothesized to lead to abnormal engagement of learning and memory mechanisms, ultimately producing abnormal plasticity in neuronal circuits involved in motivation and decision-making. As a result, the addict becomes narrowly focused on compulsive, habitual behaviors associated with the addictive

substance. Many features of addiction are consistent with this hypothesis, such as the important role of learned associations in triggering drug craving and the activation of learning and memory circuits during craving. However, the strongest support for this hypothesis has come from studies showing that drugs of abuse can influence synaptic plasticity and its underlying mechanisms.

Studies of behavioral sensitization have linked addiction to long-term potentiation (LTP). Behavioral sensitization is an animal model for the intensification of drug craving (see Psychomotor Stimulants above). Work in the late 1980s and early 1990s showed that glutamate transmission is required for the development of behavioral sensitization [32], suggesting a parallel between sensitization and LTP, which also requires glutamate transmission for its induction (see Ch. 53). This was important for shifting addiction research towards an emphasis on plasticity and learning mechanisms. Many studies have now demonstrated that common signaling pathways are activated by drugs of abuse and learning [6]. For example, the MAP kinase/ERK pathway, critical for learning and memory, is increasingly implicated in plasticity related to addiction. In addition, recent years have witnessed an explosion of research demonstrating that drugs of abuse influence LTP and long-term depression (LTD) in neuronal pathways related to addiction [47, 48]. For example, the development of behavioral sensitization to cocaine is correlated with LTP at excitatory synapses onto dopamine neurons of the VTA. This provides a transient increase in excitatory drive to the dopamine neurons, triggering downstream adaptations in nucleus accumbens and other forebrain regions. Other drugs of abuse (amphetamine, nicotine, ethanol and morphine) and stress have in common with cocaine the ability to produce LTP in VTA dopamine neurons [49]. This commonality may explain cross-sensitization between drug classes, and between stress and drugs. Cannabinoids and nicotine may influence LTP directly, through mechanisms described in previous sections. Psychomotor stimulants may do so indirectly through their effects on protein kinases and other signaling molecules involved in LTP [47]. While these and other findings strongly suggest that LTP participates in the cascade that leads to addiction, LTP is not permanent. An important goal of current research, considered in the next two sections of this chapter, is to understand how relatively short-lived adaptations such as LTP may lead to the long-lasting, and perhaps permanent, behavioral abnormalities that are associated with addiction.

Drugs of abuse have profound effects on transcription factors and gene expression. These effects are clearly important for long-lasting changes in brain function. Two transcription factors strongly implicated in addiction are CREB and ΔFosB [23]. CREB is important for learning and memory and is activated by phosphorylation. Addictive drugs influence many of the signal transduction pathways that regulate its phosphorylation state (see Ch. 26). ΔFosB isoforms are highly stable products of the *fosB* gene that accumulate in nucleus accumbens and dorsal striatal neurons during chronic drug treatment, enabling novel patterns of gene expression. Changes in gene expression initiated by these transcription factors contribute to 'sensitizing' adaptations that promote drug-taking as well as 'compensatory' adaptations that oppose drug-taking (e.g. dynorphin upregulation, see Opiates above). However, CREB and ΔFosB levels return to normal following weeks or months of drug withdrawal, so these transcription factors are not directly responsible for maintaining more persistent changes associated with addiction.

Persistent adaptations may involve changes in the structure of dendrites and dendritic spines. Dendritic spines are the postsynaptic contact site for most excitatory synapses in the brain. Structural changes in dendrites and spines were originally described after experience-dependent forms of plasticity, such as exposure to an enriched environment, and in association with LTP. It is now appreciated that structural plasticity also occurs after repeated exposure to psychomotor stimulants, nicotine and morphine [50]. For example, both cocaine and amphetamine increase spine density and dendritic branching in the nucleus accumbens and prefrontal cortex (Fig. 56-7). These structural changes, which are among the most long-lasting adaptations reported in response to repeated drug exposure, may initiated by some of the more transient adaptations discussed in previous sections. For example, ΔFosB may partly mediate cocaine-induced increases in spine density in the nucleus accumbens [23]. Drugs of abuse may initiate synaptic remodeling through their effects on LTP (Fig. 56-5). This is based on evidence that LTP is an initial trigger for activity-dependent changes in the structure of synapses (Ch. 53).

An important challenge is to understand the relationship between neuronal plasticity in identified pathways and specific behavioral alterations that underlie addiction. For example, plasticity in specific pathways presumably explains how drug-associated cues acquire a heightened ability to control behavior in drug addicts. If we identify the pathways that are rewired, and understand the pharmacology of systems that regulate these pathways, perhaps pharmacological treatments can be devised to reduce craving and relapse.

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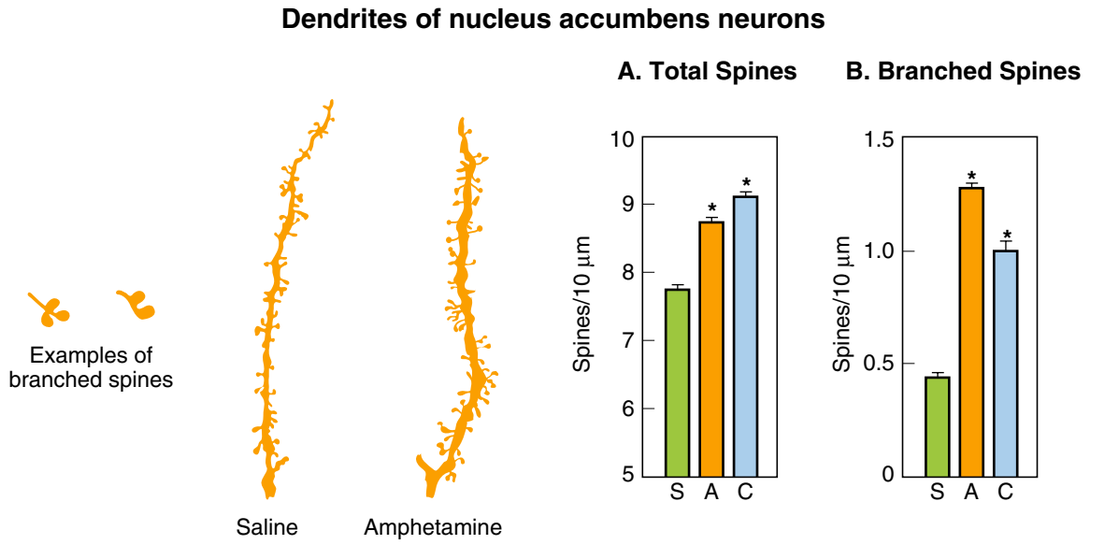

FIGURE 56-7 Repeated exposure to amphetamine or cocaine increases spine density and the number of branched spines in medium spiny neurons, the major cell type of the nucleus accumbens. Left: camera lucida drawings of representative dendritic segments. Rats received 20 injections of saline (*S*), amphetamine (*A*) or cocaine (*C*) over 4 weeks and were then left undisturbed for about 1 month prior to analysis. Adapted from Robinson, T. E. and Kolb, B., *Eur. J. Neurosci.* 11; 1598–1604, 1999.

REFERENCES

1. Substance Abuse: The Nations Number One Health Problem (2001) The Robert Wood Johnson Foundation (http://www.rwjf.org/publications/SubstanceAbuseChartbook. pdf).
2. Robinson, T. E. and Berridge, C. The psychology and neurobiology of addiction: an incentive-sensitization view. *Addiction* 95, Suppl. 2: S91–S117, 2000.
3. Koob, G. F. and Le Moal, M. Drug abuse: hedonic homeostatic dysregulation. *Science* 278: 52–58, 1997.
4. Di Chiara, G. Drug addiction as dopamine-dependent associative learning disorder. *Eur. J. Pharmacol.* 375: 13–30, 1999.
5. Everitt, B. J., Dickinson, A. and Robbins, T. W. The neuropsychological basis of addictive behaviour. *Brain Res. Rev.* 36: 129–138, 2001.
6. Hyman, S. E. and Malenka, R. C. Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat. Rev. Neurosci.* 2: 695–703, 2001.
7. Jentsch, J. D. and Taylor, J. R. Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control of behavior by reward-related stimuli. *Psychopharmacol.* 146: 373–390, 1999.
8. Kelley, A. E. and Berridge, K. C. The neuroscience of natural rewards: relevance to addictive drugs. *J. Neurosci.* 22: 3306–3311, 2002.
9. Wise, R. A. Dopamine, learning and motivation. *Nature Rev. Neurosci.* 5: 483–494, 2004.
10. Volkow, N. D., Fowler, J. S., Wang, G. -J. and Swanson, J. M. Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. *Molec. Psychiat.* 9: 557–569, 2004.
11. Schultz, W. and Dickinson, A. Neuronal coding of prediction errors. *Annu. Rev. Neurosci.* 23: 473–500, 2000.
12. Kalivas, P. W. and McFarland, K. Brain circuitry and the reinstatement of cocaine-seeking behavior. *Psychopharmacol.* 168: 45–56, 2003.
13. See, R. E., Fuchs, R. A., Ledford, C. C. and McLaughlin, J. Drug addiction, relapse, and the amygdala. *Ann. N. Y. Acad. Sci.* 985: 294–307, 2003.
14. Cardinal, R. N., Parkinson, J. A., Hall, J. and Everitt, B. J. Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci. Biobehav. Rev.* 26: 321–352, 2002.
15. Shaham, Y., Erb, S. and Stewart, J., Stress-induced relapse to heroin and cocaine seeking in rats: a review. *Brain Res. Rev.* 33: 13–33, 2000.
16. Piazza, P. V. and Le Moal, M. The role of stress in drug self-administration. *Trends Pharmacol. Sci.* 19: 67–74, 1998.
17. Kreek, M. J. and Koob, G. F. Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend.* 51: 23–47, 1998.
18. Wilson, S. J., Sayette, M. A. and Fiez, J. A. Prefrontal responses to drug cues: a neurocognitive analysis. *Nat. Neurosci.* 7: 211–214, 2004.
19. Williams, J. T., Christie, M. J. and Manzoni, O. Cellular and synaptic adaptations mediating opioid dependence. *Physiol. Rev.* 81: 299–343, 2001.
20. Levac, B. A., O'Dowd, B. F. and George, S. R. Oligomerization of opioid receptors: generation of novel signaling units. *Curr. Opin. Pharmacol.* 2: 76–81, 2002.
21. De Vries, T. J. and Shippenberg, T. S. Neural systems underlying opiate addiction. *J. Neurosci.* 22: 3321–3325, 2002.

22. von Zastrow, M. A cell biologist's perspective on physiological adaptation to opiate drugs. *Neuropharmacol.* 47: 286–292, 2004.
23. Nestler, E. J. Historical review: molecular and cellular mechanisms of opiate and cocaine addiction. *Trends Pharmacol. Sci.* 25: 210–218, 2004.
24. Wilens, T. E., Faraone, S. V., Biederman, J. and Gunawardene, S. Does stimulant therapy of attention-deficit/hyperactivity disorder beget later substance abuse? A meta-analytic review of the literature. *Pediatrics* 111: 179–185, 2003.
25. Rocha, B. A. Stimulant and reinforcing effects of cocaine in monoamine transporter knockout mice. *Eur. J. Pharmacol.* 479: 107–115, 2003.
26. Sulzer, D., Sonders, M. S., Poulsen, N. W. and Galli, A. Mechanisms of neurotransmitter release by amphetamines: a review. *Prog. Neurobiol.* 75: 406–433, 2005.
27. Fleckenstein, A. E., Gibb, J. W. and Hanson, G. R. Differential effects of stimulants on monoaminergic transporters: pharmacological consequences and implications for neurotoxicity. *Eur. J. Pharmacol.* 406: 1–13, 2000.
28. Brown, J. M. and Yamamoto, B. K. Effects of amphetamines on mitochondrial function: role of free radicals and oxidative stress. *Pharmacol. Ther.* 99: 45–53, 2003.
29. Wang, G. J., Volkow, N. D., Chang, L., Miller, E., Sedler, M., Hitzemann, R., Zhu, W., Logan, J., Ma, Y. and Fowler, J. S. Partial recovery of brain metabolism in methamphetamine abusers after protracted abstinence. *Am. J. Psychiatry* 161: 242–248, 2004.
30. Parrott, A. C. Recreational Ecstasy/MDMA, the serotonin syndrome, and serotonergic neurotoxicity. *Pharm. Biochem. Behav.* 71: 837–844, 2002.
31. Vezina, P. Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. *Neurosci. Biobehav. Rev.* 27: 827–839, 2004.
32. Wolf, M. E. The role of excitatory amino acids in behavioral sensitization to psychomotor stimulants. *Prog. Neurobiol.* 54: 679–720, 1998.
33. Vanderschuren, L. J. M. J. and Kalivas, P. W. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacol.* 151: 99–120, 2000.
34. Schlicker, E. and Kathmann, M. Modulation of transmitter release via presynaptic cannabinoid receptors. *Trends Pharmacol. Sci.* 22: 565–572, 2001.
35. Maldonado, R. and Rodriguez de Fonseca, F. Cannabinoid addiction: behavioral models and neural correlates. *J. Neurosci.* 22: 3326–3331, 2002.
36. Piomelli, D. The molecular logic of endocannabinoid signaling. *Nature Rev. Neurosci.* 4: 873–884, 2003.
37. Gerdeman, G. L. and Lovinger, D. M. Emerging roles for endocannabinoids in long-term synaptic plasticity. *Brit. J. Pharmacol.* 140: 781–789, 2003.
38. Dani, J. A. and de Biasi, M. Cellular mechanisms of nicotine addiction. *Pharm. Biochem. Behav.* 70: 439–446, 2001.
39. Picciotto, M. R. and Corrigall, W. A. Neuronal systems underlying behaviors related to nicotine addiction: neural circuits and molecular genetics. *J. Neurosci.* 22: 3338–3341, 2002.
40. Fagen, Z. M., Mansvelder, H. D., Keath, J. R. and McGehee D. S. Short and long-term modulation of synaptic inputs to brain reward areas by nicotine. *Ann. N. Y. Acad. Sci.* 1003: 185–195, 2003.
41. Laviolette, S. R. and van der Kooy, D. The neurobiology of nicotine addiction: bridging the gap from molecular to behavior. *Nature Rev. Neurosci.* 5: 55–65, 2004.
42. Woodward, J. J. Ethanol and NMDA receptor signaling. *Critical Rev. Neurobiol.* 14: 69–89, 2000.
43. Weiss, F. and Porrino L. J. Behavioral neurobiology of alcohol addiction: recent advances and challenges. *J. Neurosci.* 22: 3332–3337, 2002.
44. Davies, M. The role of $GABA_A$ receptors in mediating the effects of alcohol in the central nervous system. *J. Psychiatry Neurosci.* 28: 263–274, 2003.
45. Krystal, J. H., Petrakis, I. L., Mason, G., Trevisan, L. and D'Souza, D. C. *N*-methyl-D-aspartate receptors and alcoholism: reward, dependence, treatment, and vulnerability. *Pharmacol. Therap.* 99: 79–94, 2003.
46. Nichols, D. E. Hallucinogens. *Pharmacol. Therapeutics* 101: 131–181, 2004.
47. Wolf, M. E., Sun, X., Mangiavacchi, S. and Chao, S. Z. Psychomotor stimulants and neuronal plasticity. *Neuropharmacol.* 47, Suppl. 1: 61–79, 2004.
48. Gerdeman, G. L., Partridge, J. G., Lupica, C. R. and Lovinger, D. M. It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends Neurosci.* 26: 184–192, 2003.
49. Saal, D., Dong, Y., Bonci, A. and Malenka, R. C. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron* 37: 577–582, 2003.
50. Robinson, T. E. and Kolb, B. Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacol.* 47, Suppl. 1: 33–46, 2004.

CHAPTER

57

Pain

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INTRODUCTION: THE PAIN PATHWAY

The neural pathway responsible for the sensation of pain begins with high-threshold primary sensory neurons, called nociceptors because they are receptive only to noxious peripheral stimuli. Nociceptors have cell bodies outside the central nervous system (CNS) in dorsal root ganglia (DRG) and peripheral axons that innervate the surface and deep tissues of the body. The central axons of nociceptors enter the CNS and synapse on second order neurons within the spinal cord (Fig. 57-1). Some second-order neurons send outputs that activate flexor motor neurons to produce a brisk withdrawal response (the flexion reflex), and others elicit the sensation of pain by projecting to the somatosensory cortex via the thalamus [1]. The dysphoric, unpleasant aspect of pain is generated by pathways that also originate in the spinal cord and activate the prefrontal cortex, amygdala and nucleus accumbens, and is accompanied by changes in autonomic function, sleep-cycle and mood [2].

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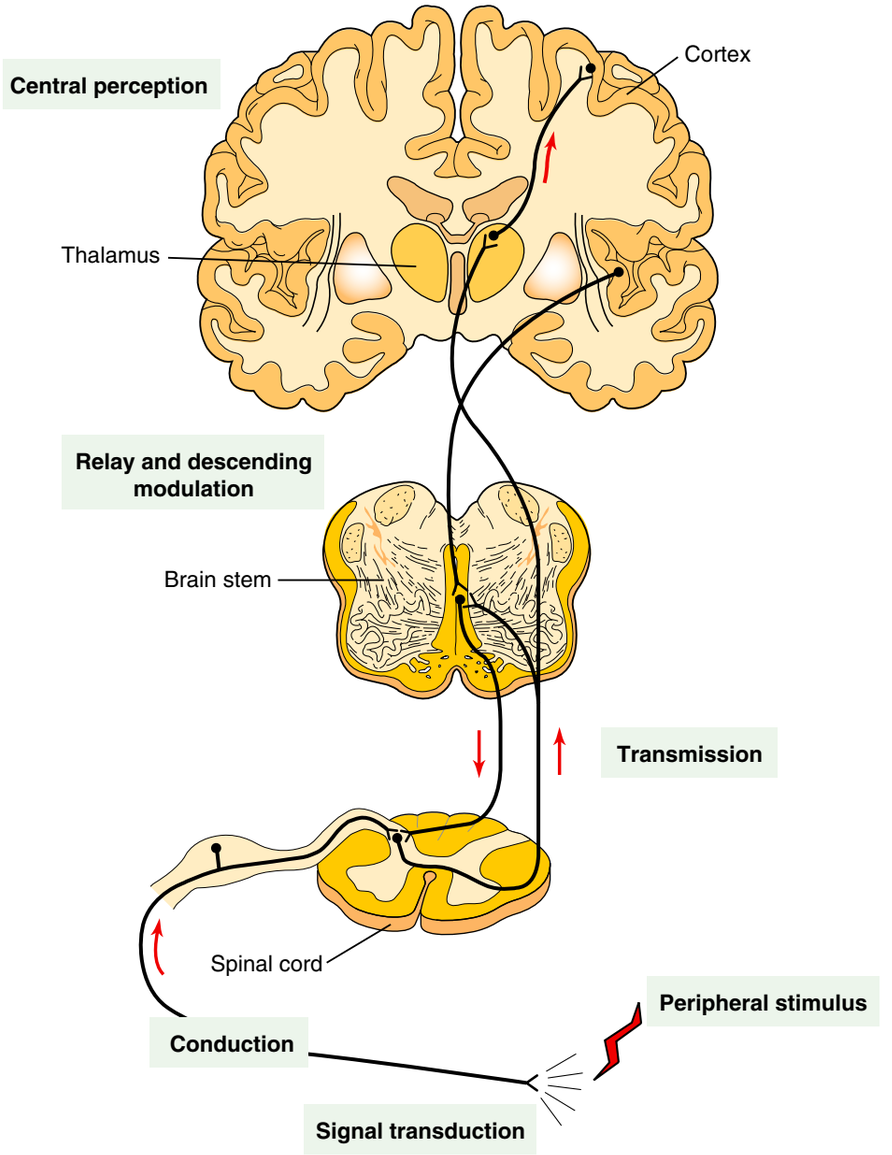

FIGURE 57-1 A diagrammatic representation of the major neural pathway responsible for pain.

In this chapter we highlight the mechanisms and neurochemical basis for nociceptive pain as well as the pain that occurs clinically after tissue injury (inflammatory pain) or nerve damage (neuropathic pain).

PRIMARY SENSORY NEURONS

Sensory detection of a noxious stimulus is known as nociception. The perception of such a stimulus, nociceptive pain, is essentially the 'ouch pain' we experience in response to a pinprick or touching a hot or very cold object. Nociceptive pain is an important physiological protective mechanism that warns of impending tissue injury, and activates reflex and sensory responses to avoid it.

Primary sensory neurons sense pain and convey it to the spinal cord. Nociceptors have unmyelinated (C fiber) or thinly myelinated (Aδ) axons, while the low-threshold Aβ-fiber mechanoreceptors involved in tactile and proprioceptive perception have large myelinated sensory neurons (Table 57-1). The peripheral terminals of primary sensory neurons convert changes in the environment into neuronal activity by transducing mechanical (Ch. 51), thermal or chemical stimuli into ion fluxes across their

TABLE 57-1 Classification of primary afferents

	Fibre class	Role	Activation threshold	Conduction velocity	Cell body diameter	Cell count in dorsal root ganglia
Large calibre myelinated	Aα	Proprioception	Low	Fast	Large (>40 μm)	20%
	Aβ	Touch	Low	Fast	Large (>40 μm)	20%
Medium calibre myelinated	Aδ	Nociception	High	Medium	Medium (25–40 μm)	5%
Small calibre unmyelinated	C	Nociception	High	Slow	Small (<25 μm)	>70%

membrane, producing a generator potential [3]. This transduction is mediated by specialized receptor ion channels that are located on the nociceptor terminal and determine the threshold and specificity of a sensory neuron's response (Fig. 57-2). While some nociceptors are unimodal, activated only by a mechanical, thermal or chemical stimulus, most are polymodal activated by a variety of these intense stimuli. Nociception involves detection of high and low temperature extremes, mechanical stimuli, the presence of chemicals released by damaged cells and extrinsic chemical irritants (Fig. 57-2). Some nociceptors have such a high threshold that they cannot normally be activated by noninjurious stimuli (silent nociceptors).

The molecules that transduce noxious heat or cold are members of the transient receptor potential (TRP) receptor family. TRP proteins (Table 57-2) form tetrameric nonselective cation channels within the plasma membrane, allowing sodium and calcium ion influx [4]. The TRPV3 channel is activated at temperatures between 31 and 39°C, TRPV1 at 43°C, and TRPV2 at 52–55°C. The heat pain threshold in humans is 43°C, suggesting that TRPV1 has a particular role in its determination. TRPV1 and TRPV3 are expressed in C fibers as well as in Aδ fibers, while TRPV2 is found only in Aδ fibers. TRPV1 is activated by capsaicin, the pungent ingredient in chili peppers, giving these fruits their characteristic hot taste. TRPV4 has an activation temperature below 27°C and may play a role in innocuous heat (warm) perception. TRPV3 and 4 channels are localized in skin keratinocytes, suggesting that they act in a paracrine fashion, detecting change in temperature in the epidermis and then, by releasing an intermediary signaling molecule such as ATP, activate sensory fibers through purinoreceptors [5].

The TRPM8 channel is activated by temperatures between 28°C and 8°C and by menthol, producing the cooling effect of this compound. TRPA1 is also activated by noxious cold (<18°C) and by pungent isothiocyanate compounds in horseradish (wasabi) and mustard, as well as cinnamaldehyde (the active ingredient of cinnamon). Temperature detection over the entire physiological range is likely to be achieved by combined actions of the TRPs, with some functional specificity achieved by mixing TRP subunits to produce heteromeric channels [5].

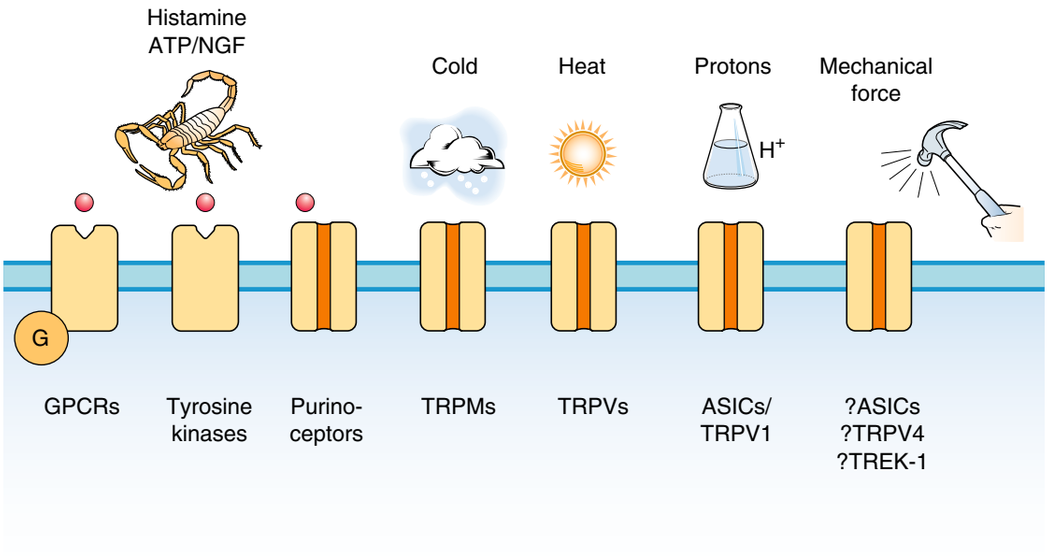

FIGURE 57-2 Noxious chemical, thermal and mechanical stimuli activate specific high-threshold receptors and ion channels that lead to inward currents in the peripheral terminals of nociceptors.

