Developmental Neurobiology

Edited by
Greg Lemke

DEVELOPMENTAL NEUROBIOLOGY

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DEVELOPMENTAL NEUROBIOLOGY

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TABLE OF CONTENTS

Contributors xi

Preface xvii

SECTION I: NEURAL INDUCTION, PATTERN FORMATION, AND CELL SPECIFICATION

Neurulation
A Lawson and G C Schoenwolf 3

Neural Induction in Chicks
C Linker and C D Stern 10

Morphogens: History
L Wolpert 16

Sonic Hedgehog and Neural Patterning
H Roelink 21

Floor Plate Patterning of Ventral Cell Types: Ventral Patterning
E Dessaud, J Briscoe, and K Hill 29

Wnt Pathway and Neural Patterning
R M Twyman 39

Bone Morphogenetic Protein (BMP) Signaling in the Neuroectoderm
C M Mizutani and E Bier 45

Retinoic Acid Signaling and Neural Patterning
G Duester 55

Hox Genes Expression
M Parrish, C Nolte, and R Krumlauf 61

Anterior–Posterior Spinal Cord Patterning of the Motor Pool
J S Dasen 72

Neural Patterning: Midbrain–Hindbrain Boundary
S Scholpp and M Brand 80

Midbrain Patterning
S Agarwala and C W Ragsdale 87

Forebrain Development: Prosomere Model
L Puelles 95

Forebrain: Early Development
F Cavodeassi, M Kapsimali, S W Wilson, and R M Young 100

Forebrain Development: Holoprosencephaly (HPE)
E S Monuki 105

Neural Patterning: Arealization of the Cortex
D D M O'Leary and T T Kroll 116

Neural Patterning: Eye Fields
M Noda, H Takahashi, and H Sakuta 125

Notch Pathway: Lateral Inhibition
A Chitnis 131

Notch Signal Transduction: Molecular and Cellular Mechanisms
A Miyamoto and G Weinmaster 140

Helix–Loop–Helix (bHLH) Proteins: Proneural
J E Johnson and S M Glasgow 150

Helix–Loop–Helix (bHLH) Proteins: Hes Family
R Kageyama, T Ohtsuka, R Ohsawa, and J Hatakeyama 156

Sox Gene Expression
S R Hutton and L H Pevny 165

Transcriptional Networks and the Spinal Cord
S L Pfaff and T Macfarlan 172

Transcriptional Silencing
R M Twyman 178

Motor Neuron Specification in Vertebrates
M-R Song and S L Pfaff 184

Lissencephaly
T Pramparo and T Wynshaw-Boris 192

Molecular Anatomy of the Mammalian Brain
M A Zapala, C Barlow, and I Hovatta 199

Terminal Differentiation: REST
S Aigner and G W Yeo 206

Drosophila Apterous Neurons: From Stem Cell to Unique Neuron
S Thor 213

Macroglial Lineages
A M Butt 218

Dopaminergic Differentiation
A Rosenthal and J C Lin 221

Olfactory Neuron Patterning and Specification
C L Beites, S Kawauchi, and A L Calof 226

Oligodendrocyte Specification
W D Richardson 238

Hair Cell Differentiation
C Puligilla and M W Kelley 245

Retinal Development: An Overview
H R L Napier and B A Link 251

Retinal Development: Cell Type Specification
F L A F Gomes and M Cayouette 259

Neural Stem Cells: Ocular
T A Reh 266

Neural Crest
H M Young, R B Anderson, and D F Newgreen 271

Schwann Cell Development
R Mirsky and K R Jessen 282

Schwann Cells and Axon Relationship
R D Fields 293

Neural Crest Cell Diversification and Specification: ErbB Role
K-A Nave and M H Schwab 298

Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation
M Wegner 306

Neural Crest Cell Diversification and Specification: Melanocytes
K Bismuth and H Arnheiter 312

Autonomic Nervous System Development
D F Newgreen, M J Howard, and R Nishi 321

Autonomic Neuroplasticity: Development
P G Smith 333

Enteric Nervous System Development
M D Gershon 341

Sexual Differentiation of the Brain
D G Zuloaga, C L Jordan, and S M Breedlove 348

Sexual Differentiation of the Central Nervous System
R B Simerly 354

SECTION II: AXON GUIDANCE AND SYNAPTOGENESIS

Chemoaffinity Hypothesis: Development of Topographic Axonal Projections
T McLaughlin and D D M O'Leary 365

Topographic Maps: Molecular Mechanisms
A C Lin and C E Holt 372

Axonal Pathfinding: Theoretical and Computational Models
D Mortimer and G J Goodhill 381

Axon Guidance: Guidance Cues and Guidepost Cells
L Ma and K Shen 387

Axon Guidance by Glia
A Faissner 393

Corticospinal Development
J H Martin, K M Friel, I Salimi, and S Chakrabarty 403

Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems
F Wang 415

Growth Cones
J A Raper 422

Optic Nerve, Optic Chiasm, and Optic Tracts
R A Rachel and B E Reese 427

Activity in Visual Development
M B Feller and E S Ruthazer 433

NMDA Receptors and Development
M A Phillips and M Constantine-Paton 438

Optic Tectum: Development and Plasticity
S C Sharma and K A Patil 449

Dendrite Development, Synapse Formation and Elimination
H Cline 456

Axon Guidance: Morphogens as Chemoattractants and Chemorepellants
J B Thomas and S Yoshikawa 460

Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh)
F Charron and M Tessier-Lavigne 465

Axonal Pathfinding: Netrins
S W Moore, T E Kennedy, and M Tessier-Lavigne 472

Axonal Pathfinding: Extracellular Matrix Role
P Letourneau 480

Postsynaptic Development: Neuronal Molecular Scaffolds
E Kim 487

Postsynaptic Specialization Assembly
C Sala and A El-Husseini 495

Presynaptic Development and Active Zones
N E Ziv and C C Garner 502

Presynaptic Development: Functional and Morphological Organization
D Atasoy and E T Kavalali 512

Development of Drosophila Neuromuscular Junctions
B Berke and H Keshishian 520

Neuromuscular Junction (NMJ): Mammalian Development
C R Slater 529

Neuromuscular Junction (NMJ): Postsynaptic Basal Lamina
M J Werle 538

Neuromuscular Junction: Neuronal Regulation of Gene Transcription at the Vertebrate
H R Brenner and D L Falls 544

Neuromuscular Junction: Synapse Elimination
R R Ribchester 553

Schwann Cells and Plasticity of the Neuromuscular Junction
Z Feng and C-P Ko 561

Glia and Synapse Formation: An Overview
N J Allen and B A Barres 567

GABA$_A$ Receptors: Developmental Roles
Y Ben-Ari 573

SECTION III: NEUROGENESIS, NEUROTROPHISM, AND REGENERATION

Cerebral Cortex: Symmetric vs. Asymmetric Cell Division
G Fishell and C Hanashima 585

Differentiation: The Cell Cycle Instead
A Philpott 592

Programmed Cell Death
R Oppenheim and C Milligan 599

Autonomic and Enteric Nervous System: Apoptosis and Trophic Support During Development
R O Heuckeroth 607

Autophagy and Neuronal Death
P G H Clarke 615

Neurotrophins: Physiology and Pharmacology
J M Conner and M H Tuszynski 622

Enteric Nervous System: Neurotrophic Factors
M J Saffrey 628

Glial Growth Factors
B M Reuss 634

Neural Stem Cells: Adult Neurogenesis
C Zhao and F H Gage 641

Neurogenesis in the Intact Adult Brain
G Kempermann 648

Stem Cells and CNS Repair
R Jandial, A Persson, A Ho, M L Levy, and E Y Snyder 653

Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging
G M Bernal and D A Peterson 662

Synaptic Plasticity: Neuronal Sprouting
J Nilsen 666

Axonal Regeneration: Role of Growth and Guidance Cues
M Bähr and P Lingor 671

Spinal Cord Regeneration and Functional Recovery: Strategies
J W Fawcett 681

Peripheral Nerve Regeneration: An Overview
P M Richardson 687

Index 691

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PREFACE

This volume contains selected chapters from the *New Encyclopedia of Neuroscience* that address key features of the development of nervous systems. They are organized to cover events in neural development that occur from early to late – from the first induction and segregation of neural cells and tissues from non-neural epithelia, through the axial patterning of neural anlagen and the specification of neural cell types, to axon guidance and the organization of axonal connections, adult versus embryonic neurogenesis, and nervous system degeneration and repair.

The 93 chapters are loosely grouped into three sections – *Neural Induction, Pattern Formation, and Cell Specification*, *Axon Guidance and Synaptogenesis*, and *Neurogenesis, Neurotrophism and Regeneration*. These groupings notwithstanding, there are often important connections between chapters in different sections, in keeping with the frequent use of the same molecular and cellular strategies by neural cells at different times and for different purposes in development.

There has been an explosion of knowledge in developmental neuroscience over the last two decades. In general, this explosion has been driven by (a) the increasingly prominent application of genetics and molecular biology to longstanding developmental questions; and (b) the development and application of new cell labeling and imaging methods, many of which exploit a cornucopia of fluorescent proteins. These methodological advances, together with the conceptual integration of experimental models across *Drosophila*, *C. elegans*, zebrafish, mice, and humans, have allowed us to answer a variety of longstanding ('classical') questions. These include the molecular mechanisms underlying the formation of the retinotectal and other topographic maps, the composition and interaction of the sets of transcription factors that specify distinct subsets of neurons and glia, the molecules that attract axons to appropriate targets and repel them from inappropriate sites, the mechanisms by which synapses are established and consolidated, and the events that promote neurogenesis in the adult rain. At the same, this new work has, in several instances, raised unanticipated scientific questions that now must be answered.

Many different phenomena are addressed in the chapters of *Developmental Neuroscience*, by authors who have made important contributions to our current understanding of these phenomena. For example, the volume begins with discussions of neurulation by G. C. Schoenwolf and A. Lawson, and neural induction by C. D. Stern and C. Linker. It also contains chapters on the history of morphogens by L. Wolpert, on the role of Hox genes in neural development by R. Krumlauf, M. Parrish, and C. Nolte, and on arealization of the neocortex by D. D. M. O'Leary and T. T. Kroll. The molecules and mechanisms underlying Notch-Delta signal transduction are addressed by G. Weinmaster and A. Miyamoto, motor neuron specification is discussed by S. L. Pfaff and M.-R. Song, and dopaminergic neuron differentiation by A. Rosenthal and J. C. Lin. Advances in our understanding of topographic mapping are summarized by C. E. Holt and A. C. Lin, of growth cone dynamics by J. A. Raper, and of dendrite development by H. Cline. Netrins are addressed by M. Tessier-Lavigne, T. E. Kennedy, and S. W. Moore, Drosophila neuromuscular junction formation by H. Keshishian and B. Berke, programmed cell death by R. Oppenheim and C. Milligan, and adult neurogenesis by F. H. Gage and C. Zhao. Since the authors of these and many other chapters have pioneered the science under discussion, readers of *Developmental Neuroscience* will very much benefit from the perspectives of the people who have made the science happen.

Greg Lemke

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NEURAL INDUCTION, PATTERN FORMATION, AND CELL SPECIFICATION

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Neurulation

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Phases and Stages of Neurulation

The central nervous system is one of the first organ systems to develop in vertebrate embryos. The process by which this occurs is called neurulation. Neurulation is a developmental event that results in formation of the neural tube, the early hollow rudiment of the adult central nervous system. Once formed, the neural tube differentiates into a rostral portion, the future brain, and a caudal portion, the future spinal cord. The future brain further divides into three main swellings, the forebrain, midbrain, and hindbrain, and further subdivision occurs throughout the rostrocaudal extent of the neural tube with the formation of segmental swellings called neuromeres.

Neurulation occurs in two sequential phases termed primary and secondary neurulation. Primary neurulation occurs in four clearly defined but overlapping steps. In the first step, the neural plate is formed from epiblast, one of two primitive layers composing the early embryo. In the second step, the neural plate undergoes shaping, extending its length and narrowing its width. In the third step, the neural plate bends to form a neural groove, flanked by bilateral neural folds. In the final step, the tips of the neural folds meet and fuse in the dorsal midline to generate the neural tube. Secondary neurulation also involves a series of steps. In the first step, a solid mass of cells, derived from the tail bud, forms. This solid mass is called the medullary cord. In the second step, the medullary cord cavitates to form multiple lumina. In the final step, all lumina coalesce into a single, central lumen bounded by the walls of the so-called secondary neural tube. The processes of primary and secondary neurulation give rise to different rostrocaudal levels of the neural tube. Primary neurulation forms the entire brain and most of the length of the spinal cord (down to about the lumbosacral level), whereas secondary neurulation forms the tailmost portion of the spinal cord. Because much more is known about the cellular and molecular mechanisms of primary neurulation, compared to secondary neurulation, and because most of the neural tube forms during primary neurulation, further discussion herein focuses only on primary neurulation. Also, the following discussion of the tissue and cellular bases of primary neurulation is based principally on the chick, because this model system has been the main one used to unravel the morphogenetic mechanisms underlying neurulation.

Tissue and Cellular Events Underlying Primary Neurulation

The neural plate is formed during gastrulation when epiblast cells located rostral to and beside Hensen's node and the cranial portion of the primitive streak respond to signals from the node by a process known as neural induction. The epiblast cells undergo apicobasal thickening to generate a neural plate, which at this initial stage of development is broad mediolaterally and short rostrocaudally. Once the epiblast is committed to such a neural fate, formation of the neural plate becomes autonomous to the committed cells and does not require the presence of either Hensen's node or nonneural ectodermal cells.

Neural plate shaping is the process by which the broad and short neural plate becomes narrowed transversely and elongated rostrocaudally (**Figures 1(a)–1(c)**). This is achieved through a convergent-extension movement of the neural plate. A convergent-extension movement consists of a simultaneous rostrocaudal lengthening and mediolateral (transverse) narrowing of the neural plate (**Figure 1(d)**). The neural plate also undergoes further apicobasal thickening during its shaping.

The neural plate begins to bend while neural plate shaping is underway. This commences in the cranial region first, and in the spinal cord region later. The process resembles the closing of a door as it rotates around its hinges. The midline of the neural plate remains fixed during bending, creating a median hinge point (MHP) along essentially the entire rostrocaudal extent of the neuraxis, whereas the lateral aspects of the neural plate are elevated gradually, forming the neural groove and incipient neural folds (i.e., the initial folds at the lateral margins of the neural plate) (**Figures 2(a)–2(c)**). Subsequently, additional bending occurs in the neural plate close to its junction with the epidermal ectoderm, resulting in the formation of two dorsolateral hinge points (DLHPs) (**Figures 3(a)–3(c)**). This is seen at prospective cranial levels of the neuraxis, as well as the extreme caudal end of the future spinal cord region. The morphological hinge points are localized regions where the neural plate is anchored to adjacent tissues – the MHP to the prechordal plate mesoderm and notochord and the DLHPs to the adjacent surface ectoderm of the

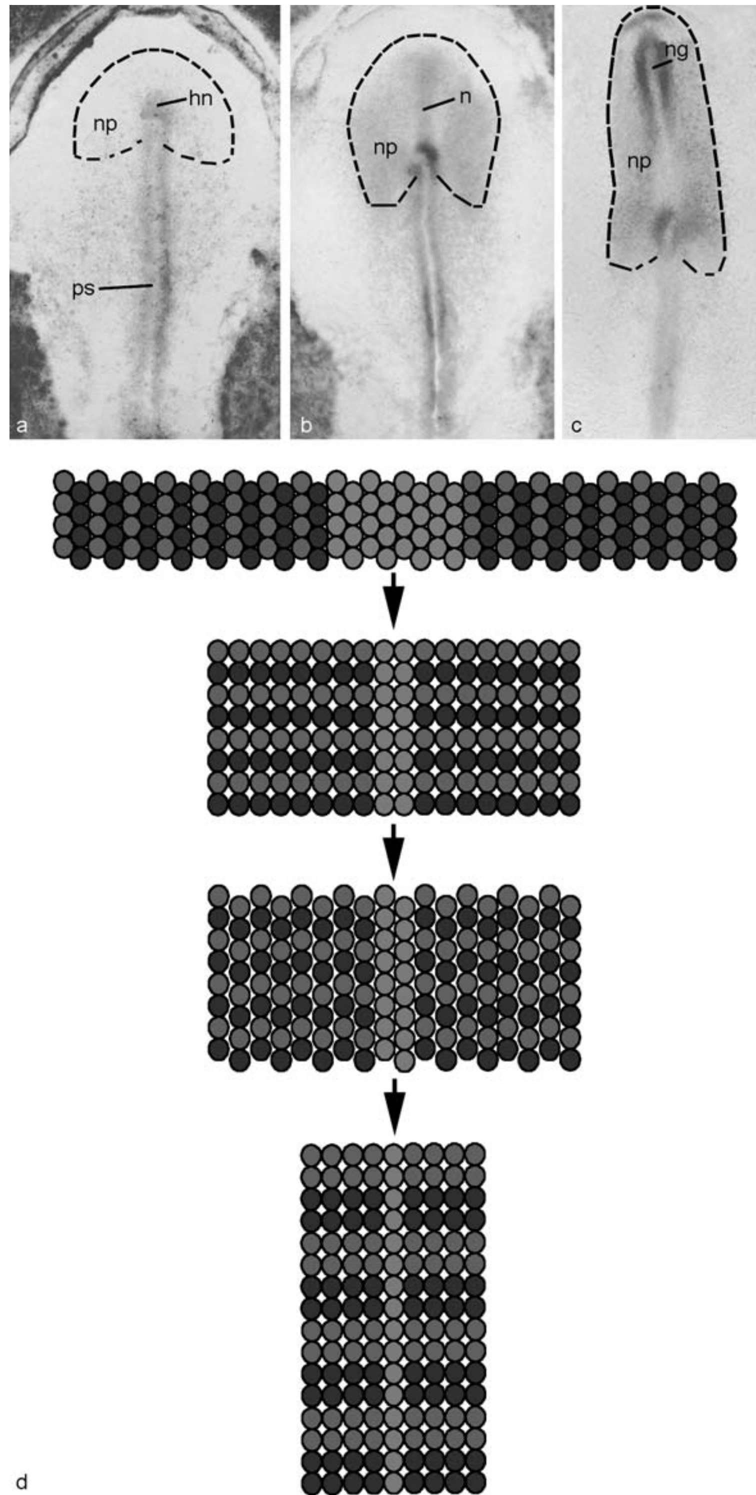

Figure 1 Whole-mounts of chick embryos undergoing primary neurulation, viewed from the dorsal surface (a–c), and a model of neuroepithelial cell rearrangement during neural plate shaping (d). (a) The neural plate (np) has just formed; its approximate borders are outlined (hn, Hensen's node; ps, primitive streak). (b) The neural plate is undergoing shaping; its approximate borders are outlined (n, notochord). (c) The neural plate is initiating bending, establishing a neural groove (ng), while still undergoing shaping. (d) Cell rearrangement occurs during shaping of the neural plate, thereby increasing its length while decreasing its width; median hinge point neural plate cells, yellow; lateral neural plate cells, red and green.

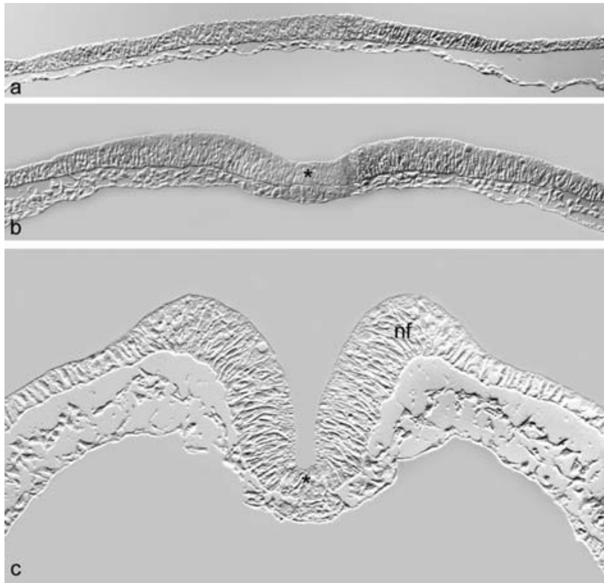

Figure 2 (a–c) Cross-sectional views showing formation of the median hinge point (*) and elevation of the incipient neural fold (nf) at the future midbrain level of the chick. The light micrographs are transverse plastic sections at three sequential stages.

neural folds. Thus hinge points stabilize the neural plate during bending. Bending about the DLHPs results in convergence of the definitive neural folds, directing their tips medially until they meet in the dorsal midline in readiness for fusion (**Figure 3(c)**). Elsewhere (i.e., throughout most of the length of the spinal cord), where DLHPs are not formed, bending occurs about the MHP only, bringing the apical surfaces of the neuroepithelial cells into apposition with each other (**Figure 4**). This results in a temporary occlusion of the spinal canal. The absence of the DLHPs, and therefore neural fold convergence, in the spinal cord is one of the major differences between neurulation at cranial and spinal cord levels of the neuraxis.

The neural folds are located at the lateral margins of the neural groove and include the junction of the neural plate with the adjacent epidermal ectoderm. The incipient neural folds first appear as the neural plate folds about the MHP. Later, as folding occurs about the DLHPs, the definitive neural folds become evident. Each neural fold is double-layered, composed of an inner neuroepithelial layer and an outer epidermal ectodermal layer. Four key events occur in sequence during formation and morphogenesis of the neural folds. They are epithelial ridging, kinking, delamination, and apposition (**Figure 5**). Epithelial ridging is characterized by the formation of a ridge at the prospective outer epidermal–inner neuroepithelial transition zone (**Figure 5(a)**). It results from the difference in heights of the epidermal ectoderm (ee) and

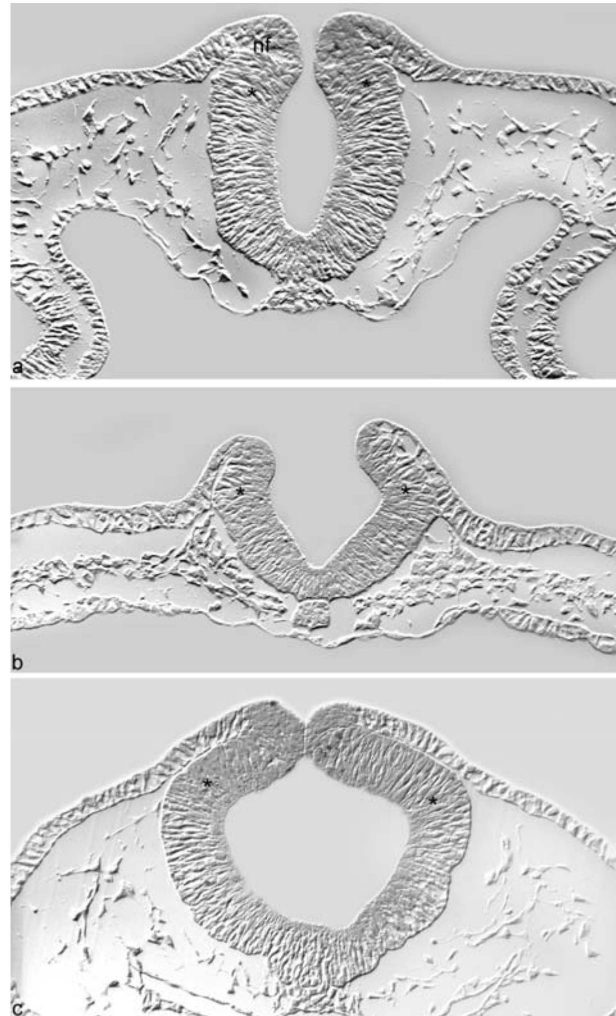

Figure 3 Cross-sectional views showing formation of the dorsolateral hinge points (*), formation and convergence of the definitive neural folds (nf), and closure of the neural groove in the chick. The light micrographs are plastic transverse sections through the future midbrain (a) and hindbrain (b) levels at one stage, and through the future forebrain level (c) at a later stage.

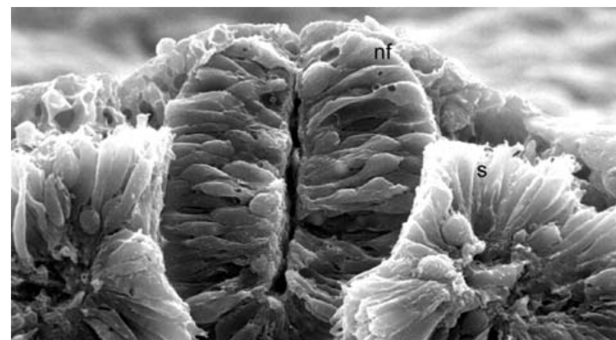

Figure 4 Scanning electron micrograph showing apposition of the neural folds (nf) in the chick at the spinal cord level (level of the seventh somites, S) of the neuraxis. Fusion of the neural folds is in progress and the neurocele is occluded.

neuroepithelial (NE) cells, and it initiates the formation of the neural folds. The NE cells increase their heights, whereas the ee cells decrease theirs. The next step is epithelial kinking (**Figure 5(a)**), defined as the formation of a concave curvature centered at the prospective ee–NE interface. The main feature here is a change in cell shape within the incipient neural folds; the cells here have constricted bases and expanded apical ends, and are described as inverted wedge-shaped, in sharp contrast to the morphology of the cells at the hinge points, which are described as wedge-shaped. The third event is epithelial delamination (**Figure 5(b)**), defined as the splitting of a single epithelium into two epithelial layers. The process results in the formation of a linear interface at the ee–NE transition zone between the two epithelial layers. Delamination occurs by cavitation and the deposition of extracellular matrix at the interface. The cells then reorient radially with respect to the interface and acquire intercellular junctions between either adjacent ee cells or adjacent NE cells, but never across the interface between ee and NE cells. The final event of neural fold morphogenesis is epithelial apposition (**Figure 5(c)**). This is the increase in width of the interface formed during epithelial delamination. The hallmark of this stage of neural fold morphogenesis is a mediolateral expansion of the NE layer, coupled with an apicobasal flattening of the ee layer. Regional differences exist in the degree of epithelial apposition along the rostrocaudal extent of the neuraxis. Generally, where DLHPs are formed and convergence of the neural folds occurs, as seen at forebrain and midbrain levels, apposition is extreme. However, where true DLHPs do not form, as at rostral spinal cord levels, epithelial apposition is lacking.

The final stage of primary neurulation involves the closure of the neural groove. Of all of the steps of avian neurulation, this is the step that has been least explored. Following neural fold convergence, the tips of the neural folds are brought into apposition with each other in the dorsal midline where fusion occurs. In the chick embryo, neural groove closure commences in the prospective midbrain region before progressing rostrally to involve the forebrain, and caudally to involve the hindbrain and spinal cord.

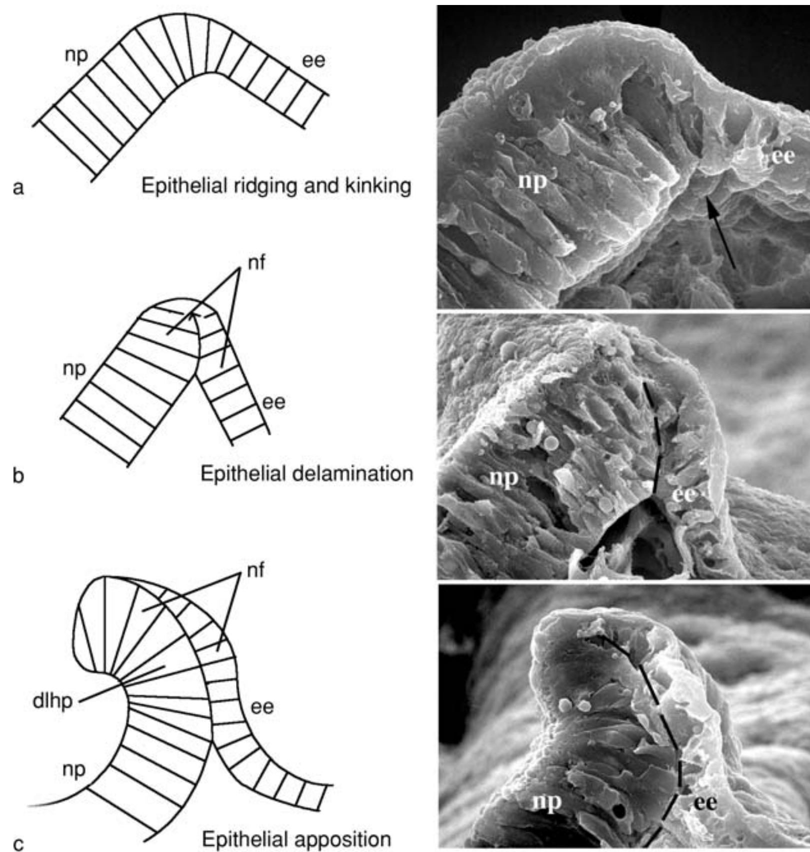

Figure 5 Drawings and scanning electron micrographs showing the four key events of neural fold formation and morphogenesis at the future brain level in the chick embryo: epithelial ridging and kinking (a, b), epithelial delamination (b), and epithelial apposition (c). dlhp, dorsolateral hinge point; ee, epidermal ectoderm; nf, neural fold; np, neural plate. Arrow on micrograph indicates the neural ridge and point of kinking (a). Dashed lines on micrographs indicate the neural fold interface (b, c).

This leaves the rostral and caudal ends of the neural tube temporally open; these openings are known as the rostral and caudal neuropores, respectively. At the tip of each neural fold at most rostrocaudal levels, the epidermal ectoderm caps the neuroepithelium. Apposition of the neural folds, therefore, brings the two epidermal ectodermal layers in contact with each other. Hence, fusion of the neural fold commences with the epidermal ectoderm. As fusion progresses, the newly formed epidermal ectoderm layer delaminates from the neuroepithelium. This brings the neuroepithelium layers of each fold into apposition for fusion of the neuroepithelium to commence. Neural fold fusion is, therefore, described as a double fusion involving two epithelial layers.

Cell Behaviors Generate Tissue Forces in Primary Neurulation

Formation of the neural plate in the chick embryo occurs through cell palisading, which is apicobasal thickening owing to cell elongation. The neural plate so formed consists of a pseudostratified columnar epithelium with two principal cell types, namely, spindle-shaped or fusiform, and wedge-shaped or flask-shaped, as well as other cell types (spherical and inverted wedge-shaped) present in far fewer numbers. The increase in the heights of neuroepithelial cells during neural plate formation is attributed largely to the activity of paraxial microtubules aligned along the long axes of the cells. But other factors such as cell packing and changes in cell–cell adhesion may also contribute to the increase in thickness of the neural plate.

Shaping of the neural plate is largely the result of convergent-extension movements generated by cells within the neural plate. The cell behaviors that account for this movement during shaping of the neural plate in the chick embryo include neuroepithelial cell rearrangement and oriented or nonrandomized cell division. Neuroepithelial cells undergo approximately two rounds of rearrangements (i.e., cell–cell intercalation) during neurulation, narrowing the width of the neural plate by about half during each round, while simultaneously doubling its length with each round (**Figure 1(d)**). The process is believed to be driven by interkinetic nuclear migration, as well as by cell protrusive activity. Additionally, lengthening of the neural plate is achieved by oriented neuroepithelial cell division. On average, chick neuroepithelial cells undergo two to three rounds of cell division over a 24 h period, the plane of cleavage being at right angles to the orientation of the mitotic spindles. In about half of these divisions, daughter cells are placed in the long axis of the neural plate, thereby contributing to its rostrocaudal extension.