TABLE 57-2 Transient receptor potential channels

Subtype	Other names	Threshold (°C)	Other activating stimuli	Channel selectivity (P_{Ca}/P_{Na})	Chromasomal location	Tissue localization
TRPV1	VR1	>43	Capsaicin, anandamide, PIP_2 extracellular H^+, 2APB	10 ($P_{Mg}/P_{Na}\approx5$)	17p13	DRG
TRPV2	VRL1	>53	2APB	3	17p11	DRG, CNS
TRPV3		>30	Insulin growth factor-1, 2APB	-10	17p13	DRG, keratinocytes, CNS
TRPV4	OTRPC4	>27	Hypotonicity, phorbol esters, anandamide	6	12p24	Keratinocytes, muscle, kidney, CNS
TRPM8		<25	Menthol, icilin	1-3	2q37	DRG
TRPA1	ANKTM1	>18	Icilin, mustard oil	1.4 $P_{Ca}\approx P_{Mg}$	8q13	DRG, fibroblasts

Receptors that transduce mechanical stimuli are members of the Degenerin family. Analysis of mutant *C. elegans* responses to light touch led to the detection of several mechano-sensory abnormal or *mec* genes. These genes, MEC-4 and MEC-10, are members of the Degenerin (DEG) receptor family, and form a hetero-tetrameric nonselective cation channel that is gated by mechanical tension. DEG channels are tethered to a specialized extracellular matrix around the touch-sensitive cell. In addition, the channel is anchored inside the cell to a microtubule network via accessory proteins. A mechanical shearing movement of these two scaffolding structures relative to one another provides the energy needed for DEG channel gating [6]. DEG genes show significant homology with mammalian acid-sensing ion channels (ASIC) and amiloride-sensitive epithelial Na^+ channels (ENaCs). One of these, DRASIC, is expressed only in DRG neurons. While ASIC2- and ASIC3-null mutant mice display deficits in mechanosensation, their specific role in mechanotransduction in sensory neurons remains controversial, and it is possible that another channel is responsible, such as one of the stretch-sensitive TRP channels or the TREK-1 potassium channel [6].

Several molecules participate in chemical stimulus detection. Histamine released from mast cells directly stimulates a small subset of C-fiber sensory neurons through its Gq-linked H1 GPCR receptor to contribute to the initiation of itch sensation. Direct tissue damage results in the release of protons and high concentrations of ATP from injured cells. Protons directly depolarize primary afferents through the ASIC channels. ASIC3 for example, mediates the intense pain associated with cardiac ischemia (angina). ATP acts both on ligand-gated P2X2 and P2X3 channels and the P2Y2 GPCR to activate nociceptors. ATP is released from damaged cells and acts as an efficient tissue injury detector. P2X3 is also involved in detecting bladder distension, since ATP is released from the urothelium of the distended bladder and activates P2X3 receptors on sensory neuron terminals [7].

Sodium channels are key in conveying nociceptive information from the periphery to the CNS. Voltage-gated sodium-ion channels (VGSC) allow Na^+ ion influx in response to local membrane depolarization, such as the generator potential in the peripheral terminals of nociceptors. These ions determine the excitability of the membrane and mediate the propagation of action potentials from peripheral terminals to the CNS. They consist of a large α subunit (~250 kDa) and one or more accessory β subunits (33–36 kDa) (Table 57-3) [8]. Nociceptor neurons express a number of VGSC α subunits, including some found only in these cells (Na_V 1.8 and 1.9). Local anesthetics are nonselective sodium-channel blockers, and specific blockade of sensory-neuron-specific sodium channels could lead to analgesics with potentially few if any side-effects.

The sodium channel α subunit spans the membrane 24 times. These transmembrane domains are split into four groups, each with six spanning segments. The four structurally homologous domains (I–IV) come together to form a membrane-spanning pore; in addition, domain IV contains a voltage sensor and domain III an ion selectivity filter [9]. The fourth spanning segment (S4) of each domain plays the pivotal role in channel opening. These regions possess multiple positively charged residues and undergo conformational change in response to depolarization. A single aromatic amino-acid within domain I confers binding and sensitivity to the puffer-fish toxin tetrodotoxin (TTX). The sensory neuron specific channels Na_V1.8 and 1.9 are TTX resistant. Sodium channel β subunits target and anchor the channels to the plasma membrane, they consist of a single transmembrane region and are glycosylated on the *N*-terminal extracellular domain. These glycoproteins accelerate inactivation kinetics and shift the voltage dependence of steady-state inactivation.

DORSAL HORN

The dorsal horn acts as a gate, regulating the flow of peripheral activity. In 1965 Melzack and Wall launched the spinal gate-control theory, arguing that pain represents the outcome of a balance of excitatory and inhibitory influences operating on the dorsal horn of the spinal cord. Forty years later, much has changed, particularly the realization that nociceptive pain is the expression of activation of a highly specialized and specific sensory

TABLE 57-3 Sodium channels

α-Subunit type	Other names	TTX sensitivity (nM)	Activation threshold	Inactivation rate	Chromasomal location	Tissue localization
$Na_v1.1$	Brain I	-	Low	Fast	2q24	CNS, DRG, motor neurons
$Na_v1.2$	Brain IIA	18	Low	Fast	2q23-24	CNS
$Na_v1.3$	Brain III	15	Low	Fast	2q24	Embryonic nervous system, adult CNS, injury induced DRG/CNS
$Na_v1.14$	SkM1	5	Low	Fast	17q23-25	Skeletal muscle
$Na_v1.5$	H1	1,800	Low	Medium	3p21	Heart, embryonic DRG
$Na_v1.6$	SCP6, PN4	1	Low	Fast	12q13	DRG, motor neurons, CNS
$Na_v1.7$	PN1	2	Low	Fast	2q24	Mostly DRG, some CNS (all cells)
$Na_v1.8$	SNS, PN3	>100,000	High	Slow	3p22-24	Exclusively DRG (80% small cells, 20% large cells)
$Na_v1.9$	NaN, SNS2, PN5	39,000	Low	Noninactivating	3p21-24	Almost exclusively DRG (small cells only)
β-Subunit type			**Chromasomal location**			**Tissue localization**
$Na_vβ1.1$			19q13			CNS, muscle
$Na_vβ1.2$			11q23			CNS
$Na_vβ1.3$			11q24			CNS
$Na_vβ1.4$			11q23			mainly DRG, CNS and muscle

pathway, not of changing patterns of activity in large and small afferents. Nevertheless, it is increasingly clear that both inflammatory and neuropathic pain reflect major alterations in excitation and inhibition in the spinal cord, some transient and some persistent. It is the balance of these positive and negative influences which determines the degree of information that is relayed to higher centers and therefore defines the level of perceived pain.

The dorsal horn is the first site of synaptic transfer in the nociceptive pathway. Aδ- and C-fiber nociceptors in common with most neurons in the CNS utilize glutamate as their fast transmitter (Fig. 57-3). Glutamate binds to ionotropic AMPA and NMDA receptors, as well as to metabotropic glutamate receptors expressed by dorsal horn neurons. The AMPA receptors produce fast excitatory postsynaptic potentials that signal the onset, duration, intensity and location of peripheral noxious stimuli. More intense or sustained C-fiber nociceptor activation results in the release of neuropeptide neuromodulators (substance P and calcitonin gene-related peptide or CGRP) that produce slow synaptic potentials via NK1, CGRP1 and CGRP2 receptors. The latter receptors , by temporal and spatial summation, can generate a sustained postsynaptic depolarization. This depolarization removes the voltage-dependent block by Mg^{2+} of NMDA receptor ion channels (see Ch. 15), thereby producing a short-latency, short-lasting activity-dependent increase in responsiveness called *windup*. In windup, the response to successive repeated noxious stimuli gets bigger with repetition [10].

The release of transmitters from C fibers is mediated by presynaptic, voltage-gated *N*-type calcium channels (Ca(V)2.2). Omega conotoxins found in mollusks that block these channels have an analgesic action when given locally into the spinal cord. Voltage-gated calcium channels (VGCC) comprise pore-forming α subunits plus accessory α2δ and β subunits. A class of novel anticonvulsants that bind to the α2δ subunits (gabapentin and pregabalin) have analgesic activity and modulate transmitter release (Fig. 57-3). Transmitter release is also regulated by presynaptic inhibition produced by the transmitter GABA G-protein-coupled receptors, acting on $GABA_A$ ion channels and $GABA_B$ GPCRs (see also Ch. 16). Other presynaptic inhibitory GPCRs (see Ch. 19) include the cannabinoid CB1 receptor and the three opioid μ, δ and κ GPCRs (MOR, DOR and KOR; discussed in detail in Ch. 56). GABA also produces postsynaptic inhibition, as does glycine, which acts mainly on GlyR α3 containing receptors [1] (Fig. 57-3). Inhibitory circuits are activated either tonically or phasically in response to afferent and descending inputs from the brainstem, and thereby control the transfer of output from the spinal cord [12].

THE BRAIN

Nociceptive information is integrated in the brain. Nociceptive signals reach the brain through spinal projections to the brainstem, hypothalamus, thalamus and ventral forebrain. Two major ascending pathways convey nociceptive signals from the dorsal horn to the thalamus; these are the lateral and the medial (or anterior) spinothalamic tracts (STT). The majority of STT neurons project to the contralateral thalamus. Direct spinobulbar terminations are concentrated in the parabrachial nucleus, midbrain periaqueductal gray (PAG), reticular formation and catecholaminergic nuclei of the brainstem. Functional imaging in humans shows that the primary (S1) and secondary (S2) somatosensory cortex, the insula, anterior cingulate gyrus and prefrontal cortices are all activated in association with the sensation of pain [13].

Descending systems modify the spinal processing of nociceptive input. Supraspinal control of nociceptive signaling is relayed through the PAG, which integrates input from a number of sources: the frontal cortex, insula,

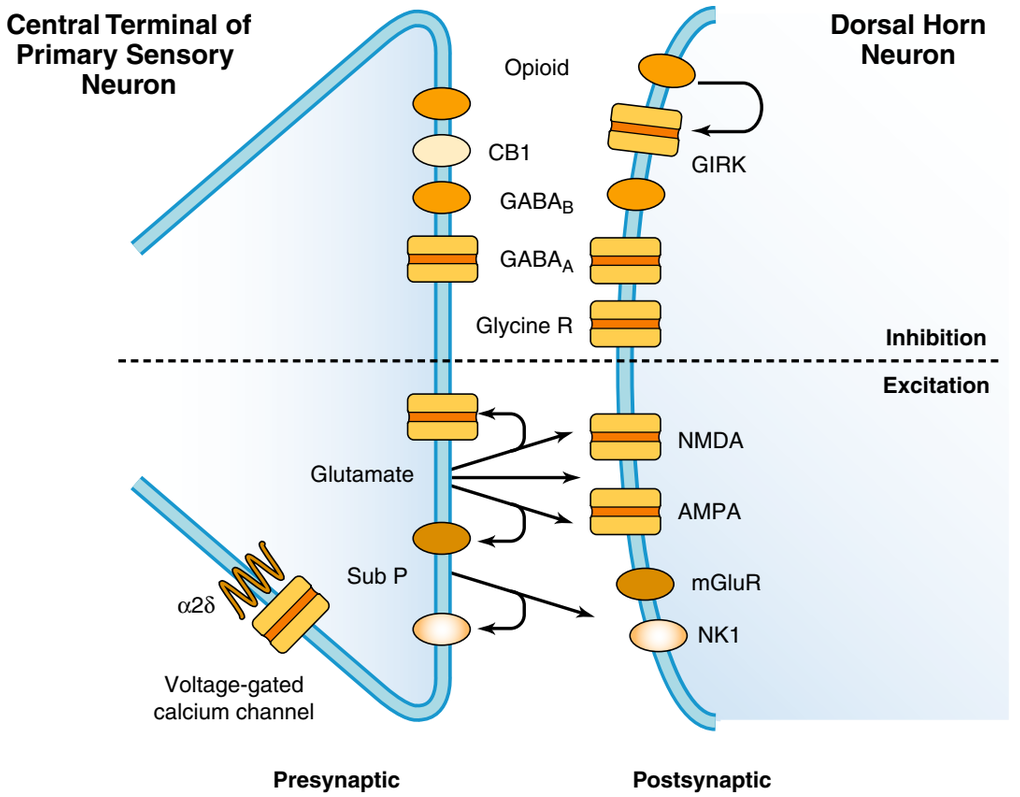

FIGURE 57-3 Transmission between primary sensory and dorsal horn neurons is subject to pre- and postsynaptic excitatory and inhibitory influences.

amygdala, hypothalamus and brainstem nuclei. The PAG does not project directly to the dorsal horn, but rather via the rostral ventromedial medulla (RVM) and the pontomesencephalic noradrenergic nuclei. The RVM is the major source of descending serotonergic input (Ch. 13) that modulates nociceptive transmission in the spinal cord [14].

Endogenous opioids and opioid receptors inhibit pain responses. The DRG, spinal dorsal horn, PAG, RVM, and the amygdala are rich in opioid G-protein-coupled receptors. Local injection of morphine into each of these areas produces analgesia. Opioid receptors (a) decrease the release of transmitters presynaptically by reducing calcium ion influx, and (b) hyperpolarize postsynaptic neurons by opening G-protein-coupled inward-rectifying potassium channels (GIRKs). Five endogenous ligands for the MOR and DOR receptors have been identified: leucine (leu)-enkephalin, methionine (met)-enkephalin, β-endorphin, and endomorphins I and II. A sixth endogenous opioid peptide, dynorphin, binds with high affinity to KOR. Orphanin is a 17-amino-acid neuropeptide similar to dynorphin and acts on the opioid-receptor-like receptor 1. The precursor molecule of orphanin, pronociceptin, gives rise to another peptide with analgesic effects, nocistatin. Neurons synthesizing endogenous opioid peptides are present along the neuraxis and in the DRG. It is still not clear what activates release of the endogenous opioids, such as in life-threatening stress situations (fight or flight) and the placebo reaction, which can produce effective analgesia. MOR agonists such as morphine produce powerful analgesia because they act on multiple sites. However, the role of opiates as analgesics is complicated by tolerance, side-effects such as sedation and nausea, and because of their euphoric actions, a high potential for abuse [15]. Further details concerning peptides and opioids and their receptors are found in Chapters 18 and 56.

CLINICAL PAIN

Clinical pain is characterized by the presence of spontaneous pain or hypersensitivity to pain-provoking stimuli. Hypersensitivity includes pain produced by low-intensity stimuli that normally only elicit an innocuous sensation (*allodynia*), or an exaggerated response to a noxious stimulus (*hyperalgesia*). There are two distinct forms of clinical pain, the pain that occurs after tissue injury or inflammatory diseases (*inflammatory pain*) and the pain associated with a lesion or disease of the nervous system (*neuropathic pain*). Although the mechanisms responsible for the initiation and maintenance of these pains differ, they are both characterized by heightened

pain sensitivity, with a reduction in threshold and increased responsiveness. Unlike nociceptive pain, which reflects short-lasting activation of a specific sensory pathway, *clinical pain* is generally long-lasting and the consequence of a remarkable plasticity of the nervous system due to post-translational changes and altered trafficking of receptor ion channels, altered transcription, neuron loss and novel synaptic inputs [16].

INFLAMMATORY PAIN

Inflammatory pain results from changes both in primary sensory and dorsal horn neurons. The alterations in primary sensory neurons fall into two broad categories: (a) a reduction in threshold and an increase in the response of the peripheral terminals of nociceptors (*peripheral sensitization*), and (b) an alteration in transmitter content modifying synaptic input to the spinal cord. In the dorsal horn, peripheral inflammation results in an increase in membrane excitability and synaptic efficacy, which is the phenomenon of *central sensitization* [12].

Peripheral sensitization lowers nociceptor activation threshold. Tissue damage and peripheral inflammation result in the release and production of inflammatory mediators, including cytokines, chemokines, growth factors, kinins and prostanoids that sensitize the nerve terminal rather than directly activating it [17]. Prostaglandins and inflammation are discussed in Ch. 33. Such sensitizers include bradykinin acting through B1 and B2 GPCRs, prostanoids (notably prostaglandin E2) that bind to four EP GPCRs, and nerve growth factor (NGF) via the membrane tyrosine kinase, trkA receptor. These receptors activate intracellular signal pathways that converge on calcium-dependent protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA) (see Ch. 6). Two major substrates for these kinases are the TRPV1 heat sensor and the $Na_V1.8$ sodium channel. Phosphorylation of TRPV1 reduces the temperature at which it is activated and this is responsible for heat hypersensitivity after sunburn, while phosphorylation of $Na_V1.8$ alters its kinetics, increasing the excitability of the membrane and thereby the responsiveness of the terminal. TRPV1 is also sensitized by phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis after phospholipase C activation and by protons. Peripheral sensitization is restricted to the site of tissue damage (zone of primary hyperalgesia).

NGF, in addition to its direct sensitizing action in the peripheral terminal, is retrogradely transported to the cell body, where, via the ERK MAPK and CREB pathways, it increases expression of substance P (sub P), CGRP, BDNF, ASIC and $Na_V1.8$, and produces an increase in TRPV1 translation. $Na_V1.8$, ASIC and TRPV1 are shipped to the periphery, where their increased levels further augment peripheral sensitivity. After peripheral inflammation, substance P and BDNF are expressed in low threshold myelinated Aβ fibers (normally they are expressed only in C fibers) and this phenotypic switch contributes to an altered synaptic drive to the dorsal horn [16].

Central sensitization results in hyper-responsive dorsal horn neurons. Second-order sensory neurons in the dorsal horn respond to high-level nociceptive input with an increase in excitability that outlasts the stimulus and that is termed *central sensitization* [10]. Glutamate receptors play a critical role in central sensitization (Fig. 57-4). Normally, glutamate released from primary afferent terminals only activates AMPA and mGluRs on dorsal horn neurons, because the NMDA receptor ion channel is blocked by a magnesium ion, preventing influx of sodium and calcium ions (see Ch. 15). Synaptic input through AMPA receptors and activation of NK_1 by substance P lead to membrane depolarization, the opening of voltage-gated calcium channels, and a rise in intracellular calcium, which triggers a phosphorylation cascade within dorsal horn neurons involving the ERK MAPK, protein kinase A (PKA), calcium-calmodulin-dependent protein kinase II and protein kinase C (PKC) (Ch. 24). Downstream of PKC, cell adhesion kinase β (CAKβ) and Src are also activated. Src and Fyn, phosphorylate serine and tyrosine residues on the NMDA receptor (Ch. 23). As a consequence, the magnesium block is removed and channel-open time is increased, and therefore synaptic-released glutamate acting on the NMDA receptor provides a powerful additional source of postsynaptic activation. NMDA-receptor activation itself allows calcium ion influx into the cell that further augments signal transduction within the dorsal horn neuron. ERK activation also leads to phosphorylation of A-type potassium channel Kv4.2, which increases membrane excitability. Apart from a change in ligand- and voltage-gated ion-channel properties induced by these activity-dependent post-translation changes, the intracellular kinase cascades also increase the trafficking of AMPA and NMDA receptors from a cytosolic site to the synaptic membrane (see trafficking in Ch. 9). Synaptic efficacy increases, and previously subthreshold inputs now drive the postsynaptic neuron — the dorsal horn cell is thereby sensitized and hyper-responsive — representing a central mechanism that plays the major role in establishing post-injury pain hypersensitivity.

Features of central sensitization are pain in response to normally innocuous tactile stimuli, and the spread of pain sensitivity beyond the site of tissue injury. Central sensitization plays a major role in acute post-traumatic pain, and also in migraine, neuropathic pain (see below) and some diffuse chronic pain syndromes, such as fibromyalgia and irritable bowel syndrome. In these conditions, which have no detectable peripheral trigger, an autonomous central sensitization may be the pathology, increasing the gain in neuronal activity in the CNS and thereby producing abnormal responses to normal inputs.

The activity-dependent changes in dorsal horn that constitute acute or immediate central sensitization are

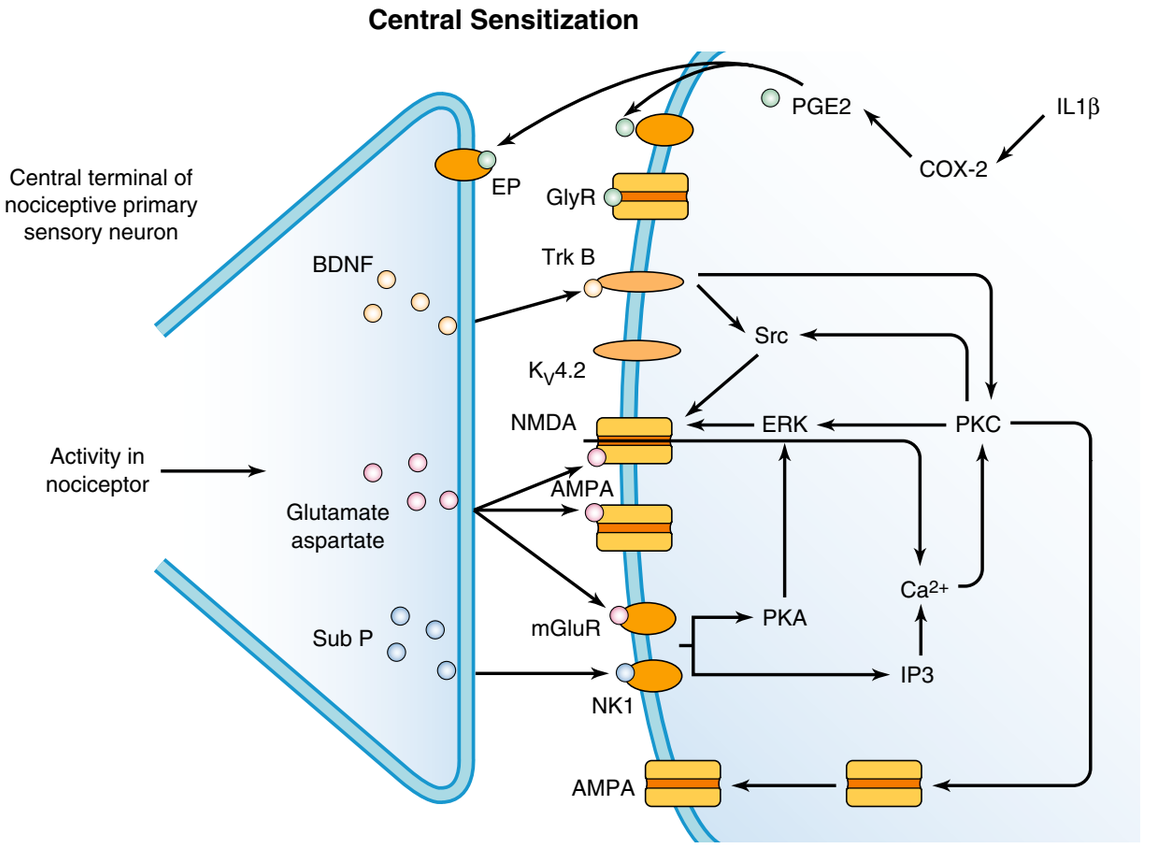

FIGURE 57-4 High levels of activity in nociceptors induce long-lasting increases in the excitability of postsynaptic dorsal horn neurons, the phenomenon of central sensitization. Initially, these are the result of post-translational processing with phosphorylation of ion channels and receptors, altering receptor kinetics as well as trafficking to the membrane. Later transcriptional changes contribute to the sustained excitability, including induction of COX-2 leading to PGE2 production.

induced within minutes, and typically last for an hour or so. However, transcription-dependent changes then kick in and produce alterations in excitability that can last for several days. These include increased expression of the sodium channel $Na_V1.3$, the NK1 receptor, dynorphin and COX-2 in spinal neurons [18]. PGE2 produced as a result of central COX-2 induction results in an increase in transmitter release, a direct depolarization of dorsal horn neurons and a blockade of glycine receptors. All of these result in a marked increase in neuronal excitability. Altered transcription results from increased expression or activation of transcription factors, or the removal of transcription repressors, driven by some of the same signal transduction pathways, such as the MAPKs, that drive the post-translational changes that underlie central sensitization [10]. Some of these transcription-dependent changes are activity-dependent, driven by synaptic input and restricted to localized regions of the spinal cord (e.g. dynorphin). Others are mediated by humoral signals initiated by peripheral inflammation and central cytokine production (e.g. COX-2) and are much more widespread (see Ch. 33).

Prostanoids are key modulators of inflammatory pain. Peripheral inflammation increases prostanoid levels at the site of inflammation and this contributes directly to inflammation and peripheral sensitization (prostanoids and inflammation are discussed in Ch. 33). Peripheral inflammation also increases central prostanoid levels as a result of the central induction of COX-2, to mediate widespread changes in pain sensitivity as well as fever, anorexia, altered mood and sleep patterns [19].

Basal levels of prostanoids are important for homeostatic functions in several tissues, including the kidney, gastric mucosa and platelets. Prostanoids derive from arachidonic acid that is liberated from phospholipids in the cell membrane by the action of phopholipase A2 enzymes (PLA2s). Cyclo-oxygenase (COX) is the enzyme that catalyzes the first two reactions of the prostaglandin pathway leading to the formation of PGH2, an intermediate metabolite. Tissue-specific isomerases (prostaglandin synthases) metabolize PGH2 into the prostaglandin isoforms PGE2, PGD2 and PGF2, prostacyclin (PGI2) or thromboxane (TxA2), which exert their biological actions

via GPCRs, classified as DP, EP, FP, IP and TP receptors according to their respective prostanoid ligand. Two isoforms of cyclo-oxygenase exist. COX-1 is expressed constitutively, and generally produces prostanoids in response to physiological processes requiring continuous regulation. In contrast, COX-2 is inducible and typically produces prostanoids that mediate responses to physiological stress such as injury, infection and inflammation. COX-2 is induced peripherally and in the CNS as an immediate early gene at both transcriptional and translational levels by the proinflammatory cytokines TNFα and IL1β via the NFκB transcription factor and the MAP kinase signaling pathways (see Ch. 26).

The anti-inflammatory and analgesic benefits of non-steroidal anti-inflammatory drugs (NSAIDs) derive from inhibition of the COX-2 isoform in the periphery and, it is increasingly recognized, within the CNS. NSAIDs that specifically inhibit COX-2 activity while sparing COX-1 may be beneficial through their anti-inflammation effect while having reduced risk of certain side-effects, particularly gastric erosions and bleeding. COX-2 selective drugs may, however, increase cardiovascular side-effects.

NEUROPATHIC PAIN

A broad variety of diseases may cause neuropathic pain. The majority of diseases associated with neuropathic pain involve the peripheral nervous system. These diseases include: traumatic injuries; hereditary, metabolic, inflammatory or paraneoplastic neuropathies; and infections. However, neuropathic pain can also be caused by injuries or disorders affecting the spinal cord or the brain (*central neuropathic pain*): tumors; stroke; epilepsy; and neurodegenerative disorders [20]. Genetic factors appear to contribute to inter-individual differences in the susceptibility to neuropathic pain.