Bending of the neural plate involves two main events, neural plate furrowing and neural plate folding. Furrowing of the neural plate involves the formation of longitudinal furrows at the three morphological hinge points (one MHP and two DLHPs) (**Figure 6**). Furrowing is driven by changes in neuroepithelial cell shapes at the hinge points. A significant number of neuroepithelial cells at the MHP (70%) and DLHPs (55%) become wedge-shaped during furrowing (**Figure 5**). In contrast, less than 35% of neuroepithelial cells outside the hinge points become wedge-shaped during neurulation, with a majority of these cells remaining spindle-shaped. Furrowing at the hinge points, therefore, is generated through neuroepithelial cell wedging. Neuroepithelial cell wedging is achieved through both apical constriction and basal expansion of neuroepithelial cells. These processes are likely mediated by contraction of apical bands of microfilaments and translocation of cell nuclei to the bases of the cells during interkinetic nuclear migration, respectively. The signal for furrowing at the MHP comes from the underlying notochord.

Unlike furrowing, folding of the neural plate is mediated by the lateral nonneuroepithelial tissues, principally the epidermal ectoderm, assisted by the mesoderm, endoderm, and the extracellular matrix underlying the neural plate. The cell behaviors in the epidermal ectoderm responsible for folding include cell flattening, oriented cell division, and cell–cell intercalation.

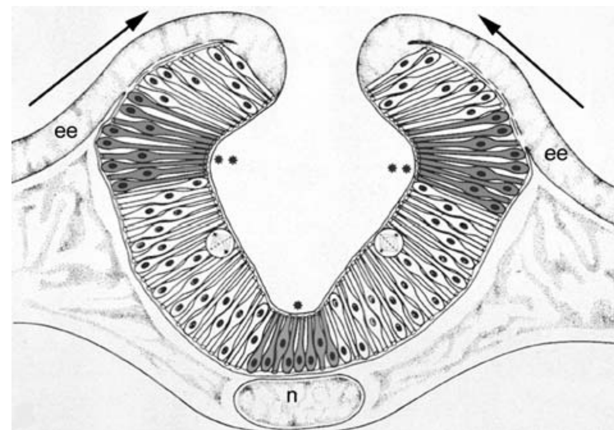

Figure 6 Drawing illustrating the cooperative (hinge point) model of bending of the chick neural plate. Neuroepithelial cell wedging within the hinge points is indicated by red (median hinge point) and blue (dorsolateral hinge points). Arrows indicate mediolateral expansion of the epidermal ectoderm. Single asterisk indicates furrowing associated with the median hinge point; double asterisks indicate furrowing associated with the dorsolateral hinge points; ee, epidermal ectoderm; n, notochord.

Both Intrinsic and Extrinsic Tissue Forces Drive Primary Neurulation

Several studies demonstrate that the forces driving neurulation reside both within the neural plate (intrinsic), as well as outside of this structure (extrinsic). Intrinsic forces are responsible for neural plate shaping and furrowing, and they are generated by behaviors of neuroepithelial cells, such as changes in cell shape, position, and number. In contrast, extrinsic forces generated outside the neuroepithelium account for neural plate folding and neural groove closure. Experimental evidence implicates the epidermal ectoderm in generating a major extrinsic motive force for folding of the neural plate. First, separation of the neural plate and underlying layers from the epidermal ectoderm and underlying layers results in shaping and furrowing of the neural plate, but not folding. Second, removal of the neural plate and underlying layers, leaving intact epidermal ectoderm and underlying layers, results in medial expansion of the epidermal ectoderm. This provides direct evidence of a medially directed epidermal expansion. Third, removal of the epidermal ectoderm, leaving the underlying mesoderm and endoderm intact, stops folding, whereas folding occurs normally after removal of the mesoderm and endoderm beneath the epidermal ectoderm while leaving the latter intact. Finally, when epidermal ectodermal cells at the ee–NE transition zone are tagged with a fluorescent marker and their movement is followed over time, the labeled cells thin and spread medially during neural fold morphogenesis. Primary neurulation thus requires both intrinsic and extrinsic forces acting in concert.

Toward a Molecular Understanding of Primary Neurulation

Progress in understanding the molecular basis of primary neurulation has occurred on two fronts: neural induction and shaping and bending of the neural plate. In regard to the former, the neural plate is now known to be the default state of the ectoderm, and induction of the neural plate actually involves suppressing the formation of the epidermal ectoderm (neural induction is not considered further herein). In regard to shaping and bending of the neural plate, about 100 mutations in the mouse have been identified that result in defective shaping and/or bending and, consequently, result in neural tube defects (NTDs); thus, these mutations provide insight into which genes are involved in both normal and abnormal neurulation. Because these form-shaping events of neurulation are driven by changes in cell behavior, as discussed earlier, it is not surprising that mutation of cytoskeletal, extracellular matrix/cell adhesion, cell cycle, and cell death genes results in NTDs. Neurulation is a highly choreographed morphogenetic series of events that must be precisely timed and coordinated across multiple tissues. This presumably involves signaling among tissues. It is the hope of studies using mouse mutations that such signaling pathways will be identified, ultimately leading to an understanding of the molecular basis of neurulation and the formation of NTDs in both animal models and ultimately in humans. In the following sections, we discuss what has been learned about the molecular basis of shaping and bending of the neural plate.

Shaping and Bending of the Neural Plate

Planar-Cell Polarity Pathway and Convergent Extension

As discussed earlier, convergent extension plays a major role in neurulation. Recent studies have revealed that convergent extension is regulated by the Wnt (wingless) signaling pathway. During development, epithelial sheets become polarized not only apicobasally but also within the plane of the epithelium. In *Drosophila*, the planar-cell polarity (PCP) pathway functions in this latter polarization of the epithelium. Thus, for example, the orientation of wing hairs is established by the PCP pathway. In vertebrates, the PCP pathway is required for proper orientation of stereociliary bundles in the outer hair cells of the mouse inner ear, and for convergent extension during gastrulation and neurulation. How are the PCP and Wnt signaling pathways related?

The *Drosophila* PCP pathway consists of several core proteins that collectively act to convert an extracellular polarity cue into specific changes in the cytoskeleton. These core proteins are now known to be components of the Wnt signaling pathway, and orthologs of several of the *Drosophila* components are conserved in vertebrates. Thus, convergent extension during gastrulation and neurulation is blocked in loss-of-function mutations of the cytoplasmic protein dishevelled in *Xenopus* and its two orthologs in mouse (dishevelled 1 and 2). Wnt signaling involves both a so-called canonical Wnt pathway and a noncanonical Wnt pathway. The PCP pathway utilizes the noncanonical pathway in which certain Wnts, such as Wnt11, bind to their receptors (known as frizzleds). Several other proteins, including dishevelled, must interact in this pathway for proper signaling (and, consequently, for proper convergent extension) to occur. In addition to double dishevelled 1 and 2 mutants, four other mouse mutants exhibit convergent-extension defects: circletail, crash, spin cycle, and loop-tail.

Loop-tail mice have a mutation in the ortholog of the strabismus/van gogh-like gene (*Vangl1*, *Vangl2*), which encodes a transmembrane protein that interacts with dishevelled. Both crash and spin cycle mice have a mutation in the ortholog of the *Drosophila* protocadherin flamingo gene called *Celsr1*. In *Drosophila*, flamingo is required for PCP signaling. Circletail mice have a mutation in the ortholog of the *Drosophila* scribble gene (*Scrib*). Scribble interacts with strabismus. Thus, obtaining an understanding of the PCP pathway in *Drosophila* has had a surprising result – a better understanding also of vertebrate gastrulation and neurulation, and, potentially, a better understanding of how NTDs form in humans.

Actin-Binding Proteins and Apical Constriction

Several actin-associated proteins, when genetically ablated in mice, result in NTDs. One of these, the actin-binding protein shroom, has received considerable study. Overexpression of shroom in cultured epithelial cells is sufficient to cause apical constriction. Shroom causes apical constriction by altering the distribution of F-actin to the apical side of epithelial cells and regulating the formation of a contractile actomyosin network associated with apical intercellular junctions. When shroom is inactivated in *Xenopus* embryos, hinge point formation is drastically altered and neural tube closure fails to occur, providing further evidence for a role of cell shape changes in generating intrinsic forces important for neurulation.

Toward the Prevention of Neural Tube Defects

Neurulation is a complex developmental process that often goes awry in human embryos, leading to neural tube defects, serious congenital anomalies that are severely debilitating and sometimes life threatening. Although the tissue and cellular bases of neurulation are now well understood, the challenge for future studies will be to dissect the molecular basis and provide molecular candidates for mutational analysis in humans. Once such candidate genes are discovered, early and accurate detection of NTDs will be a realty, and their total prevention may be on the horizon. Although the underlying mechanisms remain unclear, periconceptional folic acid supplementation has been shown to reduce the incidence of NTDs in human infants dramatically. Thus, by understanding the process of neurulation better, the hope remains that some day NTDs, severely debilitating birth defects, may no longer occur.

See also: Forebrain Development: Holoprosencephaly (HPE); Forebrain Development: Prosomere Model; Midbrain Patterning; Neural Crest; Neural Crest Cell Diversification and Specification: Melanocytes; Neural Crest Cell Diversification and Specification: ErbB Role; Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation; Neural Patterning: Arealization of the Cortex.

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Neural Induction in Chicks

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Introduction – Neural Induction as a Complex Process

Embryonic development involves the progressive restriction of the fates of initially pluripotent cells to different cell types, arising at the correct locations in the embryo and at the right time. Surprisingly, only a few signaling pathways (as few as seven or eight different classes) appear to be involved in all of these cell fate choices. As in music, where an infinite number of tunes can be played by arranging only a few different notes in precise combination, order, and duration, the timing of the sequence of signaling events is critical in directing cells to their ultimate fates.

Over the past two decades, we have learned considerably about the roles of these different signaling pathways, in large part due to research on embryos of the South African clawed toad frog, *Xenopus laevis*. This species lays large eggs which do not change in volume during early development, permitting genes to be misexpressed or their function inhibited in the entire embryo or a part thereof. This is done by injection of RNAs encoding a gene product of interest, or inhibitory, mutant versions of it (including antisense morpholino oligonucleotides), into one of the cells (blastomeres) arising during the first few cell divisions, and studying the consequences at a later stage of development. This approach has been particularly powerful in identifying the critical signaling pathways that influence cell fate choices in the early embryo. However, because all signaling pathways are used repeatedly during development, the usefulness of the misexpression approach is limited to the study of the very earliest developmental events. It was this approach that led to the identification of fibroblast growth factors (FGFs) and Nodal (a member of the transforming growth factor-β family) as critical players in the induction of the mesoderm, when misexpression and loss of function approaches were combined with the 'animal cap assay' designed by Pieter Nieuwkoop to study this particular inductive event in the late 1960s. The animal cap assay involves isolation of the animal pole of the blastula-stage embryo (which does not normally contribute to mesoderm), which is then cultured in a neutral medium – the cap can be cut from embryos injected with control RNAs or with experimental constructs, or can be treated with protein factors added to the medium.

Although the animal cap assay was designed specifically to study mesoderm induction (precisely because it does not normally contribute to this tissue), it has also been extended to study neural induction and to other, somewhat later developmental events. This approach, along with other experiments (summarized in **Figure 1**), led to the influential 'default model,' which proposes that inhibition of bone morphogenetic protein (BMP; another member of the transforming growth factor-β family) signaling is sufficient to cause animal pole cells to adopt a neural fate. However, BMP signaling is also critical for other patterning and inductive events that take place either earlier than or at the same time as neural induction, making it rather difficult to separate these processes from a direct role purely in neural induction. In particular, inhibition of BMP is first required to specify the 'dorsal' side of the whole embryo (where gastrulation will begin) and where Spemann's organizer will arise. Then, different levels of BMP specify different types of mesoderm: the higher the level, the more ventral the mesoderm. The mesoderm arises from the equatorial region of the embryo. Therefore, treatments that alter the levels of BMP signals from the earliest stages of development will affect all of these processes and the assay will reveal the cumulative effects of the treatment on all of the intervening events as well as any indirect consequences.

An additional problem with studying neural induction using this approach is that the animal cap does contain cells whose normal fate is to contribute to part of the nervous system (especially neural crest, but also some central nervous system, depending on the size of the cap excised). It is therefore impossible to exclude the possibility that by the time it is isolated, the cap has already received some of the signals required by cells to be specified as neural. Thus, the animal cap assay makes it impossible to distinguish a truly 'instructive' induction from a merely 'permissive' event that is part of a more complex cascade.

It is therefore not surprising that, although there is considerable evidence consistent with the proposals of the default model (summarized in **Figure 1**), some experiments in both the chick and in the frog (especially those that do not rely exclusively on early misexpression and on the animal cap assay) are not so consistent and reveal more complexity (summarized in **Figure 2**). In *Xenopus*, inhibition of BMP signaling in one early blastomere (A4) whose progeny does not include any part of the nervous system is not sufficient to induce expression of neural markers. In addition, when the FGF pathway is inhibited with a dominant-negative FGF receptor or with a chemical

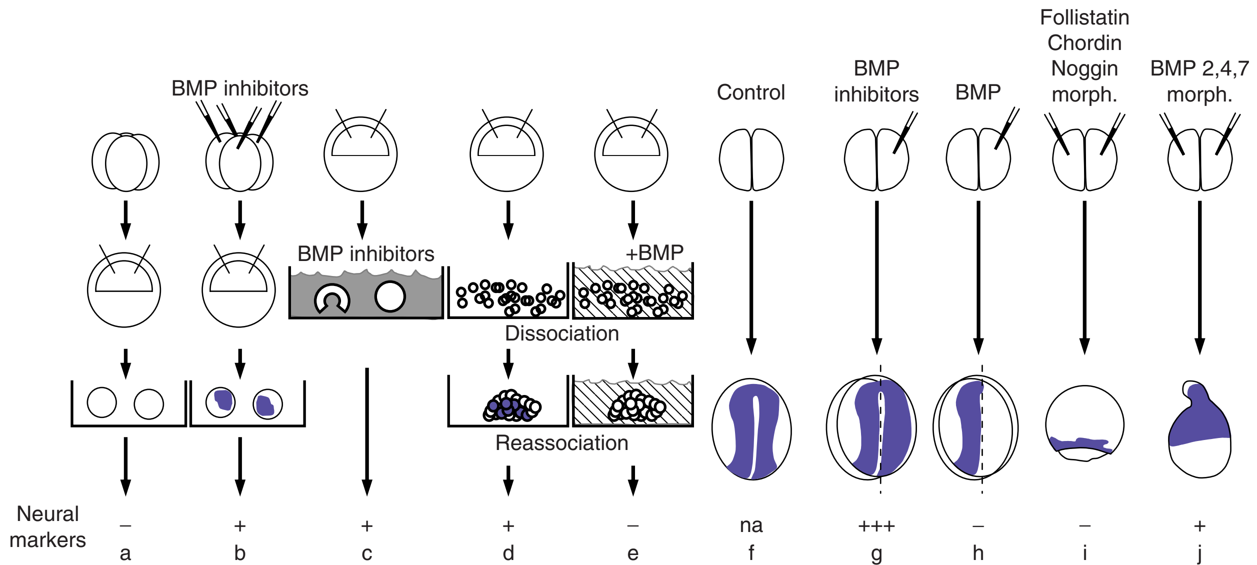

Figure 1 Experimental evidence consistent with the default model. Control animal caps (a) will develop as epidermis; neural markers (+, expressed; –, not expressed; na, not applicable) are expressed when caps are excised from embryos injected with constructs encoding bone morphogenetic protein (BMP) inhibitors (b), or when a BMP antagonist is added to the medium in which caps are cultured (c). Dissociation followed by reaggregation of animal cap cells causes neural differentiation (d), while addition of BMP proteins to the culture medium reverts this effect (e). In whole embryos, the neural plate (blue) develops from the dorsal side (f); when BMP inhibitor constructs are injected into one of the blastomeres at the two-cell stage, the neural plate is expanded in the injected side (g), while injection of BMP at the same stage leads to a drastic reduction in the size of the neural plate (h). Early depletion of BMP antagonists, by injection of antisense morpholinos against Noggin, Chordin, and Follistatin in the whole embryo, leads to expression of BMP molecules in the neural territory and causes severe ventralization of the whole embryo and almost complete abrogation of the neural plate (i). The opposite experiment (j), depletion of three BMPs (BMP2, BMP4, and BMP7), which effectively eliminates BMP signaling in the whole embryo, produces dorsalized radial embryos expressing neural markers throughout the ectoderm.

inhibitor (SU5402), animal caps from embryos injected with a BMP antagonist no longer form neural tissue, suggesting that FGF signals are required in addition to BMP inhibition to specify neural fates. Interestingly, in the ascidian *Ciona intestinalis* (a basal chordate), it is FGF rather than BMP inhibition that is responsible for inducing neural fates, suggesting that a requirement for FGF in neural induction is an ancestral feature of this process.

In the chick embryo it is possible to introduce DNA constructs or morpholinos at virtually any chosen stage of development and in precisely selected groups of cells. In addition, the chick embryo contains a very large region which never contributes to any part of the nervous system in normal embryos, although it is competent to do so in response to grafts of the organizer (Hensen's node, at the tip of the primitive streak, which is analogous to the amphibian dorsal lip of the blastopore). Experiments (**Figure 2**) combining grafts of the organizer with a time-course analysis of gene expression at intervals following the graft first revealed that neural induction is a relatively long process: if an organizer is grafted and then removed after different periods of time, it can be shown that a period of contact as long as 13 h is required before the cells that had been exposed to organizer signals express definitive central nervous system (CNS) markers (such as the transcription factor Sox2). Within a shorter time, the organizer does induce transient expression of some markers, such as Sox3 (which appears after about 3 h) and the 'early response to neural induction' marker (ERNI; which is induced after just 1 h). Despite the induction of these markers, however, cells expressing them revert to a nonneural fate and do not express Sox2 or form a neural plate if the organizer is removed. Inhibition of BMP after 5 h of contact with the organizer can, however, stabilize the expression of Sox3, but is still not sufficient for induction of either Sox2 or of a neural plate.

The evidence suggesting that BMP inhibition is not sufficient for neural induction in the chick embryo also includes the fact that the timing of expression of the BMP antagonists implicated in this process in the frog (such as Chordin, Noggin, and Follistatin) does not entirely fit with the period during which the chick organizer is able to induce neural markers: Chordin is expressed in the organizer at a stage when this tissue can no longer induce, while Noggin and Follistatin are not present at all at the inducing stages and appear only in nonorganizer tissue at later stages. Likewise BMP4 and BMP7 (the BMPs implicated as the inhibitors of neural fate in the frog) are not ubiquitously present

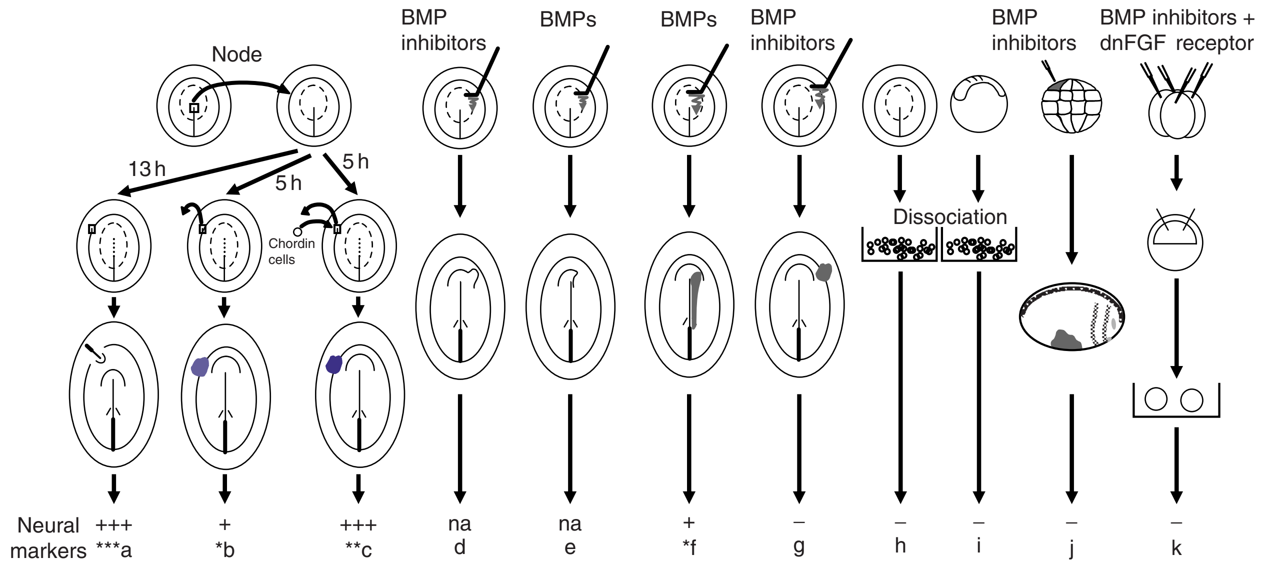

Figure 2 Experimental evidence inconsistent with the default model. Analysis of the time course of neural induction, after grafts of the organizer (Hensen's node), shows that while 13 h of contact between the organizer and the host ectoderm is required for full neural induction (a), 5 h of contact induces transient expression of early neural markers (e.g., Sox3 and ERNI) (b). Contact for 5 h is also required to sensitize the ectoderm to bone morphogenetic protein (BMP) inhibitors such as Chordin (c). Manipulation of BMP levels at the border of the neural plate affects the size of the neural plate: when Chordin (BMP antagonist) is expressed at this border, the neural plate is expanded (d); misexpression of BMP4 at this site causes narrowing of the neural plate (e). Overexpression of BMP in the neural plate territory does not affect the expression of early neural markers (f) (although it does abolish Sox2 expression at a later stage), while inhibition of BMP in the nonneural ectoderm does not induce neural markers (g). Dissociation of either chick epiblast (h) or of zebra fish animal caps (i) does not lead to neural differentiation. In *Xenopus*, injection of BMP antagonists at the 32-cell stage into a blastomere (A4) that is fated to give rise predominantly to ventral epidermis is not sufficient to trigger neural marker expression in the progeny of the injected cell (j). Animal caps excised from embryos injected with both a dominant-negative fibroblast growth factor (dnFGF) receptor and BMP antagonists (Chordin or Noggin) do not express neural markers. *, Expression of only early neural markers; **, expression of early and definitive neural markers; ***, induction of a neural plate; na, not applicable.

throughout the early embryo before neural induction as they are in *Xenopus*. Finally, inhibition of BMP (by Chordin, Noggin, or intracellular antagonists) is not sufficient to induce any neural marker in the chick, even when combined with or preceded by application of FGFs to the same cells. However, inhibition of BMP is undoubtedly required for neural induction, since when BMP4 is misexpressed in the prospective neural plate it prevents expression of Sox2 (but not Sox3). Moreover, when BMP levels are manipulated at the border of the neural plate (prospective neural crest/placode territory, somewhat equivalent to the animal cap of *Xenopus*) the size of the neural plate is affected: higher levels of BMP narrow the neural plate, while lower levels expand it.

Experiments using explants of prospective neural plate or prospective epidermis from pregastrula-stage embryos, either cultured alone or in the presence of various peptide growth factors, suggested that in addition to FGF and BMP inhibition, inhibition of Wnt signaling may also be important. However, in whole embryos, it is still impossible to impart neural identity to competent cells even with a combination of all three of these signals.

These results suggest that neural induction is likely to be the result of a more complex and relatively long cascade of sequential signaling events, involving both FGF signals and inhibition of BMP, but also other signals which are still unknown. They also suggest that cells at the anterior and lateral borders of the neural plate are particularly sensitive to the level of BMP signaling.

The Timing of Neural Induction

The classical experiments of Spemann and Mangold (published in 1924) and subsequent studies in various species suggested that neural induction probably occurs at the gastrula stage, after the mesoderm has been specified and patterned to some extent. However, experiments in the chick using explanted tissues as well as studies of the expression patterns of very early 'preneural' markers suggested that cells are already started along the cascade that will lead to the acquisition of neural fates at much earlier stages, even preceding gastrulation and before the organizer forms. ERNI and Sox3 are two such markers. Both are induced by, and require, FGF, but neither this

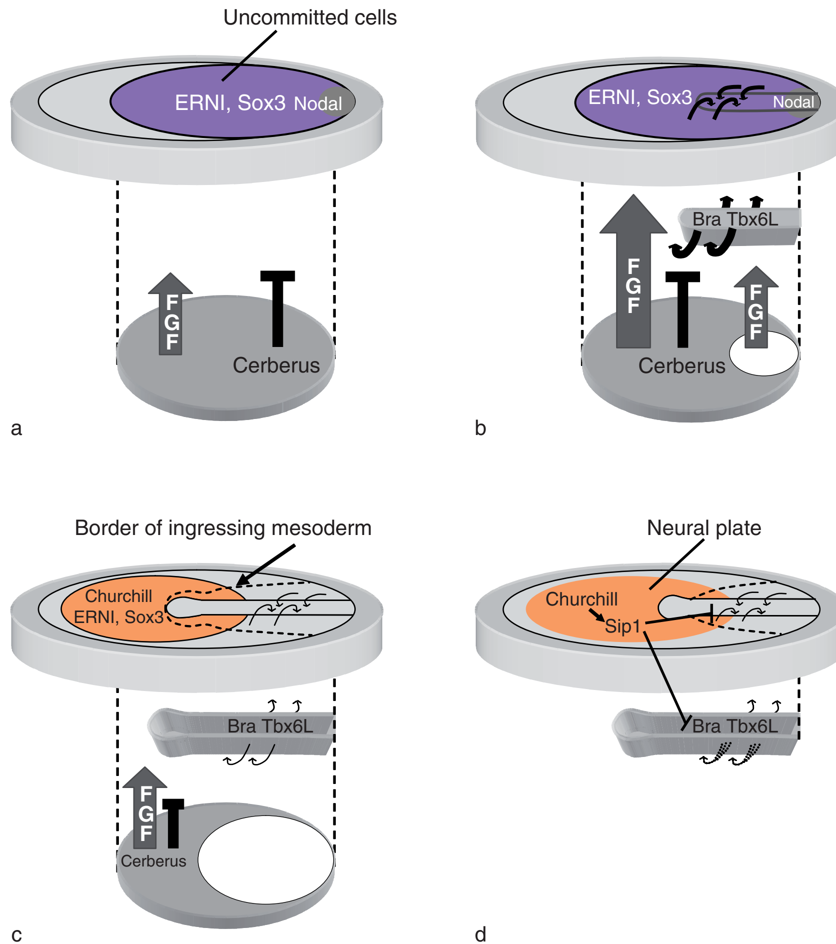

Figure 3 A model for the sequence of events that initiate neural induction in the chick embryo. (a) Before gastrulation, the epiblast (upper disk) is induced to express the early, 'preneural' markers ERNI and Sox3 by fibroblast growth factor (FGF) secreted by the extraembryonic endoderm (hypoblast; lower gray disk). At this time the posterior (right) margin of the epiblast expresses Nodal, but its activity is blocked by its inhibitor Cerberus, which is also produced by the hypoblast. (b) Gastrulation begins when the hypoblast (and Cerberus expression) moves away from the Nodal-expressing region: this releases Nodal signaling, which cooperates with FGF to induce the formation of the primitive streak. Cells ingress from the surface of the epiblast into the primitive streak to form mesoderm and endoderm. (c) Continued exposure of the epiblast that still remains on the surface to FGF from the hypoblast now induces expression of the zinc finger transcription factor Churchill. (d) A target gene activated by Churchill is *Sip1*, which encodes another zinc finger transcription factor that blocks two genes (*Brachyury* and *Tbx6L*) required for ingression of cells into the primitive streak. This effectively ends the process of gastrulation next to the anterior parts of the primitive streak and therefore causes *Churchill*$^{+}$/*Sip1*$^{+}$ cells to remain on the surface and become committed to a neural plate fate.

signal nor the expression of these early markers is sufficient for neural plate formation (**Figure 3**). At this early stage, FGF8 (the most likely candidate for an initial inducing signal) is expressed in an extraembryonic tissue (called the hypoblast in chick, rabbit, and human embryos, the equivalent of which is the anterior visceral endoderm (AVE) in the mouse); this tissue can induce the expression of these early preneural markers when grafted to an ectopic site, even though neither the chick hypoblast nor the mouse AVE can induce a neural plate in the same assay. It is therefore likely that neural induction is initiated by signals from the hypoblast/AVE but that acquisition of a neural plate fate occurs only after the initial induction has been reinforced and completed by signals from other tissues, most likely to be the organizer itself and its derivatives (such as the notochord, prechordal mesendoderm, and perhaps paraxial mesoderm). Importantly however, the early organizer contains both the early and the later signals (or the cells that are a source of the latter), explaining why grafts of the organizer are sufficient to induce a complete nervous system even if it does not normally do all this in the embryo.

FGF8 can induce expression of ERNI, Sox3, and Churchill (a zinc finger transcription factor, which in normal embryos is expressed in the forming neural plate from the end of gastrulation) in a competent

region of the chick embryo that is not fated to contribute to the neural plate. However, these three markers are induced at different times following exposure to FGF: ERNI is induced after 1–2 h and Sox3 after 3 h, and Churchill induction requires 4–5 h. Loss-of-function experiments suggest that both Sox3 and Churchill are functionally required for neural induction to produce a neural plate expressing Sox2. These findings suggest that even a single initial signal can trigger events that follow each other in time. Although we do not yet understand the mechanisms that regulate the timing of onset of expression of these different markers, this is likely to involve complex interactions and feedback mechanisms within the responding cells. It may well turn out that, as in music, the duration of each signal is just as important as the sequence in which they are experienced by cells.

Not Just a Decision between Neural Plate and Epidermis

In the experiments of Spemann and Mangold and those that followed them, the organizer was usually grafted ventrally, adjacent to a region of the embryo whose fate is normally to give rise to epidermis. This led to the generally assumed view that neural induction is a decision between these two fates: skin as opposed to nervous system. However, a study of the expression and functions of early 'preneural' markers suggested that, in addition, neural induction also requires cells to decide between mesodermal and neural fates, close to the midline of the embryo. Functional studies of transcription factor Churchill suggested that it encodes a transcriptional activator, the target of which is another zinc finger transcription factor called Sip1 (Smad-interacting protein-1), which in turn inhibits expression of the primitive streak gene *Brachyury*, required for ingression of cells to form mesoderm. Therefore the onset of Churchill expression marks cells that will no longer ingress to form mesoderm and which will therefore remain on the surface of the embryo, where they can form a neural plate if they receive the remaining signals required to complete the process. Interestingly, Sip1 was originally isolated as a direct partner of Smad-1, a critical effector of BMP signaling, suggesting perhaps that Churchill/Sip1 are part of a mechanism required to sensitize cells to levels of BMP signals, perhaps explaining why cells that have not been exposed either to an organizer or to FGF signals for at least 5 h do not appear to be responsive to BMPs or their antagonists. Thus, neural induction comprises not only a decision between neural plate and epidermis at its lateral boundary, but also the decision to stop ingressing (gastrulation) so that cells can remain on the surface to give rise to neural plate, at the midline. These results are summarized as a model in **Figure 3**.

Importantly, exposure to FGF and absence (or low levels) of BMP are both required along with Nodal signaling to initiate gastrulation and for specifying the organizer. Although the hypoblast/AVE do produce FGF8 and BMP antagonists, both of these tissues paradoxically inhibits primitive streak formation in both chick and mouse embryos. This is due to their expression of Cerberus and Lefty-1, which inhibit Nodal. Thus, FGF signals are required in parallel to specify mesendoderm and for the initiation of neural induction, but different cells within the epiblast are destined to form mesendoderm and neural plate. It is the duration and timing of these signals as well as the position of the cells when they receive them that determine which of these fates (mesendoderm, neural plate, or epidermis) they will adopt.

Taken together, these findings reveal several distinct stages in the neural induction process: first, an FGF signal induces, sequentially, expression of ERNI, Sox3, and Churchill. Then, Churchill (via Sip1) inhibits ingression of cells, preventing them from forming mesendoderm and causing them to stay on the surface. These surface cells become sensitive to both BMP inhibition and to other signals required for formation of a Sox2-expressing, mature neural plate. BMP inhibition stabilizes the expression of early markers (Sox3) but is not sufficient to induce Sox2.

Conclusions – Neural Induction Is Not Yet a Solved Problem

The experimental findings discussed here reveal that neural induction is likely to be the result of a sequence of signaling events rather than a single signal. Although BMP inhibition is required for cells to acquire a neural plate fate, this is insufficient, and other signals, including FGF (but not only this factor), are also required. Moreover, formation of a neural plate also requires mechanisms that cause cells to remain superficial in the embryo and prevent them from gastrulating. While some of these signals are starting to be uncovered, it is becoming clear that there are several other pathways that must be involved. These other pathways still remain to be found, as are the precise interactions between these different pathways, which are likely to be very complex.

The timing and spatial pattern of expression of the earliest marker, ERNI – which is initially expressed in a large territory, including the entire future neural plate, and is downregulated just before the appearance of Sox2 expression – raise the possibility that ERNI might act as an antagonist of commitment to neural plate identity. However, BMP inhibitors and Wnt

antagonists (either alone or when combined with each other and with FGF) are not sufficient to downregulate ERNI expression. Among the many questions that still remain open about neural induction, it will be particularly interesting to explore whether the signals that cause the downregulation of ERNI expression are the missing signals required alongside FGF and BMP inhibition to commit cells to a neural plate fate, and which trigger initial expression of Sox2.

See also: Bone Morphogenetic Protein (BMP) Signaling in the Neuroectoderm; Neural Patterning: Eye Fields.

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Morphogens: History

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Introduction

Morphogens refer to substances thought to be involved in the patterning of cells during embryonic development. It is a term coined by Alan Turing in 1952 for a substance whose distribution by diffusion could determine the development of cells which would respond differently to different concentrations of the morphogen. But the patterning of cells in relation to varying concentrations of a substance, particularly graded concentrations, has a long history. Of particular importance is how the position of cells in the developing embryo is specified so that they develop in an appropriate manner. It is still not clear whether diffusible morphogens provide cells with positional information and so pattern a tissue during development.

Gradients and Polarity

In 1888, Wilhelm Roux, an embryologist, reported that when one of the cells at the two-cell stage of the frog embryo was killed, the remaining cell formed a half embryo. This was interpreted to mean that different structures were already specified to develop from the two cells. But then Hans Driesch, just 3 years later, set out to repeat the experiment on sea urchin embryos. Contrary to his expectation, he found that half an embryo could give rise to a small but normal whole embryo. This was the first example in a developing system of what is known as regulation. It showed that there is a fundamental distinction between prospective and possible fates of cells – the possible fates being greater.

Further experiments by Driesch on the sea urchin embryo at a later stage led him to believe that the prospective fate of any cell in the early embryo was determined by its position in relation to the whole embryo. The 'whole' was related to the three Cartesian axes that could be drawn on the embryo – the x, y, and z axes. So in his model, each cell had its position defined. He argued that no matter which part of the early embryo is removed, it will develop normally. This is not true, but he ignored evidence to the contrary.

His model was what he called a harmonious equipotential system; the fate of any cell, or group of cells, was a function of the size of the embryo and it relative position. Driesch rejected any chemical theories to explain his complex model and proposed the concept of 'entelechy,' a vitalistic mechanism. There was no further progress along these lines, but he had emphasized the importance of position in embryonic development.

For any system based on coordinates or position, polarity plays a key role, for it can determine the direction in which a coordinate can increase or decrease. Interest in polarity initially came from studies on regeneration. Thomas Hunt Morgan in 1905 was the first to clearly state the relationship between gradients and polarity. In studying the regeneration of earthworms he put forward the idea of gradients of formative stuffs. He found, for example, that the further back a cut is made, the longer it takes to regenerate a head. "...We might speak of the cells of the worm containing some sort of stuff that is more or less abundant in different parts of the body. The head stuff would gradually diminish as we pass posteriorly...." He came to a similar conclusion studying the regeneration of *Tubularia*: "We may assume that the gradation of the material is of such a kind that the hydranth forming material decreases from the apical towards the basal end." But Morgan did not pursue these important ideas on polarity and gradients.

They were, however, taken up by Child in 1911. Studying regeneration in planaria, he concluded, "All these facts indicate that a graded difference of some sort in the dynamic processes exists along the axis.... It is this dynamic gradation along the axis, together with the complex of correlative conditions associated with it, which I regard as constituting physiological polarity. According to this idea polarity is not a condition of molecular orientation, but is essentially a dynamic gradient in one direction...."

Child went on to propose that the gradient was in metabolism, and he introduced the concept of dominance, drawing an analogy between the organism and the state – both require authority. The dominant region at one end determines this metabolic gradient by determining the rates of reaction at other levels. He gave no indication of how this was done. In support of his ideas he used evidence from experiments in which poisons such as cyanide had less effect on regions with the highest metabolism and so they would die last. All the references in his 1915 book are, with one exception, to his own work. Even as late as 1941 he was still constrained by metabolism. He argued that "...if decrease in concentration of amount of a certain substance or substance-complex occurs in one direction along a gradient, there must be increase in concentration or amount of another substance or substances unless there is a decrease in

volume." It was only when metabolism was replaced with the concept of information that new ideas emerged.

Developmental biologists were at this time becoming interested in gradients in eggs and paid no attention to the work of Morgan or Child. For example, in 1915 the Swedish embryologist Runnstrom studied the effect of different ions on the development of the sea urchin embryo and concluded that there was a chemical material expressed along the axes of the embryo and this "....material is localised in a way that different parts of the embryo have different concentrations. It creates a concentration gradient. The phenomenon of polarity is an expression of the presence of this concentration gradient." Later studies by Runnstrom and Horstadius provided further evidence for gradients. One of the most important experiments in 1935 which illustrated the quantitative nature of gradients along the main axis of the sea urchin embryo – the animal vegetal axis – involved showing that in order for normal development to occur, the number of vegetal cells that were required to be added to the animal pole fragment was greater that that required for a fragment from a slightly more vegetal region.

At this time another approach to embryonic development involved induction and the concept of the embryonic field. Spemann in the 1930s discovered induction of a new axis and the organizer in the amphibian embryo. One part of the embryo, the dorsal lip of the blastopore, the site where gastrulation begins, could, when grafted to another embryo, induce almost a complete second axis. This was a most influential discovery. Yet as early as 1909 it had been shown that the head region of hydra could, when grafted to the body of another animal, induce the formation of a new axis. Spemann also introduced the field concept, referring to different fields of organization in the embryo.

Perhaps the clearest views of gradients, some 30 years after their initial conception, came from Huxley and de Beer in 1934 based largely on studies on regeneration. Their rules were that the origin of polarity is to be sought in external factors, though in some cases it may already be polarized; in regeneration the apical, or head, region is the first formed and is autonomous; and the apical region influences adjacent regions and prevents them forming an apical region.

In 1938 he lectured on gradient theory, but one of the problems he recognized was understanding how quantitative differences along the gradient could give rise to qualitative differences, namely, complex cell patterns. How could discontinuities arise from a continuous gradient? A possible solution came from Dalcq and Pasteels. Considering early amphibian development, they introduced two factors: the yolk gradient V and a cortical factor C which was graded dorsoventrally. They then defined the product CV as the morphogenetic potential as well as the concept of a threshold. How parts of the embryo developed was then determined by whether CV was below or above a threshold.

Other specific models were developed, and that of Rose in 1968 was the first gradient model to generate a pattern. He suggested that the genesis of a cellular pattern could be the consequence of a hierarchy of self-limiting reactions, together with a spread of inhibition form one differentiating region to others further down the gradient. The gradient determined the rate of differentiation, with the reactions proceeding fastest at the high point of the gradient. The reaction that predominates is then that closest to the top of the hierarchy which has not yet been inhibited. Each reaction is self-limiting by virtue of the inhibitor it produces. However, there was no evidence for the proposed inhibitors.

Insects were to provide crucial information on the possible role and nature of gradients. The gradient concept gained considerable support from Klaus Sander's 1960 experiments on the axial body pattern of the leafhopper *Euscelis* embryo, with the results on the reversal of the segment sequence providing strong evidence for gradients. At about the same time, Locke had shown that the ripple patterns in the cuticle of the insect *Rhodnius* could be explained by a gradient along the segment.

Hidegard Stumpf interpreted these results in terms of a concentration gradient in a substance produced at one margin and destroyed at the other – the first clear statement as to how a concentration gradient could be set up. Using the abdominal segments of *Galleria*, which are divided axially into three parts, she then did experiments to determine whether this pattern was based on a gradient. She concluded that this was the case and that the gradient had two functions: to orient the scales in a direction of decreasing concentration, and to provide the cells with specific concentrations to determine their fate. At the same time, and quite independently, Lawrence had proposed a gradient model for the epidermis of the insect *Oncopeltus* based on the orientation of the hairs and bristles. His gradient model was based on a gradient in sand together with active transport against diffusion.

Positional Information

Again quite independently, the author put forward the concept of positional information similar to that

proposed by Stumpf, on the basis of the French flag problem. I had been very impressed that sea urchin embryos could develop a normal pattern of tissue proportions over an eightfold size range. Again, *Hydra* could have a normal pattern over a large range of sizes. The French flag problem addressed how a line of cells, each of which could differentiate into a blue, white, or red cell, could develop the French flag pattern – one-third blue, one-third white, one-third red. Moreover, this pattern would develop irrespective of the number of cells in the line. A clear solution was that each cell knew its position in the line of cells and then could differentiate in an appropriate manner to give the French flag. One way cells could have their position specified was by a gradient in some substance whose concentration was fixed at both ends of the line, one high, the other low, and that varied linearly between the two ends – simple thresholds would give the flag reliably. A very attractive feature of positional information was that it could account for genetic mosaics in the wing and antenna of the fruit fly *Drosophila*.

Curt Stern had shown that mosaics of leg and antenna tissue developed according to where they were along the proximo-distal axis – they had the same positional values but interpreted them differently because of their position along the main body axis.

In 1952 Alan Turing, the famous mathematician, code breaker, and inventor of computers, published a totally new approach to pattern formation. It was essentially a self-organizing mechanism for setting up a pre-pattern in a diffusible substance that could cause structures to form at specific sites.

> It is suggested that a system of chemical substances, called morphogens, reacting together and diffusing through a tissue, is adequate to account for the main phenomena of morphogenesis. Such a system, although it may originally be quite homogeneous, may later develop a pattern or structure due to an instability of the homogeneous equilibrium, which is triggered off by random disturbances. Such reaction-diffusion systems are considered in some detail in the case of an isolated ring of cells, a mathematical convenient, though biologically unusual system. It is found that there are essentially six different forms it might take. In the most interesting form, stationary waves appear on the ring. It is suggested that this might account, for instance, for the tentacle pattern of Hydra and for whorled leaves.

This approach has been extended by the reaction diffusion models of Gierer and Meinhardt which can set up and regulate axial gradients.

One feature of what might be systems in which positional information is involved is that they are relatively small, none being greater than 0.5 mm maximum linear dimension, and there are typically fewer than 30 cells along an axis where position is thought to be specified. The time required to specify position appears to be on the order of hours. This led Crick to propose the existence of a diffusible morphogen for setting up positional fields. A simple model would have the morphogen produced locally at a source at one end, and then it would spread by diffusion along the axis. The resulting concentration gradient could provide positional information.

By far the best evidence for such a gradient in a morphogen patterning a developmental system is the bicoid gradient in the *Drosophila* egg. The gradient in this protein is maternal and runs from anterior to posterior. However, it acts at a time when the embryo is multinucleate, there are no cell wall membranes for it to cross, and the nuclei share a common cytoplasm. At a specific concentration it activates the gene hunchback in the nuclei in the anterior region. Another example in the early embryo relates to the dorso-ventral axis. For cellular systems things are very much more complicated.

Positional signaling by morphogens has been quite widely investigated over the past two decades but it has not been established that this mechanism provides the basis for pattern formation in any multicellular system. In principle it can account for the pattern of digits in the developing wing of the chick embryo. At the posterior margin of the limb bud is a signaling region, the polarizing region, that produces a putative morphogen, the protein sonic hedgehog. The normal pattern of digits from posterior to anterior is 4, 3, 2. The polarizing region is posterior to the future digit 4. If an additional polarizing region is grafted to the anterior margin of the bud, the resulting pattern of digits is 4, 3, 2, 2, 3, 4. The best evidence that the signal is graded comes from the observation that manufacturing a reduced signal by grafting anteriorly a small number of polarizing tissue cells results in a reduced response; if only a small piece is grafted, then the pattern is 4, 3, 2, 2. This fits nicely with a simple graded diffusible signal of sonic hedgehog, but there is no good evidence to support it. Recent studies have shown that the time that the cells are in contact with the polarizing region also plays a role. Again, the elegant model for specification of neuronal subtypes in the vertebrate ventral neural tube is based on a gradient in sonic hedgehog, but direct evidence is still lacking.

Another well-studied multicellular system is the imaginal wing disc of the *Drosophila* embryo. The wing disc is divided into anterior and posterior compartments. The diffusible molecule Hedgehog is expressed in the posterior compartment, and this causes diffusible Decapentaplegic (DPP) to be expressed at the compartment boundary. DPP is indeed

distributed in a long-range concentration gradient; DPP moves without preferential direction at a speed of more than four cells per hour through the target tissue, in spite of which the shape of the gradient remains stable. However, DPP extracellular diffusion alone does not explain its distribution as a stable gradient and that receptor-mediated endocytosis is essential for DPP long-range movement. Although there are some indications that at specific DPP thresholds the genes omb and spalt are turned on, it is notable that even in this well-studied case there is scant if any reliable quantitative evidence for a gradient-dependent positional gene activation.

In early *Xenopus* embryo there is a presumed gradient of an activin-like morphogen activity. At high concentrations of activin the gene goosecoid is activated while Xbra is turned on at lower concentrations. This has been demonstrated by placing a bead of activin in the middle of a group of animal cap cells: goosecoid is turned on nearer the bead than Xbra. These results have been interpreted in terms of the morphogens binding to receptors. However, there is no evidence for an actual diffusion-mediated gradient of activin in the embryo.

One could list many other examples, with the conclusion that no clear case of a canonical morphogen has been found. But even in principle, could diffusing morphogens by themselves specify positional information? We do not know how precise specification of position has to be, but it could be at the single cell level. There are major difficulties. The molecular concentrations involved are invariably small, hence there is an intrinsic chemical noise. Candidate morphogens and their receptors are very dilute, and they operate in a complex physicochemical environment. This potentially makes morphogen propagation extremely unreliable. The extracellular matrix most likely will present a variety of binding sites for the morphogen, trapping it. There are indeed numerous examples of interaction between proposed morphogens and other extracellular proteins. In addition, the extracellular space is tortuous. It has been estimated that this can increase the effective diffusion times as much as fivefold. Many cellular factors can be expected to perturb and/or actively modulate these various constraints. There is also almost always some endocytosis of a diffusing molecule that binds to cell receptors, and this together with tortuosity makes a precise determination of morphogen concentrations problematic. Worse still, the binding of the morphogen to the receptors can have a very significant effect on the distribution and can even prevent a gradient in receptor occupation as receptors become saturated.

It may be that cell–cell interactions play a key role in specifying positional information. Cell–cell interactions figure prominently in all mechanisms relating to tissue polarity. Favorite model systems for studying planar polarization are the *Drosophila* wing and eye. It is widely assumed that one or several (some opposed) gradients are involved. Gradient-distributed molecules such as Dachsous, Four-jointed, and Fat have been identified. Remarkably, while all these candidate players are growth factors, all are membrane proteins; they intervene in cell adhesion and communication, but likely not by being transmitted between cells.

There is a model for polarity in the abdominal epidermis of *Drosophila* based on cell interactions which also provides the cells with positional information – no morphogen playing a key role. The proteins Four-jointed, Dachsous, and Fat are involved in setting up a gradient of an unknown factor X, which determines Frizzled activity. Reading the gradient are Frizzled itself, Prickle, and VanGogh/Strabismus, enabling cells to compare their level of X with that of their neighbors and to set their value as an average of these. On the other hand, there is a model for planar cell polarity based purely on cell-to-cell interactions and local (cell-scale) graded distributions, where factor X is dispensed with, and hence no global positional information is imparted to cells. The model posits that a weak Frizzled activity gradient is read by asymmetric molecular complexes built at cell interfaces around the cadherin Flamingo. Polarization happens robustly over hundreds of cell diameters even with much noise in the Frizzled gradient. It is probable that these last two models have the potential, if suitably extended, to provide positional information.

See also: Axon Guidance: Morphogens as Chemoattractants and Chemorepellants.

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Sonic Hedgehog and Neural Patterning

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Ventralization of Naive Neural Epithelium by Signals from the Notochord and Prechordal Plate

During early development, the neural tube starts out as an epithelial sheet, the neural plate. At this early stage of development, cells in the neural plate epithelium are largely identical. Over time, this epithelial sheet rounds up to form the neural tube, and differentiated neural cells appear at stereotypic times and positions. Classic transplantation experiments have shown that the nature of the differentiated cells arising from the neural tube is dependent on molecular cues in the environment that are initially released by tissues surrounding the neural tube. In particular, the ectoderm overlying the neural tube at its dorsal aspect and the notochord located below its ventral aspect are prime sources of dorsalizing and ventralizing signals, respectively, for cells in the neural tube (**Figure 1**).

The notochord is a direct derivative of the node and is, like the node, an important signaling center in the developing embryo, both for the adjacent somites and for the overlying neural tissue. Transplantation of a notochord to a position dorsal to the neural tube causes a mirror image duplication of the ventral half of the neural tube, indicating that signals derived from the notochord are responsible for the induction of ventral cell types in the neural tube. The principal mediator of the inductive properties of the notochord is the signaling molecule Sonic hedgehog (Shh). Similarly, ectodermal explants can induce dorsal cell types in neural plate tissue, and it appears that most of the dorsalizing signals released by the ectoderm are members of the bone morphogenetic protein (BMP) family of signaling molecules.

Superimposed on the signals mediating the dorsal–ventral pattern are signals that mediate positional information along the anterior–posterior axis, restricting the subsequent development of the neural plate to unique identities, such as forebrain, midbrain, hindbrain, and spinal cord. Shh plays an important role in patterning of the neural tube, and at least early in development, but its main function is the induction of ventral cell types in the developing neural tube. The absence of Shh has little effect on patterning along the anterior–posterior axis, indicating that other signals are involved in inducing differentiation along that axis.

Shh Patterns the Developing Spinal Cord

The role of Shh in neural tube patterning has been most extensively studied in the developing spinal cord since this structure represents the simplest and best understood part of the central nervous system. In the spinal cord, it has become obvious that the role of Shh extends well beyond the induction of motor neurons.

In the ventral–medial aspect of the spinal cord, distinct cell types can be recognized. At the ventral midline is the floor plate, which itself starts expressing Shh. At increasing distances from the floor plate, five distinct groups of neurons can be identified: V3 interneurons, motor neurons, and V2, V1, and V0 interneurons. The ventral neural tube is subdivided into five progenitor domains by the combinatorial expression patterns of at least seven distinct homeodomain proteins. These progenitor domains generate the five ventral neuronal subtypes – V0–V3 interneurons and motor neurons. The homeodomain proteins that mediate this code are divided into class I and class II homeodomain proteins. The class I factors are induced by BMP signals originating from the dorsal neural tube, and the class II homeodomain proteins are induced by Shh. Different thresholds of induction of the class I and class II homeodomain proteins, combined with the mutual antagonism of pairs of these proteins, result in precise domains in the ventral neural tube characterized by stereotypic combinations of class I and class II homeodomain proteins. The combinations of class I and class II homeodomain proteins determine the type of neurons into which these precursor cells will differentiate (**Figure 2**).

The loss of the Shh gene in mice causes a severe embryonic lethal phenotype characterized in the neural tube by the almost complete absence of ventral cell types, resulting in a ventral expansion of intermediate/dorsal cell types to all positions in the neural tube (**Figure 2**). This loss of ventral cell types is evident along the entire anterior–posterior axis of the embryo. In the developing spinal cord, the loss of Shh results in the failure of all ventral cell types to form in the developing neural tube. The loss of multiple cells types, as a consequence of the absence of a single signaling molecule, supported the idea that Shh is present as an activity gradient away from its source, which is instrumental in the induction of pattern along the dorsal–ventral axis of the developing neural tube.

Shh Is a Morphogen

Immediately after its induction from the ectoderm, the neural epithelium is homogeneous without overt

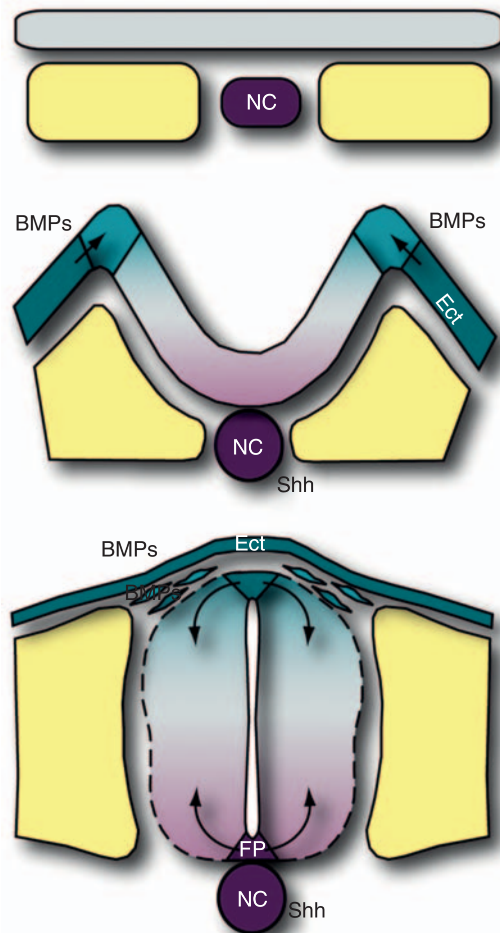

Figure 1 Neurulation diagram. The neural plate receives ventralizing signals from the notochord (NC) and dorsalizing signals from the adjacent/overlying ectoderm (Ect). Shh derived from the Notchord and floor plate (FP) is distributed in a ventral-to-dorsal gradient, and BMP activity derived from the ectoderm and dorsal neural tube is distributed in a dorsal-to-ventral gradient.

dorsal or ventral cell differentiation. Shh released from the notochord causes stereotypical changes in the overlying neural epithelium (**Figure 3**). At the position where the notochord is in direct contact with the neural plate, the floor plate is induced. The floor plate is a nonneuronal structure in the ventral midline of the neural tube which concomitant with its induction also begins to express Shh (**Figure 4**). At longer distances from the notochord and floor plate, motor neurons and interneurons are induced (**Figures 2** and **4**). Importantly, all these inductive events are mediated by Shh and not secondary signals induced by Shh. The explanation by which Shh can induce several distinct cell types is deceptively simple: Close to the sources (notochord and floor plate), cells in the neural epithelium are exposed to a high concentration of Shh, and this concentration gradually declines further away from the source due to limited transport and the presence of local 'sinks,' places where Shh is degraded (**Figure 3**). The resulting ventral-to-dorsal concentration gradient of Shh is interpreted by the cells in the neural epithelium such that cells exposed to a high concentration of Shh differentiate in floor plate cells and cells exposed to intermediate concentrations differentiate into motor neurons, whereas dorsal to the motor neurons, cells differentiate into interneurons in response to low levels of Shh.

Experimental support for this model came from experiments using explants from the neural plate in tissue culture. In these explants, all ventral cell types could be induced using increasing concentrations of a soluble form of Shh, ShhNp. Very high concentrations (>10 nM) resulted in the induction of floor plate cells. At decreasing ShhNp concentration thresholds, the predominant differentiation in the neural tube explants was V3 interneurons, motor neurons, and V2–V0 interneurons. The latter cell types require a Shh concentration less than 1 nM.

Although this morphogen model provides a reasonable explanation of how a single inducer can cause a stereotypic pattern in a bilaterally symmetrical way with columns of specific cells running parallel to the floor plate, a complication is caused by the physical properties of Shh since its mature form has two lipophilic modifications. First, an autocatalytic proteolysis necessary to yield an active N-terminal Shh peptide (ShhNp) results in the addition of a cholesterol moiety to the C-terminus of ShhNp. Second, an acyltransferase facilitates the addition of a palmitic acid. These two lipophilic moieties cause Shh to be associated with membranes. This membrane association is not easily reconciled with the free diffusion thought to be critical in creating a morphogenic gradient. Two models have been put forward to explain how ShhNp exerts its action at multiple cell diameters away from the source. One model proposes the formation of ShhNp of soluble multimers that diffuse through the extracellular space. The second model proposes an active mechanism whereby membrane-associated ShhNp is actively transported from cell to cell – a mechanism termed planar transcytosis (**Figure 5**).

The membrane association of Shh requires the existence of a regulated mechanism of export. Dispatched (Disp) is a transmembrane protein that contains a sterol-sensing domain and its expression is required in cells synthesizing the sterol-modified Shh but not necessary for the secretion of a form of Shh lacking the sterol conjugation. *Disp1* null mice resemble *Shh* null mice, indicating that Disp1 is required for proper Shh signaling. Since *Disp1* null cells are equally sensitive to a mutant form of Shh that is not membrane

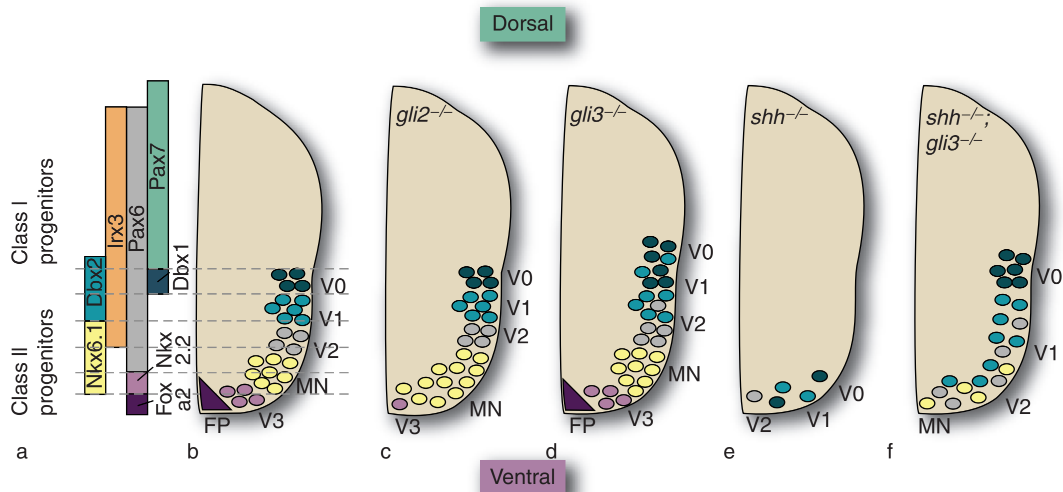

Figure 2 Diagram of class I/class II expression domains and the corresponding regions of ventral neuronal differentiation. A gradient of Shh signaling in the ventral spinal cord activates expression of class II progenitor proteins such as Nkx2.2 and Nkx6.1 while repressing expression of class I progenitor proteins such as Pax7, Pax6, Dbx1, Dbx2, and Irx3 (a). A gradient of BMP activity from the dorsal spinal cord is also able to alter the expression of progenitor proteins such as Pax6 and Pax7. Mutually repressive interactions between class I and class II proteins result in defined boundaries of protein expression, and the ensuing expression pattern of progenitor proteins within discrete regions establishes domains of neuronal differentiation. For example, the expression of Dbx2, Irx3, and Pax6, but not Dbx1, gives rise to the V1 progenitor domain (pV1) from which V1 interneurons (V1) differentiate. Differentiation of ventral neurons along the dorsoventral axis is marked by expression of Engrailed1 (En1) in V1 interneurons. *gli1*$^{-/-}$ mice have phenotypically wild-type spinal cords (b), whereas loss of *gli2* results in a loss of floor plate (FP) and most V3 interneurons (c). *gli3*$^{-/-}$ mice have a mild spinal cord phenotype with a dorsal expansion and intermingling of V0–V2 interneurons (d). Deletion of *shh* results in a loss of most ventral cell types (e), most of which are restored upon additional removal of *gli3* (*shh*$^{-/-}$; *gli3*$^{-/-}$) (f). This suggests that much of the *shh*$^{-/-}$ spinal cord phenotype is mediated by Gli3. MN, motor neurons.

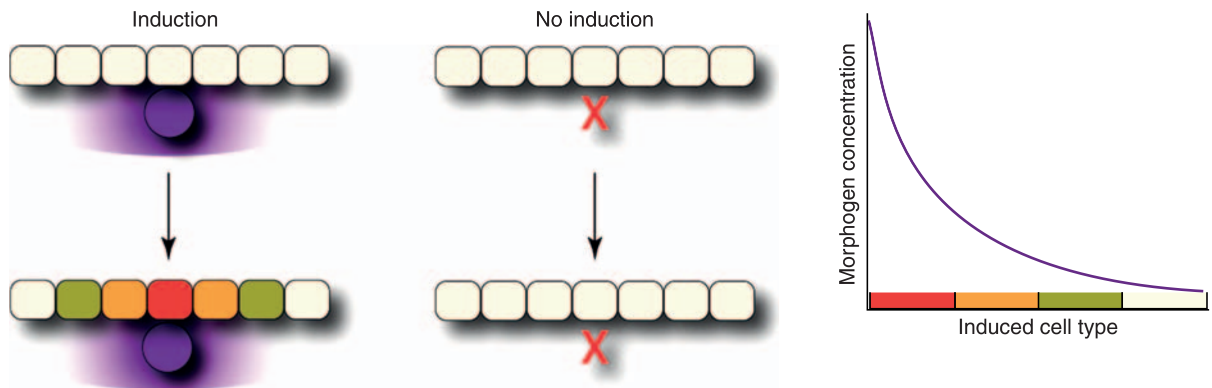

Figure 3 Diagram of morphogen action. Shh released from the notochord (purple) establishes a gradient along the neural plate. The local gradient is interpreted by cells in the neural plate which undergoes a bilaterally symmetric differentiation. In the absence of a notochord or Shh signaling, ventral cell types are not indicated (X).

bound, it has been postulated that Disp1 only plays a role in the cells that produce Shh, possibly mediating the release of Shh multimers from Shh source cells. It remains a possibility that Disp1 also plays this role in cells that have internalized Shh as part of their response. Disp1 could be an integral part of the planar transcytosis pathway by facilitating the export of Shh of cells that have taken it up and making Shh available to neighboring cells.