Injured axons may develop spontaneous and repetitive firing known as ectopic activity. Injured primary afferents develop spontaneous action potential discharges that occur uncoupled from receptor stimulation in the periphery (Fig. 57-5). These ectopic discharges can be evoked by protons, cytokines or mechanical stimulation of the injured nerve. Another source of ectopic activity are pacemaker currents produced in the plasma membrane

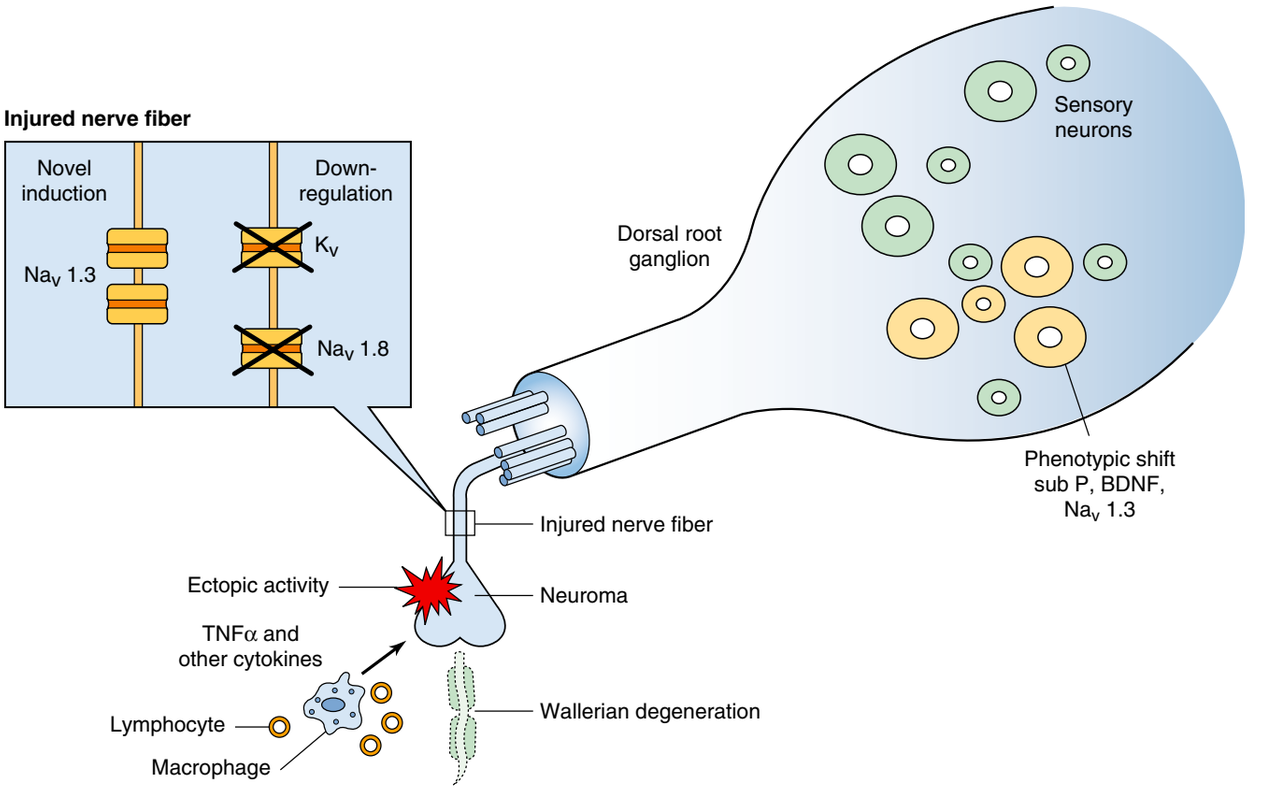

FIGURE 57-5 When severed nerve fibers fail to regenerate, a neuroma is formed. Neurotrophic factors and cytokines released at the neuroma site promote the development of ectopic activity. Changes in the composition of plasma membrane ion channels include a downregulation of $Na_V1.8$ and KV while $Na_V1.3$ is induced. In addition, injured (and neighboring uninjured) primary afferents acquire a new phenotype, producing signaling molecules such as BDNF and substance P.

as a result of changes in ion channel expression and trafficking. After nerve injury, expression of $Na_V1.8$ and $Na_V1.9$ sodium channels decreases, paralleled by a loss of TTX-resistant sodium currents. This contrasts with induction of $Na_V1.3$, a TTX -sensitive sodium channel normally only expressed in DRG neurons during development [21]. Rectifier type K_V channels regulate the pattern and frequency of action potentials by determining membrane repolarization. In large myelinated DRG neurons, K_V channels are expressed as $K_V1.1$, $K_V1.2$ and $K_V\beta2.1$, whereas $K_V4.1$ predominates in small-diameter neurons [22]. Following peripheral nerve injury the expression of K_V is reduced by more than 50%. Together with the injury-induced upregulation of $Na_V1.3$ the decrease in K_V produces a heightened responsiveness of primary sensory afferents. The $\alpha2\delta$ subunit of high-threshold voltage-gated calcium channels is upregulated many-fold by nerve injury, which explains why antiepileptic drugs that target this protein have efficacy in treating neuropathic pain.

Ectopic activity in C fibers elicits central sensitization in the dorsal horn, contributing to the generation of tactile allodynia, a prominent feature of neuropathic pain.

Incomplete nerve lesions provoke changes in the membrane excitability of afferents spared by the injury. These effects are the consequence of an altered cellular and signaling environment at the lesion site and in the DRG. Wallerian degeneration of axotomized nerve fibers involves degeneration and proliferation of Schwann cells, and an invasion of macrophages (discussed in Chs 30 and 36). Nerve injury is accompanied by a release of trophic factors at the injury site and by skin keratocytes in the innervation territory of the injured nerve. Schwann cells, invading macrophages and satellite cells in the DRG produce cytokines including TNFα, to activate uninjured primary afferents to activate them. Nerve injury alters the trafficking of sodium channels in intact neurons. A lesion of neighboring nerve fibers induces an increased integration of $Na_V1.8$ distally in the peripheral axon of intact nociceptors, leading to enhanced excitability.

Sensory neurons transform their phenotype. Microarray gene expression analysis reveals that many hundreds of transcripts change in the DRG after peripheral nerve injury [23]. Injured and neighboring uninjured sensory neurons dramatically change their phenotype following a nerve lesion, with alterations in the expression levels of many genes and *de novo* synthesis of other genes. Because of the phenotypic changes in A fibers, with novel expression of substance P and BDNF, these afferents, which normally do not produce central sensitization, begin to do so.

Spinal disinhibition allows more nociceptive signal input. Following peripheral nerve injury there is a reduction in the GABAergic component of postsynaptic inhibitory currents caused by a degeneration of GABAergic interneurons [24] (Fig. 57-6). This loss of inhibition (disinhibition) results in an overall increase in the excitability of dorsal horn neurons. The degeneration of inhibitory interneurons is due to an excitotoxic effect of primary afferent ectopic activity on dorsal horn neurons [26].

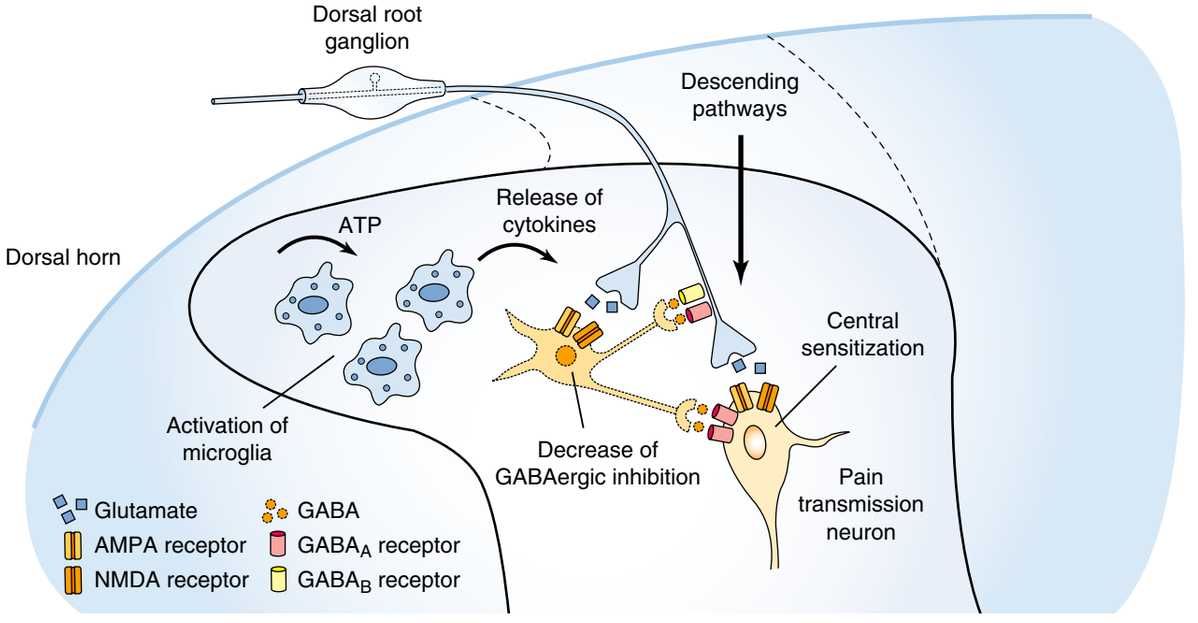

FIGURE 57-6 Central sensitization of dorsal horn neurons and a loss of inhibition after nerve injury result in altered processing of sensory input. ATP activates microglia through the P2X4 receptor. The resulting release of cytokines contributes to the development of pain through a mechanism of action that is, as yet, unknown.

Peripheral nerve injury provokes a marked neuroimmune reaction. Peripheral nerve injury also results in an activation of microglia and astrocytes in the dorsal horn of the spinal cord [25]. Activated glia and invading macrophages release cytokines including IL-1β, IL-6 and TNFα. The purinergic receptor $P2X_4$ (Ch. 17) expressed by activated microglia and activated by ATP contributes to neuropathic pain [26]. Modulation of the immune response can attenuate pain in experimental conditions; however, long-term clinical immunosuppression is hampered by its side-effects.

CONCLUSION

Pain is not a homogenous sensory state. Instead, there are several distinct types of pain: nociceptive pain, a physiological warning system mediated by activation of a specific high threshold sensory pathway; and two forms of clinical pain characterized by spontaneous pain and hypersensitivity, inflammatory and neuropathic pain. Elucidation of the molecular mechanisms in the peripheral and central nervous systems responsible for pain perception has enabled an understanding of how currently available analgesics such as morphine-like opioids and nonsteroidal anti-inflammatory drugs produce their effects, and is also revealing targets for new drugs with the potential for greater efficacy and reduced side-effects. It is increasingly clear that a feature of pain is that it reflects the modifiability or plasticity of the nervous system. A high-threshold system, switched on only by very intense stimuli can, after injury, be initiated by normally innocuous stimuli. Nociceptive pain is protective. The hypersensitivity associated with inflammatory pain can aid healing and repair by minimizing further stimulation of an injured body part, although in the case of chronic inflammatory pain, such as rheumatoid arthritis, the pain does not contribute to healing and is pathological. Neuropathic pain also lacks useful function; it represents maladaptive changes within the nervous system. The pathological changes underlying chronic inflammatory and neuropathic pain effectively represent a disease state.

REFERENCES

1. Willis, W. D. and Coggeshall, R. E. *Sensory Mechanisms of the Spinal Cord.* New York: Kluwer Academic, 2004, pp1–962.
2. Price, D. D. Central neural mechanisms that interrelate sensory and affective dimensions of pain. *Mol. Interv.* 2: 392–403, 339, 2002.
3. Julius, D. and Basbaum, A. I. Molecular mechanisms of nociception. *Nature* 413: 203–210, 2001.
4. Clapham, D. E., Montell, C., Schultz, G. and Julius, D. International Union of Pharmacology. XLIII. Compendium of voltage-gated ion channels: transient receptor potential channels. *Pharmacol. Rev.* 55: 591–596, 2003.
5. Jordt, S. E., McKemy, D. D. and Julius, D. Lessons from peppers and peppermint: the molecular logic of thermosensation. *Curr. Opin. Neurobiol.* 13: 487–492, 2003.
6. Sukharev, S. and Corey, D. P. Mechanosensitive channels: multiplicity of families and gating paradigms. *Sci. STKE* 2004: re4, 2004.
7. North, R. A. P2X3 receptors and peripheral pain mechanisms. *J. Physiol* 554: 301–308, 2004.
8. Wood, J. N. and Baker, M. Voltage-gated sodium channels. *Curr. Opin. Pharmacol.* 1: 17–21, 2001.
9. Catterall, W. A., Goldin, A. L. and Waxman, S. G. International Union of Pharmacology. XXXIX. Compendium of voltage-gated ion channels: sodium channels. *Pharmacol. Rev.* 55: 575–578, 2003.
10. Ji, R. R., Kohno, T., Moore, K. A. and Woolf, C. J. Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci.* 26: 696–705, 2003.
11. Harvey, R. J. *et al.* GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. *Science* 304: 884–887, 2004.
12. Woolf, C. J. and Salter, M. W. Neuronal plasticity: increasing the gain in pain. *Science* 288: 1765–1769, 2000.
13. Rainville, P., Bushnell, M. C. and Duncan, G. H. Representation of acute and persistent pain in the human CNS: potential implications for chemical intolerance. *Ann. N.Y. Acad. Sci.* 933: 130–141, 2001.
14. Fields, H. L. Pain modulation: expectation, opioid analgesia and virtual pain. *Prog. Brain Res.* 122: 245–253, 2000.
15. Waldhoer, M., Bartlett, S. E. and Whistler, J. L. Opioid receptors. *Annu. Rev. Biochem.* 73: 953–990, 2004.
16. Scholz, J. and Woolf, C. J. Can we conquer pain? *Nat. Neurosci* 5(Suppl): 1062–1067, 2002.
17. McCleskey, E. W. and Gold, M. S. Ion channels of nociception. *Annu. Rev. Physiol.* 61: 835–856, 1999.
18. Samad,,T.A. *et al.* Interleukin-1-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 410: 471–475, 2001.
19. Samad, T. A., Sapirstein, A. and Woolf, C. J. Prostanoids and pain: unraveling mechanisms and revealing therapeutic targets. *Trends Mol. Med.* 8: 390–396, 2002.
20. Dworkin, R. H. *et al.* Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. *Arch. Neurol.* 60: 1524–1534, 2003.
21. Lai, J., Porreca, F., Hunter, J. C. and Gold, M. S. Voltage-gated sodium channels and hyperalgesia. *Annu. Rev. Pharmacol. Toxicol.* 44: 371–397, 2004.
22. Gutman, G. A. *et al.* International Union of Pharmacology. XLI. Compendium of voltage-gated ion channels: potassium channels. *Pharmacol. Rev.* 55: 583–586, 2003.
23. Costigan, M. *et al.* Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci.* 3: 1–18, 2002.
24. Moore, K. A. *et al.* Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *J. Neurosci.* 22: 6724–6731, 2002.
25. Watkins, L. R. and Maier, S. F. Glia: a novel drug discovery target for clinical pain. *Nat. Rev. Drug Discov.* 2: 973–985, 2003.
26. Tsuda, M. *et al.* P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424: 778–783, 2003.

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CHAPTER

58

Neuroimaging

J. Eric Jensen

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Weiguo Ye

MAGNETIC RESONANCE IMAGING (MRI) 940
MRI has become the preferred modality for medical and diagnostic imaging due to the superior resolution and contrast in the images 940
The real power of MRI is the ability to exploit the inherent NMR properties of different tissues and tissue pathologies 940
Volumetric and morphological brain measurements are made with MRI 940
Diffusion tensor imaging (DTI) yields information on white matter tracts 941
Signal-intensity analysis yields further insight into cellular pathology 941
Magnetization-transfer imaging (MTI) reveals pathology through signals retrieved from 'bound' protons 941
Functional magnetic resonance imaging (fMRI) maps brain function based on changes in regional blood flow 941

MAGNETIC RESONANCE SPECTROSCOPY (MRS) 942
The overall physical principles and characteristics of magnetic resonance spectroscopy (MRS) are identical to those of MRI 942
Multinuclear MRS studies have demonstrated alterations in brain of schizophrenic patients 943
MRS research is increasingly used to indicate the regions of brain affected in addiction/alcoholism 943
MRS can provide a snapshot into the metabolic processes of brain tumors 944
Phosphorus MRS studies of depressed subjects strongly indicate that this disorder arises from altered brain energy metabolism 944
Hydrogen MRS has revealed unique and consistent metabolic characteristics in the brains of patients with probable AD 944
Hydrogen and phosphorus MRS have proven to be useful tools in the characterization of several epilepsies 944

POSITRON EMISSION TOMOGRAPHY (PET) AND SINGLE PHOTON EMISSION COMPUTED TOMOGRAPHY (SPECT) 944
PET and SPECT are both functional imaging methods that use externally administered radionuclide-labeled substances to image *in vivo* physiological processes in 3-D space 944

APPLICATIONS OF PET AND SPECT FOR BRAIN FUNCTION 945
Measurements of regional cerebral blood flow by PET and of cerebral perfusion by SPECT often detect functional abnormalities before CT or MRI identifies morphological abnormalities 945
The PET method is a valuable tool for the estimation of regional glucose and oxygen metabolic rates and cerebral blood flow 946
PET and SPECT combined with principles of receptor binding permit imaging of receptors in the intact brain 946

CNS DRUG DESIGN AND PRECLINICAL/CLINICAL DRUG DEVELOPMENT 948
The site of drug action and receptor distribution are being investigated 949
The mechanism of drug action may be elucidated by studying its binding 949
Drug dosing is estimated based on proportions of receptor occupancy by the drug 949

CLINICAL APPLICATIONS OF PET AND SPECT 949
The metabolic features of brain tumors can be imaged using PET 949
Brain infarction was the first clinical application of SPECT 949
PET scans with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) have an established role in the localization of epileptic foci in patients being evaluated for epilepsy surgery 949
Correlations of cognitive functions with PET or SPECT imaging are under investigation 950
PET and SPECT imaging are used to study Parkinson's and Alzheimer's diseases 950

The primary focus of this chapter is to provide an introduction to the main imaging modalities and the current biomedical research that is currently underway in the field of modern neuroimaging. Since the focus of this chapter is on neurobiology and neurochemistry, only MRI/MRS

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and PET/SPECT are covered, since these modalities are most directly involved in neurobiological and neurochemical research. Other imaging methods, including X-ray CT, ultrasound and electroencephalography, are not covered, since their primary research use is beyond the scope of this chapter. After reading this chapter, you should have a firm grasp of the basic workings of each imaging technique and a broad, working knowledge of the applications and current research possibilities offered by each method.

MAGNETIC RESONANCE IMAGING (MRI)

MRI has become the preferred modality for medical and diagnostic imaging due to the superior resolution and contrast in the images. MRI has several advantages over X-ray CT, in that MRI does not result in any accumulated ionizing radiation dose to the subject, and it can detect spinal lesions, such as multiple sclerosis, that would otherwise go undetected in X-ray CT (see Appendix: Brief history of medical imaging, and refs [1–9]). However, MRI does pose a minimal risk to the subject in several ways.

1. The radiofrequency pulses involved in MRI cause thermal heating of the tissues, and are thus subject to FDA limits on the amount of RF power that is transmitted to a subject during a medical scan. The RF power unit is specified as the specific absorption rate (SAR) and is measured in watts per kilogram of body tissue (W/kg tissue). Powers that exceed this level put the subject at risk of tissue damage incurred as a result of the tissue's inability to remove the heat through blood flow.
2. The rapid and repetitive application of magnetic field gradients during MRI give rise to a very high rate of change of magnetic field. If this rate of change is fast enough, it can begin to cause neural stimulation of the muscles, causing involuntary twitching.
3. Very little is known about the short-term and long-term effects of exposure to high magnetic fields.

Although the power of research MRI/MRS machines for human use can be as high as 9.4 T, the FDA has imposed a limit of 3 T for routine clinical human use. Industrial manufacturers of MRI equipment are very careful to conform to all the FDA guidelines regarding magnetic field strength, gradient speed and RF power (see Appendix: Basic principles of MRI).

The real power of MRI is the ability to exploit the inherent NMR properties of different tissues and tissue pathologies. The main MR parameters are known as: longitudinal-relaxation time-constant (T1), describing the time for the magnetization to recover to its equilibrium along the main-field axis after RF perturbation; transverse-relaxation time-constant (T2), describing the transient decay of the rotating (MR signal-producing) magnetization from all nuclei in the sample after RF perturbation: and susceptibility-influenced transverse time-constant (T2*) which is identical to T2 with the additional influence of main magnetic-field inhomogeneities. Each parameter offers its own array of information-abundant measurements. In T1-weighted MR images, the ability to resolve discrete structures, tissues or pathology, is greatly enhanced by the additional contrast (ability to distinguish separate structures based solely on their differing intensity, independent of pixel size) resulting from tailoring the MR image sequence specifically to this MR parameter (Fig. 58-1). For further T1 contrast, enhancement of pathological-tissue contrast-agents can be safely delivered to the patient through injection. The contrast agent contains T1 enhancing molecules which are large enough to remain within healthy blood vessels and not to be taken up into healthy tissue, but small enough to diffuse through damaged vessels into damaged or necrotic tissues, such as infarcted, inflamed or cancerous regions. The contrast agent accumulates in these pathological tissues, and shows up as a bright region in the T1-weighted image. Similarly, T2 and T2*-weighted images can reveal unique, underlying structure or pathology that would otherwise be invisible with nonweighted images (Fig. 58-1).

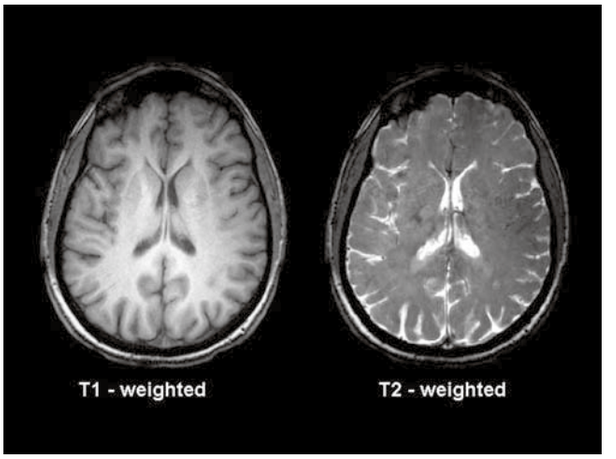

FIGURE 58-1 T1- and T2-weighted images of a normal human brain at 4 Tesla field strength. Gray and white matter is clearly differentiated in the T1-weighted scan, whereas cerebral–spinal fluid (CSF) is well differentiated from the rest of the brain in the T2-weighted image. Both image sets allow for the segmentation of gray matter, white matter and CSF for analysis.

Volumetric and morphological brain measurements are made with MRI. Modern MRI can produce detailed, high-contrast images of the entire brain in relatively short scan times with no invasive procedures or radioactivity. Thus, it is the modality of choice for detailed neuroanatomic studies in various neuropsychiatric disorders. Many groups have addressed the challenge of how to quantify subtle structural characteristics in the brain that appear to differ consistently between comparison groups. Methods such

as tissue segmentation can accurately measure the amount of gray and white matter in either a voxel, a discrete brain region or the entire brain [10]. With this analysis, investigators have uncovered neural processes that contribute to the understanding of mental illness and addiction. For example, magnetic resonance imaging reveals enlarged ventricles in the brains of schizophrenic patients [11], signifying neuronal atrophy and loss. Gray-matter volume reductions in schizophrenia have also been reported in the temporal, cerebellar and anterior-cingulate regions [12, 13]. Aside from parcellelation of tissue-types in the brain, MRI allows the detailed delineation of separate neuroanatomic structures and measurement of their dimensions in the brain. In addition, morphometric techniques are used to normalize the brain images from different subjects into a common space or coordinate system, to correct for the wide variation in brain anatomy between subjects [10]. This method can reveal subtle neuroanatomic features that are unique to neuropsychiatric patients and that may go undetected by conventional means. Consistent alterations in the neuromorphology of the anterior cingulate gyrus and the hippocampus in schizophrenia have also been shown by using high-resolution MRI morphology techniques [14–16] (see also Ch. 54).

Diffusion tensor imaging (DTI) yields information on white matter tracts. Multidimensional quantitation of various neuroanatomic structures in bulk concentrates on macroscopic brain structure and development in neuropsychiatric illness. However, these methods provide little information on the subtle organization of the tissue within these structures and interconnecting tissue. DTI, a form of MRI that exploits the Brownian motion of water molecules, can provide valuable information on the fine structure and organization of white-matter tracts that connect the various brain components. With a carefully tailored sequence of RF pulses and gradients, the fine structure of white-matter tracts can reveal quantifiable information pertaining to the degree of coherent organization (or lack of organization) in separate brain structures. Tissue that looks normal on a conventional MRI scan, may prove to be completely different with DTI, thus making this modality a promising new tool for research. Indeed, DTI has proven to be very effective in the evaluation of brain ischemia, resulting from stroke and/or traumatic brain injury [17]. White-matter abnormalities, such as in multiple sclerosis MS, can be detected and characterized with DTI. Demyelination and axonal loss in MS would likely go undetected with conventional MRI [17]. In neuropsychiatric disorders such as schizophrenia and Alzheimer's disease, as well as addictive disorders like alcoholism, DTI can reveal disconnective and disorganized fiber tracts in the interconnecting white-matter circuitry linking the various regions of the brain, caused either by abnormal brain development or neuronal-toxic damage [17–19].

Signal-intensity analysis yields further insight into cellular pathology. T1- and T2-weighted MRI imaging can differentiate pathology in brain tissues based on analyzing the signal-intensity (SI) of each pixel in the image. T1 and T2 are MR parameters that are affected by the molecular environment of the water in brain tissue. The physical basis for this is described in the Appendix in the accompanying CD-ROM. Abnormalities in the cyto-architecture of axons or in myelin structure can lead to differing image intensity in a T-weighted image. SI in the white matter of T1-weighted MRI images is decreased in multiple sclerosis, while changes in the SI of the corpus callosum in schizophrenia and obsessive-compulsive disorder (OCD) have been reported [10]. White-matter hyperintensities (WMH) detected in brain MRI scans of patients with psychiatric illness show a trend for increased prevalence of WMH in young psychiatric patients at increased risk for cardiovascular disease, rather than an increased rate of WMH in any particular psychiatric illness [20].

Magnetization-transfer imaging (MTI) reveals pathology through signals retrieved from 'bound' protons. With MTI, off-resonance pulses are used to retrieve MR signal from the population of 'bound', or not-freely-mobile protons that are found in macromolecules, fiber tracts and myelin. In conventional MRI, the signal originates from the freely mobile protons in water molecules. With a carefully designed MTI sequence, quantifiable MR signal from the 'bound' protons in various neural tissues can be acquired through transfer of some magnetization from water to produce a quantifiable image of the degree of magnetization transfer (see complete details in Appendix: Basic principles of MRI). This measurable degree of magnetization transfer can reveal underlying abnormalities in tissue pathology, thus making it a valuable research tool. The abnormal myelination that occurs in white matter in MS can be readily detected with MTI. Although not yet consistently utilized in neuropsychiatry, MTI studies in schizophrenia suggest quantifiable differences in magnetization transfer in the parieto-occipital cortex and corpus callosum [10].