Shaping the Shh Gradient

Regardless of whether Shh travels from cell to cell or diffuses long distances, it interacts with components in the extracellular space and these molecules can shape the Shh gradient (**Figure 4**). Known molecules that bind Shh in the extracellular space are Patched1 (Ptc1, a part of the Shh receptor complex), Scube2, Hhip, the related molecules CDO and BOC, and HSPGs (**Figure 6**).

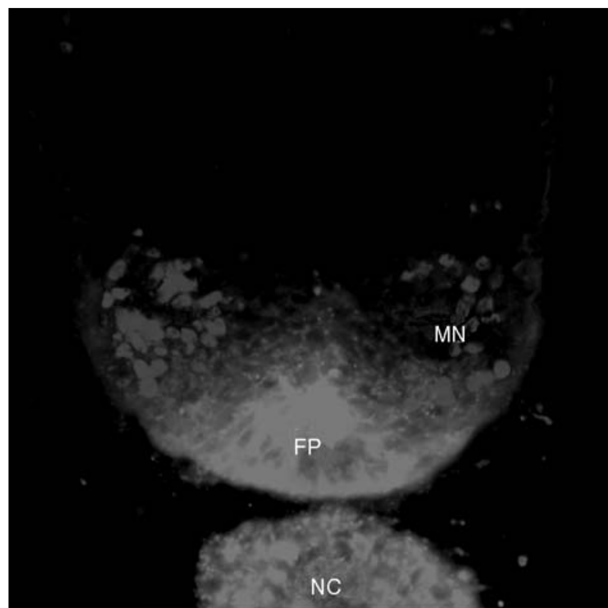

Figure 4 Shh and motor neuron distribution in the developing spinal cord. Cross-section of an embryonic chicken spinal cord, with the ventral/medial aspect shown. Significant amounts of Shh are present in its sites of synthesis, the floor plate (FP) and notochord (NC), but detectible levels of Shh are present in the ventral neural tube in the domain where motor neurons (MN) are differentiating.

Ptc1 plays a particularly interesting role in the distribution of Shh. Ptc1 binds Shh and regulates the activation of the Shh response. However, this binding also results in trafficking of the Ptc1/Shh complex to endosomes and lysosomes, providing a 'sink' for Shh. This role of Ptc1 in sharpening the Shh gradient is exacerbated by the observation that Ptc1 expression is upregulated by the Shh response, increasing the sink effect on the Shh gradient. CDO and BOC are transmembrane, cadherin-associated immunoglobulin superfamily members that bind Shh and are required for the presentation of Shh to Ptc1 and thus for initiating the Shh response. The range of Shh signaling is increased in mice that lack the feedback activities of Ptc1 and also when Hhip (hedgehog interacting protein) is absent. Thus, a Shh-dependent antagonism, mediated by Ptc1 and Hhip, is critical for normal dorsal–ventral development of the neural tube. In contrast, the loss of Scube2, a molecule present in the extracellular space of the neural plate, decreases the efficacy of Shh signaling. However, it is unclear whether scube2 acts by inhibiting the activity of BMPs, and thus reducing the activity of this Shh antagonist, or alternatively by enhancing the range of Shh signaling, possibly by binding to Shh resulting in a complex that is easier to transport in the extracellular space.

A rather complex picture arises with regard to the forms of Shh present in the extracellular space, where there appear to be multiple distinct molecules around molecules that bind to Shh and they are required either for a normal response or for normal distribution. Given that due to its lipophilic modifications, Shh is a nonsoluble molecule that nevertheless travels considerable distances, it is not surprising that many molecules are involved in this process. The nature of the complexes or multimers of Shh that travel from cell to cell remains obscure.

The Shh Response

The Shh receptor complex consists of at least two proteins, Ptc1 and Smo. Ptc1 is a large protein with 12 transmembrane segments sharing structural similarity with Disp1, whereas Smo may be an atypical member of the G-protein-coupled receptor family. Ptc1 is the ligand-binding component and in the absence of Shh it inhibits Smo activity, which is the signaling component that activates the Shh response pathway (**Figure 6**). Smo is constitutively active in *Ptc1*-deficient cells, and in *Ptc1*-deficient embryos the whole neural tube resembles the floor plate. Although the precise mechanism by which Ptc1 inhibits Smo is unknown, there is evidence that Ptc1 functions as a pump of small, vitamin D-like molecules that inhibit Smo. Consistent with this is the Shh-induced trafficking event culminating in the segregation of Smo into an endocytic compartment not containing Ptc1, which is a critical event in the initiation of the Shh response. Most likely, this process involves the function of intraflagellar transport proteins, indicating that transport along microtubules is an integral part of the Shh response. When segregated away from the inhibitory effect of Ptc1, Smo activates the Shh response likely via an inhibitory G-protein.

Inside the responding cells, three Gli proteins (Gli1–3) which are zinc finger-containing transcription factors, are the transcriptional effectors of the Shh response. In the absence of Shh signaling, Gli2 and Gli3 are proteolytically processed into repressors of Shh target genes. A protein complex that includes SuFu tags Gli3 for cleavage at a proteosome (**Figure 6**). An early event in the transduction of the Shh response involves the phosphorylation of this multiprotein complex within 15–30 min after Shh binding. These phosphorylation events result in the inhibition of Gli2 and Gli3 cleavage, and thus more of the activator forms of Gli2 and Gli3 are present at the expense of the cleaved inhibitor forms. The vast majority, if not all, of the effects of Shh signaling are mediated by Gli2 and Gli3. A simple model emerges of how cells interpret the Shh activity gradient. In the absence of Shh, all Gli3 is converted into the repressor

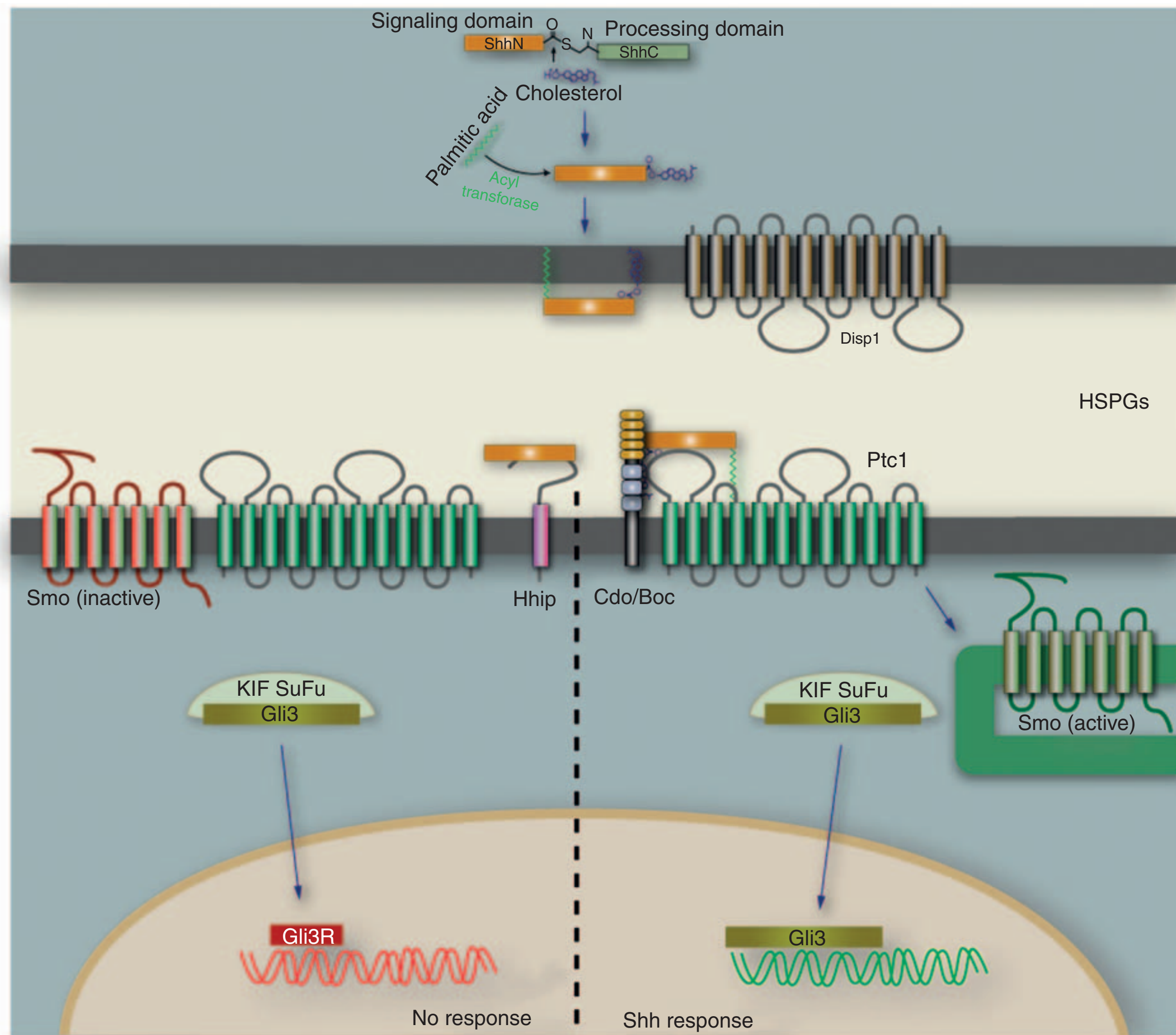

Figure 5 Models of Shh transport mechanisms. The two fundamental mechanisms are diffusion and planar transcytosis. The pathways have in common that Shh is released from the source by Disp1. The first pathway would involve the diffusion of a multimeric, soluble form of Shh across the developing neural epithelium. The second pathway evokes the activity of Ptc1 and Disp1 to create a 'bucket brigade' for Shh, effectively handing over internalized Shh to neighboring cells, which do the same.

form, and with increasing levels of Smo activation, the ratio of Gli2 and Gli3 activator forms over the Gli3 repressor form increases in a graded manner, reflecting the Shh morphogenetic gradient.

The phenotypes in the spinal cord caused by the loss of Gli2 and Gli3 are relatively mild (**Figure 2**). In the absence of Gli2, the floor plate and adjacent cells fail to form, but motor neurons are present, although their domain spans the ventral midline. The loss of Gli3 has no significant effect in the developing spinal cord, although an expansion of ventral cell types can be observed in the developing brain. These relatively minor effects might seem difficult to reconcile with the dramatic phenotype observed in the *Shh* null mice. However, Shh signaling would have two independent effects on Gli activation: The ventral-to-dorsal Shh gradient would establish a ventral-to-dorsal gradient of activated Gli2 as well as a dorsal-to-ventral gradient of Gli3 repressor activity. This would result in a somewhat redundant situation in which loss of either gene causes a much milder phenotype than the loss of Shh or Smo. However, the opposing gradients of activated Gli2 and the Gli3 repressor form are required for normal dorsal–ventral patterning in the neural tube.

Is Shh Required for Neural Tube Patterning?

The severity of the phenotype caused by Smo and Shh loss-of-function phenotype makes a compelling argument that Shh is critically important for the induction

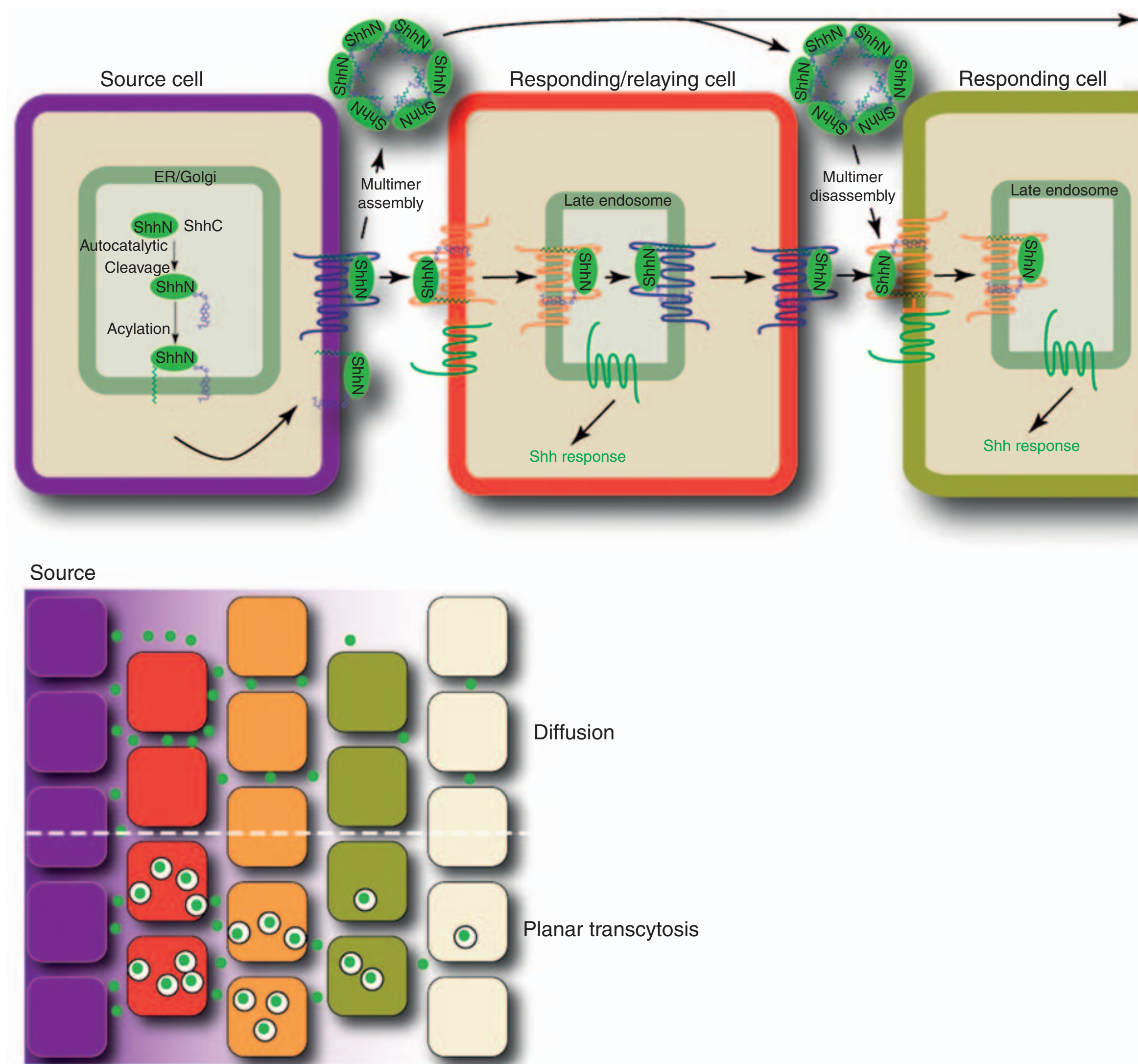

Figure 6 Diagram of Shh synthesis, posttranslational modification, export, and response. Normal processing of full-length Shh results in a molecule with N-terminal palmitic acid and a C-terminal cholesterol moiety. Disp1 is involved in the excretion of Shh. On the recipient cell, Hhip prevents binding to the receptor, whereas CDO/Boc are required for presentation of Shh to Ptc1. In the absence of hedgehog, Patched represses the activity of Smo. Gli3 is cleaved and converted to a repressor in a process that involves SuFu and scaffold proteins. The Patch-mediated inhibition of Smo is released upon binding of Shh to Patched. Gli3 is converted to a transcriptional activator and activates the transcription of hedgehog responsive genes.

of ventral cell types in the neural tube. However, in the absence of Shh signaling, some of the loss of ventral cell types can be rescued when Gli3 is also absent. This is consistent with the repressor activity mediated by Gli3 in the absence of an activated Shh response (**Figure 2**). In other words, the loss of Shh signaling can be partially overcome by removing the main repressor of the Shh response as well. In the absence of an activated Shh response and addition of Gli3 repressor activity, some ventral neurons such as motor neurons differentiate in the developing spinal cord. This demonstrates that the repressor activity of Gli3 is almost single-handedly responsible for the phenotype observed in the Shh or Smo null mice. Although the most ventral cell types, such as floor plate and V3 interneurons, are not present in such mice, other ventral cell types do appear, although not in the distinct dorsoventral domains characteristic for normal embryos. Nevertheless, this indicates that Shh-independent positional signals are present in the neural tube, and likely candidates for this activity are members of the BMP family, which are expressed in the dorsal neural tube and are predicted to be distributed in the neural tube in a dorsal-to-ventral gradient. It thus appears that although the Shh signaling system is not required for the formation of most

ventral cell types in the neural tube, the precise segregation of the differentiated cells into specific domains required the graded Shh signal.

During normal development of the neural tube, dorsally derived BMP signals antagonize the Shh response and vice versa. Activation of the BMP response is sufficient to induce dorsal cell types, whereas activation of the Shh response induces ventral cell types. Together, these overlapping and antagonistic gradients ensure a very robust mechanism that mediates dorsal–ventral patterning in the neural tube.

Shh and Patterning of the Brain

The forebrain (prosencephalon) is the most anterior aspect of the neural tube, and it is divided by the zona limitans intrathalamica into anterior and posterior parts. The prechordal plate is the rostral-most extension of the notochord, and it is located ventral to the anterior forebrain. The importance of the prechordal plate was already appreciated more than 70 years ago, when ablation experiments showed that the loss of the prechordal plate causes cyclopia, a disorder in which instead of two bilateral eyes, a single midline eye is present, as well as an array of associated malformations such as a single, undivided cerebral hemisphere (holoprosencephaly). The midline eye presents a barrier for the migration of the frontonasal process into its appropriate position to form the nose, philtrum, incisors, and part of the palate. Instead, this tissue accumulates in a proboscis–like structure above the single midline eye. The single midline eye is a consequence of the failure of the formation of ventral midline tissue in the anterior forebrain, which normally separates the eye fields into two bilateral domains. Homozygous loss of Shh causes cyclopia as well, indicating that much of the ventralizing activity of the prechordal plate is mediated via the Shh response pathway. As in the spinal cord, in the absence of Shh signaling the ventral forebrain acquires a more lateral phenotype.

Cyclopia is invariably associated with holoprosencephaly, a condition characterized by a single undivided cerebral hemisphere. However, holoprosencephaly is a much more developmental abnormality. Nevertheless, a principal cause of holoprosencephaly is an absent or attenuated Shh response. In line with the morphogenetic function of Shh, an attenuated Shh response can result in some loss of ventral structures, reflected in the face by malformations such as a single midline incisor, the absence of the nasal septum, and hypotelorism (closely spaced eyes). Holoprosencephaly is highly associated with these birth defects. It is unclear why the failure of the ventralizing activities of Shh to function normally is so closely correlated with holoprosencephaly, which involves the dorsal midline of the telencephalon, seemingly far away from the sources of Shh. In the ventral forebrain, prechordal plate and notochord are required for the induction of the hypothalamus, which is involved in many of the homeostatic functions of the organism.

Notochord-derived Shh also induces ventral structures in the midbrain, the tegmentum. Importantly, the ventral tegmentum is where dopaminergic neurons are localized in the substantia nigra. These neurons are preferentially lost in Parkinson's disease. Obviously, much research is being directed to recreate dopaminergic neurons *in vitro*, and invariably these approaches rely on the activation of the Shh response to generate such neurons from stem cells.

The third cranial (oculomotor) nerve also emerges from the ventral midbrain, and the motor neurons innervating the muscles that move the eyes are located in nuclei just ventral to the cerebral aqueduct. The oculomotor nuclei contain the most rostrally located motor neurons that connect to striated, voluntary muscles. Caudal to the oculomotor nuclei are a series of nuclei in the midbrain and hindbrain, which contain the motor neurons innervating the muscles in the face and neck, all of which require notochord and floor plate-derived Shh for their induction.

Small Molecule Inhibitors of Smo

More than a decade of research has demonstrated that Shh is critical for the formation of ventral cell types in the developing neural tube, and the nature of these ventral cell types is dependent on the position along the anterior–posterior axis. At any position along this axis, Shh functions as a morphogen and as such is responsible for the induction of a variety of cell types. However, because the loss of Shh signaling either through the loss of the ligand or through the loss of Smo causes the embryos to die before birth, not much is known about many of the later events mediated by Shh in neural development since this analysis requires the generation of multiple conditional knockouts.

An alternative approach has been made possible with the use of small molecule inhibitors of Smo, such as cyclopamine. Due to its ability to cause cyclopia in offspring of ruminants, this compound, which was isolated from *Veratrum californicum*, has served as a lead compound in the identification of several inhibitors of Smo. These molecules are used to address the requirement for Shh signaling at later stages of neural development as well as in normal function of neural stem cells.

See also: Anterior-Posterior Spinal Cord Patterning of the Motor Pool; Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh); *Drosophila* Apterous Neurons: from Stem Cell to Unique Neuron; Floor Plate Patterning of Ventral Cell Types: Ventral Patterning; Olfactory Neuron Patterning and Specification; Retinoic Acid Signaling and Neural Patterning; Wnt Pathway and Neural Patterning.

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Floor Plate Patterning of Ventral Cell Types: Ventral Patterning

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Introduction

Interest in understanding the patterning of the vertebrate neural tube stems from its complex yet highly organized structure and its central role in the life and behavior of animals. Here we concentrate on the strategies and molecular mechanisms by which neuronal subtype identity is assigned in ventral regions of the spinal cord – the region of the central nervous system (CNS) that contains the neurons which control and coordinate motor output. At early stages of embryonic development, five distinct subtypes of neurons can be molecularly identified in the ventral spinal cord; among these are four types of interneurons (V0–V3) and somatic motor neurons (MNs). Each neuronal population arises from blocks of proliferating progenitors that occupy stereotyped locations along the dorsal/ventral axis of the neural tube (**Figure 1**). These domains of progenitor cells can be distinguished by their expression of distinct molecular markers, including a set of transcription factors, notably homeodomain-containing (HD) proteins and basic helix–loop–helix (bHLH) proteins. The combinatorial expression profile of these proteins delineates five populations of progenitor cells, each of which generates one of the identified neuronal subtypes; consequently, the early spinal cord develops as a dorsal/ventral array of neuronal subtypes. We review the mechanisms involved in the establishment of this pattern of neurogenesis; in particular, we describe the function of the Sonic hedgehog (Shh) signaling pathway, which appears to act as a graded morphogen in the ventral neural tube to control pattern formation.

Evidence That Notochord-Secreted Signals Pattern the Dorsoventral Axis of the Neural Tube

Studies initiated in the 1920s suggested that, rather than containing intrinsic self-organizing capacity, the major determinant of dorsoventral (D/V) polarity is the mesoderm surrounding and underlying the neural tube. From these studies, the notochord was identified as one of the mesodermal structures from which instructive signal(s) emanate that direct D/V patterning of the developing neural tube (**Figure 2**). In the absence of the notochord, floor plate cells – the characteristic wedge-shaped cells that are generated at the ventral midline of the neural tube – were missing, whereas in embryos containing two notochords a duplication of floor-plate-like structures was evident.

The same principles were reinforced in subsequent experiments using chick embryos, from the 1950s onward. Moreover, these experiments indicated that contact between the notochord and neural tube was essential for floor plate formation. Ensuing experiments in which extra notochords were grafted into chick embryos confirmed the inductive effect on the floor plate, and added an extra dimension; neuroblasts (presumptive motor neurons) were also induced by the notochord but at a distance from the position of the graft (**Figure 2(b)**). In addition, the floor plate was found to display similar inductive properties to the notochord, indicating that the floor plate also has capacity to promote the generation of both more floor plate cells and MNs. These experiments led to the conclusion that the notochord and floor plate are the source of a signal(s) that induced different cell types and that the distance from the source of this signal(s) determines the cell type generated.

Shh Patterning of the Neural Tube

We now know a considerable amount about the molecules involved in D/V patterning and the understanding of their action relies to a significant degree on the foundation provided by these early embryological experiments. In the search for the signal providing instructive cues to the ventral neural tube, a secreted protein, Sonic hedgehog, was identified. This protein is an ortholog of the *Drosophila* segment polarity gene product, Hedgehog (Hh), a signaling molecule involved in the development of several embryonic tissues. Consistent with this, Shh is expressed in many developing tissues, including the notochord and floor plate. The period when Shh is expressed in these structures corresponds to the time when neural tube polarity is being established. Since the discovery of the Hh family of signaling molecules, much effort has been employed in identifying the components of the signaling pathway, and an understanding of the mechanism of signal transduction is beginning to emerge. Transduction depends on two transmembrane proteins: Patched 1 (Ptc1), the receptor which binds Hh proteins, and Smoothened (Smo), which is responsible for transmitting Hh signals intracellularly (see later).

A range of loss-of-function experiments support the idea that Shh corresponds to the signal that

provides instructive cues to control the generation and arrangement of distinct neuronal populations in the neural tube. In mouse embryos harboring a targeted null allele of Shh, there is a loss of MNs and other ventral interneuron subtypes; concomitantly molecular markers normally restricted to dorsal aspects of the spinal cord expand toward the ventral midline. Furthermore, antibodies raised against Shh that block its ability to bind Ptc1 also disrupt patterning of the ventral neural tube and inhibit the ability of notochord to induce ventral neuronal subtypes. Together, these studies suggest that production of Shh is necessary for correct formation of ventral neuronal subtypes *in vivo*.

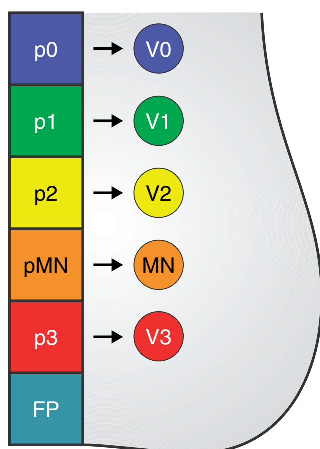

Figure 1 Organization of the ventral neural tube: progenitor domains and ventral neuronal cell types and their fates in the spinal cord (schematic of the ventral half of the neural tube). Five progenitor domains are found in stereotyped positions within the ventricular zone of the ventral neural tube, lying dorsal to the floor plate (FP). These progenitor domains – p0, p1, p2, p3, and pMN – will generate the corresponding neuronal cell types – V0 to V3 interneurons and motor neurons (MN) – involved in motor output control.

Experiments carried out *in vitro* using explants of naive neural tissue provided further understanding of the mechanism of Shh activity. These studies indicated that exposure to varying concentrations of Shh protein induced the populations of neurons characteristic of the ventral neural tube. Incremental two- to threefold changes in Shh concentration led to the generation of different neuronal subtypes; moreover, a correlation between the *in vitro* concentration of Shh necessary to induce each neuronal class and their position of generation *in vivo* was apparent (**Figure 3**). Thus, neurons generated in progressively more ventral regions of the neural tube required correspondingly higher Shh concentrations for their induction. These data indicated that in addition to being necessary for their development, Shh is sufficient to induce ventral interneurons and motor neurons in neural tissue, suggesting that Shh functions in a graded manner to impart positional identity. Thus, cells in the ventral neural tube are exposed to a ventral^{HIGH}–dorsal^{LOW} concentration gradient of Shh, and the concentration of Shh to which a progenitor cells is exposed determines the neuronal subtype generated (**Figure 3**).

A key prediction of this model is that Shh should operate as a long-range signal, acting directly on cells at a distance from its source. Several lines of evidence indicate that this is indeed the case. First, Shh protein is detectable several cell diameters away from the producing cells of the floor plate (**Figure 3**). Second, a dominant active version of Smo, a component of the signaling pathway, is sufficient to induce ectopic expression of ventral markers in the neural tube. Third, blockade of Shh signaling, by either cell autonomous removal of Smo or ectopic expression of a mutated version of Ptc1 (the Shh receptor) that acts as a dominant inhibitor of signaling, blocks the generation of ventral neurons, which instead develop

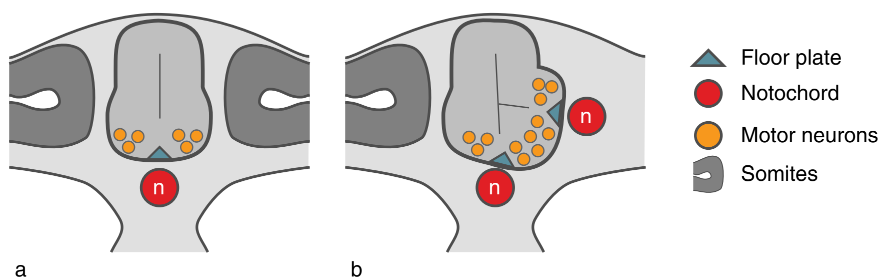

Figure 2 Notochord-secreted signal(s) influence patterning of the neural tube. (a) The notochord (n) is an axial mesodermal structure that underlies the caudal neural tube. The floor plate is formed in the ventral neural tube, close to the notochord, and motor neurons are generated in the ventral horns of the spinal cord, at a distance from the notochord. (b) Grafting an ectopic notochord next to the neural tube induces an ectopic floor plate at a short distance, whereas at a greater distance excess motor neurons are generated. These experiments suggest the existence of an instructive secreted signal(s) from the notochord that can control the patterning of the ventral neural tube.

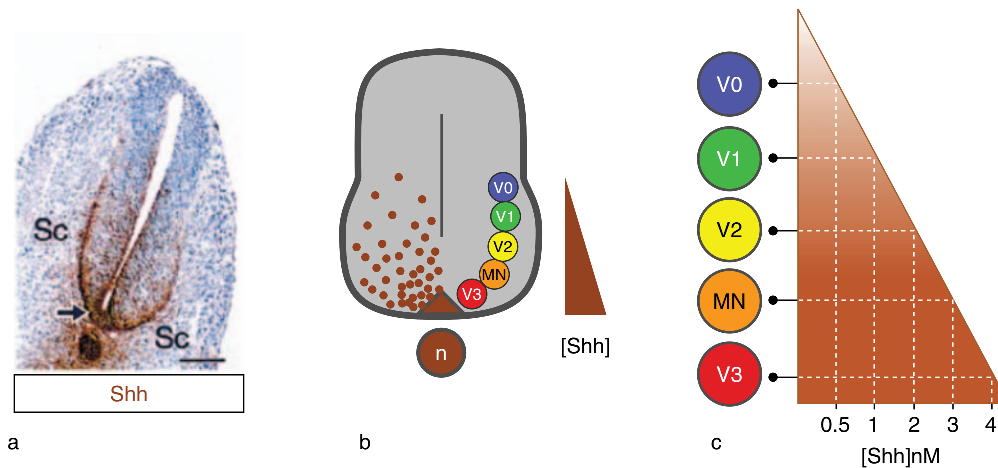

Figure 3 Sonic hedgehog (Shh) diffuses in the ventral neural tube and controls neuronal cell fate in a concentration-dependent manner. (a) Staining for Shh in E10.5 mouse embryo. Shh protein can diffuse through the ventral half of the neural tube to establish a ventral-to-dorsal gradient (high levels of protein are detected in the Shh-secreting floor plate and lower levels in the intermediate neural tube). (b) Schematic of Shh distribution, illustrating the graded distribution of the protein in the developing neural tube, together with the relative position of newborn ventral neuronal cell types (interneurons, V0–V3; motor neurons, MN). (c) Naive neural plate chick explants can generate the five ventral neuronal cell types, depending on exposure to Shh. Two- to threefold increments in Shh concentration allow the induction of ventral interneurons and motor neurons, suggesting that Shh protein is acting as a morphogen, patterning the ventral neural tube. (a) Reproduced with permission from Gritli-Linde A, Lewis P, McMahon AP, et al. (2001) The whereabouts of a morphogen: Direct evidence for short- and long-range activity of hedgehog signaling peptides. *Developmental Biology* 236: 364–386.

with characteristics of neurons located in the dorsal neural tube. Together, these results suggest that Shh acts directly on cells at a distance through its receptors, rather than indirectly through a relay signal.