Functional magnetic resonance imaging (fMRI) maps brain function based on changes in regional blood flow. The emergence of functional magnetic resonance imaging (fMRI) is proving to be a breakthrough in modern imaging research [21]. fMRI is based on susceptibility-induced signal-intensity changes in the MR image that are directly related to the iron-containing hemoglobin concentration in the tissue, and therefore it is a measure of regional blood flow. With this powerful modality, brain function can be imaged in real time, thus providing a window into the nature of neural motor, cognitive and emotional processing. Indeed, fMRI has revealed reduced response of the auditory cortex to external auditory stimuli in hallucinating schizophrenic patients, along with altered temporal cortex activation during a word presentation paradigm [21]. In other fMRI studies in schizophrenia, amygdyla activation during emotional cues was absent, compared to very predictable activation of this region in a healthy comparison group [21]. Schizophrenia patients

also exhibit either reduced or dysfunctional activation of the frontal cortical regions, compared with controls, during cognitive memory-activation tasks, lending additional support to explaining schizophrenia as an inability to adequately activate the frontal brain region when confronted with a cognitive task [22] (see detailed discussion of schizophrenia in Ch. 54).

MAGNETIC RESONANCE SPECTROSCOPY (MRS)

The overall physical principles and characteristics of magnetic resonance spectroscopy (MRS) are identical to those of MRI. In fact, magnetic resonance spectroscopy can simply be thought of as just another way of expressing the NMR signals that are recorded during an NMR experiment. However, MRS is designed to measure extremely small NMR signals of H1, C13, or P31 nuclei in specific amino acids, energy metabolites and membrane lipid components (see Appendix: Basic principles of MRS, Table 58-1). MRI measures much larger signals of water hydrogen (see details in Appendix: Basic principles of MRS, Table 58-1; Figs 58-2 to 58-4).

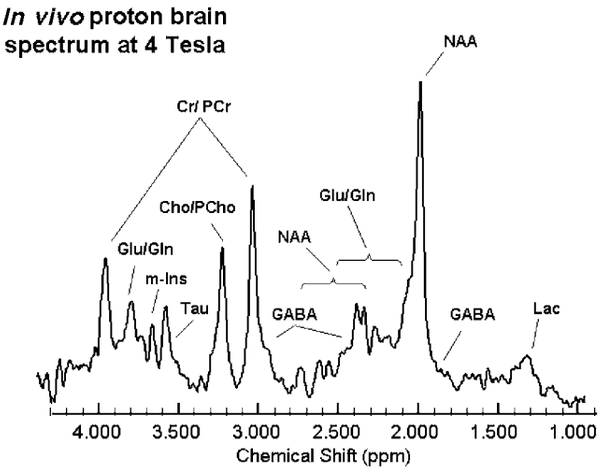

FIGURE 58-2 Hydrogen (^{1}H) spectrum from a normal human brain at 4 Tesla field strength. The spectrum is very complicated, comprised of many overlapping peaks which are difficult to resolve from each other. Spectral analysis routines make use of spectral models which are constructed from the individual spectra acquired from each biomolecule from *in vitro* solutions. This model is then fitted to the raw data and approximate concentrations for each biomolecule are extracted.

TABLE 58-1 Nuclei in MRS and common biological functions [23–25]

Nucleus	Biological function	Molecule	Abbreviation
Hydrogen(^{1}H) /Carbon (^{13}C)	Amino acids/neurotransmission/ intermediate storage/synthesis	γ-amino butyric acid	GABA
		glutamine/glutamate	Gln/Glu
		glutathione	GSH
		glycine	Gly
		acetate	Ace
		N-acetylaspartylglutamate	NAAG
		aspartate	Asp
		phenylalanine	Phe
		serine	Ser
		taurine	Tau
		threonine	Thr
		tryptophan	Trp
		tyrosine	Tyr
		valine	Val
	Energy metabolism	creatine/phosphocreatine	Cr/PCr
		adenosine-triphosphate	ATP
		glucose	Glc
		lactate	Lac
		pyruvate	
		succinate	Suc
	Membrane/lipid metabolism/ neuronal-integrity	choline/glycerophosphorylcholine/ phosphorylcholine	Cho/ PCho GPCho/
		N-acetyl aspartate	NAA
		ethanolamine/ phosphoethanolamine	Etn/PEtn
		glycerol	
		myo/scyllo-inositol	M/s-Ins
Phosphorus(^{31}P)	Energy metabolism	phosphocreatine	PCr
		adenosine triphosphate	NTP/ATP
		inorganic phosphate	Pi
	Membrane/lipid metabolism	phosphoethanolamine	PEtn
		phosphocholine	PCho
		glycerophosphoethanolamine	GPEtn
		glycerophosphocholine	GPCho
		membrane phospholipid	MP
Lithium(^{7}Li)	Lithium drug metabolism	lithium-containing pharmaceuticals	Li
Fluorine(^{19}F)	Fluorine drug metabolism	fluorine-containing pharmaceuticals	F
Sodium(^{23}Na)	Sodium distribution	inter/intra-cellular sodium	Na

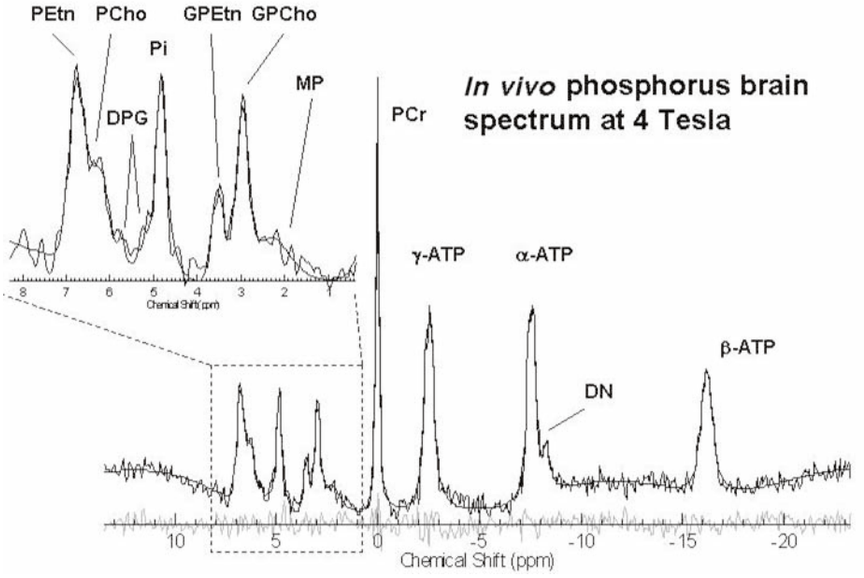

FIGURE 58-3 Phosphorus (^{31}P) spectrum from a normal human brain at 4 Tesla field strength. Unlike the hydrogen (^{1}H) spectrum, The phosphorus spectrum is not as complicated, comprised of much fewer overlapping peaks. Like hydrogen spectrum analysis, phosphorus spectral analysis routines makes use of spectral models. The inset depicts a close-up of the spectral region where membrane phospholipid metabolites are found. Energetics can be measured from extracting relative peak area ratios of ATP to PCr. Tissue pH, a measure of the acidity or alkalinity, can be derived from the relative spectral separation of Pi and PCr.

Multinuclear MRS studies have demonstrated alterations in brains of schizophrenic patients. Phosphorus MRS studies, looking at both first-onset-unmedicated and chronic-medicated schizophrenia, consistently report alterations in the phospholipid profile in the frontal and temporal cortex [26–30]. Typically, phosphomonoester (PME) levels are lower than in controls, and phosphodiesters (PDE) levels are higher in schizophrenia, but opposite directionality in the levels of these membrane metabolite compounds also have been reported [25, 30]. It is thought that membranes in schizophrenia are broken down or metabolized at a higher rate than can be produced, leading to eventual neurodegeneration and progression of symptoms over time. These phosphorus findings are complemented by the findings of altered glutamate, glutamine and NAA levels in schizophrenia, revealed with high-resolution hydrogen [31] or proton MRS studies of the anterior cingulate gyrus and thalamus [32, 33]. Overall, the hydrogen and phosphorus MRS studies in schizophrenia potentially point to a neurodegenerative process involving focal regions of the brain, emphasizing involvement of the frontal and temporal cortex, as well as the anterior cingulate gyrus, thalamus and cerebellum [34] (see Ch. 54).

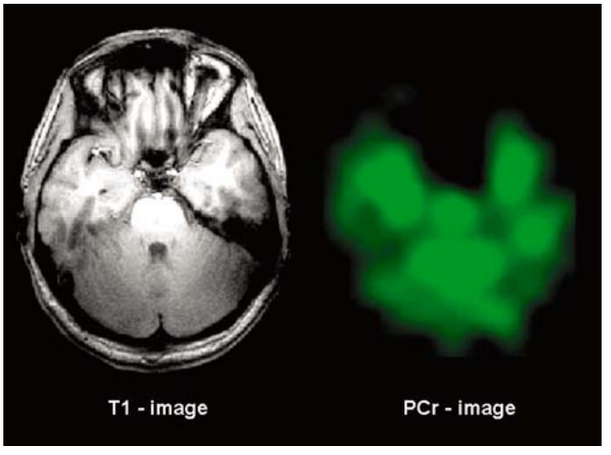

FIGURE 58-4 Chemical-shift image of PCr (right) in a normal human brain at 4 Tesla field strength. Shown on the left is the corresponding T1-weighted image from the same subject. Chemical-shift images can be expressed differently. Here, the peak area of PCr is expressed in terms of image intensity. However, for each voxel in the image, an entire phosphorus spectrum can be extracted, and all metabolites measured.

MRS research is increasingly used to indicate the regions of brain affected in addiction/alcoholism. The increasing use and availability of illicit narcotics, such as heroin and cocaine, as well as alcohol, in modern society, hardly needs mention. MRS research in addiction provides a major avenue for identifying the anatomic regions of brain involved, and the molecular pathology, with the hope of eventually developing more effective strategies in drug treatment programs [35]. Studies with phosphorus MRS have revealed transient alterations in phospholipid metabolism and phosphoenergetics in the brains of opiate-dependent patients undergoing methadone-maintenance therapy. These data show a consistent pattern of increasing

levels of PCr and decreasing levels of phosphodiesters and phosphodiesters in the brain during acute and long-term methadone-maintenance therapy, compared to initial therapy and withdrawal from heroin use [36–38]. Interestingly, phosphorus MRSI indicates that the changes are focused in the frontal lobes, a finding consistent with MRS research in other neurospsychiatric disorders (see the detailed discussion of addiction in Chapter 56).

MRS can provide a snapshot into the metabolic processes of brain tumors. Imaging research has investigated the biochemical functioning of brain tumors. Tumor biopsies can provide valuable cellular structural, histological and genetic information about the nature of malignant tissue, but MRS methodology allows analysis of the metabolic processes within tumor tissue, as well as measurement of the effects of treatment with chemotherapy and radiation. Hydrogen MRS of brain tumors *in vivo* have revealed marked differences in the ratios of NAA, Cho and Cr, compared to healthy tissue [39–42]. Elevated mobile lipid levels detected with hydrogen spectroscopy suggest tumor necrosis, and thereby provides a useful prognostic tool.

Phosphorus MRS studies of depressed subjects strongly indicate that this disorder arises from altered brain energy metabolism. Like any other debilitating mental illness, depression and bipolar disorders cost millions of dollars annually in treatment and loss of productivity (see discussion in Ch. 55). Two phosphorus MRS studies show very consistent and similar results, with reduced levels of nucleoside triphosphates (NTP), the main source of energy in living tissue [43, 44]. What is interesting is that this finding seems to contradict the expectation that NTP levels in living tissue remain absolutely constant, regardless of the metabolic demand placed on the tissue, with PCr providing the buffer to maintain steady NTP levels. This research has prompted more in-depth questions, and given rise to interesting theories concerning mitochondrial dysfunction as the possible underlying cause in depression, as well as other neuropsychiatric disorders (see discussions in Ch. 55).

Hydrogen MRS has revealed unique and consistent metabolic characteristics in the brains of patients with probable AD. Although Alzheimer's disease (AD) is not formally diagnosed until postmortem brain examination, it can be tentatively diagnosed during life as being 'clinically probable AD' (see Ch. 47). Alzheimer's shares common symptoms with other dementias, and is often difficult to diagnose based only on symptoms. Two recent studies have shown that ratios of *N*-acetyl-aspartate to creatine (NAA/Cr) are reduced in probable AD, indicative of either neuronal loss or neuronal atrophy [45–47]. The other consistent finding in these studies is increased ratios of myoinositol to creatine (MI/Cr), suggesting enhanced neuronal gliosis and/or altered myoinositol metabolism in probable AD.

Hydrogen and phosphorus MRS have proven to be useful tools in the characterization of several epilepsies. Epilepsy is a neurological condition in which sudden, uncontrollable firing of neurons in the brain causes seizures. The debilitating aspect of this disorder is the unpredictability and possible injury or death resulting from these seizures. Epilepsy is divided into several subtypes, each with their own set of symptoms and characteristics (see Ch. 37). In patients with juvenile myoclonic epilepsy (JME), for example, reduced ratios of NAA/Cr have been found in bilateral thalami, whereas hydrogen MRS of idiopathic generalized epilepsy (IGE), has shown increased glutamine and glutamate (Glx) levels and decreased NAA levels in the frontal lobes of patients compared with healthy volunteers, suggesting increased neuronal excitability (increased Glx) and neuronal loss or dysfunction (reduced NAA) in IGE [48–52].

POSITRON EMISSION TOMOGRAPHY (PET) AND SINGLE PHOTON EMISSION COMPUTED TOMOGRAPHY (SPECT)

PET and SPECT are both functional imaging methods that use externally administered radionuclide-labeled substances to image *in vivo* physiological processes in 3-D space. These substances may be radionuclide-labeled ligands, metabolites, drugs, nondiffusible tracers, CO_2, H_2O and O_2. Both methods involve the measurement of gamma radiation, emitted from radioactive atoms or processes resulting from the decay of these atoms, with energies of 511 kilo electron volts (keV) in the case of PET or in the order of hundreds of keV in the case of SPECT. Both methods have the unique characteristic of employing the tracer principle, which permits high sensitivity for functional measurements, usually much greater than that achieved by the MR magnetic resonance imaging technique, because with PET and SPECT individual molecules, selected for their specificity, are being imaged as they exhibit their specific functional action. These physiological measurements are used not only to measure normal biochemical processes, but those in disease and those related to therapy. Such measurements include: regional blood flow/perfusion; blood volume; glucose and oxygen metabolism; pre-, post- and intrasynaptic neuroreceptor-transmitter activity; receptor density and affinity; neurotransmitter transporter density; neuronal enzyme activity; and the delivery of drugs across the blood–brain barrier into areas of interest and which bind to molecules of interest, such as receptors or transporters. In addition, these methods can be used to image gene expression and effects of gene therapy. (For detailed technical comparisons, see Appendix: PET versus SPECT: similarities and contrasts, Table 58-2.)

SPECT is routinely available in most nuclear medicine clinics throughout the world, whereas PET is more specialized, but is increasingly available in countries with high levels of medical technology especially with the advent

TABLE 58-2 Typical radionuclides employed in PET/SPECT and physical properties

Isotope	PET/SPECT	Decay mode (%)	Half-life (min)	Energy (Mev) (max/most abundant)	Range in H_2O mm (half-value thickness)	Production: cyclotron (C), reactor (R), generator (G)
^{11}C	PET	β^+ 100	20.1	0.97	4	C
^{18}F	PET	β^+ 97 EC 3	109.8	0.64	2	C and R
^{15}O	PET	β^+ 100	2.04	1.74	8	C
^{13}N	PET	β^+ 100	9.996	1.20	5	C
^{62}Cu	PET	β^+ 97 EC 3	9.7	2.92	14	G ^{62}Zn
^{82}Rb	PET	β^+ 95 EC 5	1.3	3.4	17	G ^{82}Sn
^{68}Ga	PET	β^+ 89 EC11	68	1.899	9	G ^{68}Ge
^{99m}Tc	SPECT	IT 100	360	0.140	4,600 t	G ^{99}Mo
^{123}I	SPECT	EC100	780	0.159	4,700 t	C
^{131}I	SPECT	β^- 100	8 (days)	0.192	6,300 t	C/R
Annihilation gamma	PET	—	—	0.511	7,100	(see above)

EC = electric capture
IT = isomeric transition
t extrapolated from ref. [53]

of clinical uses. In either case, the principle consists of chemically linking a radioactive atom with water, a gas, or any ligand or pharmaceutical used in tracer quantities, which is then inhaled by the subject or injected intravenously. Virtually all areas of the human body can be visualized, but for purposes of this chapter we will emphasize the CNS.

APPLICATIONS OF PET AND SPECT FOR BRAIN FUNCTION

Knowledge about brain function has been revolutionized by PET and SPECT radiopharmaceuticals that measure specific biochemical processes or proteins in living animal or human brain. The challenge is to develop these radiotracers so that they can cross the blood–brain barrier (BBB) and still maintain sensitivity and specificity in binding to the target. (For technical details with respect to the different functions measured, pharmaceuticals, resolution, limitations, imaging equipment and relative risks, see Appendix: Advantages and disadvantages of PET and SPECT, Table 58-3 [54, 55]; for basic radiochemistry for PET, SPECT [56–59] and for validation of radiotracers [60–62] see Appendix: Fundamentals of radiotracer methodology and Fig. 58-5).

Measurements of regional cerebral blood flow by PET and of cerebral perfusion by SPECT often detect functional abnormalities before CT or MRI identifies morphological abnormalities. The function of the cerebral tissue depends critically on the regional cerebral blood flow (rCBF) and rate of metabolism (see Ch. 31). Impairment of blood flow or the rate of consumption of O_2 or glucose constitutes a pathological condition which can be

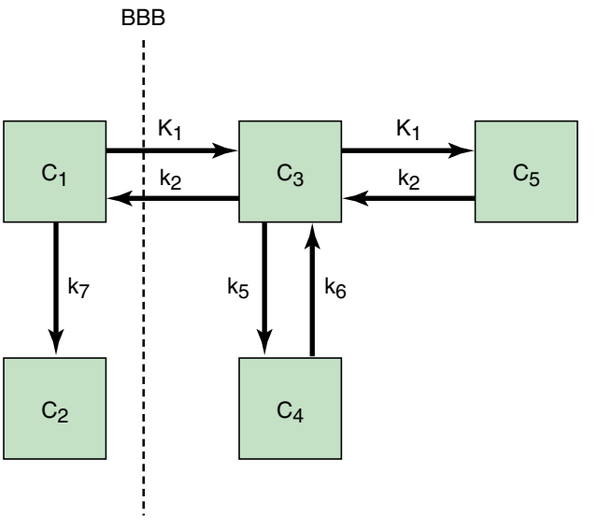

FIGURE 58-5 This is a typical co-compartmental model used in PET or SPECT dynamic image. Compartments such as C_1 and C_2 could be native plasma activity and metabolites respectively. After the radiotracer crosses the blood–brain barrier and enters compartment C_3 this could be the free or precursor pool, C_5 could be the product of either receptor binding or the final step for the measurement of same metabolism, e.g. glucose-6-phosphate, and product of C_4 could be a secondary binding site. For example, the rate constants such as K_1 are (typically) blood flow and tracer extraction. k_2 is typically the dissociation from the blood supply, k_7 same metabolism, k_3 and k_4 the association and dissociation to the receptor or to the final product enzyme step. For example in k_5, k_6 reversibly steps to another receptor. This compartment model could be easily used for FDG glucose metabolism, enzyme activity (dopadecarboxylase) or receptor binding say to D_2 (C_5) or serotonin (C_4) sites. It could even be used for blood flow, except that C_3, C_5, and C_4 would be collapsed to one compartment, and C_1 and C_2 would collapse to a second compartment. Models such as these with the blood–brain barrier of plasma on the one side and the brain compartments on the other are typically used in PET and SPECT quantitation. The ultimate goal is to simplify these methods but initially this is what is necessary for the appropriate quantification of any physiological process using these PET/SPECT imaging methods.

detected on a regional basis in three dimensions by these methods [63].

Radiolabeled agents used to assess brain perfusion by SPECT. Examples include Technetium-99m-hexamethyl propylamine oxime (^{99m}Tc-HMPAO), which is a lipid-soluble macrocyclic amine, permitting rapid diffusion across the blood–brain barrier (BBB). Once it crosses the BBB, ^{99m}Tc-HMPAO is converted into a hydrophilic compound in the presence of intracellular glutathione, which is incapable of rapid back-diffusion. Technetium-99m-bicisate (bicisate ethyl cysteinate dimer, ^{99m}Tc-ECD) is another lipophilic agent that can enter the brain by rapid diffusion. It is converted in the brain by an esterase to a negatively charged complex that cannot diffuse back across the BBB. Brain uptake of both ^{99m}Tc-HMPAO and ^{99m}Tc-ECD are rapid, and reach their maxima within 5–10 minutes of injection.Their regional distributions are proportional to rCBF but do not represent parametric units of rate of blood flow.

The PET method is a valuable tool for the estimation of regional glucose and oxygen metabolic rates and cerebral blood flow. The measurement of glucose metabolic rate is based on the principle that the phosphorylation of glucose to glucose 6-phosphate catalyzed by hexokinase is rate-limiting. The antimetabolite analog 2-deoxyglucose competes with glucose for entry into the cells and phosphorylation by hexokinase, but 2-deoxyglucose 6-phosphate is not metabolized further in the glycolytic sequence. ^{18}F-2-deoxyglucose (^{18}F-DG) is used in tracer quantities and enters brain along with glucose. ^{18}F-labeled 2-deoxyglucose 6-phosphate accumulates in the cells where formed, and the amount accumulated is a measure of the rate of glucose metabolism (see Ch. 31). An example of an FDG image is given in PET (Fig. 58-6). Examples of FDG use for epilepsy, cognitive function and brain tumor are given below. In addition, the short half-life of oxygen-15 ($t^1/_2$=2.1 min) allows for rapid sequential studies of cerebral blood flow using $H_2{}^{15}O$.

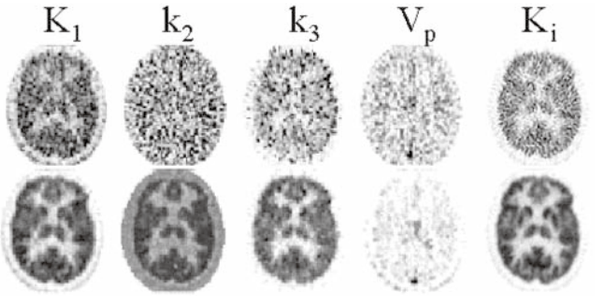

FIGURE 58-6 FDG. The parametric images of K_1, k_2, k_3, V_p. K_1 (ml/min/g) is the forward transport rate constant of FDG from plasma across the blood–brain barrier (BBB) to brain tissue, k_2 (1/min) is the reversed transport across the BBB, k_3 (1/min) is the phosphorylation rate constant, and V_p (ml/g) is the fractional plasma volume in tissue. The images were generated by conventional weighted nonlinear regression (top row) and nonlinear ridge regression with spatial constraint (bottom row). The K_i (K_1 $k_3/(k_2+ k_3)$) was calculated after pixel-wise model fitting. (Yun Zhou *et al.* Improved parametric image generation using spatial–temporal analysis of dynamic PET studies. *Neuroimage*, 15: 697–707, 2002.)

PET and SPECT combined with principles of receptor binding permit imaging of receptors in the intact brain. Over 20 years ago, in a number of almost simultaneous studies involving PET and SPECT, researchers developed the technique of radiolabeling receptor ligands that had a high affinity for CNS receptors. Although this initially began with an antagonist to the D_2 dopamine system, radiolabeled agonists for the muscarinic cholinergic system are now also employed. Many of these radiotracers are successfully used because of the ability to replace a stable carbon (^{12}C) with a radioactive positron emitter (^{11}C) or a fluorine (^{18}F). Alternatively, derivatizing the substance, for example by adding an ^{11}C, ^{18}F or ^{123}I where the atom did not exist, causes a minor modification to the pharmacologic affinity of these ligands. By radiolabeling ligands with PET or SPECT isotopes, however, the labeled ligands allow external imaging and, most importantly, reflect directly much of the drug action that is associated with neuroreceptor binding and neurotransmission. (For a detailed discussion, see Appendix: Fundamentals of radiotracer methodology.)

As shown in Figure 58-7, the *in vivo* efficacy of receptors on the absiccal axis correlates with the *in vitro* binding values (IC50) obtained by measures of binding to tissue homogenates. Since radioligands with a higher affinity as well as higher selectivity tend to underlie the principles of receptor labeling it has an important role in potential drug efficacy (see the section on drug development below).

Quantification of neuroreceptors with PET/SPECT. The typical process consists of identifying an appropriate ligand that is known already to bind *in vitro* or *ex vivo* animal models. Then the radiosynthetic process for providing a positron- or SPECT-emitting radiotracer is obtained. The latter is often a difficult task, and only a very small fraction of such ligands can be labeled in this fashion. Once this is obtained, the radiotracer must then be studied first in small animals and then in nonhuman primates to examine its usability. At any stage, species differences can result in lack of penetration into the brain or rapid metabolism [64]. The validation studies in small animals and baboons typically consist of *in vitro* studies, including saturability and competition with other chemically different but pharmacologically related drugs. Once receptor criteria have been established, the radiotracer is considered appropriate for quantification either in nonhuman primates or in human beings. Of course, the appropriate safety profiles including toxicology and radiation dosimetry must be carried out [64]. One of the newest advances in PET instrumentation is the development of dedicated cameras for imaging rats and mice and nonhuman primates.

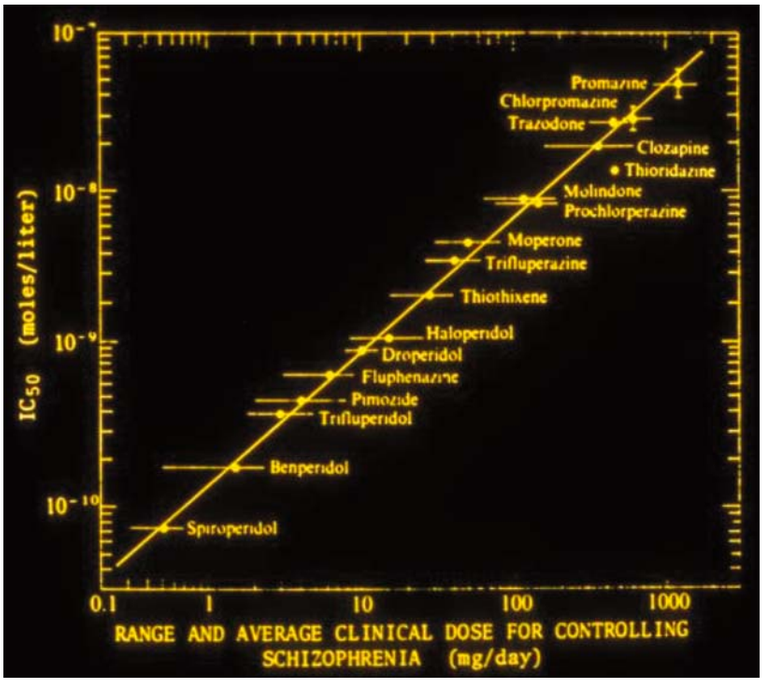

FIGURE 58-7 The IC_{50} values (ordinate) are the concentrations of the antipsychotic drugs that reduce the stereospecific component of ^{3}H-haloperidol binding by 50%. The abscissa indicates the average values (and ranges) of doses used for schizophrenia. (From Seeman, P. *et al.* Antipsychotic drug doses and neuroleptic/dopamine receptors. *Nature* 261: 717–719, 1976)

This methodology provides resolution of approximately 1 mm. Thus, not only *in vitro* but *in vivo* data from genetic knockouts and other animal models can be obtained with PET and more recently SPECT imaging [65].

Neuroreceptor ligands for PET. As a direct result of work in the 1960s and 1970s predominantly in the dopamine system (Fig. 58-8), a number of high-affinity and clinically potent ligands such as spiroperidol (spiperone) were employed for the first PET human imaging studies in 1983 with [^{11}C]3-*N* methylspiperone. Interestingly in that same year, the first SPECT radioligand ^{123}I-QNB and the first measurement of presynaptic dopamine ^{18}F-fluorodopa were developed as well. Since then, a myriad of radioligands has been developed for multiple dopamine and serotonin subtypes and other neuroreceptor transmitter systems. It is this rich number of radioligands that has allowed many of the clinical applications which are described later in this chapter.

Neuroreceptor ligands for SPECT. Neuroreceptor ligands make neuroreceptor imaging available, due to their properties of selectivity, affinity and reversibility of receptor binding. Therefore, the receptor density and dynamic measurement of neurotransmission can be obtained non-invasively, which helps us to understand neuropsychiatric conditions. Currently, the commonly used neuroreceptor ligands for SPECT are *Iodine-123-QNB, Iodine-123-IBZM, Iodine-123-epidepride.*

Iodine-123-QNB (quinuclidinyl benzilate) is a ligand for the cerebral postsynaptic acetylcholine muscarinic receptor, and has been used to image these receptors in the brains of normal subjects and patients with Alzheimer's disease [66–68]. Both *Iodine-123-IBZM* and *Iodine-123-epidepride* have been developed for dopamine D_2 receptor imaging and studies showed that I-123-epedepride is superior to I-123-IBZM [69, 70], and may prove useful in studies of Parkinson's disease and other movement disorders as well as in schizophrenia. Iodine-123 labeled ligands have been used for imaging the serotonin receptors, such as *I-123-5-I-R91150* [71, 72] for 5-HT_2A receptor and *I-123-beta-CIT* [73] for serotonin transporter. These research radiotracers, however, have not yet reached the stage of routine clinical practice.

^{99m}Tc- labeled imaging agents: ^{99m}Tc has a suitable energy and half-life, is readily available and is suitable for large-scale clinical use. *^{99m}Tc-TRODAT-1* is a recently developed radiotracer that selectively binds to the dopamine transporters (DAT) [74, 75], and has been used for studies of Parkinson's disease and attention deficit syndrome [76, 77]. A study has shown that it is possible to synthesize a ^{99m}Tc-based ligand for the 5-HT_{2A} receptor [78]. More recently, SPECT radioligands for the nicotinic cholinergic system have been developed (Fig. 58-8).

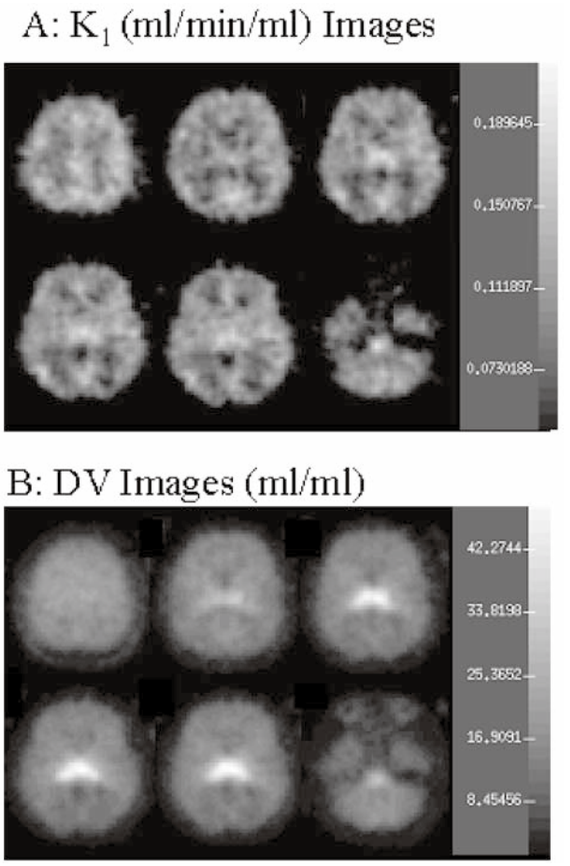

FIGURE 58-8 (**A**) SPECT parametric images of K_1 (ml/min/min), the transport rate constant from plasma component to the brain. (**B**) Distribution volume (DV(ml/ml)), the ratio of brain activity to plasma at steady-state estimated from a healthy control subject using dynamic ^{123}I-5-I-A-85380 (^{123}I-5-I-A) SPECT study. (From Zhou, Y., Brasic J. R., Musachio J. L. *et al.* [^{123}I]5-A-85380 dynamic SPECT studies in normals: kinetic analysis and parametric imaging. *Nuclear Science Symposium Conference Record*, Institute of Electrical and Electronics Engineers (IEEE), Inc. 2001; 3, pp. 1335–1340.)

CNS DRUG DESIGN AND PRECLINICAL/CLINICAL DRUG DEVELOPMENT

It is now possible to image not only postsynaptic, but presynaptic and intrasynaptic neurotransmission (Fig. 58-5). Presynaptic sites, such as the dopamine transporter and the serotonin transporter; the presynaptic dopamine vesicular transporter (VMAT-2) and the acetylcholine transporter; extrasynaptic sites such as the enzymes which break down neurotransmitters, e.g. MAO A and MAO B; with radioligands that bind to post or pre-synaptic sites, i.e. dopamine competing with radioligands such as ^{11}C raclopride (see Fig. 58-9) (PET (Fig. 58-10) can be measured under basal conditions or following drugs which either decrease (e.g. AMPT) or increase (e.g. intravenous amphetamine) intrasynaptic dopamine.

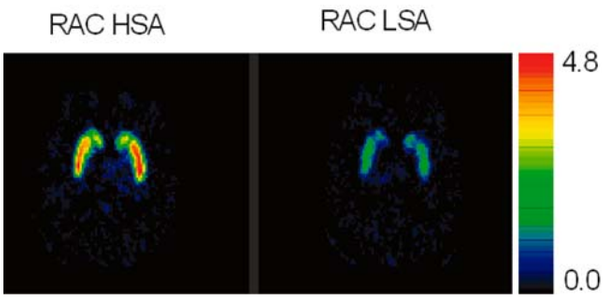

FIGURE 58-9 These images are generated from a healthy normal control subject using high-specificity activity (HSA) ^{11}C-raclopride (left) and low-specificity activity (LSA) ^{11}C-raclopride (right) by linear regression with spatial constraint method to estimate binding potential, as per the method of Zhou Y. *et al.* Linear regression with spatial constraint to generate parametric images of ligand-receptor dynamic PET studies with a simplified reference tissue model. *Neuroimage* 18: 975–989, 2003.

The myriad number of sites that can now be imaged has led to applications in multiple disorders, with the ability to study multiple components of these disorders. Some of the most important applications have included looking at the pathophysiology of disorders by examining abnormalities compared to healthy controls, and monitoring disease progression without or with medication. The most striking examples of this have included schizophrenia or abnormalities of multiple sites [79]; substance abuse [80] including cocaine and methamphetamine abuse; and Parkinson's disease [81, 82]. Thus, for example, in the case of schizophrenia, evidence that may be integrated with both chronic and phasic dopamine changes originally described by electrophysiologically processes might in fact explain finding elevated D_2 receptors and increased dopa decarboxylase measurements with ^{18}F-fluorodopa, and elevation of intrasynaptic dopamine challenge following psychostimulants such as amphetamine. Conversely, a number of studies in chronic cocaine users have shown decreases in intrasynaptic dopamine as well as D_2 receptor binding and density. Remarkable changes have been observed by measuring the dopamine transporter in Parkinson's disease, which may precede severe clinical symptoms, and this is now being

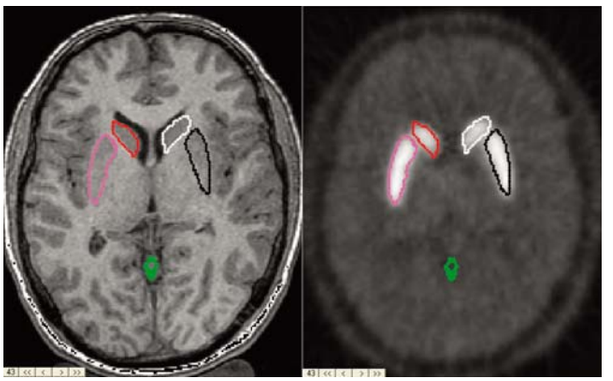

FIGURE 58-10 This image shows the volume of interest (VOI) drawn on MRI (left) matches the VOI on the PET scan (right) with ^{11}C-rclopride using MatLab program.

actively used to monitor other major neuropsychiatric disorders such as depression [83] and dementia.

The site of drug action and receptor distribution are being investigated. An additional application in studying neurochemical changes and their relationship to treatment include the use of neuroreceptor imaging for drug design and preclinical drug development. Therefore, such applications for use in CNS drug design include:

1. Radiolabeling of a candidate drug to observe its distribution within brain;
2. Determining the underlying rationale for studying a neuroreceptor system, e.g. the dopamine hypothesis for cocaine abuse and schizophrenia;
3. Understanding the mechanism of action of various drugs, especially those that act on neuroreceptors systems;
4. Guidance for therapeutic drug dosing in patient studies by estimating receptor occupancy preclinically or in normal volunteers [94].

We will briefly elaborate on the last two of these applications in our evolving understanding of actions of drugs and rational drug dosing.

The mechanism of drug action may be elucidated by studying its binding. An example of mechanism of action is the study of the dopamine transporter inhibitor GBR12909 as a possible drug for substance abuse and psychostimulant abuse. It was suggested by results from microdialysis studies in animals that this drug would increase basal dopamine but inhibit psychostimulant dopamine release. The drug was subsequently studied in a nonhuman primate PET study by Villemagne *et al.* [95]. Here the reduction in binding of the radiotracer [^{11}C] raclopride was diminished following administration of the drug GBR12909, while the basal levels of dopamine increased. Therefore, this drug could potentially be used to treat psychostimulant abuse, but reduce cocaine craving or amphetamine craving.

Drug dosing is estimated based on proportions of receptor occupancy by the drug. PET and SPECT neuroreceptor imaging now has a major role in helping determine what is the most likely therapeutic dose, and what dose should be used in clinical trials of drugs whose action is receptor-mediated. Examples of such drugs include anxiolytics, antipsychotics, some antidepressants acting through the serotonin transporters, and anxiolytics acting through various receptor systems, such as NK1 and GABA. It has been shown for antipsychotics as well as for a number of antidepressants, that 60–80% receptor antagonism of the dopamine receptors or of the serotonin transporters is associated with therapeutic plasma levels of the particular drug [96]. Hence the receptor occupancy plotted against plasma levels of parent drug showing the 'window of therapy zone' can be derived in nonhuman primate studies and normal volunteers to guide dosing in patient populations and early Phase 1 studies with normal volunteers for alternate FDA approval. Such studies not only are of great economic value, by leading to cost saving, but are of great clinical importance in saving needless overdosing or underdosing of human subjects when developing clinical trials. This principle of using imaging as a biomarker or surrogate marker has the most important role in imaging of receptors.

CLINICAL APPLICATIONS OF PET AND SPECT

The metabolic features of brain tumors can be imaged using PET. These measurable features include glucose metabolism, blood flow and oxygen consumption. FDG-PET can be used to monitor the rate of glucose uptake and level of hexokinase gene expression throughout the body, and has been widely used as a surrogate marker [31]. FDG is extensively utilized in PET neuroimaging. FDG PET imaging helps to identify malignant brain tumors as the increased glucose metabolism of the malignancy is reflected in the PET image. The co-registration of the PET imaging with the MRI (or CT) helps to give information on the prognosis. PET images co-registered with MRI (or, more recently, using combined PET and CT, i.e. PET/CT scanners [97] are also used to test for the presence of residual and recurrent tumors afters surgery and to monitor patients for degeneration of slowly growing, low grade tumors into rapidly growing, high grade malignancies. [97a, 98].

Brain infarction was the first clinical application of SPECT. Decreases in relative cerebral perfusion were imaged by SPECT for diagnosis. Decreased rCBF is visualized in the form of decreased signal on SPECT and PET. The sensitivity and specificity of brain SPECT for infarct localization are 85.5% and 97.6%, respectively [99]. Blood-flow imaging is useful in the evaluation of response to therapy in patients with cerebrovascular diseases [100].

PET scans with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) have an established role in the localization of epileptic foci in patients being evaluated for epilepsy surgery. Epilepsy is one of the most common neurological disorders, which is a clinical condition of recurrent seizures, resulting from excessive, uncontrolled electrical activity in the brain [101, 102] (see Ch. 37). With recent advances in neuroimaging, management of the drug-resistant focal epilepsy now increasingly relies on neurosurgery to resect the 'epileptic focus'. FDG-PET scans between seizures (interictal) is the more common approach. The region of hypometabolism was also the location of the recorded electronic seizure onset [103]. The decision regarding seizure surgery depends on a concordance of data from

multiple investigations, including video EEG, neuropsychological tests, MRI, ictal and interictal SPECT and PET. FDG-PET may also be useful in directing placement of intracranial electrodes for presurgical evaluation of refractory seizures.

Correlations of cognitive functions with PET or SPECT imaging are under investigation. Regional brain function is tightly coupled to regional energy metabolism and regional cerebral blood-flow (rCBF), and brain energy is normally produced by glucose metabolism [104, 105] (see Ch. 31). PET and SPECT have the ability to noninvasively image regional cerebral energy metabolism and/or blood flow, to allow localization of cerebral function in humans by using different radiotracers. For example, ^{18}F-FDG PET allows the determination of regional cerebral glucose ($rCMR_{glu}$) metabolism and function in human brain [106, 107], and ^{15}O-H_2O PET and ^{99m}Tc-HMPAO SPECT can determine rCBF [111].

Close relationships between the mean CBF and cognitive function have been observed [108]. For example, in patients with Parkinson's disease and Alzheimer's disease, their cognitive function impairment is significantly negatively correlated with rCBF [109–111].

PET and SPECT imaging are used to study Parkinson's and Alzheimer's diseases.

Parkinson's disease (PD) is a progressive disorder consisting of a variable combination of symptoms of tremor, rigidity, postural imbalance and bradykinesia[112, 113]. It causes disability in most individuals that cannot be completely controlled with available medications (see Ch. 46). Current pharmacologic treatments for PD are all symptomatic, and aimed at either restoring dopaminergic tone or blocking excessive glutamatergic or cholinergic activity. Neuroimaging techniques developed in the past decades have greatly aided in the diagnosis of PD by measuring residual presynaptic dopamine cell markers [114]. More importantly, today's neuroimaging can provide insights into the natural history of PD.

SPECT and PET are both established modalities for monitoring disease progression and providing the rationale for testing new drugs as putative neuroprotective or neurorestorative agents. The radioligands which target dopaminergic system for both SPECT and PET have been widely used to evaluate patients with PD, including Iodine-123-epidepride, ^{18}F-dopa, ^{11}C-VMAT2, and dopamine transporter ligands [115–120]. Studies with Iodine-123-β CIT SPECT and ^{18}F-DOPA PET demonstrated that at the time of emergence of PD symptoms there is approximately 40–60% reduction of radiotracer uptake in the striatum and putamen [121–123].

Alzheimer's Disease (AD) is the most important and most common terminal disease in ageing populations [124, 125] (see Ch. 47). Its prevalence is continuing to increase with increased human life expectancy. Mental degeneration is insidious, and progressive memory loss is the most important symptom. The current treatment options remain limited, so early diagnosis is now a key problem with this disease, since new therapeutic agents are likely to be more effective when given early in the course of the disease [126].

Brain SPECT of AD patients typically shows bilateral posterior temporal and parietal hypoperfusion. The areas of reduced perfusion are secondary to the reduced brain metabolism in areas of neuronal depletion. This scintigraphic pattern of bilateral decreased posterior parietal–temporal perfusion has a predictive value of greater than 80% for AD [63]. The sensitivity and specificity of brain SPECT for the diagnosis of AD are 86% and 96%, respectively [127].

AD also produces a typical pattern of glucose metabolic derangement on FDG PET scans, which consists of selective hypometabolism in the posterior–parietal and temporal lobes [128]. The sensitivity and specificity of the visual assessment of PET has recently been evaluated in 284 patients with dementia, 146 of whom had a 2-year follow-up, and 138 had pathological confirmation [129]. A most promising approach is radiolabeled dyes that bind to amyloid plaques, one of the pathological signs of AD. If developed fully it promises potentially earlier detection and monitoring of AD [130].

APPENDIX

Brief history of medical imaging. From the first discovery of X-rays by Wilhelm Roentgen in 1895, medical neuroimaging has progressed rapidly over the last century as an invaluable tool for the detection, characterization and treatment of countless neuropathological and neuropsychiatric disorders. Medical research has also embraced the fascinating science of medical neuroimaging as a powerful tool to probe the inner workings of the brain on a biological, biochemical and biophysical level. Indeed, the X-ray soon produced the first image, leading to the eventual development of modern X-ray computed tomography (X-ray CT) [1, 2] Computer-assisted tomographic image reconstruction, in which a two-dimensional image 'slice' through the object is created from a series of multi-angle one-dimensional image 'projections', was originally utilized in other well-known imaging modalities, which came to be know as Positron Emission Tomography (PET) and Single-Photon Computer-Assisted Tomography (SPECT) [3]. Unlike X-ray CT, in which a beam of X-rays from an external source are passed through the object and attenuation data recorded, PET and SPECT record the emission of photons originating from within the object, introduced via a decaying radioisotope, from multiple angles around the object. Whereas X-ray CT is very useful in providing static attenuation information about internal structures, and is the standard in any well-equipped hospital, PET

and SPECT provide information pertaining to the internal distribution of the radioactive label, therefore giving these imaging modalities the ability to trace and record the uptake and metabolism of the radiolabeled biochemicals *in vivo*. This functional aspect of PET and SPECT makes them an invaluable probe for investigating the workings of many biological and biochemical systems *in vivo*.

In the 1930s, two physicists working independently reported that certain nuclei placed in a magnetic field absorbed energy transmitted at their specific precessional frequency in the field, and emitted energy when they were allowed to return to their original energy state. In 1952 Felix Bloch and Edward Purcell were awarded the Nobel Prize in Physics for their discovery, giving birth to magnetic resonance spectroscopy [4, 5]. Soon, it was discovered that several species of nuclei in magnetic fields were sensitive to perturbation by radiofrequency (NMR-visible), and that the signal they emitted in return contained detailed information on the structure and composition of the molecules in which the nuclei were contained. Compounds, organic and inorganic alike, had a unique spectral signature, and the signals from hydrogen, phosphorus carbon and other biologically prevalent nuclei in these compounds each had distinct properties and characteristics. With this powerful tool, known commonly as nuclear magnetic resonance spectroscopy (NMRS), scientists could begin to investigate the *in vivo* biochemical workings of live specimens. In the 1970s, a physician from Brooklyn proposed to use NMR as a tool for characterizing and distinguishing different tissue pathologies in medical diagnosis [6]. At approximately the same time, a professor of chemistry in New York published the first water-proton magnetic resonance image of two water-filled tubes, work that would eventually earn Paul Lauterbur the Nobel Prize in physics [7]. This new medical imaging modality would come to be known as magnetic resonance imaging (MRI), and would prove to become one of the most powerful medical and research tools of the modern age. With Damadian's proposal of pathological characterization of tissue types based on their NMR properties, combined with modern image acquisition/reconstruction technology, MRI remains at the forefront of modern medical imaging.

Technical developments in MRI have since spawned numerous other breakthroughs in the field of NMR. In the early 1980s, it was demonstrated that a spatial image of the complete spectrum of a sample could be created, by slightly altering the acquisition and reconstruction parameters of the established MRI experiment [8]. Unlike conventional MRI, which relies on the magnitude of the water resonance signal to create an image, magnetic resonance spectroscopic imaging (MRSI) combines spectroscopy with imaging, and has since become a staple of modern MRS research. Magnetic resonance imaging finally entered the functional realm in the late 1980s, when it was discovered that the iron content of hemoglobin in blood caused enough of a distortion of the local magnetic field, that the resultant signal fluctuation could be detected in an MR image [9]. This effect, known as the BOLD (blood-oxygenation-level dependent) effect is most apparent when the oxygenated hemoglobin content of the blood in a given region changes over time, causing a proportional change in image intensity. With the advent of rapid imaging techniques, functional magnetic resonance imaging (fMRI) can record the transient activation and inactivation of brain regions that occur during the execution of a given task, be it motor, cognitive or emotional.

Basic principles of MRI. The NMR signal in magnetic resonance imaging originates from the rapidly precessing hydrogen nuclei in the sample when they are placed in the main, static magnetic field (B_0). Each nucleus can be thought of as a tiny bar magnet with two magnetic poles, therefore each having a magnetic moment or vector. Any nucleus within the sample can exist in two distinct energy states, corresponding to the direction of its magnetic moment (low-energy), or opposite to the direction of the main magnetic field vector (high-energy). In a sample with many nuclei, there will always be a population of nuclei in the high-energy state, and another population in the low-energy state. In the presence of the main magnetic field, there will be an excess of nuclei forced into the high-energy state, thus giving rise to a net magnetic moment to some of all nuclei in the entire sample. It is this excess of high-energy nuclei (approximately one nucleus in a million) that gives rise to the observed NMR signal. Each hydrogen nucleus precesses (similar to a top) about the main magnetic field vector at a specific frequency, determined by the strength of the main field (measured in Tesla) and an inherent property of the nucleus called the gyromagnetic constant (γ). For hydrogen, γ is 42.577 megahertz per Tesla (MHz/T) and the nucleus would therefore precess at 4T field strength, for example, at 170.3 MHz (170,300,000 precessions per second). The precessional frequency, of a nucleus as it relates to main field strength and gyromagnetic constant is known as the Larmor frequency, as described by the equation, ω - γB_0, where ω is the Larmor frequency [5]. In order to extract a measurable signal from the sample, energy must be transmitted into the sample by way of a radiofrequency (RF) pulse delivered at the Larmor, or resonant, frequency of the hydrogen nucleus. Once the RF pulse is terminated, the system of nuclei returns back to its lower-energy state, emitting a small but detectable RF signal that can be recorded. It is in this signal that detailed spatial and spectral information is contained that can be extracted to form a MRI image. In MRI, spatial information about the sample is introduced into the NMR signal with the application of magnetic field gradients in three dimensions. The gradients, which vary linearly in space in three dimensions, cause a known and momentary linear grade of the main magnetic field.

In one spatial dimension (usually denoted as the 'slice-select' direction), a selective RF pulse with a certain

resonant frequency and bandwidth perturbs only those nuclei precessing within the specified frequency range of the pulse. By varying the center frequency of the RF pulse and either the bandwidth of the RF pulse, or the magnitude of the applied gradient, one can adjust the spatial position and thickness of the planar image 'slice', containing the perturbed nuclei. In addition to the slice-selective gradient, another gradient is applied in one of the planar dimensions, usually denoted as the 'phase-encode' gradient, and is stepped incrementally in magnitude with a predetermined step-size, range and number which determine the spatial field-of-view (FOV) that will be sampled in the MR image, as well as the degree of spatial resolution (the ability to delineate separate structures from each other) that will be obtained in the final MR image.

The application of this series of phase-encode gradients links the resultant phase of each nucleus with its position in space. While the NMR signal after each gradient step is digitally recorded, a third gradient is simultaneously applied in the perpendicular planar dimension ('readout-gradient') with a pre-determined and fixed time of duration and strength, again, dictating the FOV and spatial resolution of the MRI image sampled in that dimension. Although applied in different manners, the readout and phase-encode gradients achieve identical end-results. Throughout this entire (and repetitive) procedure, the recorded digital NMR signals are entered into a data matrix known as 'k-space'. This recorded data set contains all of the spectral and spatial phase information needed to construct the final MR image. The sample has, in essence, been discretely sampled into its individual spatial frequency components.

Structures at given locations within the sample are now each described as an oscillating signal with a unique amplitude, phase and frequency in k-space. A mathematical operation called a fast Fourier transform, performed on the k-space matrix, extracts the original amplitude and location of each structure within the sample, representing the original structure as a rectilinear, two-dimensional matrix of discrete elements, or pixels (picture–elements), each varying in gray-scale intensity depending on the amount of signal at that spatial location. Standard clinical and research MR images are usually described as 128 or 256 pixels per dimension, and achieve submillimeter pixel resolution. At this resolution, fine structures such as cerebellar striations or small vessels can be resolved in the brain.

Basic principles of MRS. The overall physical principles and characteristics of magnetic resonance spectroscopy (MRS) are identical to those described previously in the MRI section. In fact, magnetic resonance spectroscopy can simply be thought of as just another way of expressing the NMR signals that are recorded during an NMR experiment. Whether it is an MRI or and MRS experiment, virtually all of the same equipment is used and all of the basic NMR principles still apply. The prime difference that separates basic MRS from modern-day MRI is that in MRS, the entire NMR signal is recorded in the absence of a readout gradient (although in more-recent rapid MRSI experiments, spectral/spatial encoding gradients are applied during NMR signal readout). However, in MRS (unlike MRI), some aspects of the NMR signal are much more emphasized and are of extreme importance in acquiring clinically or experimentally relevant MRS data.