A Transcription Factor Code and Neural Cell Fate

The ability of graded Shh signaling to directly control D/V patterning in the ventral neural tube raised the question of how positional identity is imposed on progenitor cells and how this determines neuronal subtype identity. Progenitors respond to Shh signaling by regulating the expression of a series of transcription factors, which, with the exception of the bHLH protein Olig2, are members of the homeodomain protein family, including Pax7, Pax3, Dbx1, Dbx2, Pax6, Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2, and Irx3. These transcription factors play a crucial part in patterning the ventral neural tube and can be split into two groups according to their response to Shh: class I, the genes of which are repressed by Shh signaling, and class II, the genes of which are activated by Shh (**Figure 4(a)**). Furthermore, within the same class, genes respond in a concentration-dependent manner to Shh, such that the repression of class I genes with more ventral limits of expression requires higher levels of Shh signaling compared to more dorsally restricted class I genes. Conversely, class II proteins that have a broad domain of expression in the ventral neural tube are induced by lower concentrations of Shh than are class II proteins that have a more ventral limit of expression. In addition, class I and class II genes can be grouped in pairs based on two prerequisites: (1) the ventral limit of expression of a class I protein coincides with the dorsal limit of the paired class II protein and (2) the pair of class I and class II factors display mutual cross-repression.

Graded Shh Controls Class I and Class II Protein Expression

Evidence that graded Shh signaling establishes the transcription factor code emerged from studies of the expression patterns of Nkx2.2 and Pax6. In the developing ventral neural tube, the class I protein Pax6 and the class II protein Nkx2.2 have a mutually exclusive expression pattern, with the most ventral progenitors expressing Nkx2.2, while Pax6 is expressed in progenitors situated dorsal to the boundary of Nkx2.2 (**Figure 4(a)**). *In vitro* experiments demonstrated that the expression of both of these proteins is regulated by Shh signaling in a concentration-dependent manner. In the absence of Shh, explants express Pax6; exposure to concentrations of Shh above a given threshold results in the repression of Pax6 and the concomitant induction of Nkx2.2. Importantly, the mutually exclusive relationship between Pax6 and Nkx2.2 is

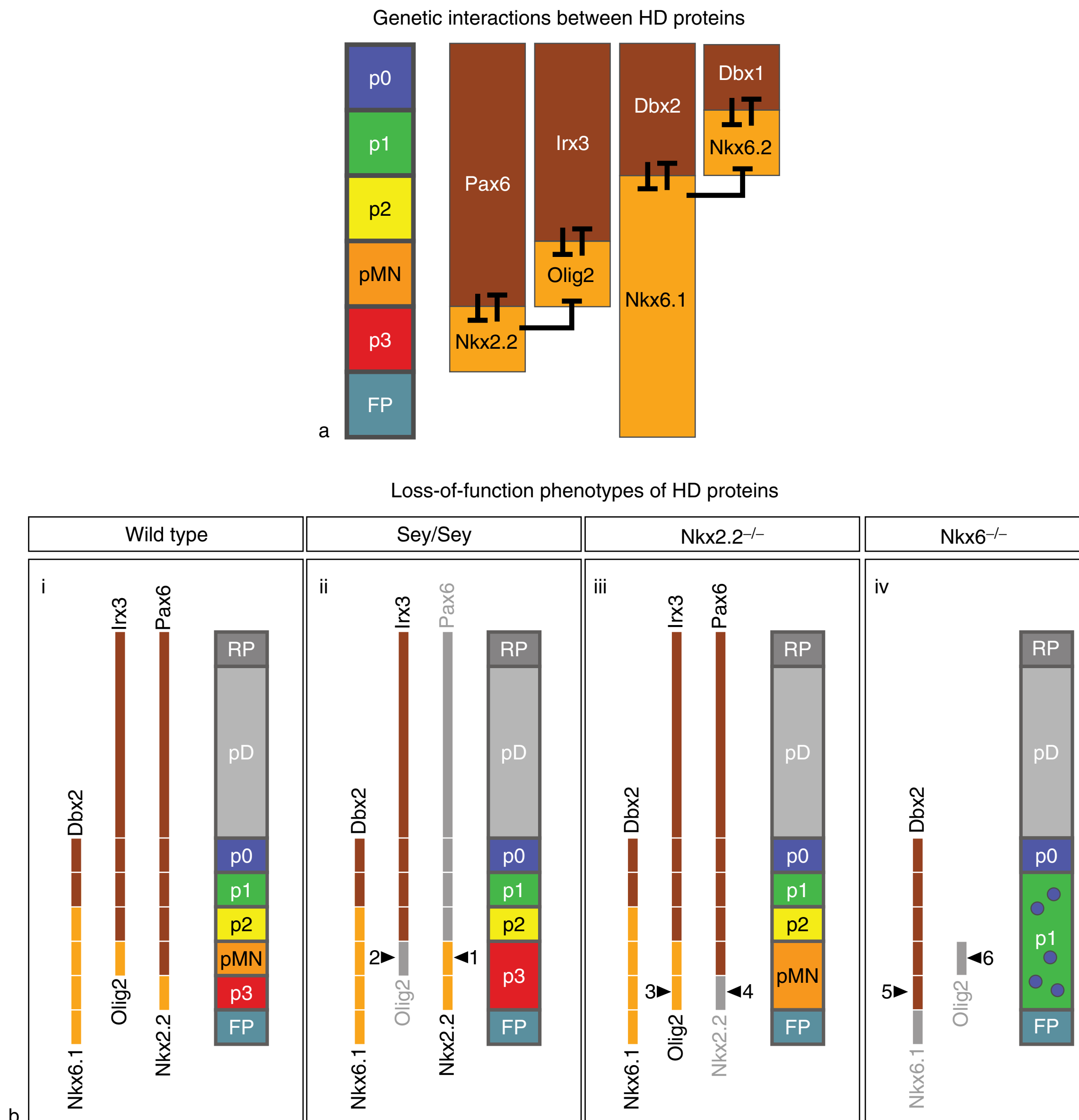

Figure 4 A transcription factor code and patterning of the ventral neural tube. (a) Combinatorial expression of homeodomain-containing (HD) proteins Pax, Irx3, Nkx, and Dbx, together with the basic helix–loop–helix transcription factor Olig2, distinguishes five ventral progenitor domains in the caudal neural tube. HD proteins can be subdivided into two groups according to their response to Sonic hedgehog (Shh) signaling, class I (brown) and class II (orange) proteins, which are inhibited or activated by Shh, respectively. Cross-repression between selected pairs of proteins controls the formation of boundaries between progenitor domains. (b) Cross-repression between pairs of HD proteins controls ventral neural tube patterning. Compared with wild-type embryos (i), in the absence of Pax6 (Sey/Sey; ii), there is a dorsal expansion in Nkx2.2 expression (1) that leads to the inhibition of Olig2 expression (2) and results in the absence of the motor neuron progenitor (pMN) domain and a concomitant expansion of the p3 domain. Conversely, in the absence of Nkx2.2 (iii), there is a ventral expansion of Olig2 (3), but not of Pax6 (4; see text for more details), leading to an expansion of pMN at the expense of p3. Another example of a pair of HD proteins is illustrated with Nkx6.1 and Dbx2 (iv). In double mutants lacking Nkx6.1 and Nkx6.2 (Nkx6$^{-/-}$), there is a ventral expansion of Dbx2 (5) and a downregulation of Olig2 (6). As a consequence, p2, pMN, and p3 domains are lost and p1 progenitors occupy most of the ventral spinal cord. There is also a sporadic expression of Dbx1 (blue circles) in this ectopic p1 domain due to the loss of inhibition by Nkx6.2 (RP, roof plate; pD, dorsal spinal cord domain). Expression patterns of Irx3, Pax6, and Nkx2.2 in these mutants have not been determined.

maintained in these experiments in a cell autonomous manner. Furthermore, addition of anti-Shh antibodies promotes Pax6 expression and inhibits Nkx2.2 expression, even in the presence of Shh. Similar findings have been documented for other class I and class II proteins. Additional signaling molecules have also been implicated in modulating the expression of the progenitor-expressed

transcription factors (see later) and these may act by either influencing the Shh signal transduction pathway or by directly regulating target gene expression.

Cross-Repression between Class I and Class II Proteins Establishes the Mutually Exclusive Expression Profiles

The regulation of class I and class II protein expression by graded Shh signaling is not alone sufficient to explain how the discrete, sharp boundaries of gene expression arise in the neural tube. Gain- and loss-of-function studies indicate that selective cross-repressive interactions between pairs of class I and class II proteins expressed in adjacent domains are important for establishing the all-or-none changes in gene expression that characterize progenitor domain boundaries. This first became apparent for Nkx2.2 and Pax6. Gain-of-function experiments indicate a cross-repressive interaction between Pax6 and Nkx2.2. Moreover, expression of Nkx2.2 expands dorsally in mutant mice lacking Pax6 (**Figure 4(b)**). This results in a decrease in MN production coupled with an expansion in Nkx2.2-produced V3 neurons. Pax6 is therefore required for accurate positioning of the boundary between MN and V3 progenitors, and for correct production of the neurons from these progenitors. The absence of Pax6 also results in defects in the generation of other ventral neuronal subtypes, in particular V1 and V2 neurons, suggesting that Pax6 has further roles in the control of neuronal subtype identity in the neural tube. Additional studies indicated that in embryos lacking Nkx2.2 there was a loss of V3 neuronal subtype identity, and a ventral shift in the MN progenitor marker Olig2 and subsequently in the generation of MNs (**Figure 4(b)**). Pax6 expression remained unchanged, however, possibly due to the presence of Nkx2.9, another Nkx family member, which is expressed in a similar domain to Nkx2.2.

Evidence from a second set of class I and class II proteins lends support to the idea of reciprocal cross-repressive interactions. The class II proteins Nkx6.1 and Nkx6.2 adjoin the ventral boundaries of the class I proteins Dbx2 and Dbx1, respectively (**Figure 4(a)**). In double-knockout embryos lacking both Nkx6 proteins, a ventral expansion of Dbx2 expression was evident (**Figure 4(b)**). Furthermore, forced expression of Nkx6.1 cell autonomously repressed Dbx2. Conversely misexpression of Dbx2 resulted in the down-regulation of Nkx6.1. A similar relationship between a third pair of proteins, Olig2 and Irx3, is also apparent (**Figure 4(a)**). With the exception of Pax6, the class I and class II proteins act as transcriptional repressors in neural progenitors, raising the possibility of direct interactions between the proteins and promoters of class I and class II genes.

Control of Neuronal Identity by Graded Shh Signaling

The regulation of class I and class II proteins ultimately leads to the establishment of the progenitor domains p0, p1, p2, pMN, and p3, defined by the expression of distinct combinations of transcription factors, which generate the five neuronal subtypes (**Figure 4(a)**). Gain- and loss-of-function experiments support the hypothesis that the progenitor transcription factor expression profile specifies the identity of neurons generated from each progenitor domain. The forced expression of a class I or class II protein in the neural tube and targeted inactivation of individual class I or class II proteins change the fate and position of generation of individual neuronal subtypes in a manner predicted by the normal profile of transcription factor expression.

Overall, these studies suggest a model to explain ventral patterning of the neural tube. Crucially, the initial activation or repression of the class I and class II proteins by graded Shh signaling imparts positional identity to progenitor cells. The reciprocal repression between pairs of class I and class II proteins provides a mechanism to convert the gradient of Shh signaling into discrete all-or-none changes in gene expression that may account for the formation of sharp boundaries between adjacent progenitor domains. Each domain then generates a specific neuronal subtype. The repressive interactions between progenitor expressed transcription factors could also serve to consolidate progenitor domain identity, relieving a requirement for a prolonged period of Shh signaling to maintain gene expression domains. Significantly, *Drosophila* orthologs of several of the class I and class II proteins have been demonstrated to play a similar role in allocating regional identity in ventral nerve cord of the developing embryo, raising the possibility that this strategy represents a fundamental feature of nervous system patterning of many extant animals.

Additional Signals Involved in Dorsoventral Patterning of the Neural Tube

Although the evidence confirms the importance of Shh signaling in controlling spatial patterning within the neural tube, other extracellular signals also contribute to correct D/V patterning. During neural induction, progenitors are exposed to fibroblast growth factors (FGFs), originating from the presomitic mesoderm, the regressing node, and the neural plate. FGF signaling acts as an inhibitor of neural differentiation, ensuring cells remain progenitors. FGFs also inhibit the expression of many of the

progenitor transcription factors and it is not until cells have emerged from the influence of FGFs that they mature and begin to express class I and class II proteins. Conversely, retinoic acid (RA) is expressed anteriorly to FGF, in paraxial mesoderm adjacent to the neural tube, and RA is necessary for neural differentiation and progenitor transcription factor expression. RA promotes class I protein expression, therefore counteracting the ventralizing effects of Shh. A third signaling pathway is also implicated in D/V patterning – bone morphogenetic proteins (BMPs). Several members of the BMP family are expressed in the dorsal pole of the neural tube, where they play a role in specifying dorsal neuronal fates. In addition, BMP proteins appear to oppose Shh-mediated ventralization of the neural tube, limiting the dorsal extent of the ventral neural tube. Hence, precise coordination of ventral neural patterning depends on the complex interplay between several signaling pathways, the details of which are only now beginning to be understood.

Formation and Maintenance of Shh Gradient

The central role of graded Shh signaling in the control of D/V patterning in the neural tube has focused attention on how the production and distribution of Shh are regulated. For this, knowledge of the concentration and shape of the gradient is required; parameters such as the rate at which Shh is released from its source, the speed of its spread, and its stability are necessary but difficult to obtain in embryos *in vivo*. Additional complexity to this problem is introduced by the attachment of lipid moieties to Shh and the presence of extracellular proteins that bind to Shh.

Posttranslational Modification and Release of Hedgehog Proteins

The Hh precursor protein is posttranslationally processed to a mature Hh protein by an autocatalytic cleavage that removes the carboxyl (C)-terminus. As part of this reaction, a cholesterol moiety is covalently attached to the C-terminal end of the processed protein. Shh is also acylated on its amino (N)-terminus, for example, by addition of palmitate; the end result is a Shh protein linked to two hydrophobic groups. Although these posttranslational modifications are not absolutely required for Hh activity, as illustrated by the induction of several Hh-target genes with recombinant forms of Shh that lack lipid moieties, the lipid modifications appear to affect the spread and/or activity of Shh in neural tissue. The secretion of cholesterol modified Hh appears to require the transmembrane protein, Dispatched-1 (Disp1), as mutations in this gene result in alterations in neural tube patterning consistent with a lack of Shh released. Palmitoylation of Shh also appears essential in vertebrate neural tissue. Prevention of palmitate addition to Shh protein by mutation of the target cysteine (Shh^{C25S}) leads to a severe deficiency in ventral neural tube patterning, reminiscent of embryos lacking Shh protein. Similar effects are also observed in mice deficient in Skn, the murine ortholog of the product of the *Drosophila ski* gene, which catalyzes Hh palmitoylation. Thus, the hydrophobic modifications of Shh have important and complex effects on Shh function *in vivo*.

Hedgehog-Binding Proteins and Feedback Loops

In addition to lipidation, Hh-binding proteins also modify Hh activity. One important regulatory strategy used by many signaling systems is negative feedback, where signal transduction leads to upregulation of inhibitors of the pathway to attenuate signaling. Hh uses such feedback regulation and inhibition is mediated by several Hh-binding proteins. Among these are Hh receptor Ptc1 and an additional protein, Hh-interacting protein 1 (Hhip1). Both Ptc1 and Hhip1 are upregulated by Shh signaling in the ventral neural tube (**Figures 5(a)** and **5(b)**). Evidence that upregulation of Ptc may be involved in limiting the spread of Hh proteins initially came to light in experiments in *Drosophila* which demonstrated an extended range of Hh in the absence of Ptc. Similar observations were later made in the vertebrate neural tube, where a dorsal-to-ventral change in neural fate was noted in cells dorsal to clusters of cells unable to respond to Hh. Elegant studies in mouse subsequently documented the role Ptc1- and Hhip1-mediated Shh sequestration plays in patterning the ventral neural tube (**Figure 5(c)**). In these experiments, a transgenic mouse strain expressing a low basal level of Ptc1 from a heterologous promoter (MtPtc1) was used. This was sufficient to suppress unregulated Smo activity; however, in the absence of endogenous Ptc1 and Hhip1 genes, MtPtc1 failed to restrain the range of Shh signaling, resulting in a ventralization of the neural tube (**Figure 5(c)**). Thus, the upregulation of Ptc1 and Hhip1 in response to Shh signaling normally acts to limit the range over which the ligand can diffuse (**Figure 5(b)**). The appearance of intermediate phenotypes in compound heterozygote mutants for both Ptc1 and Hhip1 indicates that this mechanism utilizes both Ptc1 and Hhip1 in a dose-dependent manner.

Two further transmembrane Hh-binding proteins, Cdo and Boc, have been identified as both targets and regulators of Shh signaling. Both are expressed in the dorsal spinal cord – a domain with low level of Ptc1

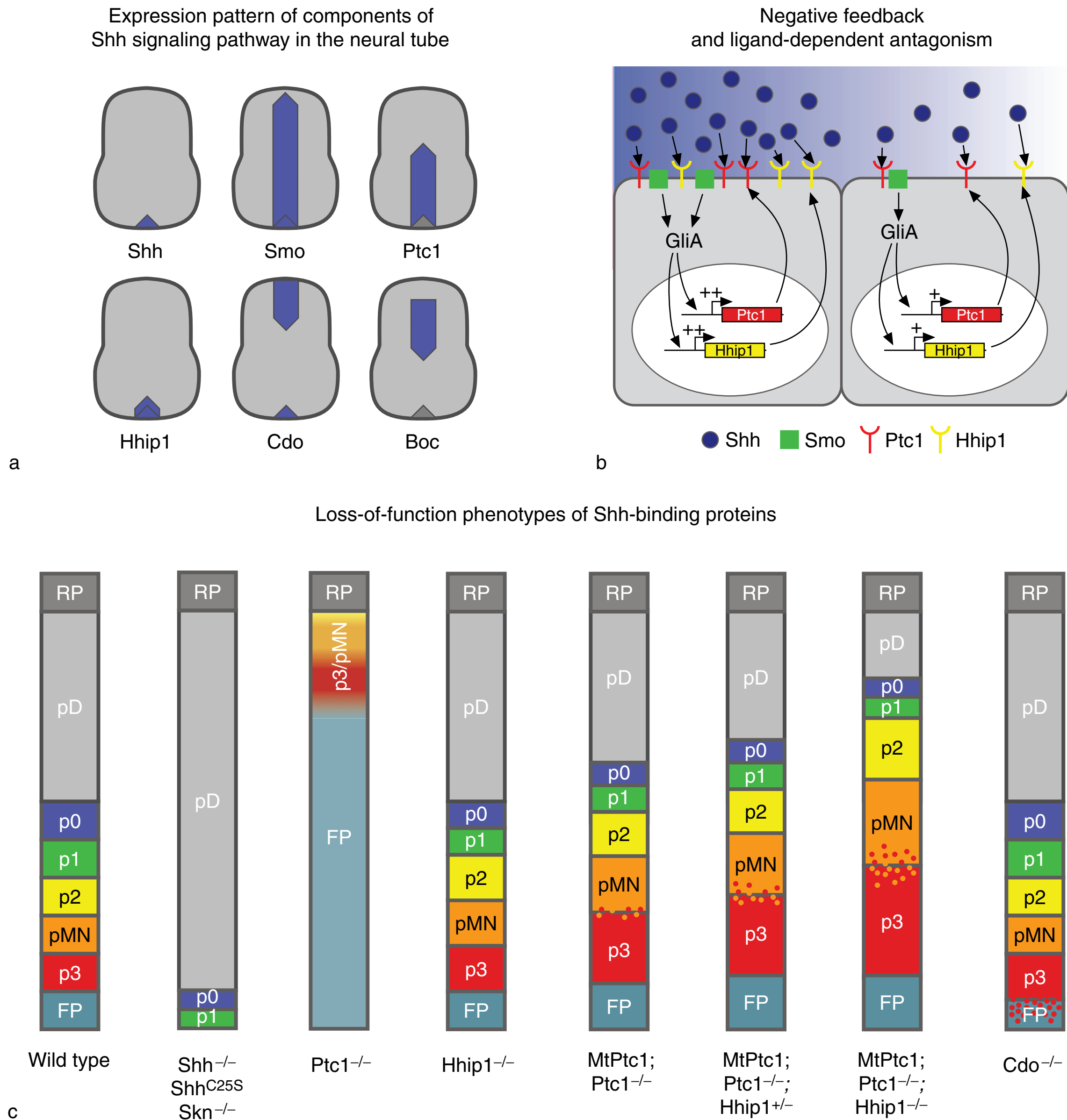

Figure 5 Regulation of the Sonic hedgehog (Shh) gradient is important for dorsoventral patterning of the neural tube. (a) Shh is expressed by the notochord (not shown) and the floor plate. Shh-binding proteins are found along the dorsoventral axis of the neural tube; Smoothened (Smo) and Patched1 (Ptc1) are expressed in a ventral-to-dorsal gradient in the spinal cord, together with Hhip1, found in the ventralmost part of the spinal cord. Cdo and Boc are expressed in the dorsal spinal cord; in addition, Cdo is also expressed in the floor plate. (b) Schematic of the control of the Shh gradient by the negative feedback loop triggered by Shh-binding proteins Ptc1 and Hhip1. Shh signaling enhances the expression of its receptors Ptc1 and Hhip1 through Gli transcriptional activators (GliA). The more Shh signaling received by a cell, the more it will increase the expression of Ptc1 and Hhip1. As a result, cells located near the source of Shh express higher levels of Ptc1 and Hhip1 (and also GliA) compared to cells located far away from the Shh source. The two Shh-binding proteins sequester Shh, thus limiting the range of Shh spread. (c) Abrogating the production and posttranslational modifications of Shh results in a dramatic dorsalization of the neural tube as observed in $Shh^{-/-}$ mice or mutants lacking Shh palmitoylation (Shh^{C25S} and $Skn^{-/-}$). In the absence of Ptc1, there is high Shh activity along the dorsoventral axis due to the lack of Smo inhibition. This is not observed in $Hhip1^{-/-}$ embryos. When Ptc1 is ubiquitously expressed at low level under the control of the metallothionein promoter (MtPtc1) and Ptc1/Hhip1 ligand-dependent antagonism is abolished, the distance of Shh spread is increased and consequently ventralization of the spinal cord results. This phenotype is dependent on the quantity of Hhip1, as illustrated by the comparison between MtPtc1;$Ptc1^{-/-}$ with MtPtc1;$Ptc1^{-/-}$;$Hhip^{+/-}$ and MtPtc1;$Ptc1^{-/-}$;$Hhip^{-/-}$. In parallel, this perturbation in Shh gradient decreases the precision in ventral patterning, as the boundary between p3 and pMN is less precise. There is also a lack of precision in the boundary between the floor plate (FP) and the p3 domain in the absence of Cdo, together with a reduction of the FP and a ventral expansion of the p3 domain. RP, roof plate; pD, dorsal spinal cord domain; pMN, motor neuron progenitor.

and Hhip1 – and Cdo is also expressed in the floor plate (**Figure 5(a)**). Gain- and loss-of-function experiments indicate that Cdo and Boc cell autonomously enhance Hh signaling. Given their expression profiles, it is possible that Cdo increases Shh signaling where the highest Shh signaling levels are required (e.g., in the floor plate). Conversely, at the fringes of the Hh target field (e.g., in intermediate and dorsal regions of the neural tube), where Ptc1 and Hhip1 levels are expected to be low, Cdo and Boc may sensitize cells to even low levels of Shh protein. Ectopic expression of Cdo and Boc led to a non-cell autonomous expansion of dorsal genes in cells lying dorsal to expressing cells, consistent with the idea that these proteins bind Shh, impeding its ability to spread further through tissue. Together, these data begin to indicate that the extracellular spread of Shh is the subject of finely controlled regulation which establishes the appropriate distribution of Shh that ensures the accurate supply of positional information to progenitors residing in the ventral neural tube.

Intracellular Mediators of Shh-Dependent Neural Tube Patterning: A Major Role of Gli Proteins

In receiving cells, intracellular signal transmission depends on two transmembrane proteins: Patched 1 (Ptc1), as already mentioned, the receptor which binds Hh proteins, and Smoothened (Smo), which is responsible for transducing Hh signals intracellularly (**Figure 6(a)**). In the absence of Shh, Ptc1 inhibits Smo activity, and binding of Shh to Ptc1 releases this inhibition, allowing intracellular signal transduction. Although the exact mechanism of signal transmission downstream of Smo is still the subject of study, several key components of the pathway have been identified. In vertebrates, Suppressor of Fused (Sufu) has a central role in Shh signaling, as gene disruption of Sufu in mice leads to potent ligand-independent activation of the Hh pathway, mimicking the defect in neural tube patterning observed in Ptc1 mutant embryos. Several other factors, including Rab23 (a vesicle transport family protein) and a number of factors involved in the assembly and/or function of a cell's primary cilium, have also been implicated in Shh signal transduction.

Gli Proteins and Dorsoventral Patterning

Although the mechanism of signal transduction remains to be clarified, the evidence suggests that the signal culminates in the regulation of a family of zinc finger-containing transcriptional effectors known, in vertebrates, as the Gli transcriptional regulators (Gli1, 2, and 3). These control target gene expression. All three Gli genes are expressed in the neural tube and several studies have begun to examine the regulation of Gli gene activity and the roles they play in neural tube patterning. Gli1 is expressed in the ventralmost neural tube, and its expression is dependent on Shh signaling. Gli1 appears to act solely as a transcriptional activator. In contrast, Gli2 and Gli3 are expressed in neural tissue prior to neural tube closure; later on, the expression pattern of Gli3 becomes confined to intermediate and dorsal spinal cord regions (**Figure 6(b)**). Analogous to the regulation of the *Drosophila* Gli ortholog, Cubitus interruptus (Ci), Shh signaling appears to control the transcriptional activity of Gli2 and Gli3. The C-terminally deleted versions of Gli2 and Gli3 proteins are transcriptional repressors, and in the absence of Shh signaling Gli3 is processed to this transcriptional repressor form. On Shh binding to Ptc1, the formation of Gli3 repressor is blocked and the transcriptional activation potential of Gli3 is revealed. In the case of Gli2, exactly how Shh controls transcriptional activity remains unclear, but may involve regulation of Gli2 stability or transcriptional potency by posttranslational modification.

In the mouse, targeted deletion of Gli2 leads to a failure in the generation of floor plate and the adjacent p3 domain and a concomitant ventral expansion in the production of MNs (**Figure 6(c)**). Cell types located dorsal to the pMN are, however, unaffected. This suggests that Gli2 is required for specifying the cell types that require the highest levels of Shh signaling; in support of this, analysis of mutants for Gli2 crossed with Ptc1 mutants indicates that expansion of the floor plate observed in the absence of Ptc1 is abolished when Gli2 is also absent. Surprisingly, although ectopic Gli1 expression is able to induce ventral cell types, no neural defects have been detected in Gli1 mutant mice. However, a compound mutant lacking both Gli1 and Gli2 has more severe defects than do Gli2$^{-/-}$ mutants. Moreover, the neural tube defects seen in Gli2 mutant embryos can be rescued by replacing Gli2 with Gli1.