In MRS, the NMR signals of interest are typically very small, and in some cases are difficult to differentiate from the ambient noise in the recorded NMR signal. For example, the metabolites that are measured with hydrogen spectroscopy are approximately 1/10,000 the strength, or amplitude, of the hydrogen water signal that is recorded in standard MRI (see **Fig. 58-2**). The NMR signals in MRS of other nuclei (i.e. phosphorus) are even less sensitive and more susceptible to corruption by ambient NMR noise. Signal-to-noise ratio (S/N), the ratio of measurable NMR signal over the baseline NMR noise, is therefore a fundamental and limiting parameter of the quality and measurability of the signal in MRS. Modern-day MR systems are usually equipped with specialized hardware that is extremely sensitive to the weak signals recorded in MRS, for maximum S/N. Aside from very low *in vivo* concentrations and inherent nuclear properties, other prime factors that greatly influence the S/N in MRS are the T1, T2 and T2* parameters described previously. Magnetic resonance spectroscopy protocols must therefore be very fine-tuned to the specific nucleus and metabolites under study, as well as the target-organ and pathology. **(Table 58-1.)**

In MRI an image is produced that allows clear visual examination of the structures and/or pathology of interest, thus making it an invaluable tool in radiology. However, in diagnostic imaging, a thorough visual examination by a trained radiologist usually suffices, with little or no attempt at actually quantifying the data. In MRS, quantifying the data and obtaining a hard measurement of metabolite concentrations is the ultimate goal. When the recorded NMR signal is Fourier transformed in MRS, a spectrum results, representing a series of resonant lines, or 'peaks' across a spectral bandwidth (expressed in Hz or ppm) where these spectral lines represent different biomolecules in the sample. Magnetic resonance spectroscopy is based on a phenomenon known as 'chemical-shift', in which the surrounding electronic environment about a nucleus within a molecule acts to partially shield the nucleus from the main magnetic field. This shielding effect results in a very slight change in the local magnetic field that the nucleus experiences, thus causing it to precess at a slightly different frequency. Nuclei contained in different molecules will each have precessional frequencies that are slightly different from one another, manifested as separate spectral lines. A single molecule can have multiple resonant lines, depending on its composition, structure and another phenomenon called *J-coupling*, which refers to the influence that exists between neighboring nuclei, affecting their resonant line patterns. When many biomolecules exist in the sample, as is the case *in vivo*, a very complicated

spectrum of overlapping resonant lines results, making it very difficult to assign any given peak to its corresponding biomolecule, as well as quantifying the area under each overlapping peak. It is for this reason that scientists have strived to work at higher magnetic field strengths, since both S/N and spectral resolution (the ability to resolve overlapping resonant lines) are increased at high field. By deriving the area under each spectral line and correcting for the many factors affecting this area, the concentrations of the corresponding biomolecules in the sample can be calculated (Figs 58-2, 58-3). Spectral quantitation is an active area of research and many complex strategies and algorithms have been developed to perform this difficult task. However, they will not be discussed here.

The ability to spatially localize the NMR signal to a specific structure within a sample is of utmost importance in MRS. Spatial localization in MRS can be separated into two categories, single-voxel MRS and multivoxel MRS, each having its own set of advantages and disadvantages. In single-voxel MRS, a voxel (volume–element) of chosen dimensions is placed over the region of interest, guided by MR images. The MRS sequence parameters are then optimized for this specific location and the NMR signal recorded. Like the slice-selective gradient and RF pulse previously described, the single MRS voxel is defined in three dimensions, using a combination of orthogonal and intersecting slice-selective gradients and RF pulses. The advantages of this technique (there are many methods to achieve a single voxel) are the ability to optimize all of the MRS sequence parameters for this location, resulting in maximum spectra quality. The disadvantage is clear, being limited only to one or two fixed locations per scan session. In multivoxel MRS, the advantage is more information collected per scan session (Fig. 58-4). In spectroscopic imaging (MRSI), another main advantage is the ability to reposition voxels indefinitely after data collection. Spectroscopic imaging, which can be done in one, two or three dimensions, uses a combination of slice-selective and phase-encoding gradients to collect k-space and Fourier transform to localize the matrix of voxels. The disadvantage is the relative inability to optimize the MRS sequence parameters over the entire area of interest, a problem mainly due to imperfect RF coil and magnetic field homogeneity, and the rather poor voxel definition resulting from the discrete nature of the digital sampling and Fourier transform method of localization.

PET versus SPECT: similarities and contrasts. In research, PET and SPECT have very complementary roles at times. SPECT, the commonly used tool in all of nuclear medicine, primarily uses external imaged isotopes ^{123}I or ^{99m}Tc (energies of 159 and 140 (keV); half lives of 13 h and 6 h, respectively), and to a lesser extent ^{131}I (89% 191 keV and electrons of multiple energies; and half life of 8 days) as well as ^{111}In) (Table 58-2). For research purposes primarily ^{123}I and ^{99m}Tc are used. Each emits a single gamma ray, which is externally imaged using light-emitting crystals which are then detected and passed on as the radioactive signal. By rotating these crystals relative to the emissions, three-dimensional pictures can be obtained. Both ^{123}I and ^{99m}Tc are available commercially throughout the world, although the latter is more routinely available since it is available as a generator, whereas the former is typically cyclotron-produced and distributed from one or two sites per country or numbers of countries as a unit dose.

Positron emission tomography, in contrast, uses radioisotope specifically ^{11}C, ^{18}F, ^{18}O or ^{13}N. All of these have much shorter half-lives typically from two minutes up to 110 minutes. Because of the short half-life and the nature of the radioisotopic production, they must be made by cyclotron, which is either onsite or within a few miles of the location of the imaging camera (i.e. the PET camera). The production of three-dimensional images is somewhat different with PET compared to SPECT. For all of these positron emitting isotopes, the positron travels only a few millimeters (depending on each isotope) when it encounters negative electrons (the electrons that populate all living tissue), whereupon the two particles annihilate. This results in two gamma-rays, each 511 keV in energy (because of conservation of energy), and at almost 180° to each other (based on conservation of momentum). The angle of the degree to which it is not 180° relates to whether the electron or positron has some finite kinetic energy, i.e. whether they are moving very slowly or quickly relative to one another at the time of self-annihilation, in order to satisfy the conservation of momentum. These two gamma-rays, both traveling at the speed of light, as all electromagnetic radiation does, interact with detectors at approximately 180° to each other. Detectors, which measure millions of these operations at two opposing sides within a certain time window, detects each event as coming from a location within the center, e.g. the brain. After many of these processes are obtained and processed, three-dimensional images can be obtained.

At present, most PET scanners can acquire in both a two-dimensional as well as a three-dimensional mode, whereas SPECT cameras measure in a three-dimensional mode. The physical property of the dual-positron gamma-rays emission lends itself to mathematical reconstruction algorithms to produce three-dimensional images in which the calculations are much closer to exact theoretical ones than those of SPECT. This is, in part, due to the two-photon as opposed to single-photon approach. PET can now achieve resolutions, for example in animal-dedicated scanners, in the order of 1 or 2 mm. The resolution is inherently limited theoretically only by the mean free path or distance in which the positron travels before it annihilates with an electron, e.g. those in biological water 2–8 mm. SPECT, although achieving millimeter resolution with the appropriate instrumentation, cannot quite achieve these levels.

Advantages and disadvantages of PET and SPECT.
Positron emission tomography. The half-life of the isotopes also makes a difference to their application.

For example, the short half-lives of ^{15}O and ^{11}C allow multiple repeat studies within a few minutes to hours. This allows measurement of behavioral or cognitive changes (this was the original approach prior to the fMRI) as well as effects of drugs on neurotransmitter levels and occupancy, all within the same few hours or days. Even ^{18}F allows repeat studies within a few hours, whereas with SPECT imaging, at least one or two or more days are required if a drug perturbation, etc. is employed, compared to baseline, because of the interference of the prior study's radioactivity. The longer half-life of SPECT compounds, however, has several advantages. As described in the kinetic modeling section below, this allows the quantification to be simplified if the radiotracer reaches or approaches a quasi steady-state (i.e. the radioactivity in brain areas of interest compared to other brain areas or plasma is not changing). Hence, SPECT radiotracers often precede or may be preferred for kinetic reasons.

Radiopharmaceutical production: PET versus SPECT. Perhaps the biggest difference between PET and SPECT, however, exists in the variety of radiochemical processes and radiopharmaceuticals that can be developed. PET has much greater versatility, because the radioactive atoms are already smaller and much more common in biological molecules. In particular ^{11}C is often successfully employed to isotopically replace (replacing a stable ^{12}C for radioactive ^{11}C) without any modification of the chemical or pharmacological properties of the drug. Hence, it is possible to then image a radiopharmaceutical of a drug (e.g. ^{11}C cocaine for dopamine transporters or ^{11}C methylphenidate for dopamine transporter or ^{11}C MDL100907 for $5HT_{2A}$ receptors) as if one is actually imaging the native drug itself that is nonradioactive. This has tremendous power in fully understanding the pharmacokinetics and dynamics of the radiopharmaceutical, and extrapolating immediately to its unlabeled counterpart, even at therapeutic levels. Other substitutions, such as ^{11}C methylations or propylations or other alkylations, may make only moderate modifications to the pharmacology, such as changing the lipophilicity, etc. Similar to ^{18}F as an addition to a biological molecule of interest (typically around 400 molecular weight) may not cause major changes, because again its molecular weight is relatively small. So both ^{11}C and ^{18}F (the latter used in the radiolabeling of deoxyglucose, a precursor to the hexokinase step to measure regional cerebral glucose metabolism) or ^{18}F fluorodopa to measure dopa decarboxylase activity have been very successful in CNS research, and more recently in clinical applications (see below). Hence, many biological molecules, especially new CNS drugs such as antipsychotics (e.g. D_2/D_3 radioligand ^{11}C raclopride) or derivatives of neuroleptics (^{11}C-*N*-methylspiperone, a methylated derivative of spiperone, an early antipsychotic) or ^{11}C-chlorpromazine or ^{18}F-haloperidol are all examples of isotopic substitutions of drugs that bind to D_2 receptors, and have been used in the treatment of schizophrenia and other psychoses.

For SPECT, the radiolabeling with ^{123}I, ^{131}I, and ^{99m}Tc is more complex, especially for the latter where special chelates are needed to shield the charged portions of the radiopharmaceutical, which would prohibit passage across the blood–brain barrier. For example, there are no SPECT ligands for oxygen or glucose metabolism. Since both ^{123}I, ^{131}I and ^{99m}Tc are all relatively large atoms compared to PET isotopes, ^{11}C, ^{18}F, ^{13}N and ^{15}O preservation of pharmacologic properties is also difficult, but has been achieved. Indeed, measurements of the dopamine transporter, D_2/D_3, serotonin receptor (e.g. $^{123,125}I$-IodoLSD) and other radioligands have been employed for dopamine, serotonin and ^{123}I-QNB for the muscarinic cholinergic system have been successfully employed in animal and human imaging. Their long-lived properties also help to improve image quality and imaging counting statistics, since radioactivity is a statistical event requiring sufficient time to create an image.

Furthermore, some of these PET and SPECT ligands have been translated back to autoradiograms, such as ^{125}I ligands of SPECT compounds, where the β decay is detected in the film emulsions. It is of interest that, historically, *in vivo* autoradiography led to much of the imaging work, in particular both FDG and receptor imaging, so that in some ways, *in vivo* autoradiography has been extended to PET and SPECT external imaging, but to the latter with much more flexibility. In the case of autoradiography, even if the animals are injected *in vivo*, they are eventually sacrificed and slices are then incubated with photographic film to visualize the brain structures. With PET and SPECT, nonhuman primates, humans and even rodents are imaged over and over again to allow the measurement of disease progression, treatment effect and drug perturbations; hence, it is a powerful tool for these procedures.

Strengths and limitations of PET/SPECT imaging for neurochemical applications. Because so much of neurochemistry involved tracing biochemical processes, the tracer principle, which embodies these methods, starting with the first studies of fluorodeoxyglucose and glucose metabolism and continuing with receptor and second messenger imaging, is indeed the strength of these techniques. Some inherent weaknesses, however, are described below.

Finite resolution and partial volume effects. Although this can occur in other areas of imaging such as MRS, it is particularly an issue for SPECT and PET because of the finite resolution of the imaging instruments. Resolution is typically imaged as the response of the detector crystal and associated electron to the point or line source. These peak in the center and fall off from a point source, for example, in shapes that simulate Gaussian curves. These are measures of the ability to resolve two points, e.g. two structures in a brain. Because brain structures, in particular, are often smaller than the FWHM for PET or SPECT, the radioactivity measured in these areas is underestimated both by its small size (known as the partial volume effect), but also spillover from adjacent radioactivity

from other regions which cannot be resolved from the region of interest. Hence, very small structures of the brain, such as the head of the caudate, or even smaller, such as the choroid plexus, suffer from this. However, new methodology is being developed to make these corrections. So far, many neuroscience advances have been made without these corrections, because the changes are relative between control states and treatment states.

Relative risk of ionizing radiation. Another inherent difference between PET/SPECT and MRI/MRS is that the former is referred to as ionizing radiation. Indeed, there is a greater risk associated with potential DNA, chromosome or tissue damage, but the radioactivity levels which are employed, especially for human studies, are below those that even occupationally-exposed workers are allowed to receive, and are generally considered to be of only moderate risk. Indeed, we still are not totally certain of the effects of other radiological procedures such as high field MRI (which can still potentially cause molecular excitations and changes with unknown long-term effects) or ultrasound (which can cause heating). In general all of these radiological procedures, when carefully weighed against the potential scientific benefits, are considered relatively low risk, which is why these methods have become so widely used.

Imaging equipment. Either PET or SPECT consists of rings of crystals, such as sodium iodide or other crystals (see Table 58-2) in which the gamma-radiation interacts, and the subsequent reaction results in light being emitted, which is then captured by photoelectric tubes. These transmit electrical impulses, which are detected either singly as in SPECT, or in coincidence as in PET. Additional factors that have to be considered in the quantification of these procedures include: *scatter*, which occurs with both PET and SPECT; *random* coincidences, which means two detectors going off at 180° to one another that do not originate from the same positron–electron interaction, and therefore should not be counted; and degradations in resolution due to small angular differences; and even differences in the time of travel (for example, in PET, for one of the two annihilation photons arriving at the detectors at different times). Some (such as randoms), can be corrected computationally, but others (such as scatter) are more complex, depending on the organ imaged. In fact, some instruments, although not altogether successful, have attempted to track the time of flight for one photon compared to another, both traveling at the speed of light. Another potential error is the depth of interaction — that is, precisely where the photon interacts within the crystal, so corrections for this are also being made at higher, more technical scanners.

Finally, newer scanners are being developed with both PET and another modality, such as computed tomography (X-ray CT), where a subject would be scanned first with X-ray CT for anatomy and for attenuation correction, and then imaged for their PET or SPECT measurements. Attenuation correction is necessary, because the photons, either from PET or SPECT which emerge from the body, are deflected or undergo interaction or reductions in energy as they emit from the center of the body. Hence, even a cylinder filled uniformly with radioactivity would exhibit the appearance of less activity in the center of the cylinder than at the edges, because of this attenuation effect. Corrections can be made in a number of ways including correcting using the known attenuation coefficients for water (known as calculated attenuation) or using a radioactive source which rotates around the source (typically used in PET with the germanium source) or with x-ray CT for attenuation values. All of these boost the signals, which are deeper in a human structure such as the center of the brain, to correct for this attenuation; this is largely successful and allows for an essentially uniform image when radioactivity is uniform.

New PET scanners are being developed with increasingly efficient electronics, but especially new crystals for detecting the gamma rays. These detector materials may have higher sensitivity or quantification depending on a number of factors, including their density, their effective atomic number and the delay time from the interaction to the light emission which is ultimately measured in the electronic detectors. The original sodium iodide crystals are now being replaced by those with higher density (effective Z), and a much shorter delay time, such as bismuth germinate oxide (BGO), barium fluoride (Ba F_2), and, most recently, leutetium oxyortho silicate (LSO).

The resolution and sensitivity of an imaging device depend on a number of variables, ; for example, spatial resolution in SPECT is limited primarily by collimator resolution. Typical gamma camera SPECT systems have in-plane spatial resolution in the final reconstructed image of about 12 mm for high-resolution imaging. Resolution and sensitivity in PET is given in Table 58-3.

Fundamentals of radiotracer methodology.

Basic radiochemistry for PET, SPECT — Principles of labeling with radioisotopes and typical 'successful ligands'. *Typical validation for radiochemical and radiopharmaceutical purity.* Quality control is very important to ensure the safety and efficacy of radiopharmaceuticals. One important quality parameter is the radiochemical purity of the radiolabeled product. This is defined as the fraction of the total radioactivity in the desired chemical form in the radiopharmaceutical [56]. Radiochemical impurities come from incomplete labeling, shift of equilibrium, radiolysis (β decay), temperature or pH change, exposure to light,

TABLE 58-3 Performance characteristics for the current commercial clinical PET scanners

		2D	3D
Resolution FWHM (mm)			
	Axial	4.0–5.0	3.5–6.2
	Transaxial	4.8–6.2	4.8–6.3
System sensitivity (net trues) (cps/Bq/ml)		4.9– 5.4	21.1–31.0

and air oxidation. Examples of radiochemical impurity are free $^{99m}TcO_4^-$ and $^{99m}TcO_2$ in many ^{99m}Tc-labeled compounds. Radiochemical impurities in a given radiopharmaceutical can be tested using precipitation, thin-layer chromatography (TLC), paper and gel electrophoresis, ion exchange, mini columns or HPLC and distillation [57–59].

Validation of radiotracers. In vitro *versus* in vivo *rodent study.* Depending on the function being measured, the radiotracers must be further validated pharmacologically. For example, for neuroreceptor binding ligands, it must satisfy characteristics such as saturability, i.e. carrier added (unlabeled ligand) can compete with the radiotracer; stereo specificity (i.e. known unlabelled isomers with higher or lower affinity to the receptor of interest will block the radiotracer's uptake or block it less, respectively); pharmacologic specificity (i.e. unlabeled drugs will inhibit the radiotracer proportional to their binding affinity) and manipulation of receptor site (i.e. up- or down-regulation can be demonstrated by the radiotracer). These validation studies are typically carried out i*n vivo* in rodent and small animal studies first. Together with these validation studies, especially in the case of receptor tracers, metabolism of the radiopharmaceutical is needed. This is because if they cross the blood–brain barrier the radiolabeled metabolites of these radiotracers can obscure the imaging pattern quantified and hence degrade the quantification of the function being measured. Thus, not only the metabolites in whole blood or plasma are measured, but the absence of these metabolites (in labeled or unlabeled form) crossing the blood–brain barrier is an essential part of the validation studies.

Role of mathematical modeling for quantification — differences between simple tissue time–activity curve and model parameters with examples, e.g. FDG, glucose metabolism, blood flow and receptors, measures of binding potential and receptor density. Since PET or SPECT radiopharmaceuticals ideally depict physiological processes, like many biochemical processes, the underlying kinetics must be fully understood to interpret the imaging results. This, especially during the validation phase, begins with dynamic images to obtain the entire time course of the radiotracer. This is in addition to simply quantifying the varied regions of the brain images obtained, such as in an autoradiogram and in SPECT studies. Here is an important distinction between autoradiograms in *in vitro* studies and in *in vivo* imaging. In the former, conditions are typically obtained to place the radiotracer into conditions of equilibrium by such methods as temperature, mixing, etc. In an *in vivo* study, it is not possible to manipulate these factors, of course, so either radiotracers that reach a quasi-equilibrium are employed or those that do not are analyzed using mathematical modeling or kinetic modeling techniques. These latter procedures require, not only dynamic images of the radiotracer in brain over time, but also plasma samples of radioactivity to indicate the so-called 'input function' of the radiotracer prior to entering into brain. One of the most successful and earliest models were those for two-compartment modeling involving blood flow (Kety–Schmidt model) and the for glucose metabolism (Sokoloff–Phelps–Huang method). Indeed, the Sokoloff method originally employed ^{14}C-deoxyglucose measurements and autoradiograms in rodents, but also with arterial sampling to measure the radiotracer input function. This was later translated into PET imaging using ^{18}F-FDG and modeling, using first dynamic scanning and arterial inputs, and later simplified to single-scan 'parametric images' once some of the human rate constants were established.

To calculate these measures in PET/SPECT, typically two- or three-compartment models are employed. Such models have been the basis for extension into neuroreceptor imaging of the methods described below.

In all of these models, first dynamic measurements are entered into a series of mathematical equations where rate constants, just like those in biochemical equations, are derived. These rate constants are assigned biological meaning, such as hexokinase activity in the case of glucose metabolism or dopa-decarboxylase in the case of ^{18}F-fluorodopa measurements or binding to a receptor from a tissue pool to a tissue receptor area as in the case of D_2 dopamine receptor imaging. This type of compartmental modeling has many variations, and even has procedures (such as those involving not only bolus injections of the radiotracer, but also bolus plus constant infusion) to attempt to force the radiotracer into a equilibrium state to simplify the modeling. As in the example of receptor system, if such quasi-equilibrium conditions can exist, then simpler approaches to measuring receptor density and affinity, such as those in a Scatchard analysis can apply. In other cases where there is nonequilibrium, more sophisticated dynamic kinetic modeling is needed.

The ultimate goal of these models, of course, is to estimate parameters of interest, whether it is oxygen or glucose metabolism, receptor density, enzyme activity, etc. In some cases the imaging can be ultimately simplified into single maps, such as parameter maps of glucose metabolism or receptor density. In other cases these parameters can only be obtained from deriving them from the mathematical models. In any event, this represents a relatively unique aspect of neuroimaging, which is not typically required in all aspects of MRI or MRS, and only required in specialized areas (such as where tracers are used in MRI). This is a routine procedure, however, that is needed in PET and SPECT. Despite the apparent complexity, this is also one of the greatest strengths of these imaging techniques, in that they allow the measurement of so many different types of biochemical functions.

Mathematical modeling of radiotracers of the radiotracer uptake to obtain the biological parameters of interest (receptor density, affinity, glucose metabolism, etc.) plays an extremely important role in PET and SPECT imaging, much more so than in many other areas of imaging.

This is because the processes being followed are physiological and biochemical in nature, and therefore cannot be determined typically by a single snapshot in time, such as a structural image; a time series needs to be obtained, and this time series needs to be interpreted to derive the parameters of interest. Many of the measurements have previously been initiated and often validated in animals and animal models or in *in vitro* conditions. However, there are restrictions that mean that, despite the great benefits of *in vivo* imaging (repeatability, relevance, etc.) specific conditions cannot be obtained for practical reasons. For example, in an *in vitro* assay, saturation conditions and incubations cannot be done as in the typical *in vivo* model, nor can tissues be washed clean of nonspecific binding. Thus, mathematical models are needed to infer from the observables, that is brain images over time and radioactivity in plasma over time, in order to obtain the biological parameters. Several references discussing mathematical modeling exist [60, 61]. Typical models for brain imaging, in theory, could be multiple compartments, but are for practical reasons (due to limitations of data, time points and instrumentation) typically a plasma, together with one, two or three tissue compartments. A minimum is that the one tissue compartment include the area of interest. For example, for glucose metabolism, it would be the hexokinase step. For receptor binding, it would be the receptor itself. For enzyme kinetics it would be the product of the reaction. However, for some models additional compartments might be required, such as that including nonspecific binding (e.g. in receptor binding) or the precursor pool (e.g. for glucose metabolism and FDG) or an intermediary step (e.g. as in enzyme kinetics), and hence multiple compartments might be proposed. All of these result in the need to analyze and extract the rate constants of interest, which join the various compartments, just as in the case of, say, a biochemical or organic chemistry synthetic route. These parameters are often estimated through various mathematical methods such as nonlinear regression and the solutions of differential equations, which would be beyond the scope of this chapter. Nevertheless, there are many advances, including those that describe these parameter estimates, not only to a single dynamic time–activity curve in a region of interest (e.g. over the basal ganglia), but more recently throughout the entire image on a pixel by pixel basis (e.g. typically over 256×256). Although the latter is more computationally difficult, it has a number of advantages in terms of reproducibility, test–retest and is being increasingly used.

A typical compartmental model, with multiple compartments, is given in PET, Fig. 54-5.

Another issue for quantification is the effect of the finite resolution of PET/SPECT relative to small structures being imaged. This can result in the so called 'Partial Volume Effect' which is characterized by an underestimation of the true radioactivity. This is an area of increasing research, but is not a major limitation when there is not a large difference between patients and controls in the volume of structures, or when subjects are being used as their own controls [62].

REFERENCES

1. Ambrose, J. and Hounsfield, G. Computerized transverse axial tomography. *Br. J. Radiol.* 46: 148–149, 1973.
2. Hounsfield, G.N. Computerized transverse axial scanning (tomography). 1. Description of system. *Br. J. Radiol.* 46: 1016–1022, 1973.
3. Kuhl, D.E. and Edwards, R.Q. Cylindrical and section radioisotope scanning of the liver and brain. *Radiology* 83: 926–936, 1964.
4. Bloch, F., Hansen, W. W., Packard, M. Nuclear induction. *Physical Review* 69: 127, 1946.
5. Purcell, E. M., Torry, H. C. and Pound R. V. Resonance absorption by nuclear magnetic moments in a solid. *Physical Review* 69: 37–38, 1946.
6. Damadian, R. Tumor detection by nuclear magnetic resonance. *Science* 171: 1151–1153, 1971.
7. Lauterbur, P. C. Image formation by induced local interactions — examples employing nuclear magnetic resonance. *Nature* 242: 190–191, 1973.
8. Brown, T. R., Kincaid, B. M. and Ugurbil, K. NMR chemical shift imaging in three dimensions. *Proc. Natl Acad. Sci. USA* 79: 3523–3526, 1982.
9. Menon, R. S., Ogawa, S., Kim, S. G. *et al.* Functional brain mapping using magnetic resonance imaging. Signal changes accompanying visual stimulation. *Invest. Radiol.* 27(Suppl 2): 47–53, 1992.
10. Vaibhev, A. D. and Keshavan, M. S. Newer techniques in magnetic resonance imaging and their potential for neuropsychiatric research. *J. Psychosom. Res.* 53: 677–685, 2002.
11. Besson, J. A., Corrigan, F. M., Cherryman, G. R. and Smith F. W. Nuclear magnetic resonance brain imaging in chronic schizophrenia. *Br J. Psychiat.* 150: 161–163, 1987.
12. Pantelis, C., Velakoulis, D., McGorry, P. D. *et al.* Neuroanatomical abnormalities before and after onset of psychosis: a cross-sectional and longitudinal MRI comparison. *Lancet* 361: 281–288, 2003.
13. Velakoulis, D., Wood, S. J., Smith, D. J. *et al.* Increased duration of illness is associated with reduced volume in right medial temporal/anterior cingulate grey matter in patients with chronic schizophrenia. Schizophr Res. 57: 43–49, 2002.
14. Phillips, L. J., Velakoulis, D., Pantelis, C. *et al.* Non-reduction in hippocampal volume is associated with higher risk of psychosis. *Schizophr. Res.* 58: 145–158, 2002.
15. Yucel, M., Stuart, G. W., Maruff, P. *et al.* Paracingulate morphologic differences in males with established schizophrenia: a magnetic resonance imaging morphometric study. *Biol. Psychiat.* 52: 15–23, 2002.
16. Yucel, M., Wood, S. J., Phillips, L. J. *et al.* Morphology of the anterior cingulate cortex in young men at ultra-high risk of developing a psychotic illness. *Br. J. Psychiat.* 182: 518–524, 2003.
17. Kubicki, M., Westin, C. F., Maier, S. E. *et al.* Diffusion tensor imaging and its application to neuropsychiatric disorders. *Harv. Rev. Psychiat.* 10: 324–336, 2002.
18. Lim, K. O. and Helpern, J. A. Neuropsychiatric applications of DTI — a review. *NMR Biomed.* 15: 587–593, 2002.

19. Sullivan, E. V. and Pfefferbaum, A. diffusion tensor imaging in normal aging and neuropsychiatric disorders. *Eur. J. Radiol.* 45: 244–255, 2003.
20. Breeze, J. L., Hesdorffer, D. C., Hong, X. *et al.* Clinical significance of brain white matter hyperintensities in young adults with psychiatric illness. *Harv. Rev. Psychiat.* 11: 269–283, 2003.
21. Callicott, J. H. and Weinberger, D. R. Neuropsychiatric dynamics: the study of mental illness using functional magnetic resonance imaging. *Eur. J. Radiol.*130: 95–104, 999.
22. Yurgelun-Todd, D. A., Waternaux, C. M., Cohen, B. M. *et al.* Functional magnetic resonance imaging of schizophrenic patients and comparison subjects during word production. *Am. J. Psychiat.* 153: 200–205, 1996.
23. Govindaraju, V., Young, K., Maudsley, A. A. Proton NMR chemical shifts and coupling constants for brain metabolites. *NMR Biomed.* 13: 129–153, 2000.
24. Gruetter, R., Adriany, G., Choi, I. Y. *et al.* Localized *in vivo* ^{13}C NMR spectroscopy of the brain. *NMR Biomed.* 16: 313–338, 2003.
25. Jensen, J. E., Drost, D. J., Menon, R. S. and Williamson, P. C. *In vivo* brain ^{31}P-MRS: measuring the phospholipid resonances at 4 Tesla from small voxels. *NMR Biomed.* 15: 338–347, 2002.
26. Stanley, J. A. *In vivo* magnetic resonance spectroscopy and its application to neuropsychiatric disorders. *Can. J. Psychiat.* 47: 315–326, 2002.
27. Keshavan, M. S., Stanley, J. A., Pettegrew, J. W. Magnetic resonance spectroscopy in schizophrenia: methodological issues and findings — part II. *Biol. Psychiat.* 48: 369–380, 2000.
28. Pettegrew, J. W., Keshavan, M. S., Panchalingam, K. *et al.* Alterations in brain high-energy phosphate and membrane phospholipid metabolism in first-episode, drug-naive schizophrenics. A pilot study of the dorsal prefrontal cortex by *in vivo* phosphorus 31 nuclear magnetic resonance spectroscopy. *Arch. Gen. Psychiat.* 48: 563–568, 1991.
29. Stanley, J. A., Pettegrew, J. W. and Keshavan, M. S. Magnetic resonance spectroscopy in schizophrenia: methodological issues and findings — part I. *Biol. Psychiat.* 48: 357–368, 2000.
30. Williamson, P. C. and Drost, D. J. ^{31}P MRS in the assessment of brain phospholipid metabolism in schizophrenia. In: *Phospholipid Spectrum Disorder in Psychiatry.* Eds Peet, M., Glen, I., Horrobin, D. F. UK: Marius Press, 1999, pp 45–55.
31. Blasberg, R. G. and Gelovani, J. Molecular-genetic imaging: a nuclear medicine-based perspective. *Mol. Imaging* 1: 280–300, 2002.
32. Theberge, J., Bartha, R., Drost, D. J. *et al.* Glutamate and glutamine measured with 4.0 T proton MRS in never-treated patients with schizophrenia and healthy volunteers. *Am. J. Psychiat.* 159: 1944–1946, 2002.
33. Theberge, J., Al-Semaan, Y., Williamson, P. C. *et al.* Glutamate and glutamine in the anterior cingulate and thalamus of medicated patients with chronic schizophrenia and healthy comparison subjects measured with 4.0-T proton MRS. *Am. J. Psychiat.* 160: 2231–2233, 2003.
34. Jensen, J. E., Al-Semaan, Y. M., Williamson, P. C. *et al.* Region-specific changes in phospholipid metabolism in chronic, medicated schizophrenia: ^{31}P-MRS study at 4.0 Tesla. *Br. J. Psychiat.* 2002; 180: 39–44.
35. Kaufman M. J., Levin, J. M., Christensen, J. D. and Renshaw, P. F. Magnetic resonance studies of substance abuse. *Semin. Clin. Neuropsychiat.* 1: 61–75, 1996.
36. Christensen, J. D., Kaufman, M. J., Levin, J. M. *et al.* Abnormal cerebral metabolism in polydrug abusers during early withdrawal: a ^{31}P MR spectroscopy study. *Magn. Reson. Med.* 35: 658–663, 1996;.
37. Christensen, J. D., Kaufman, M. J., Frederick, B. *et al.* Proton magnetic resonance spectroscopy of human basal ganglia: response to cocaine administration. *Biol. Psychiat.* 48: 685–692, 2000.
38. Kaufman, M. J., Pollack, M. H., Villafuerte, R. A. *et al.* Cerebral phosphorus metabolite abnormalities in opiate-dependent polydrug abusers in methadone maintenance. *Psychiat. Res.* 90: 143–152, 1999.
39. Negendank, W. and Sauter, R. Intratumoral lipids in ^{1}H MRS *in vivo* in brain tumors: experience of the Siemens cooperative clinical trial. *Anticancer Res.* 16: 1533–1538, 1996.
40. Negendank, W. G., Sauter, R., Brown, T. R. *et al.* Proton magnetic resonance spectroscopy in patients with glial tumors: a multicenter study. *J. Neurosurg.* 84: 449–458, 1996.
41. Tugnoli, V., Tosi, M. R., Barbarella, G. *et al. In vivo* ^{1}H MRS and *in vitro* multinuclear MR study of human brain tumors. *Anticancer Res.* 16: 2891–2899, 1996.
42. Tugnoli, V., Tosi, M. R., Barbarella, G. *et al.* Magnetic resonance spectroscopy study of low grade extra and intracerebral human neoplasms. *Oncol. Rep.* 5: 1199–1203, 1998.
43. Moore, C. M., Christensen, J. D., Lafer, B. *et al.* Lower levels of nucleoside triphosphate in the basal ganglia of depressed subjects: a phosphorus-31 magnetic resonance spectroscopy study. *Am. J. Psychiat.* 154: 116–118, 1997.
44. Volz, H. P., Rzanny, R., Riehemann, S. *et al.* ^{31}P magnetic resonance spectroscopy in the frontal lobe of major depressed patients. *Eur. Arch. Psychiat. Clin. Neurosci.* 248: 289–295, 1998.
45. Herminghaus, S., Frolich, L., Gorriz, C. *et al.* Brain metabolism in Alzheimer disease and vascular dementia assessed by *in vivo* proton magnetic resonance spectroscopy. *Psychiat. Res.* 123: 183–190, 2003.
46. Pettegrew, J. W., Klunk, W. E., Panchalingam, K. *et al.* Magnetic resonance spectroscopic changes in Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 826: 282–306, 1997.
47. Pfefferbaum, A., Adalsteinsson, E., Spielman, D. *et al. In vivo* spectroscopic quantification of the *N*-acetyl moiety, creatine, and choline from large volumes of brain gray and white matter: effects of normal aging. *Magn. Reson. Med.* 41: 276–284, 1999.
48. Hetherington, H. P., Kuzniecky, R, I., Pan, J. W. *et al.* Application of high field spectroscopic imaging in the evaluation of temporal lobe epilepsy. *Magn. Reson. Imaging* 13: 1175–1180, 1995.
49. Hetherington, H. P., Pan, J. W. and Spencer, D. D. ^{1}H and ^{31}P spectroscopy and bioenergetics in the lateralization of seizures in temporal lobe epilepsy. *J. Magn. Reson. Imaging* 16: 477–483, 2002.
50. Simister, R. J., Woermann, F. G., McLean, M. A. *et al.* A short-echo-time proton magnetic resonance spectroscopic imaging study of temporal lobe epilepsy. *Epilepsia* 43: 1021–1031, 2002.

51. Simister, R. J., McLean, M. A., Barker, G. J. and Duncan J. S. Proton MRS reveals frontal lobe metabolite abnormalities in idiopathic generalized epilepsy. *Neurology* 61: 897–902, 2003.
52. Simister, R. J., McLean, M. A., Barker, G. J., Duncan, J. S. A proton magnetic resonance spectroscopy study of metabolites in the occipital lobes in epilepsy. *Epilepsia* 44: 550–558, 2003.
53. Sorenson, J. A. and Phelps, M. E. *Physics in Nuclear Medicine* 2nd edn. Orlando, FL: Grune & Stratton, Inc., 1987.
54. Humm, J. L., Rosenfeld, A. and Del Guerra, A. From PET detectors to PET scanners. *Eur. J. Nucl. Med. Mol. Imaging* 30: 1574–1597, 2003.
55. Tarantola, G., Zito, F. and Gerundini, P. PET instrumentation and reconstruction algorithms in whole-body applications. *J. Nucl. Med.* 44: 756–769, 2003.
56. Theobald, A. E. Quality control of radiopharmaceuticals. In *Textbook of Radiopharmacology: Theory and Practice.* New York: Gordon & Breach, 1990, pp 115–148.
57. Frier, M., Hesslewood, S. Quality assurance of radiopharmaceuticals — a guide to hospital practice. *Nucl. Med. Commun.* Special Issue 1980.
58. Mah, G., Reilly, R. M., Wong, G. L. and Houle, S. A comparison of three methods to determine the radiochemical purity of ^{99m}Tc-hexamethylpropylene amine oxime (^{99m}Tc-HMPAO). *Nucl. Med. Commun.* 10: 733–740, 1989.
59. Saha, G. B. *Fundamentals of Nuclear Pharmacy* 4th edn. New York: Springer, 1998.
60. Huang, S., Phelps, M. E. Principles of tracer kinetic modeling in positron emission tomography and autoradiography. In *Positron Emission Tomography and Autoradiography: Principles and Applications for the Brain and Heart.* Eds Phelps, M. E., Mazziotta, J. C. and Schelbert, H. New York: Raven Press, 1986, pp 287–346.
61. Gjedde, A., Wong, D. F. Modeling neuroreceptor binding of radioligands *in vivo*. In *Quantitative Imaging: Neuroreceptors, Neurotransmitters, and Enzymes.* Eds Frost, J. J., Wagner, H. N., Jr. New York: Raven Press, 1990, pp 51–79.
62. Yokoi, F., Grunder, G., Biziere, K. *et al.* Dopamine D_2 and D_3 receptor occupancy in normal humans treated with the antipsychotic drug aripiprazole (OPC 14597): a study using positron emission tomography and ^{11}C raclopride. *Neuropsychopharmacol.* 27: 248–259, 2002.
63. Thrall, J. H. and Ziessman, H. A. *Nuclear Medicine: The Requisites* 2nd edn. St. Louis: Mosby, Inc, 2001.
64. Wong, D. F. and Pomper, M. G. Predicting the success of a radiopharmaceutical for *in vivo* imaging of central nervous system neuroreceptor systems. *Mol. Imaging Biol.* 5: 350–362, 2003.
65. Chatziioannou, A. F. Molecular imaging of small animals with dedicated PET tomographs. *Eur. J. Nucl. Med. Mol. Imaging* 29: 98–114, 2002.
66. Weinberger, D. R., Gibson, R., Coppola, R. *et al.* The distribution of cerebral muscarinic acetylcholine receptors *in vivo* in patients with dementia. A controlled study with ^{123}IQNB and single photon emission computed tomography. *Arch. Neurol.* 48: 169–176, 1991.
67. Wyper, D. J., Brown, D., Patterson, J. *et al.* Deficits in iodine-labelled 3-quinuclidinyl benzilate binding in relation to cerebral blood flow in patients with Alzheimer's disease. *Eur. J. Nucl. Med.* 20: 379–386, 1993.
68. Kemp, P. M., Holmes, C., Hoffmann, S. *et al.* A randomised placebo controlled study to assess the effects of cholinergic treatment on muscarinic receptors in Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatr.* 74: 1567–1570, 2003.
69. De Herder W. W., Reijs, A. E., de Swart, J. *et al.* Comparison of iodine-123 epidepride and iodine-123 IBZM for dopamine D_2 receptor imaging in clinically non-functioning pituitary macroadenomas and macroprolactinomas. *Eur. J. Nucl. Med.* 26: 46–50, 1999.
70. Leslie, W. D, Greenberg, C. R., Abrams, D. N. and Hobson, D. Clinical deficits in Huntington disease correlate with reduced striatal uptake on iodine-123 epidepride single-photon emission tomography. *Eur. J. Nucl. Med.* 26: 1458–1464, 1999.
71. Busatto, G. F., Pilowsky, L. S., Costa, D. C. *et al.* Initial evaluation of ^{123}I-5-I-R91150, a selective 5-HT2A ligand for single-photon emission tomography, in healthy human subjects. *Eur. J. Nucl. Med.* 24: 119–124, 1997.
72. Travis, M. J., Busatto, G. F., Pilowsky, L. S. *et al.* 5-HT2A receptor blockade in patients with schizophrenia treated with risperidone or clozapine. A SPET study using the novel 5-HT2A ligand ^{123}I-5-I-R-91150. *Br. J. Psychiatr.* 173: 236–241, 1998.
73. van Dyck, C. H., Malison, R. T., Seibyl, J. P. *et al.* Age-related decline in central serotonin transporter availability with ^{123}Iβ-CIT SPECT. *Neurobiol. Aging* 21: 497–501, 2000.
74. Kung, H. F., Kim, H. J., Kung, M. P. *et al.* Imaging of dopamine transporters in humans with technetium-99m TRODAT-1. *Eur. J. Nucl. Med.* 23: 1527–1530, 1996.
75. Meltzer, P. C., Blundell, P., Jones, A. G. *et al.* A technetium-99m SPECT imaging agent which targets the dopamine transporter in primate brain. *J. Med. Chem.* 40: 1835–1844, 1997.
76. Mozley, P. D., Schneider, J. S., Acton, P. D. *et al.* Binding of ^{99m}Tc TRODAT-1 to dopamine transporters in patients with Parkinson's disease and in healthy volunteers. *J. Nucl. Med.* 2000; 41: 584–589.
77. Dresel, S., Krause, J., Krause, K. H. *et al.* Attention deficit hyperactivity disorder: binding of ^{99m}Tc TRODAT-1 to the dopamine transporter before and after methylphenidate treatment. *Eur. J. Nucl. Med.* 27: 1518–1524, 2000.
78. Pietzsch, H. J., Scheunemann, M., Kretzschmar, M. *et al.* Synthesis and autoradiographic evaluation of a novel high-affinity Tc-99m ligand for the 5-HT2A receptor. *Nucl. Med. Biol.* 26: 865–875, 1999.
79. Wong, D. F. *In vivo* imaging of D_2 dopamine receptors in schizophrenia: the ups and downs of neuroimaging research. *Arch. Gen. Psychiatr.* 59: 31–34, 2002.
80. Volkow, N. D., Fowler, J. S. and Wang, G. J. Positron emission tomography and single-photon emission computed tomography in substance abuse research. *Semin. Nucl. Med.* 33: 114–128, 2003.
81. Seibyl, J. P. Imaging studies in movement disorders. *Semin. Nucl. Med.* 33: 105–113, 2003.
82. Varrone, A., Marek, K. L., Jennings, D. *et al.* ^{123}I β-CIT SPECT imaging demonstrates reduced density of striatal dopamine transporters in Parkinson's disease and multiple system atrophy. *Mov. Disord.* 16: 1023–1032, 2001.
83. Sargent, P. A., Kjaer, K. H., Bench, C. J. *et al.* Brain serotonin1A receptor binding measured by positron emission tomography with ^{11}C WAY-100635: effects of

depression and antidepressant treatment. *Arch. Gen. Psychiatr.* 57: 174–180, 2000.
84. Wong, D. F., Wagner, H. N. Jr, Tune, L. E. *et al.* Positron emission tomography reveals elevated D_2 dopamine receptors in drug-naive schizophrenics. *Science* 234: 1558–1563, 1986.
85. Tune, L. E., Wong, D. F., Pearlson, G. *et al.* Dopamine D_2 receptor density estimates in schizophrenia: a positron emission tomography study with ^{11}C-*N*-methylspiperone. *Psychiatr. Res.* 49: 219–237, 1993.
86. Reith, J., Benkelfat, C., Sherwin, A. *et al.* Elevated dopa decarboxylase activity in living brain of patients with psychosis. *Proc. Natl Acad. Sci. USA* 91: 11651–11654, 1994.
87. Laruelle, M., Abi-Dargham, A., van Dyck, C. H. *et al.* Single photon emission computerized tomography imaging of amphetamine-induced dopamine release in drug-free schizophrenic subjects. *Proc. Natl Acad. Sci. USA* 93: 9235–9240, 1996.
88. Breier, A., Su, T. P., Saunders, R. *et al.* Schizophrenia is associated with elevated amphetamine-induced synaptic dopamine concentrations: evidence from a novel positron emission tomography method. *Proc. Natl Acad. Sci. USA* 94: 2569–2574, 1997.
89. Gjedde, A. and Wong, D. F. Quantification of neuroreceptors in living human brain. v. Endogenous neurotransmitter inhibition of haloperidol binding in psychosis. *J. Cereb. Blood Flow Metab.* 21: 982–994, 2001.
90. Abi-Dargham, A., Rodenhiser, J., Printz, D. *et al.* Increased baseline occupancy of D_2 receptors by dopamine in schizophrenia. *Proc. Natl Acad. Sci. USA* 97: 8104–8109, 2000.
91. Pearlson, G. D., Wong, D. F., Tune, L. E. *et al. In vivo* D_2 dopamine receptor density in psychotic and nonpsychotic patients with bipolar disorder. *Arch. Gen. Psychiatr.* 52: 471–477, 1995.
92. Wong, D. F., Singer, H. S., Brandt, J. *et al.* D_2-like dopamine receptor density in Tourette syndrome measured by PET. *J. Nucl. Med.* 38: 1243–1247, 1997.
93. Singer, H. S., Szymanski, S., Giuliano, J. *et al.* Elevated intrasynaptic dopamine release in Tourette's syndrome measured by PET. *Am. J. Psychiatr.* 159: 1329–1336, 2002.
94. Wong, D. F., Potter, W. Z. and Brasic, J. Proof of concept: functional models for drug development in humans. In *Psychopharmacology. The Fifth Generation of Progress.* Eds Charney, D., Coyle, J., Davis, K. and Nemeroff, . Baltimore, Maryland: Lippincott Williams & Wilkins, 2000.
95. Villemagne, V. L., Wong, D. F., Yokoi, F. *et al.* GBR12909 attenuates amphetamine-induced striatal dopamine release as measured by ^{11}C raclopride continuous infusion PET scans. *Synapse* 33: 268–273, 1999.
96. Farde, L., Wiesel, F. A., Halldin, C. and Sedvall, G. Central D_2-dopamine receptor occupancy in schizophrenic patients treated with antipsychotic drugs. *Arch. Gen. Psychiatr.* 45: 71–76, 1988.
97. Heron, D. E., Andrade, R. S., Flickinger, J. *et al.* Hybrid PET–CT simulation for radiation treatment planning in head-and-neck cancers: a brief technical report. *Int. J. Radiation Oncology Biol. Phys.*, 60: 1419–1424, 2004.
97a. Levivier, M., Wikler, D. Jr, Massager, N. *et al.* The integration of metabolic imaging in stereotactic procedures. including radiosurgery: a review. *J. Neurosurg.* 97(5 Suppl): 542–550, 2002.
98. Wong, T.Z., van der Westhuizen, G. J. and Coleman, R. E. Positron emission tomography imaging of brain tumors. *Neuroimaging Clin. N. Am.* 12: 615–626, 2002.
99. Baird, A. E., Austin, M. C., McKay, W. J. and Donnan, G. A. Sensitivity and specificity of ^{99m}Tc-HMPAO SPECT cerebral perfusion measurements during the first 48 hours for the localization of cerebral infarction. *Stroke* 28: 976–980, 1997.
100. Ezura, M., Takahashi ,A. and Yoshimoto, T. Evaluation of regional cerebral blood flow using single photon emission tomography for the selection of patients for local fibrinolytic therapy of acute cerebral embolism. *Neurosurg. Rev.* 19: 231–236, 1996.
101. Engel, J. Jr. *Seizures and Epilepsy*. Philadelphia, PA: 1989.
102. Crawford, P. M. Epidemiology of intractable focal epilepsy. In *Intractable Focal Epilepsy*. Eds Oxbury J. M., Polkey C. E. and Duchowny, M. London: Harcourt, 2000, pp25–40.
103. Henry, T. R., Sutherling, W. W., Engel, J. Jr. *et al.* Interictal cerebral metabolism in partial epilepsies of neocortical origin. *Epilepsy Res.* 10: 174–182, 1991.
104. Carter, C. C., Eichling, J. O., Davis, D. O. and Ter Pogossian, M. M. Correlation of regional cerebral blood flow with regional oxygen uptake using ^{15}O methodology. *Neurology* 22(7): 755–762, 1972.
105. Sokoloff, L. Relationships among local functional activity, energy metabolism, and blood flow in the central nervous system. *Fed. Proc.* 40: 2311–2316, 1981.
106. Phelps, M. E., Huang, S. C., Hoffman, E. J. *et al.* Tomographic measurement of local cerebral glucose metabolic rate in humans with ^{18}F 2-fluoro-2-deoxy-D-glucose: validation of method. *Ann. Neurol.* 6: 371–388, 1979.
107. Reivich, M., Kuhl, D., Wolf, A. *et al.* The ^{18}F fluorodeoxyglucose method for the measurement of local cerebral glucose utilization in man. *Circ. Res.* 44: 127–137, 1979.
108. Shiraishi, H., Chang, C. C., Kanno, H. and Yamamoto, I. The relationship between cerebral blood flow and cognitive function in patients with brain insult of various etiology. *J. Clin. Neurosci.* 11: 138–141, 2004.
109. Yamamoto, K. Analysis of cognitive function and regional cerebral blood flow in Parkinson's disease by ^{123}I-IMP SPECT. *Rinsho Shinkeigaku* 32: 1–7, 1992.
110. DeKosky, S. T., Shih, W. J., Schmitt, F. A. *et al.* Assessing utility of single photon emission computed tomography (SPECT) scan in Alzheimer disease: correlation with cognitive severity. *Alzheimer Dis. Assoc. Disord.* 4: 14–23, 1990.
111. Imran, M. B., Kawashima, R., Awata, S. *et al.* Tc-99m HMPAO SPECT in the evaluation of Alzheimer's disease: correlation between neuropsychiatric evaluation and CBF images. *J. Neurol. Neurosurg. Psychiatr.* 66: 228–232, 1999.
112. Quinn, N. Parkinsonism — recognition and differential diagnosis. *Br. Med. J.* 310: 447–452, 1995.
113. Gelb, D. J., Oliver, E., Gilman, S. Diagnostic criteria for Parkinson disease. *Arch. Neurol.* 56: 33–39, 1999.
114. Brooks, D. J. PET and SPECT studies in Parkinson's disease. *Baillieres Clin. Neurol.* 6: 69–87, 1997.
115. Almeida, P., Ribeiro, M. J., Bottlaender, M. *et al.* Absolute quantitation of iodine-123 epidepride kinetics using single-photon emission tomography: comparison with carbon-11 epidepride and positron emission tomography. *Eur. J. Nucl. Med.* 26: 1580–1588, 1999.
116. Frey, K. A., Koeppe, R. A., Kilbourn, M. R. *et al.* Presynaptic monoaminergic vesicles in Parkinson's disease and normal aging. *Ann. Neurol.* 40: 873–884, 1996.