On the other hand, Gli3 has been proposed to function primarily as an inhibitor of Shh signaling in the neural tube. Supporting this idea, in the absence of Gli3, progenitor domains located in the intermediate region of the neural tube expand dorsally, concomitant with a switch in the identity of the neurons generated in this region. This phenotype can be corrected by a truncated form of Gli3, corresponding to the processed Gli3, suggesting that the repressor form of Gli3 is responsible for patterning in the intermediate neural tube. However, Gli3 can act as a transcriptional activator, as illustrated by its ability to partially

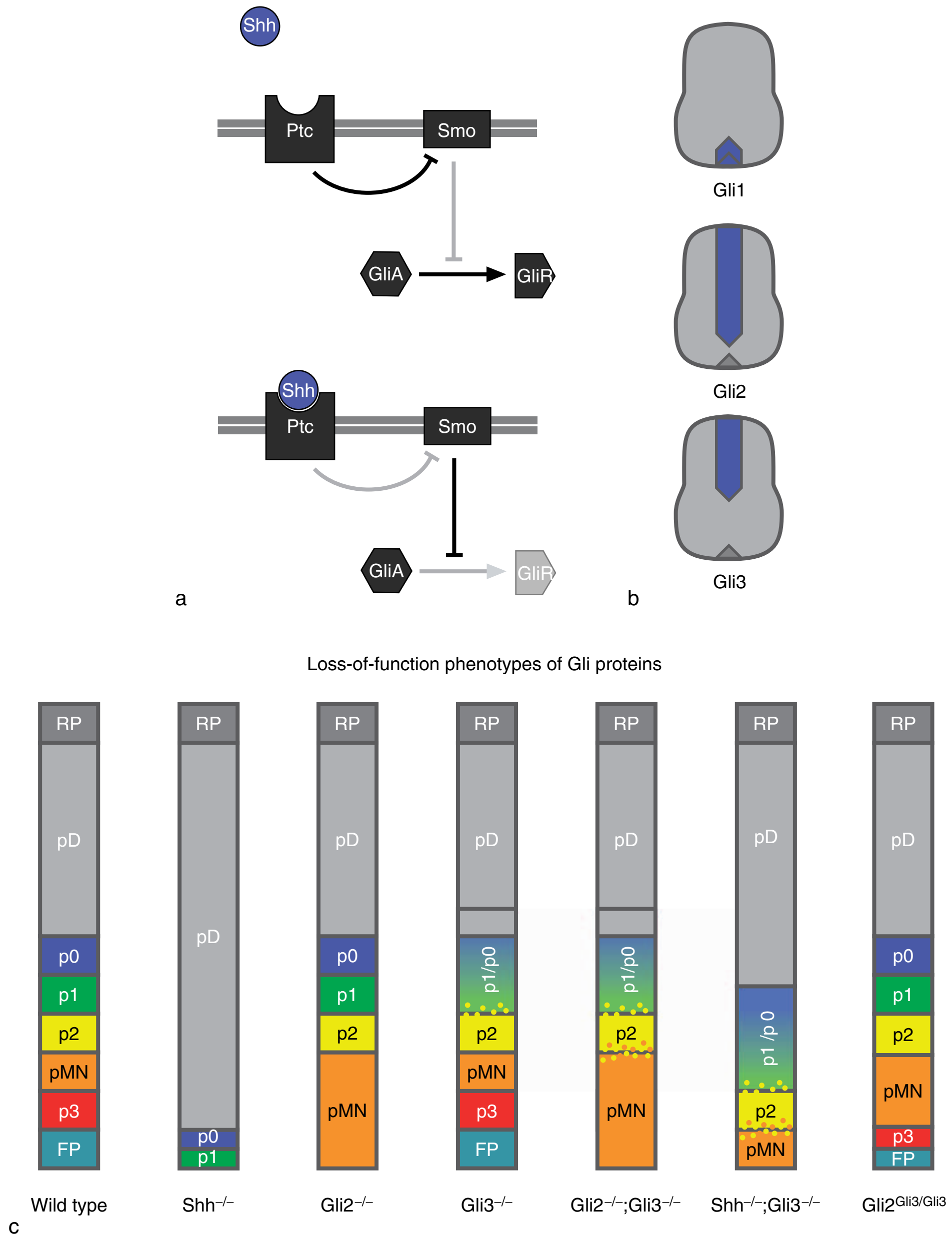

Figure 6 Gli proteins are involved in dorsoventral patterning of the neural tube. (a) In the absence of Sonic hedgehog (Shh), Patched (Ptc) inhibits the transmembrane protein Smoothened (Smo). Downstream transcriptional effectors, the Gli proteins, are thought to be processed from an activator form (GliA) to a repressor form (GliR). Shh binding to Ptc releases the inhibition of Smo, which inhibits production of inhibitory Gli proteins and promotes their transcriptional activity. (b) Schematic representing the expression of Gli proteins in the developing neural tube. (c) Abolishing Gli1 expression does not affect dorsoventral patterning. Loss of Gli2 (Gli2$^{-/-}$) results in an expansion of the ventral boundary of pMN domain together with a loss of the floor plate (FP) and the p3 domain. These results show that Gli2 is required for the patterning of the ventralmost region of the neural tube. Conversely, null mutants for Gli3 have defects in the patterning of the intermediate region of the neural tube accompanied with a mixing of the cells at the p2/p1 boundary. Double mutants for both Gli2 and Gli3 have a composite phenotype of the single mutants. Whereas Gli2 is acting as a transcriptional activator, Gli3 seems to mainly act as a repressor, as illustrated by the partial rescue of Shh$^{-/-}$ phenotype in the Shh$^{-/-}$;Gli3$^{-/-}$ mutant neural tube. However, Gli3 can also act as a transcriptional activator because it partially complements Gli2 when Gli3 is used to replace Gli2 in transgenic embryos. RP, roof plate; pD, dorsal spinal cord domain; pMN, motor neuron progenitor.

rescue the Gli2 mutant phenotype when expressed from this locus.

Importantly, abolishing Gli3 function in Shh$^{-/-}$ embryos partially rescues the severe defects in ventral neural tube patterning observed in Shh mutant embryos (**Figure 6(c)**). This confirms the inhibitory role for Gli3 in Shh signaling. It also indicates that although the spatial arrangement of neurogenesis

depends on Shh signaling, the induction of most ventral cell types can take place in the absence of Shh signaling, as long as the repressive activity of Gli3 is removed. Consistent with this, embryos lacking both Gli2 and Gli3 display a D/V pattern similar to that of Shh/Gli3 double mutants.

In zebra fish embryos, although the exact role of individual Gli genes appears to differ from that in the mouse, loss-of-function experiments indicate that the Gli proteins are also required for the reception of Hh signals. Together, these results demonstrate that Gli proteins act downstream of Shh to control ventral patterning through transcriptional regulation of target genes (**Figure 6(c)**). Moreover, the data can be accommodated by a model in which graded Shh signaling establishes a ventral-to-dorsal gradient of Gli activity that reflects the gradient of Shh activity. This Gli gradient corresponds to the sum of the transcriptional activator and repressor forms of all Gli proteins present in a responding cell. In support of this model, gain-of-function experiments in the chick suggest a tight correlation between progenitor cell fate and the level of Gli activity within a progenitor cell; furthermore, different levels of Gli activity are sufficient to emulate different levels of Shh signaling *in vivo*.

Perspective

Numerous studies show that Shh, emanating from the notochord and floor plate, patterns the ventral neural tube by controlling cell fate in a concentration-dependent manner. Several major issues remain to be resolved, including understanding how the Shh signaling pathway regulates Gli activity and how this controls the differential expression of class I and class II protein expression. Moreover, how other extracellular signals modulate and cross-talk with Shh signaling requires further investigation. In addition, the integration of D/V patterning with the anterior–posterior differences and temporal changes in neural cell fate remains to be explained. Finally, the evidence suggests that Shh signaling also controls the proliferation, adhesive properties, and survival of cells in the neural tube. The downstream targets of Shh signaling involved in these processes are not well defined, nor how these cell behaviors are coordinated with and contribute to the control of D/V patterning. Nevertheless, the progress that has been made provides a framework to understand fundamental aspects of the mechanism by which positional cues control the arrangement of distinct neuronal subtypes in the ventral neural tube.

See also: Helix–Loop–Helix (bHLH) Proteins: Hes Family; Helix–Loop–Helix (bHLH) Proteins: Proneural; Motor Neuron Specification in Vertebrates; Oligodendrocyte Specification; Sonic Hedgehog and Neural Patterning; Wnt Pathway and Neural Patterning.

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Wnt Pathway and Neural Patterning

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Introduction

The Wnt pathway is a complex signal transduction pathway triggered by secreted ligands of the Wnt family, named after its two original members, the products of the *wingless* gene from *Drosophila melanogaster* and the mammalian gene *Int-1* (now known as *Wnt-1*). Wnt signaling is involved in many different embryonic processes, including the fundamental pattern-forming stages that establish the nervous system in insects and vertebrates. Many of the components of the these developmental pathways were first identified in *Drosophila* and in the African clawed frog *Xenopus laevis* and have since been extrapolated to other organisms, including mammals. Wnt signaling is also involved in many later processes in the nervous system, including synaptic specialization, microtubule dynamics, synaptic protein organization, modulating synaptic efficacy, and regulating neuronal gene expression.

There are at least 20 distinct Wnt genes in vertebrates, organized into 12 conserved subfamilies. Not all vertebrates have orthologs of all members of the Wnt family. At the time of writing, 19 Wnt genes have been found in humans and mice and 16 in *Xenopus*. Only six of the vertebrate Wnt subfamilies have counterparts in *Drosophila*, while at least 11 are present in sea anemones, indicating selective evolutionary loss of different Wnt classes during the branching of the animal lineage.

The vertebrate Wnt proteins can be grouped by biological activity using functional assays. The overexpression of some Wnt proteins induces secondary axis formation in early *Xenopus* embryos and transforms C57MG mammary epithelial cells, while other Wnt proteins do not act in this way and can even antagonize the transforming Wnt proteins, hinting at the existence of different, competing signaling pathways. Currently, five different pathways are known to be activated by Wnt proteins: a canonical Wnt/β-catenin cascade, a divergent canonical pathway involved in synapse modeling and axon growth, the noncanonical planar cell polarity (PCP) pathway, the Ca^{2+} pathway (which is involved in the control of cell migration), and a microtubule-dependent pathway.

Secretion of the Wnt Protein

Wnt proteins are secreted from the cell and therefore carry an N-terminal signal sequence targeting them for cotranslational import into the endoplasmic reticulum. During their journey through the secretory pathway, they undergo several forms of posttranslational modification, including N-linked glycosylation and palmitoylation of specific cysteine residues. Not all Wnt proteins undergo both types of modification, but when palmitoylation occurs the added lipid moiety appears essential for Wnt function. The presence of a lipid group also makes the Wnt protein hydrophobic and insoluble, which contributes to the difficulty in purifying many Wnt proteins from cell culture media. In *Drosophila*, the product of the *porcupine* gene is required for Wnt palmitoylation and has been identified as an acyltransferase. Another gene, *wntless*, encodes a transmembrane protein which co-localizes with the Wingless protein inside the cell and is required for trafficking through the Golgi apparatus. Vertebrate orthologs of both proteins have been identified.

One of the main characteristics of Wnt proteins is their ability to function as developmental morphogens – that is, molecules that establish long-range concentration gradients that enable them to influence cell fates differently, according to the cell's position along the gradient. It has been unclear how Wnt proteins achieve such a gradient given their poor solubility, although it has been proposed that the proteins remain tethered to the plasma membrane, or to intercellular transport vesicles or lipoprotein particles. Alternatively, Wnt proteins may travel along cytonemes, which are long, thin filopodial processes that can bridge several cells. A further possibility is that extracellular heparan sulfate proteoglycans (HSPGs) may be involved in the transport of Wnt proteins and the establishment of morphogen gradients. As soon as they are secreted, Wnt proteins interact with glycosaminoglycans in the extracellular matrix and bind tightly to the cell surface, another factor which makes them difficult to isolate in cell culture. In *Drosophila*, the Wingless protein is found in specialized membrane vesicles called argosomes that are thought to be derived from lipid raft microdomains, and the incorporation of the protein into these vesicles requires HSPGs. Mutations in genes such as *dally*, which encodes components of this HSPG system, cause phenotypes similar to those resulting from mutations in *wingless*. Six HSPGs have been identified in vertebrates, and mouse knockouts confirm that Wnt signaling is disrupted in such animals.

Canonical Pathway – Wnt Receptors and Alternative Ligands

Wnt proteins bind to the cysteine-rich extracellular domain of seven-pass transmembrane receptors of the

Frizzled (Fz) family, named after the founder member identified in *Drosophila*. There are at least four Fz family genes in *Drosophila* and at least ten in vertebrates, but the complexity of signaling is much greater since different Wnt proteins can interact with more than one receptor and different Fz proteins can bind more than one ligand. Promiscuous as it is, the interaction between Wnt and Fz requires a number of additional factors, including a membrane-bound protein encoded by the *arrow* gene in *Drosophila*, and represented by two homologous lipoprotein receptor-related proteins (LRPs) called LRP5 and LRP6 in vertebrates. Wnt proteins can bind directly to LRPs and there is evidence that Wnt, Fz, and LRP form a ternary complex. Delivery of the Arrow protein depends on the presence of a molecular chaperone encoded by the *boca* gene, and in vertebrates this function is fulfilled by the homologous mesoderm development (Mesd) protein. Mutations in *arrow/Lrp* genes and in *boca/Mesd* result in phenotypes similar to those of *wingless/wnt* mutants.

The Fz/LRP receptor complex interacts not only with Wnt proteins, but also with alternative ligands that can act as agonists or antagonists to Wnt signaling. An example is Norrin, which binds to Fz4/LRP5. This is a cysteine knot protein identified through investigation of the human developmental disorder Norrie disease, which is characterized by major eye vascular defects. Another example is the family of proteins known as R-spondins. In *Xenopus*, R-spondin-2 is a Wnt agonist that synergizes with Wnt signaling to activate β-catenin in muscle development. In contrast, proteins of the Dickkopf family inhibit Wnt signaling by sequestering LRP5/LRP6 and then cross-linking them to an unrelated class of transmembrane proteins known as Kremens, thus promoting internalization. The sclerosteosis-associated SOST/sclerostin family of Wnt antagonists also acts by sequestering LRP5/LRP6. The soluble Frizzled-related proteins (SFRPs) are also Wnt antagonists, but these work by sequestering the Wnt proteins. This is possible because the SFRPs contain cysteine-rich domains that mimic the genuine Fz protein. They may either bind Wnt proteins in isolation, or form inactive complexes along with Fz. However, certain combinations can promote rather than inhibit Wnt signaling, perhaps by stabilizing the Wnt signal in a manner that preserves its ability to interact productively with Fz/LRP.

Canonical Pathway – Events in the Cytosol

The formation of a stable Wnt/Fz/LRP complex facilitates the phosphorylation and activation of a cytosolic protein called Dsh (from the *Drosophila* gene *disheveled*), which interacts with the cytosolic face of the complex. The activated Dsh protein is then able to disrupt a so-called destruction complex comprising three additional proteins: glycogen synthase kinase-3 (GSK-3), axin, and adenomatous polyposis coli (APC) protein. In the absence of activated Dsh, these three proteins would normally phosphorylate β-catenin, leading to its ubiquitinylation and proteasomal degradation. When the destruction complex is disassembled, β-catenin is stabilized and some of it can be imported into the nucleus, where it interacts with transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family to activate the transcription of target genes (**Figure 1**).

The receptor complex Wnt/Fz/LRP interacts not only with Dsh but also with axin, one of the components of the destruction complex. Axin is the scaffold for the destruction complex, so all of the other components bind directly to it. The interaction between axin and the cytoplasmic tail of LRP is mediated by direct contacts with phosphorylated residues. Axin will not interact productively with LRP unless the tail is phosphorylated on multiple serine/threonine residues, and phosphorylation occurs only when Wnt binds to the receptor complex. The phosphorylation of these residues is carried out by GSK-3 and another kinase called caseine kinase I (CKI), which is also anchored to the

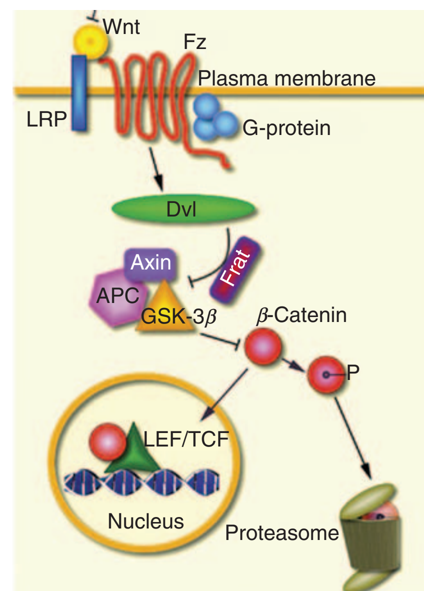

Figure 1 The canonical Wnt signaling pathway involving the destruction complex of axin, adenomatous polyposis coli (APC) protein, and glycogen synthase kinase-3β (GSK-3β), and the nuclear import of β-catenin. Fz, Frizzled; LRP, lipoprotein receptor-related protein; Dvl, Disheveled; Frat, 'frequently rearranged in advanced T-cell lymphoma'; LEF/TCF, lymphoid enhancer factor/T cell factor. Reproduced from Speese SD and Budnik V (2007) Wnts: Up- and-coming at the synapse. *Trends in Neuroscience* 30: 268–275, with permission from Elsevier.

plasma membrane. These two kinases phosphorylate different sets of serine/threonine residues on the LRP tail and both are required for transduction of the Wnt signal. The remaining component of the complex, APC protein, is an essential protein, but its precise role is unclear. It has been proposed that APC protein is required for efficient shuttling and loading/unloading of β-catenin onto the cytoplasmic destruction complex. Both APC protein and axin can be phosphorylated by their associated kinases, changing their affinity for other components of the destruction complex.

In the absence of Wnt, β-catenin is phosphorylated by CKI and GSK-3, and in its phosphorylated state it can be recognized by a transducin repeat-containing protein (β-TrCP), a component of a dedicated E3 ubiquitin ligase complex. The β-catenin protein is thus ubiquitinated and destroyed by the proteasome. In the presence of Wnt, the kinase activity of CKI and GSK-3 is inhibited, and β-catenin is able to translocate into the nucleus. Certain protein phosphatases may antagonize CKI and GSK-3, thereby promoting β-catenin stability.

Canonical Pathway – Events in the Nucleus

It is presently unclear how β-catenin is imported into the nucleus, although the process is dependent on a nuclear localization signal and β-catenin has been shown to interact with nuclear pore components. It is possible that β-catenin may shuttle from nucleus to cytoplasm in concert with axin and/or APC protein, and may be retained in the nucleus by associating with anchor proteins such as Pygopus. The role of β-catenin in the nucleus is to interact with transcription factors in the TCF/LEF family and prevent the formation of a repressive complex with proteins of the Groucho family via physical displacement. The TCF/LEF family is represented by a single protein in *Drosophila* and four paralogs in vertebrates. The replacement of Groucho with β-catenin facilitates TCF/LEF binding to the minor groove of DNA at highly conserved target sites characterized by the presence of purines on one strand and pyrimidines on the other. The binding results in the introduction of a tight kink into the DNA backbone, and allows β-catenin to bind to chromatin components such as Brahma-related gene 1 (Brg-1) protein – part of the mating-type switch/sucrose nonfermenting (SWI/SNF) chromatin remodeling complex – and histone acetylase cAMP response element-binding (CREB)-binding protein (CBP), to promote transcriptional initiation. TCF/LEF is also regulated via other pathways, and phosphorylation via the activation of mitogen-activated protein (MAP) kinase reduces its affinity for β-catenin.

The Canonical Pathway in *Xenopus* Axis Specification

Wnt signaling is critical for the establishment of dorsoventral polarity in the early *Xenopus* embryo and for laying down of the primitive anteroposterior neuraxis. The dorsal side of the embryo gives rise to a structure called the organizer, through which cells migrate to establish the anteroposterior axis. In 1989, McMahon and Moon injected mouse *Wnt-1* mRNA into the ventral blastomeres of a four-cell *Xenopus* blastula and generated an embryo with two organizers, which developed into tadpoles with a duplicated body axis. Axis duplication was also induced by Dsh, β-catenin, and a dominant-negative version of GSK-3.

In normal *Xenopus* development, the Wnt pathway is activated opposite the site of sperm entry in what becomes the future dorsal side of the embryo following cortical rotation after fertilization. It is thought that maternal Dsh protein, initially located in the vegetal region of the egg, is translocated by cortical rotation to a discrete zone of the fertilized egg, where it stabilizes β-catenin. As the embryo undergoes cleavage, cells incorporating this β-catenin-enriched cytosol activate specific genes, leading to the formation of a structure called the Nieuwkoop center, which induces the formation of the organizer in the overlying mesoderm. The ventral injection of Wnt, Dsh, β-catenin, or an inhibitory form of GSK-3 artificially activates the same genes, resulting in the formation of a duplicate organizer.

Wnt signaling is also important for the establishment of anteroposterior polarity in the neuroectoderm. The organizer expresses several Wnt antagonists, such as those encoded by *dickkopf-1*, *cerberus*, and *frzb-1*, while several Wnt proteins are expressed in the remainder of the embryo. Injection of mRNA for the antagonists leads to enlarged head development, whereas injection of wnt mRNA at this developmental stage inhibits neural induction and head induction. Although long elusive, the specific Wnt signal that triggers axis induction in *Xenopus* was identified as Wnt11 in 2005.

Wnt Signaling in Synapse Development – Divergent Pathways and Diverse Mechanisms

A number of Wnt proteins and their downstream signaling components are expressed in the developing synapse, and investigations in *Drosophila*, *Xenopus*, and mammals have revealed a previously unknown and surprisingly diverse range of signaling pathways activated by Wnt proteins. To further add to the

complexity, it appears that the same Wnt proteins can activate different downstream pathways in different parts of the central nervous system (CNS) and that different Wnt proteins can activate the same pathway in a given region of the CNS, providing scope for functional redundancy. Perhaps most exciting of all, the prominent roles of Wnt proteins in synapse formation appear to involve retrograde signaling, but there is also evidence that Wnt proteins can act as anterograde or autocrine signals.

In the mammalian CNS, various Wnt proteins are expressed during synapse development, including Wnt-3 in Purkinje cells and motor neurons, and Wnt-7a in cerebellar granule cells. These Wnt proteins can regulate axonal remodeling and presynaptic differentiation retrogradely, and do so by influencing the architecture of the cytoskeleton. For example, the addition of Wnt-7a proteins to cerebellar granule cells in culture can increase axonal branching and the clustering of synaptic vesicle proteins, and *wnt7a* knockout mice show transiently decreased axonal complexity and limited synapsin I levels. Wnt-3 is also expressed in motor neurons during synapse formation with sensory neurons, and increasing the levels of this protein results in growth cone enlargement, increased axonal branching, and increased clustering of synapsin I.

Investigation of the role of Wnt proteins in axonal remodeling has shown that a divergent form of the canonical pathway is involved (**Figure 2**). Some of the components are familiar (GSK-3, the Disheveled family protein Dvl1, and axin), while a significant role is also played by the microtubule-associated protein MAP1B. The phosphorylation of MAP1B by GSK-3 alters its affinity for microtubules and facilitates the regulation of microtubule dynamics. Wnt signaling reduces the availability of phosphorylated MAP1B and increases microtubule stability, a process mimicked by the ectopic expression of Dvl1, which is also known to be physically associated with microtubules. Therefore, it appears that Dvl1 antagonizes the effect of GSK-mediated destabilization. These effects are independent of β-catenin (i.e., the divergent canonical pathway acts directly upon the structural elements of the axons without requiring new gene expression).

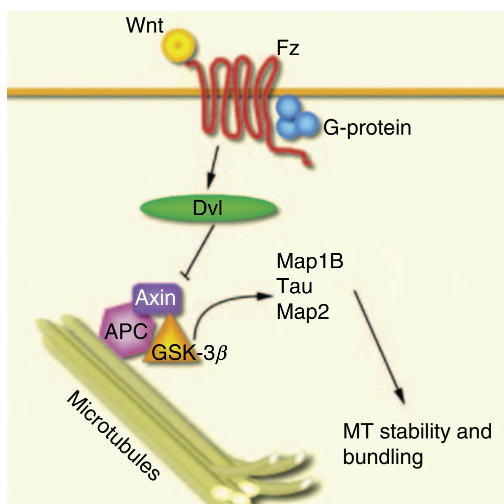

Figure 2 The divergent canonical pathway with no β-catenin and regulation of microtubule (MT) organization by the destruction complex through phosphorylation of microtubule-associated proteins 1 B (MAP1B). Fz, Frizzled; Dvl, Disheveled; APC, adenomatous polyposis coli protein; GSK-3β, glycogen synthase kinase-3β. Reproduced from Speese SD and Budnik V (2007) Wnts: Up- and-coming at the synapse. *Trends in Neuroscience* 30: 268–275, with permission from Elsevier.

A further manifestation of the Wnt signaling pathway has been identified in the hippocampus, where Wnt-7b is expressed during dendrite maturation. Experiments have shown that Wnt and Dvl1 can each increase dendritic complexity, and *dvl1* knockout mice have fewer dendritic branches. The key difference between this and the canonical pathways described earlier is that the pathways operate independently of both β-catenin and GSK-3. Instead, the Wnt/Fz/LRP complex acts through a c-Jun N-terminal kinase (JNK) signaling protein to affect cytoskeletal architecture, using the small GTPase Rac, and to regulate both actin and microtubule dynamics, in a manner similar to the planar cell polarity signaling pathway in *Drosophila* (**Figure 3**(**a**)).

Although Wnt proteins appear to function as retrograde signals to regulate the differentiation of the presynaptic compartment, there is also evidence that they might operate as anterograde or even an autocrine signals to modulate postsynaptic differentiation. Studies of the *Drosophila* neuromuscular junction show that Wingless is secreted by presynaptic boutons, and the receptor Fz2 is localized in both presynaptic and postsynaptic compartments. The release of Wingless from synaptic boutons initiates an anterograde signaling cascade, which involves yet another divergent signaling pathway in which the receptor is internalized, cleaved, and trandslocated into the nucleus in a manner dependent on the *Drosophila* homolog of the mammalian glutamate receptor-interacting protein (dGRIP), well known for its role in α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and ephrin B receptor trafficking in mammals. Postsynaptic disruption of the so-called Frizzled nuclear import (FNI) pathway (**Figure 3**(**b**)) alters the development of both presynaptic and postsynaptic specializations, suggesting that anterograde Wingless signaling might trigger

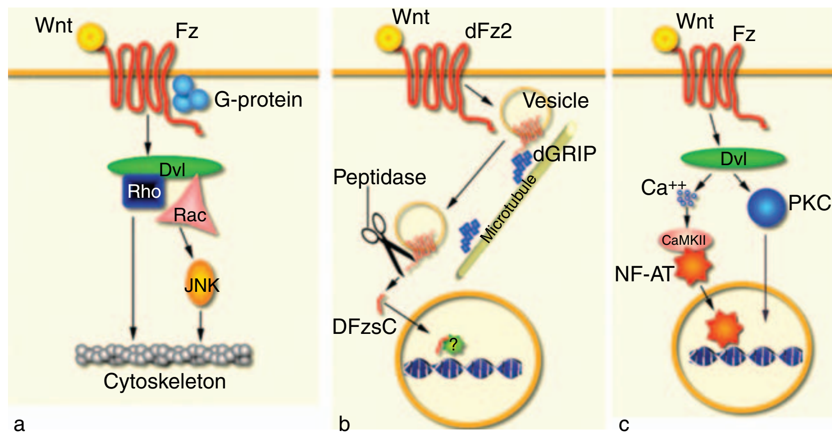

Figure 3 Three β-catenin-independent pathways: (a) planar cell polarity, (b) Frizzled nuclear import, and (c) Ca^{2+}. Fz, Frizzled; Dvl, Disheveled; JNK, c-Jun N-terminal kinase; dGRIP, *Drosophila* homolog of the mammalian glutamate receptor-interacting protein; PKC, protein kinase C; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; NF-AT, nuclear factor of activated T cells. Reproduced from Speese SD and Budnik V (2007) Wnts: Up- and-coming at the synapse. *Trends in Neuroscience* 30: 268–275, with permission from Elsevier.

further retrograde signals or even an autocrine loop to regulate presynaptic differentiation. Similar roles for Wnt proteins in postsynaptic development have been identified in vertebrates; for example, the regulation of acetylcholine receptor clustering by agrin and by muscle-specific receptor tyrosine kinase (MuSK) has been identified, the latter of which has now been shown to interact with Dvl1, linking this regulation to the Wnt pathway. However, since agrin has no effect on β-catenin accumulation, it appears that the canonical pathway is not triggered.

Wnt Signaling and Ca^{2+}

A further β-catenin-independent Wnt signaling pathway has been identified that modulates cell movements (e.g., during embryonic gastrulation), and it also plays a role in the definition of cell fate. In this pathway, the binding of Wnt to Fz/LRP leads to an increase in intracellular Ca^{2+} concentration and nuclear import of the transcription factor NF-AT (nuclear factor of activated T cells; **Figure 3(c)**). The mechanism depends on the ability of specific combinations of Wnt and Fz proteins to activate Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC). Some Fz proteins also have the ability to activate phospholipase C (PLC) and phosphodiesterase (PDE), acting through heterotrimeric GTP-binding proteins. The ability of different Wnt ligands to activate the β-catenin and Ca^{2+} pathways may reflect the preferences of different Fz proteins (e.g., rat Fz1 does not increase Ca^{2+} release or stimulate CaMKII or PKC in zebra fish embryos, whereas Fz2 does) and preferences for different coreceptors (e.g., LRP-5/LRP6 stimulates the β-catenin pathway, whereas alternative coreceptors such as Knypek and Ror2 stimulate the Ca^{2+} pathway).

Summary

Wnt signaling plays a pivotal role in the development and function of the nervous system, being required to establish the major body axes and compartment polarity in vertebrate and insect embryos, orchestrating cell migration, polarity, and fate, and controlling synaptic bouton development, axonal growth cone remodeling, and dendrite maturation, as well as a host of other processes. The canonical signaling pathway (**Figure 1**) involves the protection and subsequent nuclear import of β-catenin (Armadillo in *Drosophila*), although there is a divergent pathway, using many of the same components, that is β-catenin independent and acts directly on the organization of microtubules (**Figure 2**). There are also at least three pathways which involve Ca^{2+} as a second messenger, one involving the activation of CaMKII and protein kinase C, one involving the recruitment of heterotrimeric GTP-binding proteins to activate phospholipase C and phosphodiesterase, and, finally, one involving the planar cell polarity pathway, which signals through JNK (**Figure 3**). The particular pathways activated in any given cell, and their consequences, reflect the availability and abundance of different pathway components, particularly the Wnt ligands and Fz receptors and coreceptors, which all impact on the downstream events.

See also: Dendrite Development Synapse Formation and Elimination; Forebrain: Early Development; Morphogens: History; Neural Patterning: Midbrain–Hindbrain Boundary; Sonic Hedgehog and Neural Patterning.

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Bone Morphogenetic Protein (BMP) Signaling in the Neuroectoderm

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Introduction

The nervous system is a highly heterogeneous tissue comprising a great diversity of cell types that interconnect in complex patterns to control a myriad of conscious and unconscious behaviors. Not surprisingly, creating such an intricate system requires a series of many cellular interactions during development. Because various organisms have a wide range of different life strategies and needs, there is also a great diversity in the function and development of nervous systems across species. Notwithstanding the inherent complexity and diversity of nervous system function and development, there are remarkable parallels between the formation and function of the nervous system in organisms ranging from fruit flies and nematodes to vertebrates. In several cases, homologous gene sets play critical roles in processes such as neural induction, neurite pathfinding, synaptogenesis, action potential propagation, transmitter secretion and reception, and behavior. This high degree of conservation of basic cellular and molecular functions suggests that the common ancestor of current living metazoans had a well-formed nervous system with many of the core properties shared by diverse present-day organisms.

One of the best characterized examples of conserved pathway function in neural development is the role of bone morphogenetic protein (BMP) signaling during neural induction. During this early phase of embryonic development, BMP signaling actively represses neural cell fates in epidermal regions of the embryo. In neuroectodermal regions, BMP signaling is blocked by various BMP antagonists, which permits the default program of neural development to prevail. Because many of the pathway components required for neural induction are similarly deployed in vertebrates and invertebrates, it seems highly likely that this similarity reflects the conservation of an ancestral mechanism for specifying neural versus epidermal cell fates. BMPs also play important roles in the subsequent patterning of the nervous system along the dorsal–ventral (DV) axis. It is less clear, however, whether this latter phase of neural patterning is accomplished by homologous or convergent mechanisms. In this article, we briefly review the evidence for a conserved function of BMP signaling during neural induction and then focus on how BMPs are believed to act during neural patterning in different organisms. We propose that a unifying theme may underlie the apparent diversity of these patterning mechanisms, wherein BMPs act by a common mechanism to repress the expression of neural genes in a dose-dependent fashion. We also consider how conserved and diverse elements of neural patterning may have evolved.

Evolutionary Conservation of BMP Inhibition during Neural Induction

Nearly a century ago, Hans Spemann and Hilde Mangold showed that ventral transplantation of the dorsal lip of an amphibian embryo into a recipient embryo led to the production of a secondary neural axis. Using distinguishable host and donor embryos they demonstrated further that the dorsal lip, or Spemann organizer as it is now often called, was the source of secreted neural-inducing signals which could redirect the development of surrounding cells fated otherwise to give rise to epidermis. Since these seminal experiments, there has been great interest in isolating and understanding the function of neuralizing factors. Several neural inducers have been identified from *Xenopus* in recent years, including Noggin, Chordin (Chd), and proteins in the DAN family, which are expressed in dorsal mesodermal cells making up the Spemann organizer during late blastula and early gastrula stages. These structurally diverse neural inducers function via a common double negative mechanism by antagonizing the function of BMP signaling (**Figure 1(a)**). They bind to BMPs (BMP2/BMP4) with high affinity, preventing them from activating BMP receptors. In the nonneural ectoderm, where BMP4 is expressed at high levels, BMP signaling functions to promote epidermal fates and to repress the expression of neural genes. Similarly, in *Drosophila*, the Chd homolog known as Short Gastrulation (Sog) is expressed in the lateral neuroectoderm and blocks BMP signaling in the dorsal ectoderm. It is likely that the DV axes in vertebrate and invertebrate embryos were inverted during evolution, such that the epidermis forms ventrally in vertebrates but dorsally in invertebrates. In flies, as in vertebrates, BMP signaling represses the expression of neural genes and activates the expression of nonneural genes. It is noteworthy that in *Drosophila* significantly less BMP signaling is required to repress the expression of neural genes than to activate expression of epidermal genes. One of the genes activated by

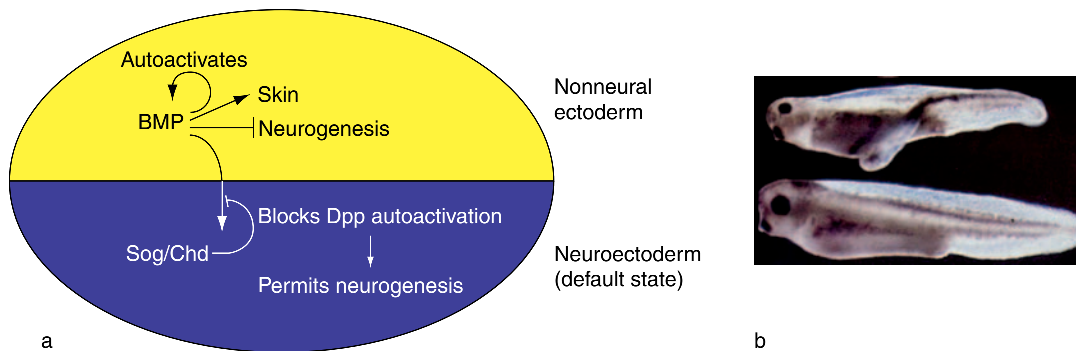

Figure 1 BMPs suppress neuronal fates in the ectoderm of vertebrates and arthropods: (a) BMP signaling in the nonneural ectoderm represses the expression of all neural genes and activates the expression of epidermal genes, including the BMP4/Dpp genes, thereby creating a positive feedback loop, referred to as autoactivation; (b) an example of the conserved nature of BMP inhibition and neural induction in *Xenopus* embryos. In (a), neural-inducing factors, such as the extracellular BMP antagonists Sog/Chd, are supplied to the neural ectoderm, where they bind to BMPs and prevent them from triggering BMP autoactivation, thereby preventing the invasive spread of BMP signaling into the neuroectoderm. This double-negative mechanism allows cells in the neuroectoderm to follow the default neural development pathway. In (b), the injection of *sog* mRNA from *Drosophila* into ventral blastomeres of early *Xenopus* embryos leads to the formation of secondary neural axes (top embryo; compare to wild-type embryo on bottom) similar to those observed in the original embryo-grafting experiments of Hilde Mangold and Hans Spemann. BMP, bone morphogenetic protein; Dpp, Decapentaplegic (the ortholog of vertebrate BMP4/2); Sog, Short gastrulation (the ortholog of vertebrate Chordin (Chd)). Adapted from Biehs B, François V, and Bier E (1996) The *Drosophila short gastrulation* gene prevents Dpp signaling from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes & Development* 10: 2922–2934; and Schmidt J, François V, Bier E, and Kimelman D (1995) The *Drosophila short gastrulation* gene induces an ectopic axis in *Xenopus*: Evidence for conserved mechanisms of dorsal-ventral patterning. *Development* 121: 4319–4328.

BMP signaling in *Drosophila* and vertebrates is the Dpp/BMP4 gene itself, which results in a positive feedback loop, referred to as autoactivation. If unopposed by BMP antagonists, BMP autoactivation can result in the invasive spread of BMP signaling into the neuroectoderm. This ectopic expression of BMP ligands leads to the repression of neural gene expression and to the activation of epidermal genes.

Conservation of the Chd/BMP signaling system extends to the functional level, as revealed in cross-species experiments. For example, injection of *Drosophila sog* mRNA into the ventral blastomeres of *Xenopus* embryos generates duplicated neural axes (**Figure 1**(**b**)) similar to those induced by injection of vertebrate Chd or by transplantation of Spemann organizer tissue. Similarly, vertebrate BMPs and BMP antagonists have the same activities in *Drosophila* as they do in vertebrate embryos. Other extracellular components of the BMP pathway identified in *Drosophila* have also been shown to play similar roles in early vertebrate embryos (**Figure 2**). For instance, embryos lacking Tolloid (Tld) and Twisted gastrulation (Tsg) activity have defects in BMP signaling. Tld is a metalloprotease that can cleave and inactivate Sog, whereas Tsg forms a trimeric complex with Sog/Chd and BMPs and modifies the BMP inhibitory function of Sog by binding to it and by generating alternative Tld cleavage products. Similarly, in vertebrates, the *Xenopus* counterpart of Tld, Xolloid (Xld), cleaves Chd in positions corresponding to two of the four sites in Sog that are cut by Tld, thereby reducing Chd activity. In addition, *Xenopus* and zebra fish homologs of Tsg also can form a ternary complex with Chd and BMPs to modulate BMP signaling.

Opposing Graded BMP and Hh Signals Pattern the Vertebrate Neuroectoderm

A wealth of embryological and genetic evidence in vertebrates indicates that, following their role in neural induction, BMPs play an important role as morphogens in organizing gene expression along the dorsal–ventral axis of the developing nervous system (note that morphogens are molecules distributed in a graded fashion that function in a dose-dependent fashion to activate or repress gene expression). Once the dorsal-most ectodermal region of the vertebrate embryo is specified as neuroectoderm (often referred to as the neural plate), these cells undergo a concerted set of bilaterally symmetric apical constrictions, causing them to fold inside the embryo by the process of invagination (also referred to more specifically as neurulation). BMP-expressing epidermal cells bordering the neural plate are thereby brought into juxtaposition to form a single coherent dorsal epidermal mass (**Figure 3**(**a**)). The invaginated neural plate forms a longitudinal cylinder, which then closes on itself and separates from the overlying epidermis to form the neural tube. The dorsal-most cells of the neural tube lie immediately below the BMP-expressing epidermis and are subsequently induced to express BMPs. This

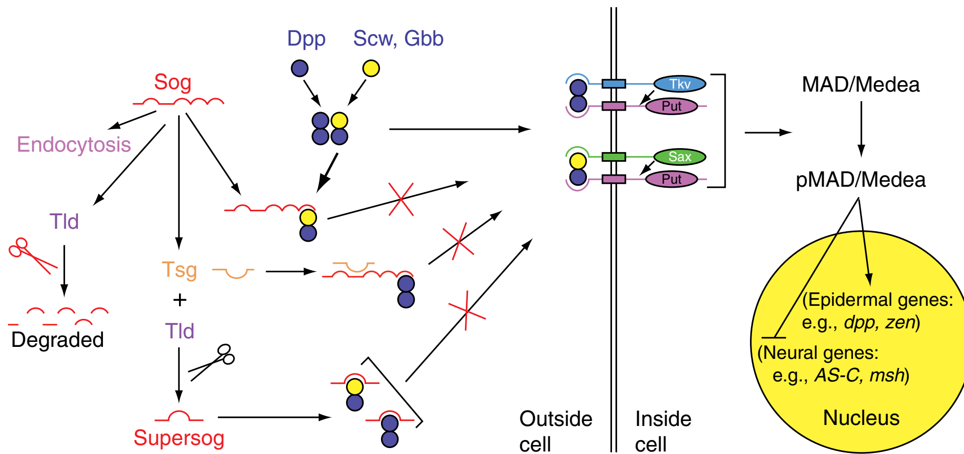

Figure 2 Extracellular regulation of BMP signaling. Diagram of BMP signaling pathway from *Drosophila,* highlighting elements that are conserved in vertebrates. BMP homodimers (e.g., Dpp-Dpp) or heterodimers (e.g., Dpp-Scw) induce the dimerization and then tetramerization of type I and type II BMP receptors. Following receptor dimerization, the type I receptor chain (e.g., Sax or Tkv receptors) phosphorylates the type II chain (e.g., the Put receptor), leading to phosphorylation of the cytoplasmic signal transducer SMAD (or MAD/Medea in *Drosophila*). Phosphorylated MAD (pMAD) then enters the nucleus, where it acts as a transcriptional cofactor to either activate gene expression (e.g., epidermal genes, including *dpp* and *zen*) or repress it (e.g., neural genes, including those of the *Achaete-Scute* complex or *msh*). Extracellular modulators of BMP signaling include Sog/Chd, which binds to Scw and inhibits peak BMP signaling mediated by the Dpp-Scw heterodimer. Tolloid is a metalloprotease that cleaves and inactivates Sog. Dpp is required as a cofactor of Tld in this cleavage reaction, both *in vitro* and *in vivo.* Tsg binds to Sog and Dpp and has been proposed to act in one of two possible ways. First, by forming a trimeric complex with Sog and Dpp-Scw heterodimers, as well as Dpp-Dpp homodimers, it broadens the BMP inhibitory range of Sog. The trimeric complex may also act as a carrier that protects BMPs from receptor-mediated endocytosis and thereby helps concentrate BMP heterodimers along the dorsal midline. The second role of Tsg is to alter the cleavage of Sog by Tld such that alternative, more broadly active forms of Sog (called Supersog) are formed. These truncated forms of Sog can bind directly to either Dpp-Dpp homodimers or Dpp-Scw heterodimers and inhibit their activity. *AS-C*, *achaete-scute* gene complex; BMP, bone morphogenetic protein; Dpp, Decapentaplegic (the ortholog of vertebrate BMP4/2); Gbb, Glass bottom boat; *msh*, *muscle specific homeobox;* Sax, Saxophone; Scw, Screw; Sog, Short gastrulation (the ortholog of vertebrate Chordin (Chd)); Tkv, Thick veins; Tld, Tolloid (the ortholog of vertebrate Xolloid (Xld)); Tsg, Twisted gastrulation; *zen*, *zerknüllt.*

dorsally restricted BMP expression is thought to lead to the formation of a BMP activity gradient, which is high dorsally and low ventrally. High levels of BMP signaling in the dorsal regions of the neural tube result in the expression of genes such as Msx1/2 and Pax7 in cells giving rise to migratory neural crest cells and sensory cells, whereas lower BMP levels result in the expression of lateral markers such as Gsh, Pax6, and Dbx1/2 in cells generating various interneurons (**Figure 4(a)**). It is not known whether BMPs act directly or indirectly to activate dorsal markers. In current models, BMPs are typically portrayed as having a direct positive role inducing gene expression, in part because a BMP-responsive enhancer region of the Msx1 gene has been shown to have binding sites for SMADs that are required for the activation of this cis-regulatory element. However, it is not clear that this element is responsible for Msx1 expression in dorsal cells of the neural tube because Msx1 is also expressed in ventral cells of the embryo during this same period.

In addition to the gradient of dorsally produced BMPs, the neural tube also receives ventral inductive cues provided by the Sonic Hedgehog (SHh) morphogen. As a consequence of the prior invagination of the mesoderm, cells derived from the Spemann organizer form a stiff longitudinal structure known as the notochord, which underlies the neural tube. These notochord cells secrete SHh and induce the neighboring ventral neural tube cells (called the floorplate) to acquire notochord-like properties, such as expression of the transcription factor HNF3β and SHh itself, which maintains its expression by a positive feedback mechanism (**Figure 4(a)**). Notochord cells also continue to express BMP inhibitors such as Noggin and Chd. SHh produced in the notochord and floorplate of the neural tube is distributed in a concentration gradient reciprocal to that of the BMP gradient (i.e., SHh is high ventrally and low dorsally). High levels of SHh result in the expression of ventral genes, such as Nkx2.2 and Nkx6.1, in cells that ultimately give rise to motor neurons, whereas lower levels of SHh lead to the expression of lateral markers.

In addition to organizing gene expression in the dorsal and ventral regions of the neural tube, BMPs and SHh also antagonize one another. For example, coexpression of BMP antagonists with limiting amounts of SHh greatly increases the ventralizing activity of SHh. Reciprocally, when BMPs are provided at levels

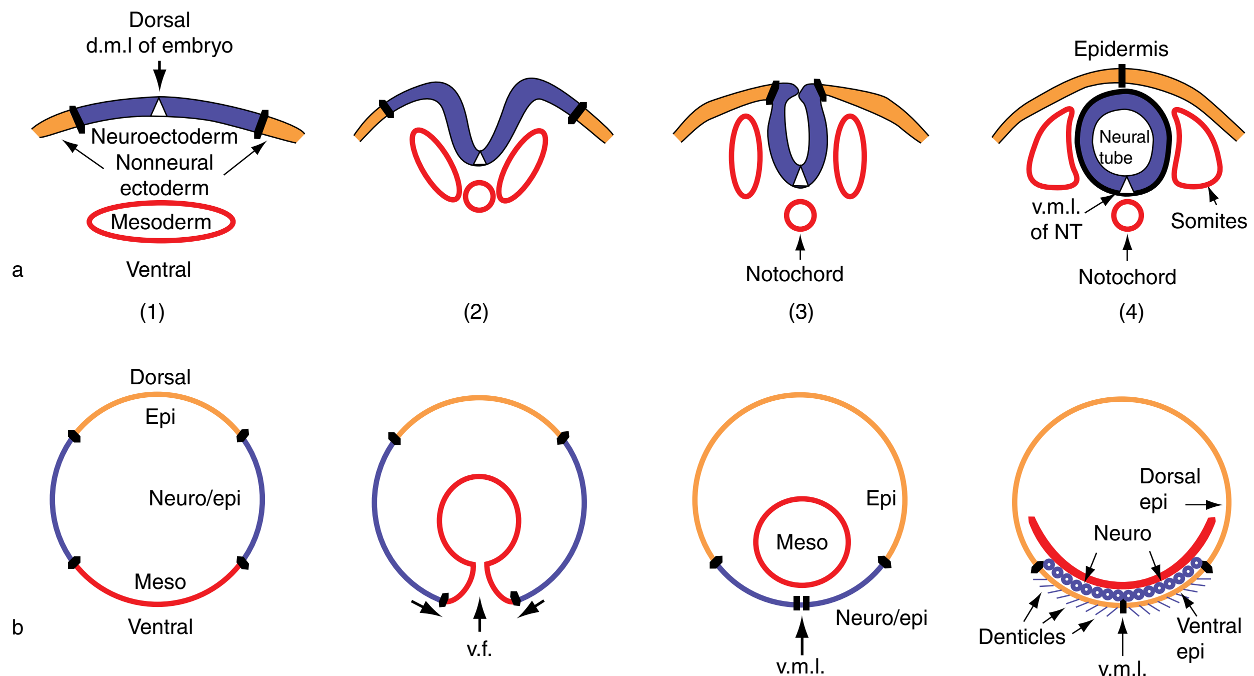

Figure 3 Early neural development in vertebrate and fly embryos, cross-sectional views of embryos: (a) neurulation in vertebrates; (b) delamination of neuroblasts in *Drosophila*. In (a), (1) is a cross section of the dorsal region of the embryo indicating the neuroectoderm (blue), or neural plate, which later invaginates along the dorsal midline. The adjacent more ventrally located nonneural ectodermal cells (orange) express high levels of BMPs and repress the expression of neural genes. Mesodermal cells (red) have already entered the interior of the embryo by this stage, by the process of involution, which begins dorsally at the blastopore and then expands ventrally. In (2) and (3), as invagination of the neural plate proceeds, its two ventral borders are brought into contact and the adjacent epithelial cells fuse into a single coherent domain as the neural ectoderm detaches to form the neural tube. In (4), The invagination of the neural plate leads to a reversal of dorsal-ventral (DV) polarity of the nervous system with respect to the primary embryonic DV axis because cells located originally at the dorsal midline of the embryo (white triangle) assume the most ventral position in the internalized neural tube. The ventral-most cells of the vertebrate neural tube, which are referred to as the floorplate, come into direct contact with a specialized mesodermal derivative known as the notochord, a rigid rodlike structure that provides support to the tadpole. Cells from the Spemann organizer give rise to the notochord. Flanking the neural tube laterally are the somites, a mesodermal tissue that gives rise to the adult bony skeleton and to muscle. In (b), (1) is a cross-section view of the early *Drosophila* embryo indicating the three germ layers along the DV axis. In (2) and (3), invagination of the mesoderm brings the left and right halves of the neural ectoderm into contact to form the ventral midline of the embryo and nervous system. In (4), neural precursor cells (neuroblasts) individually delaminate from the ectodermal epithelium and reside between the overlying epithelium and the more internal mesoderm. An important comparative point is that, due to the double inversion of the DV axis in vertebrates relative to flies, the final orientation ends up being the same. For example, the ventral midlines of vertebrates and flies are both formed by cells that were originally furthest from the source of BMPs in the nonneural ectoderm. BMP, bone morphogenetic protein; d.m.l., dorsal midline; NT, neural tube; v.f., ventral front; v.m.l., ventral midline. (See also **Figure 4**.) Adapted from Bier E (2000) *The Coiled Spring: How Life Begins*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

typical of the dorsal or lateral regions of the neural tube, they can override the induction of ventral markers by SHh.

Graded BMP-Mediated Repression of Neural Genes in *Drosophila*

Following their respective resident roles in consolidating cell fate choices within the epidermal and neural regions of the fly embryo during neural induction, Dpp and Sog play nonautonomous roles in the further subdivision of these two regions. Cells in the dorsal region of the embryo express uniform levels of *dpp* RNA and are initially equivalent because they are defined by the absence of the maternally derived Dorsal (Dl) morphogen. (Dl is a transcription factor related to mammalian nuclear factor (NF)-κB that sets up the initial DV polarity of the embryo. High levels of Dl ventrally specify the mesoderm, graded low levels of Dl define the neuroectoderm, and the absence of Dl in dorsal cells permits expression of *dpp*; see **Figure 4**(**b**).) Polarity in the dorsal region is created by Sog diffusing dorsally from the lateral neuroectoderm, where it is cleaved and inactivated by the Tld protease, which is co-expressed with Dpp in dorsal cells. The adjacent ventral source of Sog and dorsal Tld sink result in the formation of a Sog protein gradient in the dorsal region, which is high

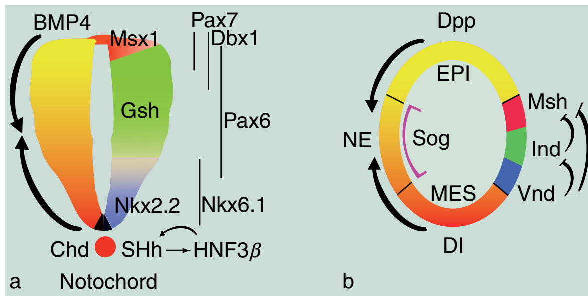

Figure 4 BMPs pattern the vertebrate and *Drosophila* neuroectoderm, cross-sectional diagrams: (a) the vertebrate neural tube, indicating the opposing gradients of BMPs and Sonic Hedgehog (SHh); (b) a blastoderm-stage *Drosophila* embryo, indicating the opposing Dpp and Dl morphogen gradients. In (a), high levels of BMPs dorsally result in the expression of the neural identity gene Msx1, and lower levels specify cells expressing lateral genes such as Gsh. High levels of SHh ventrally lead to the expression of the ventral neural identity gene Nkx2.2, whereas lower levels result in the expression of more lateral markers. The expression patterns of some other transcription factors along the dorsal-ventral (DV) axis are also indicated, including Pax7, Dbx1 (and, but not shown, Dbx2, which extends more ventrally than Dbx1), Pax6 (which also plays a conserved role in eye formation), and Nkx6.1. In (b), threshold-dependent repression mediated by the Dpp gradient helps pattern the *ind* and *msh* domains of expression, whereas concentration-dependent activation by the Dl gradient helps defining the *vnd/ind* border. An important mechanism contributing to the sharp, mutually exclusive neural domains is the ventral dominant cross-inhibition among the neural identity genes, wherein Vnd represses the expression of *ind* and *msh* and Ind inhibits expression of *msh*. Adapted from Mizutani CM, Meyer N, Roelink H, and Bier E (2006) Threshold-dependent BMP-mediated repression: A model for a conserved mechanism that patterns the neuroectoderm. *PLoS Biology* 4: e313 (online).

ventrally and low dorsally (**Figure 5(a)**). It has been proposed that this Sog gradient creates an inverse BMP activity gradient with peak levels in dorsal-most cells and lower levels in more ventral cells, which can be visualized by the *in situ* activation of the signal transducer phospho-MAD (pMAD). Sog may also carry Dpp dorsally and concentrate it along the dorsal midline. This BMP gradient results in the nested activation of a series of genes, including the transcription factors *zen*, *pannier*, and *ush*. The primary consequence of the graded activation of Dpp target genes is the subdivision of the dorsal region into two parts: a dorsal-most extra-embryonic domain (amnioserosa) and a more ventral epidermal domain. Note, however, that even the lower relative levels of BMP signaling present in the epidermal portion of the dorsal region are sufficient to repress the expression of all neural genes in those cells.

There is also evidence for a reciprocal influence of the dorsal ectoderm on patterning the lateral neuroectoderm mediated by Dpp diffusing ventrally (although diffusion of small amounts of Dpp ventrally remains to be demonstrated directly). Because Sog and a transcriptional repressor of BMP signaling known as Brinker (Brk) are expressed in the neuroectoderm, the levels of BMP signaling in neuroectodermal cells would be expected to be much lower than those in the dorsal region, where Dpp is expressed and only low levels of graded Sog are present. As a consequence of Dpp being present in limiting amounts within the neuroectoderm, its ability to repress neural gene expression becomes dosage dependent. This dosage-sensitive repression has been most conclusively studied with regard to the expression of the neural identity genes *vnd*, *ind*, and *msh*, which are required to specify the fates of the three primary rows of neuroblasts in the embryonic central nervous system (CNS) (**Figures 4(b)** and **5(b)**). *vnd*, the homolog of vertebrate *Nkx*2.2, is expressed in the ventral-most row of the neuroblasts; *ind*, the homolog of *Gsh*, is expressed in the middle row of neuroblasts; and *msh*, the homolog of Msx1/2, is expressed in the dorsal row of neuroblasts. The fact that orthologous sets of neural identity genes are expressed in the same relative ventral-to-dorsal order with regard to BMP-expressing cells in vertebrates and flies suggests that this configuration reflects an ancestral state that has been conserved during evolution (**Figure 4**). (Note that, despite the fact that the primary DV axes are inverted in vertebrates and *Drosophila* embryos, the final relative order of neural identity genes ends up being the same as a consequence of the neural DV pattern being reversed with respect to the remainder of the embryo following invagination of the neural plate. Such a secondary reversal does not take place in *Drosophila,* in which neuroblasts delaminate isotopically from the epidermis to form a subepithelial layer, as indicated in **Figure 3(b)**.)

An important regulatory feature of neural identity genes in *Drosophila* is that they cross-inhibit one another in a ventral-dominant fashion in which Vnd represses expression of both *ind* and *msh*, and Ind inhibits the expression of *msh* (**Figure 4(b)**). As Dpp diffuses ventrally, it represses expression of the intermediate neural identity gene *ind* more effectively than *msh*. This results in *ind,* but not *msh,* being repressed by BMP signaling in dorsal cells of the neuroectoderm, which are closest to the Dpp source. BMP-mediated repression of *ind* expression, in turn, relieves ventral-dominant repression of *msh* by Ind, resulting in *msh* expression in the dorsal-most domain of the neuroectoderm. Thus, as a consequence of the cross-inhibitory interactions among neural identity genes, sharp boundaries of neural gene expression domains are established in response to graded Dpp signaling along the neuroectoderm. Similar cross-regulation of neural identity genes has also been

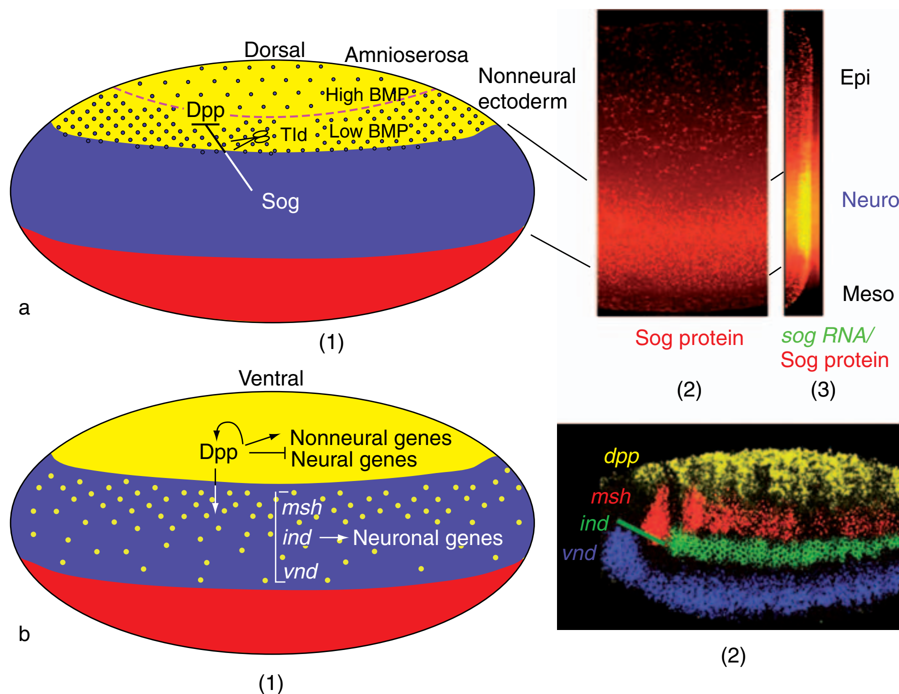

Figure 5 BMP-mediated patterning of the fly neuroectoderm: (a) reciprocal BMP gradient created by diffusion of Sog into dorsal regions and its cleavage by Tld protease in the dorsal-most cells; (b) ventral diffusion of Dpp from the dorsal ectoderm into the lateral neuroectoderm. In (a), double staining of Sog RNA and Sog protein reveals that Sog protein can be detected further dorsally forming a gradient, beyond its domain of expression within the lateral neuroectoderm ((1) and (2) show a lateral view and (3) shows a transversal view of the embryo). In (b) the ventral diffusion of Dpp forms a concentration gradient to pattern the expression domains of neural identity genes *msh* (red), *ind* (green), and *vnd* (blue) mRNA. BMP, bone morphogenetic protein; Dpp, Decapentaplegic; Sog, Short gastrulation; Tld, Tolloid. (a), (1) and (2), from Srinivasan S, Rashka KE, and Bier E (2002) Creation of a Sog morphogen gradient in the *Drosophila* embryo. *Developmental Cell* 2: 91–101. (b), (2), From Kosman D, Mizutani CM, Lemons D, Cox WG, McGinnis W, and Bier E (2004) Multiplex detection of RNA expression in Drosophila embryos. *Science* 305: 846.

observed in vertebrates, athough it remains to be determined whether they follow a ventral-dominant pattern as has been shown in *Drosophila*.

In ventral regions, it appears that the primary system involved in patterning the neuroectoderm is the opposing ventral-to-dorsal Dl gradient, which is provided maternally as previously described. Moderate levels of Dl in ventral cells activate *vnd*, whereas lower levels activate *ind*. Because Vnd represses the expression of *ind* and *msh*, the graded action of Dl results in *vnd* being expressed exclusively in ventral-most cells of the neuroectoderm and *ind* expression in the adjacent intermediate domain where the levels of Dl are too low to activate *vnd*. Although Dpp signaling can also repress the expression of *vnd* and can regulate the dorsal borders of all three neural identity genes, the border between the *vnd* and *ind* domains is established primarily by graded activation of these genes by Dl, whereas the border between *ind* and *msh* is determined primarily by the threshold-dependent repression of these genes by Dpp signaling emanating from dorsal epidermal cells.

Neural Patterning in Other Groups of Organisms

Primary insights into the mechanisms of neural induction have been provided by classical model systems such as flies, frogs, zebra fish, and mice, however, it is important to complement these studies with analyses of organisms from other phylogenetic groups. Such evo-devo studies provide two important types of information. First, cross-genome comparisons have revealed a striking degree of gene loss during the evolution of lineages that include the model systems *Drosophila* and *Caenorhabditis elegans*. Thus, finding a vertebrate gene not present in flies or other insects does not necessarily imply that the gene evolved within the vertebrate lineage following its divergence from invertebrates but, rather, that it may simply have been lost in the insect lineage. Second, one of the most interesting features of evolution is the appearance of novel structures within specific lineages which can only be understood through comparative studies using diverse organisms.

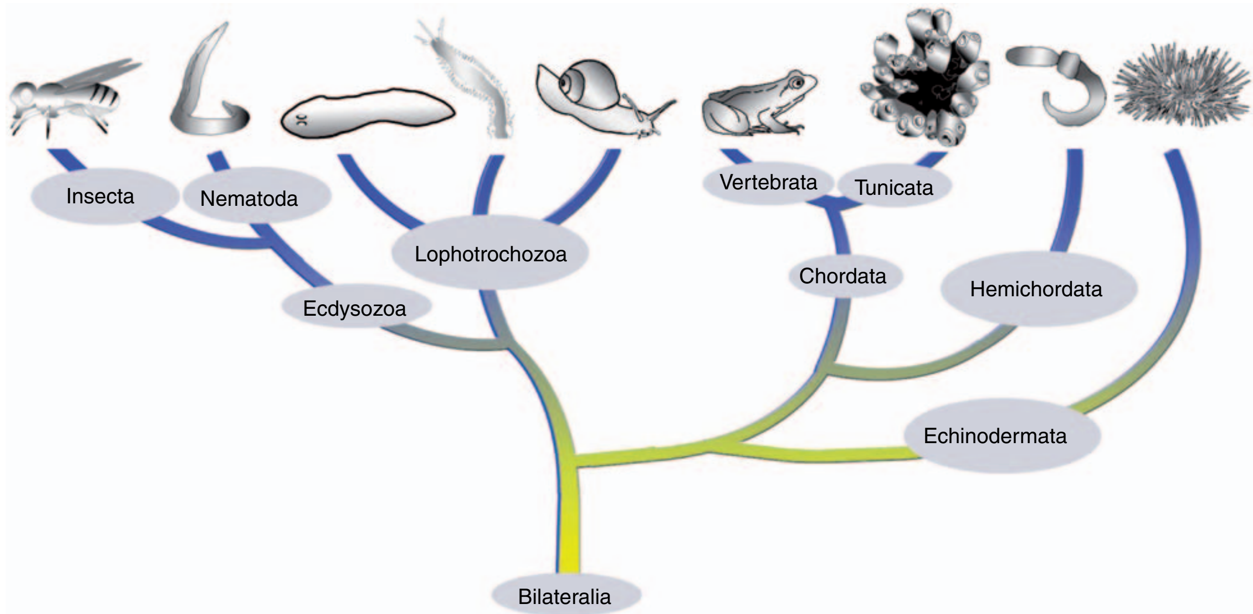

Figure 6 Simplified phylogeny of the bilateralia. Evolutionary tree indicating the relationships of the three major groups of bilateral animals. This tree is based on cladistic analysis of morphological characters and on 18S ribosomal RNA sequence-divergence data. Because of the rapid diversification of animal forms during and preceding the Cambrian radiation, the relationships at the base of the tree are not certain. The current view depicted in this tree is that the bilateralia consist of three major groups: (1) chordates (which include vertebrates and tunicates), hemichordates, and echinoderms; (2) the ecdysozoa, which includes arthropods such as insects and nematode worms such as *C. elegans*, and (3) the lophotrochozoa, which includes mollusks, flatworms, and annelid worms. The great majority of developmental studies have focused on the first two branches of the tree (chordates and ecdysozoa); there has been much less analysis of lophotrochozoan development. Further comparisons of developmental strategies and genetic pathways among these three groups will provide a much improved view of the common ancestor of the bilateralia.