117. Marek, K., Innis, R., van Dyck, C. *et al.* ^{123}I-β-CIT SPECT imaging assessment of the rate of Parkinson's disease progression. *Neurology* 57: 2089–2094, 2001.
118. Morrish, P. K., Sawle, G. V. and Brooks, D. J. Regional changes in ^{18}F-dopa metabolism in the striatum in Parkinson's disease, Part 6. *Brain* 119: 2097–2103, 1996.
119. Morrish, P. K., Sawle, G. V. and Brooks, D. J. An ^{18}F-dopa-PET and clinical study of the rate of progression in Parkinson's disease, Part 2. *Brain* 119: 585–591, 1996.
120. Morrish, P. K., Sawle, G. V. and Brooks, D. J. The rate of progression of Parkinson's disease. A longitudinal ^{18}F-DOPA PET study. *Adv. Neurol.* 69: 427–431, 1996.
121. Asenbaum, S., Brucke, T., Pirker, W. *et al.* Imaging of dopamine transporters with iodine-123-beta-CIT and SPECT in Parkinson's disease. *J. Nucl. Med.* 38: 1–6, 1997.
122. Marek, K. L., Seibyl, J. P., Zoghbi, S. S. *et al.* ^{123}I-β-CIT/SPECT imaging demonstrates bilateral loss of dopamine transporters in hemi-Parkinson's disease. *Neurology* 46: 231–237, 1996.
123. Sawle, G. V., Playford, E. D., Burn, D. J. *et al.* Separating Parkinson's disease from normality. Discriminant function analysis of fluorodopa F-18 positron emission tomography data. *Arch. Neurol.* 51: 237–243, 1994.
124. Albert, M. S. and Drachman, D. A. Alzheimer's disease: what is it, how many people have it, and why do we need to know? *Neurology* 55: 166–168, 2000.
125. Small, G. W., Rabins, P. V., Barry, P. P. *et al.* Diagnosis and treatment of Alzheimer disease and related disorders. Consensus statement of the American Association for Geriatric Psychiatry, the Alzheimer's Association, and the American Geriatrics Society. *JAMA* 278(16): 1363–1371, 1997.
126. Minoshima, S., Giordani, B., Berent ,S. *et al.* Metabolic reduction in the posterior cingulate cortex in very early Alzheimer's disease. *Ann. Neurol.* 42: 85–94, 1997.
127. Dewan, M. J. and Gupta, S. Toward a definite diagnosis of Alzheimer's disease. *Compr. Psychiatr.* 33: 282–290, 1992.
128. Jelic, V. and Nordberg, A. Early diagnosis of Alzheimer disease with positron emission tomography. *Alzheimer Dis. Assoc. Disord.* 14(Suppl 1): S109–S113, 2000.
129. Silverman, D. H., Small, G. W., Chang, C. Y. *et al.* Positron emission tomography in evaluation of dementia: regional brain metabolism and long-term outcome. *JAMA* 286: 2120–2127, 2001.
130. Klunk, W. E., Engler, H., Nordberg, A. *et al.* Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann. Neurol.* (in press).

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Glossary

AA	arachidonic acid
AADC	aromatic L-amino acid decarboxylase
AANAT	aryl alkylamine *N*-acetyltransferase
ABC	ATP-binding cassette
AC	adenylyl cyclase
ACh	acetylcholine
AChBP	acetylcholine binding protein
AChE	acetylcholinesterase
AChR	acetycholine receptor
ACTH	adrenocorticotrophic hormone
AD	Alzheimer's disease
ADP	adenosine 5′-diphosphate
AIDS	acquired immunodeficiency syndrome
AIF	apoptosis-inducing factor
AKAP	protein kinase A anchoring protein
AL	argininosuccinate lyase
ALD	adrenoleukodystrophy
ALS	amyotrophic lateral sclerosis
AMAN	acute motor axonal neuropathy
AMD	(i) acid maltase deficiency; (ii) age-related macular degeneration
AMN	adrenomyeloneuropathy
AMOG	adhesion molecule on glia
AMP	adenosine 5′-monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMP-PNP	adenylylimidodiphosphate
AMPT	α-methyl-*p*-tyrosine
ANP	atrial natriuretic peptide
AP	adaptin
APBD	adult polyglucosan body disease
APDC	2R,4R-4-aminopyrrolidine-2,4-dicarboxylate
ApoE	apolipoprotein E
APP	amyloid precursor protein
APPL	β-amyloid precursor-like protein
APRT	adenine phosphoribosyltransferase
AQP	aquaporin
ARAS	ascending reticular activating system
ARF	ADP-ribosylation factor
ARIA	ACh receptor-inducing activity
βARK	β-adrenergic receptor kinase
ARTN	artemin
AS	argininosuccinate synthetase
ASA	arylsulfatase A
AS-C	achaete–scute gene complex for sense organs
ASIC	acid-sensing ion channels
ASL	adenylsuccinate lyase
ATF	activating transcription factor
ATP	adenosine 5′-triphosphate
AZM	active zone material
BBB	blood–brain barrier
BCAA	branched chain amino acids
BDNF	brain-derived neurotrophic factor
BDZ	benzodiazepine
BH4	tetrahydrobiopterin
BMAA	β-*N*-methylamino-L-alanine
BMP	bone morphogenetic proteins
BNP	brain natriuretic peptide
BOAA	β-*N*-oxylylamino-1-alanine
BOLD	blood oxygenation level dependent
BSE	bovine spongiform encephalopathy
BTX	batrachotoxin
BuChE	butyryl cholinesterases
CA	(i) catecholamine; (ii) cornu ammonis
CACH	childhood ataxia with central nervous system hypomyelination
CAE	childhood absence epilepsy
CAM	cell adhesion molecule
CaMK	Ca^{2+}-calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3′,5′-monophosphate
CART	cocaine- and amphetamine-regulated transcript
CAT	computer assisted tomography
CBD	cortical basal degeneration
CBF	cerebral blood flow
CBP	CREB-binding protein
CBV	cerebral blood volume
CCK	cholecystokinin
CDF	cholinergic differentiation factor
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CDP	cytidine diphosphate
CDP-DAG	cytidine diphosphate diacylglycerol
Cer-Glc	glucocerebroside
CF	climbing fiber
cGMP	cyclic guanosine 3′,5′-cyclic monophosphate
CGN	*cis*-Golgi network
CGRP	calcitonin gene-related peptide
ChAT	choline acyltransferase
CHE	cholinesterase
CHT	choline transporter
CICR	Ca^{2+}-induced Ca^{2+} release
CIDP	chronic inflammatory demyelinating polyneuropathy
CJD	Creutzfeldt–Jakob disease
CK	creatine kinase
CLIP	cytoplasmic-linker protein
CLN	neuronal ceroid lipofuscinoses
CME	clathrin-mediated endocytosis
CMP	cytidine monophosphate
CMR	cerebral metabolic rate
CMR_{glc}	cerebral metabolic rate for glucose
CMRA	cerebral metabolic rate for ammonia
$CMRO_2$	cerebral metabolic rate for oxygen
CMT	Charcot–Marie–Tooth disease

CNG	cyclic nucleotide-gated channel
CNP	(i) 2′,3′-cyclic nucleotide 3′-phosphodiesterase; (ii) C-type natriuretic peptide
CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	central nervous system
rCNT	nucleoside cotransporter
CNTF	ciliary neurotrophic factor
CoA	coenzyme A
COMT	catechol-*O*-methyltransferase
COPI	coat protein
CORT	corticosterone
COX	(i) cyclooxygenase; (ii) cytochrome oxidase
CPE	carboxypeptidase E
CPEB	cytoplasmic polyadenylation element binding protein
CPK	creatine phosphokinase
CPM	central pontine myelinolysis
CPS	carbamyl phosphate synthetase
CPT	carnitine palmitoyltransferase
CRE	cAMP responsive element
CREB	CRE-binding protein
CREM	CRE-modulatory protein
CRF	corticotropin-releasing factor
CRH	corticotrophin-releasing hormone
CRP	creatine phosphate
CSF	cerebrospinal fluid
CSPG	chondroitin sulfate proteoglycans
CT	computed tomography
CT-1	cardiotrophin 1
CTP	cytidine 5′-triphosphate
DA	dopamine
DAAO	D-amino acid oxidase
DAG	diacylglycerol
DAO	diamine oxidase
DARPP-32	dopamine- and cAMP-related phosphoprotein (32 kDa)
DAT	dopamine transporter
DBH	dopamine β-hydroxylase
DBM	dopamine β-monooxygenase
DBS	deep brain stimulation
DDC	dopa decarboxylase
DHA	docosahexaenoic acid
DHAP	dihydroxyacetone phosphate
DHAP-AT	dihydroxyacetone phosphate acyl transferase
DHPG	3,5-dihydroxyphenylglycine
DHPR	dihydropteridine reductase
DHT	5α-dihydroxytestosterone
DIC	Differential Interference Contrast
DIPP	diphosphoinositol polyphosphate phosphatase
L-DOPA	3,4-dihydroxyphenylalanine
DRG	dorsal root ganglia
DTI	diffusion tensor imaging
EAAT	excitatory amino acid transporter
EAE	experimental allergic encephalomyelitis
EAN	experimental allergic neuritis
ECM	extracellular matrix
EDRF	endothelium-derived relaxing factor
EE	early endosomes
EEG	electroencephalogram
EFTU	eukaryotic elongation factor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethyleneglycotetraacetic acid
ELH	egg laying hormone
ELISA	enzyme-linked immunoabsorbant assay
EM	electron microscopy
EMG	electromyogram
EMP	epthelial membrane proteins
EOFAD	early-onset familial Alzheimer's disease
EPA	eicosapentaenoic acid
EphR	erythropoietin-producing hepatocellular receptor
EPP	evoked end-plate potential
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
ERG	early-response genes
ERGIC	ER–Golgi intermediate compartment
ERK	extracellularly regulated kinase
ERP	event-related potentials
ES	embryonic stem cell
ESI/MS	electrospray ionization/mass spectrometry
ETF	electron-transferring flavoprotein
FABP	fatty acid binding proteins
FAD	flavin adenine dinucleotide
FALS	familial amyotrophic lateral sclerosis
fCJD	familial Creutzfeldt–Jakob disease
FDC	follicular dendritic cell
FFA	free fatty acid
FFI	fatal familial insomnia
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FMRF-NH_2	Phe–Met–Arg–Phe-amide
fMRI	functional magnetic resonance imaging
FRAP	fluorescence recovery after photolysis
FRET	fluorescence resonant energy transfer
FSH	follicle-stimulating hormone
FTD	frontotemporal dementia
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAG	glycosaminoglycans
Gal	galactose
galC	galactocerebroside
GalNAc	*N*-acetylgalactosamine
GAP	GTPase-activating protein
GAP-43	growth-associated protein (43 kDa)
GAT	GABA transporter
GBS	Guillain–Barré syndrome
GC	(i) glucocorticoid; (ii) Golgi complex; (iii) guanylyl cyclase
GDF	GDI-displacement factor
GDH	glutamate dehydrogenase
GDI	varying nucleotide dissociation inhibitor
GDNF	glial-derived neurotrophic factor
GDP	guanosine 5′-diphosphate
GDPH	glycerol phosphate dehydrogenase
GEF	guanine nucleotide exchange factor
GEMSA	guanidinoethylmercaptosuccinic acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GGF	glial growth factor
GIP	GTPase inhibitory protein
GIRK	G-protein-coupled inwardly rectifying K^+ channel
GLAST	glutamate–aspartate transporter
GLC	gas–liquid chromatography
Glc	glucose
Glc-6-P	glucose-6-phosphate
GlcNAc	*N*-acetylglucosamine
GLT	glutamate transporter
GLUT	glucose transporter
GMP	guanosine 5′-monophosphate
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
GPDH	glycerol phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GRE	glucocorticoid response element
GRK	G protein receptor kinase
GS	glycogen synthetase
GSH	glutathione
GSS	Gerstmann–Straussler–Scheinker syndrome
GTP	guanosine 5′-triphosphate
GTRAP	glutamate transporter-associated protein
HCSMA	hereditary canine spinal muscular atrophy
HD	Huntington's disease
HDC	L-histidine decarboxylase
HDL	high-density lipoprotein
HGF	hepatocyte growth factor
HGPRT	hypoxanthine–guanine phosphoribosyltransferase
5-HIAA	5-hydroxyindoleacetic acid
HIF	hypoxia-inducible factor
HIOMT	5-hydroxyindole-*O*-methyltransferase
HIV	human immunodeficiency virus
HLH	helix–loop–helix transcriptional regulator
HMG	β-hydroxy-β-methylglutaryl
HMIT	H^+-myo-inositol cotransporter

HMT	histamine *N*-methyltransferase
HNPP	hereditary neuropathy with predisposition to pressure palsies
HPLC	high-performance liquid chromatography
HPPD	hallucinogen persisting perception disorder
HPRT	hypoxanthine phosphoribosyltransferase
HPTLC	high-performance thin layer chromatography
HSF	heat shock factor protein
HSP	heat shock protein
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
HVA	homovanillic acid
IAA	imidazoleacetic acid
IBMX	isobutylmethylxanthine
ICAM	intercellular adhesion molecule
IDPN	β,β′-iminodipropionitrile
IF	intermediate filaments
Ig	immunoglobulin
IGE	idiopathic generalized epilepsy
IGF	insulin-like growth factor
IKK	Iκ-kinase
IL	interleukin
IMP	inosine monophosphate
INSR	insulin receptor
IP	inositol phosphate
IPSP	inhibitory postsynaptic potential
IRD	infantile Refsum disease
IRS	insulin receptor substrate
JME	juvenile myoclonic epilepsy
JNK	c-Jun N-terminal kinase
JP	joining peptide
KA	kainic acid
αKGDH	α-ketoglutarate dehydrogenase
KRP	kinesin related protein
LBD	Lewy body dementia
LC	locus ceruleus
LCAD	long chain acyl-CoA dehydrogenase
LCHAD	long chain 3-hydroxyacyl-CoA dehydrogenase
LDCV	large dense core vesicle
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LE	late endosomes
LEMS	Lambert–Eaton myasthenic syndrome
LH	lutenizing hormone
LHRH	luteinizing hormone-releasing hormone
LIF	leukemia inhibitory factor
LNS	Lesch–Nyhan syndrome
LOAD	late onset Alzheimer's disease
βLPH	β-lipotropin
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LSD	lysergic acid diethylamide
LTD	long-term depression
LTP	long-term potentiation
LTX	latrotoxins
M6P	mannose 6-phosphate
mAChR	muscarinic acetylcholine receptor
MADA	myoadenylate deaminase
MAG	myelin-associated glycoprotein
MAGUK	membrane-associated guanylyl kinase homolog
MAL	myelin and lymphocyte tetraspan protein
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MAPKAP	MAP-kinase-activated protein kinase
MAPT	microtubule-associated protein tau
MARCKS	myristolated alanine-rich protein kinase C substrate
MAS	malate–aspartate shuttle
MASC	myotube-associated specificity component
MBO	membrane-bounded organelle
MBP	myelin basic protein
MCI	mild cognitive impairment
MDCK	Madin–Darby canine kidney
MDH_m	mitochondrial malate dehydrogenase
MDMA	3,4-methylenedioxymethamphetamine
MDR	multidrug resistance protein
MEK	MAPK/ERK activating kinase
MEKK	MEK kinase
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
MEPP	miniature end-plate potential
MERRF	myoclonus epilepsy with ragged red fibers
MF	microfilament
MG	myasthenia gravis
MH	malignant hyperthermia
t-MH	*tele*-methylhistamine
MHC	major histocompatibility complex
MHPG	3-methoxy-4-hydroxyphenylglycol
t-MIAA	*tele*-methylimidazole acetic acid
MIPP	multiple inositol polyphosphate phosphatase
MKP	MAP-kinase phosphatase
ML	mucolipidosis
MLD	metachromatic leukodystrophy
MMA	methylmalonic acid
MMN	multifocal motor neuropathy
MMP	matrix metalloproteinases
MND	motor neuron disease
MOG	myelin–oligodendrocyte glycoprotein
MPP^+	1-methyl-4-phenylpyridinium
MPS	mucopolysaccharidoses
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRP	multidrug resistant protein
MRS	magnetic resonance spectroscopy
MS	multiple sclerosis
MSD	multiple sulfatase deficiency
αMSH	α-melanocyte-stimulating hormone
MSP	macrophage stimulating protein
MSUD	maple syrup urine disease
MT	microtubule
mtDNA	mitochondrial DNA
MTI	magnetization-transfer imaging
MTOC	mictotubule-organizing center
MuSK	muscle-specific kinase
MVB	multivesicular body
NAA	*N*-acetyl aspartate
NAADP	nicotinic acid–adenine dinucleotide phosphate
NAAG	*N*-acetylaspartyl glutamate
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NAG	*N*-acetylglutamate
NALD	neonatal adrenoleukodystrophy
NANA	*N*-acetylneuraminic acid
NARP/MILS	neuropathy, ataxia, retinitis pigmentosa/maternally inherited Leigh's syndrome
NBD	nucleotide-binding domain
NC	neural crest
NCAM	neural cell adhesion molecule
NCX	Na^+/Ca^{2+} exchanger
NDF	neu differentiation factor
nDNA	nuclear DNA
NE	norepinephrine
NET	norepinephrine transporter
NF	(i) neurofilament; (ii) neurofibromatosis
NFT	neurofibrillary tangle
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NgR	Nogo receptor
NHE	Na^+/H^+ antiporter
NIPP	nuclear inhibitor of protein phosphatase
NMDA	*N*-methyl-D-aspartate
NMJ	neuromuscular junction
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric oxide synthase
NPC	Niemann–Pick disease type C
NPD1	neuroprotectin D1
NPY	neuropeptide Y

NRPE *N*-retinylidene phosphatidylethanolamine
NRPTK nonreceptor protein tyrosine kinase
NRTN neurturin
NS Nissl substance
NSAID nonsteroidal anti-inflammatory drug
NSE neuronal enolase
NSF *N*-ethylmaleimide-sensitive factor
NT neurotrophin
NTP nucleoside triphosphate
NTS nucleus tractus solitarius
OCD obsessive–compulsive disorder
OCRL oculocerebrorenal syndrome
6-OHDA 6-hydroxydopamine
OL oligodendrocyte
OLP oligodendrocyte progenitor
OLS ouabain-like substance
3-OMD 3-*O*-methyldopa
OMgp oligodendrocyte-myelin glycoprotein
OR odorant receptor
OSN olfactory sensory neuron
OTC ornithine transcarbamylase
PA phosphatidic acid
PACAP pituitary adenylyl cyclase-activating peptide
PAF platelet-activating factor
PAG periaqueductal gray
PAH phenylalanine hydroxylase
PAK p21-activated kinase
PAL peptidyl-α-hydroxyglycine α-amidating lyase
PAM peptidylglycine α-amidating monooxygenase
PARP polyADP–ribose polymerase
PAS periodic acid Schiff base
PBD peroxisome biogenesis disorders
PC (i) phosphatidylcholine; (ii) prohormone convertase
PCA *para*chloroamphetamine
PCD programmed cell death
PCP phencyclidine
PCPA *para*chlorophenylalanine
PCr phosphocreatine
PCR polymerase chain reaction
PD Parkinson's disease
PDE (i) phosphodiester; (ii) phosphodiesterase
PDGF platelet-derived growth factor
PDGFR platelet-derived growth factor receptor
PDHC pyruvate dehydrogenase complex
PDK phosphoinositide-dependent kinase
PEO progressive external ophthalmoplegia
PEP priming of exocytosis protein
PEPCK phosphoenolpyruvate carboxykinase
PET positron emission tomography
PEX peroxin
PFK phosphofructokinase
PG prostaglandin
PGK phosphoglycerate kinase
PGM phosphoglycerate mutase
PH pleckstrin-homology
PHDC pyruvate dehydrogenase
PHF paired, helically wound filament
PHM peptidylglycine α-hydroxylating monooxygenase
PI (i) phosphatidylinositol; (ii) phosphoinositide
PI3K phosphatidylinositol 3-kinase
PIP phosphatidylinositol 4-phosphate
PIP_2 phosphatidylinositol 4,5-*bis*phosphate
PI-PLC phosphoinositide-specific phospholipase C
PKA protein kinase A
PKC protein kinase C
PKG protein kinase G
PKU phenylketonuria
PLA phospholipase A
PLC phospholipase C
PLD phospholipase D
PLP proteolipid protein
PMCA plasma-membrane Ca^{2+} pump
PMD Pelizaeus–Merzbacher disease
PME phosphomonoester
PMIPs plasma membrane intergral proteins
PML progressive multifocal leukoencephalopathy
PMN progressive motor neuropathy
PMP (i) peripheral myelin protein; (ii) peroxisomal membrane proteins
PNMT phenylethanolamine *N*-methyltransferase
PNS peripheral nervous system
PNUTS phosphatase 1 nuclear targeting subunit
POMC proopiomelanocortin
PP protein phosphatase
PPN pedunculopontine nucleus
PPS pentose phosphate shunt
PRL prolactin
PrP prion protein
PrP^{Sc} scrapie prion protein
PS (i) pregnenolone sulfate; (ii) presenilin
PSA polysialic acid
PSD postsynaptic density
PSEN presenilin
PSEP postsynaptic excitatory potential
PSP progressive supranuclear palsy
PSPN persephin
PSR phosphatidylserine receptor
PTB phosphotyrosine binding domain
PTBR peripheral-type benzodiazepine receptor
PtdCho phosphatidylcholine
PtdEtn phosphatidylethanolamine
PtdIns phosphatidylinositol
PtdIns 4P phosphatidylinositol 4-phosphate
PtdIns $4,5P_2$ phosphatidylinositol 4,5-*bis*phosphate
PtdOH phosphatidic acid
PtdSer phosphatidylserine
PTEN phosphatase and tensin homolog
PTH parathyroid hormone
PTK protein tyrosine kinase
PTP protein tyrosine phosphatase
PTS peroxisomal targeting signal
PTSD post-traumatic stress disorder
PUFA polyunsaturated fatty acids
PVN paraventricular nucleus
PYK proline-rich tyrosine kinase
PZ pirenzepine
RACK receptors for activated protein kinase C
RAGS repulsive axon guidance signal
RCDP rhizomelic chondrodysplasia punctata
REM rapid eye movement
RER rough endoplasmic reticulum
RFLP restriction fragment length polymorphism
RGS regulators of G protein signaling
RME receptor-mediated endocytosis
ROCK Rho-A/Rho kinase pathway
ROS reactive oxygen species
RP retinitis pigmentosa
RPE retinal pigment epithelium
RPTK receptor protein tyrosine kinase
RRP readily reuseable pool
RTK receptor tyrosine kinase
RTN reticulons
RVM rostral ventromedial medulla
SAD seasonal affective disorder
SAG Schwann cell membrane-associated glycoprotein
SAH *S*-adenosylhomocysteine
SAICAR succinylaminoimidazole carboxamide ribotide
SAM *S*-adenosylmethionine
SAPK stress-activated protein kinase
SAR specific absorption rate
SBP serotonin-binding protein
SCa slow component a
SCAD short chain acyl-CoA dehydrogenase
SCb slow component b
SCF stem cell factor
SCHAD short chain 3-hydroxyacyl-CoA dehydrogenase
SCIP suppressed cyclic AMP-inducible POU domain protein
SCN suprachiasmatic nuclei
ScTX scorpion toxin
SDS sodium dodecylsulfate

SE	status epilepticus
SEK	SAPK kinase
SER	smooth endoplasmic reticulum
SERCA	smooth endoplasmic reticulum Ca^{2+} transporter
SERT	serotonin transporter
SES	socioeconomic status
SGLT	sodium-dependent glucose transporter
SGPG	sulfate-3-glucuronyl paragloboside
SHIP	src homology inositol phosphatase
SHMT	serine hydroxymethyltransferase
SHP	SH2 domain containing protein tyrosine phosphatase
SIF	small, intensely fluorescent cells
SLC	solute carrier
SMA	spinal muscular atrophy
SMDF	sensory and motor neuron-derived growth factor
SMN	survival motor neuron
SMP	Schwann cell myelin protein
SNAP	soluble NSF attachment protein
SNARE	soluble (*N*-ethylmaleimide-sensitive fusion protein) attachment protein receptor
SNCA	gene coding for α-synuclein
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
SOD	superoxide dismutase
SOS	son of sevenless
SPECT	single photon emission computerized tomography
SR	sarcoplasmic reticulum
SRF	serum responsive factor
SRP	signal recognition particle
SRR	synaptic re-entry reinforcement
SSRI	selective serotonin reuptake inhibitor
SSV	small secretory vesicle
ST/AR	1-stearoyl, 2-arachidonyl
STN	subthalmic nucleus
STT	spinothalamic tracts
SUR	sulfonylurea receptor
SVZ	subventricular zones
TCA	(i) tricarboxylic acid; (ii) tricyclic antidepressant
TCR	T-cell receptor
TDP	thiamine diphosphate
TGFβ	transforming growth factor β
TGN	*trans*-Golgi network
TH	tyrosine hydroxylase
THC	tetrahydrocannabinol
THIP	4,5,6,7-tetrahydroisoxolo-[5,4-*c*]-pyridine-3-ol
TLC	thin-layer chromatography
TLE	temporal lobe epilepsy
TM; TMD	transmembrane spanning domain
TNF	tumor necrosis factor
TOR	target of rapamycin
TP	trifunctional protein
TPA	(i) tissue plasminogen activator; (ii) 12-D-tetradecanoyl phorbol-13-acetate
TPH	tryptophan hydroxylase
TRC	taste receptor cell
TRE	12-D-tetradecanoyl phorbol-13-acetate response element
TRH	thyrotropin-releasing hormone
TRP	transient receptor potential
TSH	thyroid stimulating hormone
TTP	thiamine triphosphate
TTX	tetrodotoxin
UCB	unconjugated billirubin
UDP	uridine 5′-diphosphate
UTP	uridine 5′-triphosphate
VAChT	vesicular acetylcholine transporter
VAMP	vesicle-associated membrane protein
VASE	variable alternative spliced exon
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
VGLUT	vesicular glutamate transporter
VGSC	voltage-gated sodium-ion channels
VIP	vasoactive intestinal peptide
VLCAD	very long chain acyl-CoA dehydrogenase
VLCFA	very long chain fatty acids
VLDLR	very-low-density lipoprotein receptor
VMAT	vesicular membrane amine transporters
VMH	ventromedial hypothalamus
VN	veromonasal neuron
VNO	vomeronasal organ
VPA	valproate
VTA	ventral tegmental area
VWMD	vanishing white matter disease
WM	white matter
X-ALD	X-linked adrenoleukodystrophy

Amino Acids in Proteins

Symbol			Codons					
One letter	**Three letter**	**Name**						
A	Ala	Alanine	GCA	GCC	GCG	GCU		
C	Cys	Cysteine	UGC	UGU				
D	Asp	Aspartate	GAC	GAU				
E	Glu	Glutamate	GAA	GAG				
F	Phe	Phenylalanine	UUC	UUU				
G	Gly	Glycine	GGA	GGC	GGG	GGU		
H	His	Histidine	CAC	CAU				
I	Ile	Isoleucine	AUA	AUC	AUU			
K	Lys	Lysine	AAA	AAG				
L	Leu	Leucine	UUA	UUG	CUA	CUC	CUG	CUU
M	Met	Methionine	AUG					
N	Asn	Asparagine	AAC	AAU				
P	Pro	Proline	CCA	CCC	CCG	CCU		
Q	Gln	Glutamine	CAA	CAG				
R	Arg	Arginine	AGA	AGG	CGA	CGC	CGG	CGU
S	Ser	Serine	AGC	AGU	UCA	UCC	UCG	UCU
T	Thr	Threonine	ACA	ACC	ACG	ACU		
V	Val	Valine	GUA	GUC	GUG	GUU		
W	Trp	Tryptophan	UGG					
Y	Tyr	Tyrosine	UAC	UAU				

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