Current phylogenies tentatively group metazoa in one of three major groups: deuterostomes, which include chordates (vertebrates and ascidians), hemichordates, and echinoderms, ecdysozoa (which include arthropods such as *Drosophila* and nematodes such as *C. elegans*), and lophotrochozoa (which include flatworms, annelid worms, mollusks, and other shell-enclosed organisms) (**Figure 6**). Conspicuously missing among the model organisms, which have been used to define developmental paradigms, are those in the large diverse group of lophotrochozoa. Studies from additional members of the ecdysozoa and chordate lineages would also provide more generality to our current views of development and should shed light on which features are truly conserved versus which independently evolved in different lineages. We briefly summarize some current evo-devo studies in other organisms that bear on the mechanistic origins of neural induction and patterning. It is important to bear in mind, however, that each of these species is also likely to have lost genes that were present in the common ancestor of bilateral animals.

In spider embryos, the DV axis is established in a very different way than in *Drosophila* or vertebrates. A small group of Dpp-expressing mesodermal cells migrates under the epidermis, leaving a linear track of overlying epidermal cells in which BMP signaling persists and which ultimately forms the dorsal midline. The spider *sog* gene is expressed in the ventral ectoderm which gives rise to the nervous system, as in other arthropods. *sog* function is required for ventral cell-fate specification, including the nervous system, because the reduction of *sog* activity by RNA interference (RNAi) results in the spread of high BMP signaling into ventral cells and the subsequent loss of ventral structures. The invasion of BMP signaling into the neuroectoderm of *sog* RNAi spiders and its suppression of neuroectodermal fates parallel the role of BMP signaling in *Drosophila* and vertebrates. Despite the difference in how BMP signaling is established in the spider embryo, the way it is employed supports the view that an ancestral role of neural inducers was to prevent BMP from spreading into the neuroectoderm and suppressing neurogenesis.

Hemichordates, which are thought to be most closely related to echinoderms, include marine worms and other sessile marine organisms that retain only a moderate degree of DV organization as a consequence of their nearly rotationally symmetric body plans. Early during development, BMP4 and Chd/Sog are expressed in opposing domains and define a DV axis in hemichordate embryos consisting of three germ

layers and distinct domains of gene expression, except for the nervous system. Unlike vertebrates and arthropods, the nervous system in hemichordates is not condensed into either dorsal or ventral ganglia but, rather, consists of dispersed neurons which send their axons into one of two major axonal bundles, one running dorsally and the other ventrally. Because neurons form around the entire circumference of these embryos, BMP signaling does not inhibit the formation of neurons in the dorsal region, nor does BMP overexpression inhibit neuron formation elsewhere. In addition, although hemichordates have recognizable counterparts of at least *vnd/Nkx2.2* and *msh/Msx*, the homologs examined so far do not display any obvious restriction in their expression along the DV axis. One possible explanation for these observations is that the neural repressive function of BMPs in vertebrates, arthropods, and spiders arose following the separation of these lineages and that the original function of BMP signaling in bilateral ancestors may have been to establish DV polarity. Alternatively, the neural suppressive function of BMP signaling may have been lost during the course of hemichordate evolution as specialization along the DV axis became greatly simplified and the animals assumed a nearly rotationally symmetric body plan. This latter view accounts for the common pattern of neural identity gene expression in vertebrates and arthropods, as well as its potential common dosage-sensitive regulation by BMP signaling. Future experiments should resolve this question, particularly by examining the expression of neural identity genes in other chordate branches and in various lineages of the lophotrochozoa, the third major branch of the metazoan evolutionary tree. When compared to the other groups, the lophotrochozoa appear to be one of the slowest evolving groups, having lost far fewer genes present in the common bilateral ancestor and typically having ventral nerve cords similar to those in arthropods, although primitive flat worms (platyhelminths) have either diffuse nervous systems or only anterior nerve nets.

In sum, the current knowledge of neural induction in diverse bilateral embryos suggests that the role of BMPs in neural induction reflects the conservation of a mechanism that evolved from a common bilateral ancestor, although it is formally possible that this may have arisen independently in several different lineages. Clearly there are species-specific aspects that have been described, but it is not clear whether this is evidence against a common origin rather than an indication that the mechanism has been lost or highly modified in various lineages. Further analysis of additional groups should resolve these issues.

A second important evolutionary question is whether the role of BMPs in patterning neural identity also originated in a common ancestor. Indeed, because vertebrates and flies share a common set of neural identity genes expressed in the same relative order with regard to a source of BMPs and because BMPs play a prominent role in patterning the dorsal region of the nervous system in both flies and vertebrates, it seems likely that neural patterning by the BMPs was a common feature of the bilateral ancestor. Clearly, other species-specific signaling pathways are also important in DV patterning of the nervous system; for example, primary morphogens involved in ventral neural patterning appear to be different in flies (i.e., Dl) and vertebrates (i.e., Hedgehog (Hh)). Nonetheless, it is tempting to speculate that BMPs once were sufficient to pattern the entire neural DV axis. According to this hypothesis, additional signaling systems were then added to buttress patterning at the low end of the BMP gradient during the divergence of the vertebrate and invertebrate lineages. Consistent with this view are experiments on DV patterning of the mouse spinal cord. When the function of the Hh signaling pathway is completely abolished (i.e., by removing both SHh and the default repressor of the Hh pathway, known as Gli3), much of the ventral pattern is restored relative to what is lost in *SHh*-single mutants. In addition, under conditions of low-level Hh signaling, the gene-expression profile in neural-plate explants can be adjusted to ventral, lateral, or dorsal levels by adding increasing doses of BMPs, indicating again that BMPs alone are able to pattern the full DV span of neural cell fates. Thus, in the early bilateral ancestors, which are believed to have been very small (less than 2-mm long), a single BMP morphogen gradient may have been sufficient to create a pattern along the entire DV axis.

DV Inversion in Vertebrates?

The fact that DV polarity of the nervous system and the circulatory system appears to be reversed in vertebrates relative to invertebrates was noted by the renowned French comparative anatomist Geoffroy St.-Hilaire, who proposed that vertebrates were essentially upside-down invertebrates (**Figure 7(a–c)**). The patterns of gene expression in vertebrates and invertebrates summarized here have led many modern evo-devo enthusiasts to support Geoffroy St.-Hilaire's hypothesis. One possible exception to the axis inversion model, however, is the head region. Comparison of gene-expression markers for eyes such as *Pax6/eyeless* (which are thought to have played an ancestral role in specifying some properties of light-sensitive organs in metazoa), as well as genes expressed in the vertebrate hypothalamus and a potentially homologous neuroendocrine organ in

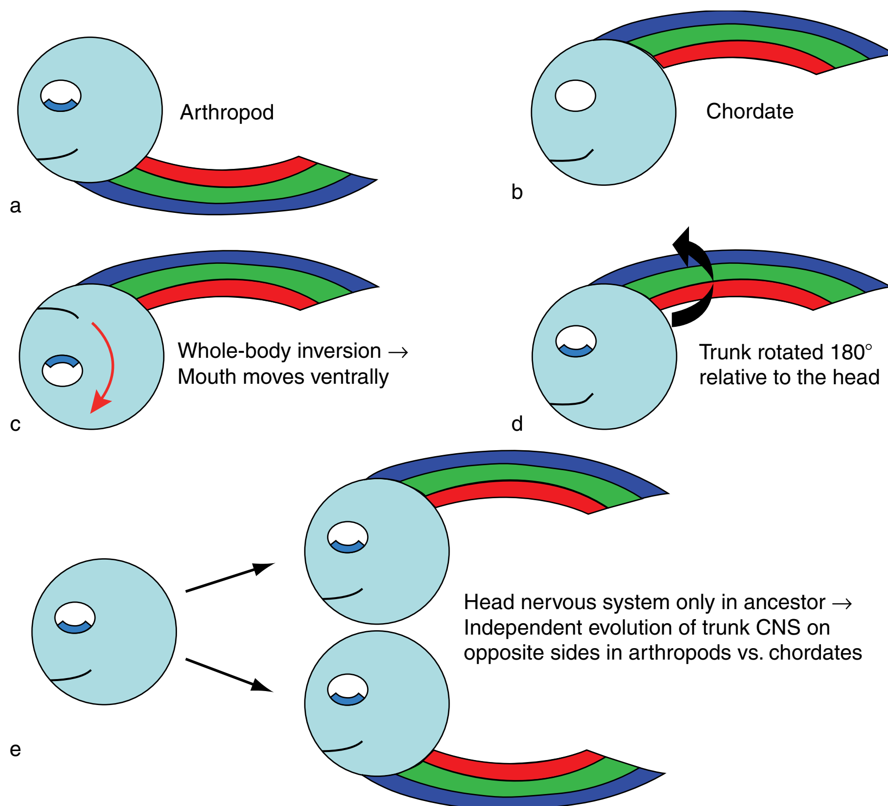

Figure 7 Possible origins of dorsal–ventral (DV) axis inversion in vertebrates: (a) invertebrate DV axis; (b) vertebrate DV axis; (c) Geoffroy St.-Hilaire model; (d) and (e), alternative models. In the early nineteenth century, Geoffroy St.-Hilaire proposed that the DV axis of vertebrates was inverted with respect to that of invertebrates, based on the opposite positions of the nervous system and heart (dorsal in flies pumping anteriorly vs. ventral in vertebrates pumping posteriorly). Several scenarios have been proposed to account for the apparent axis inversion in light of recently obtained molecular data. In the original Geoffroy St.-Hilaire model (c), the entire DV axis was inverted, followed by a ventral migration of the mouth orifice. Alternatively (d), only the trunk region was rotated by 180° with respect to the head, followed by migration of the mouth opening as well. An attractive feature of this hypothesis is that it also explains why the left and right sides of the vertebrate sensory nervous system map primarily to the opposite side of the brain. Another possibility (e) is that the last common bilateral ancestor had only a condensed anterior nervous system (or brain) and that the condensed central nervous system (CNS) trunk later evolved separately (and with opposite DV polarity). This hypothesis does not account for the similar BMP-mediated mechanisms for establishing the conserved neural patterning along the DV axis.

Drosophila, suggests that the order of DV patterning in the brain might be the same in flies and invertebrates. Thus, the relative DV patterns in the head and trunk appear to be opposite. One explanation for the apparent differences in head and trunk patterning is that the anterior brain may have evolved first from an anterior net of cells and then condensed trunk nervous systems developed later and in opposite DV orientations following the split of vertebrates and arthropods (**Figure** 7(**e**)). One argument against this model is the shared DV pattern of neural identity gene expression and the dosage-sensitive regulation of these genes by BMPs, which seems difficult to imagine having evolved twice by chance. Another possible explanation is that the inversion of neural pattern was confined to the trunk and that the body was rotated by 180° with respect to the head, which remained in a fixed DV orientation (**Figure** 7(**d**)). This hypothesis could also offer a potential explanation for an otherwise puzzling feature of the vertebrate nervous system – that the primary sensory axonal projections cross from left to right (or decussate, in the jargon). In other words, the right hand maps primarily to the left sensory cortex, as does the right eye to the left visual cortex. There is no evidence for an analogous primary cross-representation in invertebrates. For example, eyes project primarily ipsolaterally in all invertebrates examined. It is also possible that apparent differences between the head and trunk reflect a sampling bias and that further analysis of additional conserved gene sets expressed in the head will support the original Geoffroy St.-Hilaire model for the full-body axis inversion. One interesting testable prediction of the head–trunk rotation model is that genes expressed along the entire anterior–posterior (AP) axis of the nervous system in a restricted DV pattern in arthropods might have opposite split DV expression domains in the head versus trunk regions of vertebrates.

Conclusion

BMPs play a similar all-or-none role in repressing the expression of neural genes in epidermal regions in vertebrates and arthropods and then play a dosage-sensitive role in establishing a conserved pattern of neural identity gene expression during early patterning of the neuroectoderm. An important question to resolve is whether BMPs function in vertebrates as they do in flies, by using threshold-dependent repression of neural identity genes in conjunction with ventral-dominant cross-inhibition among neural identity genes. Analysis of the role on BMPs in patterning the nervous systems of other organisms will provide additional information for reconstructing the elements of neural induction present in the common bilateral ancestor. Such broadened evo-devo studies will also reveal how evolutionary novelties arise in specific lineages to give rise to the rich array of neural development and function in diverse organisms.

See also: Neural Induction in Chicks.

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Retinoic Acid Signaling and Neural Patterning

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Retinoic Acid Signaling Plays a Role in Patterning of the Posterior Central Nervous System

During gastrulation, the rostral–caudal axis of the mammalian central nervous system (CNS) is progressively laid down in an anterior to posterior fashion as primitive ectoderm in the epiblast differentiates into neuroectoderm. Various signaling pathways direct specific regions of the neuroectoderm to adopt forebrain, midbrain, hindbrain, or spinal cord fates. One of these signals is retinoic acid (RA), a lipid derived from metabolism of vitamin A (retinol). RA is not synthesized during early gastrulation, when the forebrain and midbrain fields are established (embryonic day E6.5–E7.5 in mouse embryos). However, at E7.5, RA synthesis begins specifically in the posterior paraxial mesoderm (presomitic mesoderm). From E7.5 to E8.5, mesoderm provides RA in a paracrine fashion to nearby neuroectoderm fated to become hindbrain and spinal cord, but RA does not reach the forebrain or midbrain. RA regulation of gene expression in the hindbrain helps define its patterning along the anteroposterior axis into discrete units called rhombomeres. In addition, RA control of gene expression in spinal cord neuroectoderm is required for dorsoventral patterning events leading to formation of motor neurons ventrally. Thus, RA is required for patterning of the posterior CNS (hindbrain and spinal cord), but not the anterior CNS (forebrain and midbrain).

Spatiotemporal Aspects of RA Signaling in Early Mouse Embryos

The establishment of when and where RA signaling occurs in mouse embryos has been instrumental in defining the role of RA in neural patterning. One approach has been to establish the identity and expression patterns of RA receptors and enzymes that may synthesize RA. RA serves as a ligand for three nuclear RA receptors (RARs) that bind DNA as heterodimers with retinoid X receptors (RXR). Under physiological conditions (normal dietary sources of vitamin A), RA is found only in the form known as all-*trans*-RA, which binds to RAR but not RXR, but under pharmacological conditions (vitamin A excess), the isomer 9-*cis*-RA is formed and can bind to both RAR and RXR. However, several studies have demonstrated that physiological RA signaling requires only binding of all-*trans*-RA to the RAR component of RAR/RXR heterodimers. During early stages of mouse development, expression of RARα and RARγ is widespread throughout the developing CNS, but RARβ expression is limited to hindbrain and spinal cord. Thus, RA signaling could potentially occur throughout the CNS because of the presence of RARα and RARγ. However, the location of RA signaling will also depend on the location of RA synthesis, which is not as widespread as RAR expression.

RA synthesis is a two-step process in which retinol is reversibly oxidized to retinaldehyde, followed by irreversible oxidation of retinaldehyde to RA. Several members of the alcohol dehydrogenase and short-chain dehydrogenase/reductase enzyme families catalyze oxidation of retinol to retinaldehyde in many tissues in an overlapping fashion such that it is essentially ubiquitous. In contrast, oxidation of retinaldehyde to RA is carried out by three nonoverlapping, tissue-specific retinaldehyde dehydrogenases encoded by *Raldh1*, *Raldh2*, and *Raldh3* (*Aldh1a1*, *Aldh1a2*, and *Aldh1a3* in the Mouse Genome Informatics database). These three *Raldh* genes are conserved in human, mouse, and several lower vertebrates. It has been demonstrated that *Raldh* genes determine where RA signaling occurs as mouse embryos carrying the *RARE–lacZ* RA-reporter transgene plus a null mutation of *Raldh1*, *Raldh2*, or *Raldh3* selectively lose *RARE–lacZ* expression in the location where that *Raldh* gene is normally expressed. *RARE–lacZ* expression is first observed at E7.5 in the posterior mesoderm (where *Raldh2* is expressed), as well as the adjacent posterior neuroectoderm and endoderm. As *Raldh1* and *Raldh3* are not expressed until E8.5 and E9.5, respectively, only *Raldh2* is involved in generating RA for early CNS patterning. Also, studies of null mutant embryos indicate that *Raldh1* and *Raldh3* generate RA for eye morphogenetic movements but not for CNS patterning.

Raldh2 is the only source of RA for CNS patterning, but not the only determinant of where RA signaling can occur. Selective RA degradation by P450 enzymes encoded by the *Cyp26* family has been found to limit how far anteriorly and posteriorly RA can travel from its site of synthesis by *Raldh2* in the posterior mesoderm (presomitic and somitic). Thus, RA signaling does not occur throughout the CNS during early neural patterning but only in the hindbrain and spinal cord (**Figure 1**).

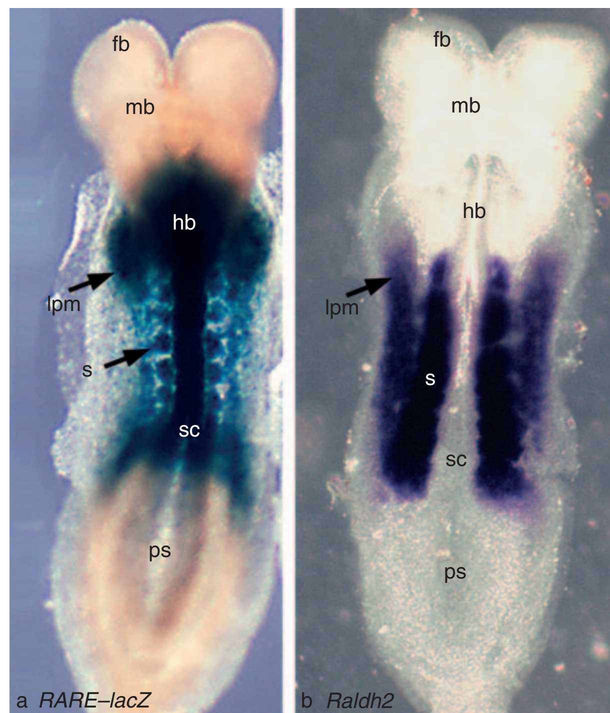

Figure 1 Retinoic acid (RA) signaling in early mouse embryos. (a) The *RARE–lacZ* RA-reporter transgene demonstrates that RA signaling activity in the central nervous system (CNS) at the six-somite stage is limited to the posterior hindbrain and spinal cord; no activity is observed in the forebrain or midbrain. (b) The only source of RA for the CNS at the six-somite stage is *Raldh2* expressed in the somitic and lateral plate mesoderm; *Raldh2* functions in a paracrine fashion by synthesizing RA in mesoderm which diffuses to the hindbrain and spinal cord neuroectoderm. fb, forebrain; hb, hindbrain; lpm, lateral plate mesoderm; mb, midbrain; ps, primitive streak; s, somite; sc, spinal cord.

RA Is Not Required for Neural Induction

In amniote vertebrate embryos, the primitive ectoderm (epiblast) consists of a pluripotent embryonic stem cell population which differentiates during primitive streak formation (gastrulation) to produce the three primary germ layers (embryonic ectoderm, mesoderm, and endoderm). As development proceeds, the primitive streak stem cell zone regresses posteriorly, and ectodermal cells emerging from the primitive streak differentiate into either neuroectoderm or epidermis in the process of neural induction. Neural induction has been found to require bone morphogenetic protein (BMP) antagonists produced in underlying mesoderm generated in the primitive streak as well as fibroblast growth factor (FGF) produced in the primitive streak. As the body axis extends, anterior neuroectoderm (forebrain/midbrain) becomes differentiated from posterior neuroectoderm (hindbrain/spinal cord) at an early stage. Posterior expansion of the hindbrain and loss of the forebrain on administration of exogenous RA to amphibian, avian, and mammalian embryos suggested that RA may be a factor normally needed to define the posterior CNS. However, as RA treatment of embryonic stem cells stimulates differentiation to neuroectoderm, it was unclear for some time whether RA is normally needed for neural induction. As ectoderm emerges from the primitive streak, *Sox1* and *Sox2*, encoding high-mobility group transcription factors, are expressed in ectoderm that has undergone neural induction to form neuroectoderm; expression occurs all along the CNS, from the forebrain to the spinal cord. *Sox1* and *Sox2* expression differentiates neuroectoderm from nonneural ectoderm, which will form epidermis. Studies on *Raldh2*$^{-/-}$ embryos has demonstrated that expression of *Sox1* and *Sox2* is not changed in the absence of RA signaling. Thus, although exogenous RA treatment has been reported to increase *Sox1* expression in mouse embryonic stem cells, endogenous RA is not required for normal induction of *Sox1* in the mouse embryo neural plate. These findings indicate that RA is not required to generate neuroectoderm. RA does, however, play a role in patterning the neuroectoderm as discussed in the section titled 'Role of RA in spinal cord dorsoventral patterning.'

Role of RA in Hindbrain Anteroposterior Patterning

During early CNS development, the hindbrain is patterned along the anteroposterior axis into segments known as rhombomeres. Studies on vitamin A-deficient quail embryos reported that a loss of the precursor for RA results in loss of rhombomeres 4–8 (r4–r8) and that the remaining hindbrain consisted of an enlarged r1–r3 joined to the anterior spinal cord. A similar phenotype was also observed in *Raldh2*$^{-/-}$ mouse embryos, vitamin A deficient rat embryos, and chick embryos treated with an RA receptor antagonist. Markers of r4 were also lost, including expression of *Hoxb1* encoding a homeobox gene and *Cyp26c1* encoding an RA-degrading enzyme.

Neural expression of *Hox* genes is limited to the posterior CNS, where these genes play essential roles in rhombomere formation. Several members of the vertebrate *Hox* gene family are direct targets of RA signaling during hindbrain development, including *Hoxb1*, whose anterior-most expression domain is in r4, located in the middle of the hindbrain. The mouse *Hoxb1* gene is regulated by an RA response element (RARE) located 3′ to the promoter that is required for early widespread induction in the posterior hindbrain up to the presumptive r3–r4 boundary, as well as another RARE located 5′ to the promoter

that has been demonstrated to be required for repression of *Hoxb1* in r3 and r5 to provide restricted expression in r4. However, *Hoxb1* r4 expression is also repressed by the homeodomain protein encoded by *vHnf1* (*Tcf2*), which is expressed in the spinal cord and posterior hindbrain up to the r4–r5 boundary. It is interesting that *vHnf1* is also inducible by RA. Studies using the *RARE–lacZ* RA reporter transgene have shown that rhombomere 3 is the anterior-most location of RA signaling during early CNS patterning but that RA activity is very transient in r3 and r4, lasting only a few hours. Transient RA activity in r3 and r4 has been found to be due to expression of RA-degrading P450s in the hindbrain. Three RA-degrading P450s encoded by *Cyp26a1*, *Cyp26b1*, and *Cyp26c1* are expressed in dynamic patterns during hindbrain development. It is now clear that the RA signal initially travels from the presomitic mesoderm (where *Raldh2* is expressed) to r3, forming a boundary next to the r2 expression domain of *Cyp26a1*. After *Hoxb1* induction, the RA boundary quickly shifts to r4–r5, coincident with induction of *Cyp26c1* in r4. Thus, during a brief period in development, RA is present in r4 to induce *Hoxb1*. Analysis of *Raldh2*$^{-/-}$ embryos has provided support for a direct role of endogenous RA signaling in *Hoxb1* induction up to r4 and repression in r3–r5 through earlier described 3′ and 5′ RAREs. Also, *Raldh2*$^{-/-}$ embryos have demonstrated that endogenous RA induces *Cyp26c1* in r4 as well as *vHnf1* posterior to the r4–r5 boundary. As *vHnf1*$^{-/-}$ embryos exhibit ectopic expression of *Hoxb1* in r5, this has provided evidence for an indirect role of RA in *Hoxb1* repression via RA induction of *vHnf1*.

It is important to note that these studies have demonstrated the existence of shifting RA boundaries in the hindbrain, as opposed to an RA gradient. *Raldh2* and *Cyp26* generate at least two distinct boundaries of RA activity along the anteroposterior axis of the hindbrain, first at r2–r3 and then at r4–r5, such that r3–r4 receives a short pulse of RA and r5–r8 receives a long pulse of RA. These two pulses of RA activity provide patterning by establishing expression of *Hoxb1* and *vHnf1* on opposite sides of the r4–r5 boundary.

Role of RA in Spinal Cord Dorsoventral Patterning

RA is required for early dorsoventral patterning of the spinal cord to generate motor neuron progenitors and later for specifying motor neuron subtype identity. RA also plays a role in assignment of positional identity of motor neurons along the anteroposterior axis of the spinal cord to generate unique neurons that innervate forelimbs, trunk, and hindlimbs. Anteroposterior effects of RA along the spinal cord are mediated primarily by regulation of *Hox* genes, similar to the role of RA in the hindbrain. In contrast, dorsoventral patterning of the spinal cord involves several different transcription factors, including the homeobox transcription factors *Pax6* and *Nkx6.1*, which are expressed dorsally and ventrally, respectively. In the region where overlapping expression of *Pax6* and *Nkx6.1* occurs (future ventral horns), the basic helix–loop–helix transcription factor *Olig2* is expressed. Genetic studies in the mouse have demonstrated that *Olig2* is required for spinal cord motor neuron differentiation. Secreted signals important for establishing spinal cord dorsoventral patterning include *Shh* expressed ventrally, *Bmp* expressed dorsally, and RA synthesized in the adjacent somitic mesoderm. A lack of RA synthesis in *Raldh2*$^{-/-}$ mouse embryos results in a loss of *Pax6* and *Olig2* expression in the spinal cord, whereas *Nkx6.1* expression remains, suggesting that *Nkx6.1* does not require RA for expression. This requirement for RA during spinal cord patterning is conserved between mammalian and avian embryos, and studies on avian embryos suggest that *Pax6* and *Olig2* are both direct targets of RA action. RA is thus needed as an additional trigger, along with *Pax6* and *Nkx6.1*, to allow induction of *Olig2*, which then stimulates undifferentiated spinal cord neuroectoderm to acquire a ventral motor neuron cell fate.

Early studies in avian embryos suggested that *Raldh2* may be responsible for synthesizing RA needed for spinal cord motor neuron differentiation as *Raldh2* is expressed in somitic mesoderm adjacent to the neural tube. Genetic studies have now shown that *Raldh2* is responsible for all RA detected posteriorly in mouse embryos at E8.5 and that *Raldh2* is necessary for *Olig2* expression in the spinal cord. The extent of RA distribution from sites of *Raldh2* expression during spinal cord development has been examined in mouse embryos. *RARE–lacZ* RA–reporter expression is observed in trunk mesoderm and throughout the dorsoventral axis of the spinal cord, demonstrating that RA synthesized by *Raldh2* in the somites can travel to all portions of the spinal cord. These findings provide evidence that RA functions exclusively in a paracrine fashion during early spinal cord patterning. As RA is not localized to any particular region of the spinal cord, this may explain its ability to induce not only *Olig2*, which is limited to the developing motor neuron domain located ventrally, but also *Pax6*, which is expressed more widely across the dorsoventral axis of the neural tube.

Development of *Raldh2*$^{-/-}$ embryos can be substantially rescued by a low-dose maternal dietary RA

supplement that returns embryonic RA levels to approximately the same level observed in wild-type embryos. Such treatment can rescue *Olig2* expression in *Raldh2*$^{-/-}$ embryos. It is interesting that *RARE–lacZ* expression in rescued *Raldh2*$^{-/-}$ embryos occurs throughout the spinal cord and posterior hindbrain, similar to wild-type, but unlike wild-type, there was no detection of *RARE–lacZ* in the somitic mesoderm, which is normally the source of RA. This phenomenon is clearly dose dependent as large doses of exogenous RA will induce *RARE–lacZ* in all cells of the embryo. These findings suggest that the low dose of exogenous RA entering the embryo was not distributed evenly. RA may be preferentially sequestered in the posterior CNS as it is known that cellular RA binding protein II (*Crabp2*) is expressed highly in the spinal cord and hindbrain but not in mesoderm. As the normal site of RA synthesis in the somitic mesoderm did not exhibit RA signaling activity in *Raldh2*$^{-/-}$ embryos under these rescue conditions, it may be that RA does not need to perform an additional function in the mesoderm during spinal cord dorsoventral patterning; the somitic mesoderm is simply acting as a source of RA. The rationale for such RA signaling may be to coordinate motor neuron differentiation with mesodermal differentiation along the anteroposterior axis as cells emerge from the primitive streak.

Many of the above observations were made possible through the use of mouse embryos carrying the *RARE–lacZ* transgene, which enables one to follow where endogenous RA signaling activity occurs in wild-type embryos and where RA signaling occurs in *Raldh2*$^{-/-}$ embryos rescued by maternal RA treatment. Such experiments have not been possible in avian, amphibian, or fish embryos because of lack of a transgene that can serve as an RA reporter during the early stages of neural development. This highlights the importance of the mouse in studies designed to understand the mechanism of RA action in the CNS.

RA Antagonism of *Fgf8* Expression in the Primitive Streak Controls Posterior Patterning

FGF signaling controlled by *Fgf8* is particularly important in the mouse as it is necessary for neural induction in the posterior CNS as well as generation of mesoderm during gastrulation. Early studies suggested that presomitic mesoderm emerging from the primitive streak provides another signal needed for differentiation of the posterior CNS, that is, a signal that antagonizes posterior FGF signaling mediated by *Fgf8*. Recent studies in avian, mouse, and zebra fish embryos indicate that RA generated by *Raldh2* is the presomitic mesodermal factor that antagonizes *Fgf8* action. The concept of embryonic tissue differentiation occurring in regions of opposing RA and FGF signals was originally proposed in studies of proximodistal outgrowth of chick limb buds where *Raldh2* and *Fgf8* also function to generate opposing RA and FGF signals.

Fgf8 is normally expressed in the primitive streak, but its expression is extinguished anteriorly as cells exit the streak. Studies in avian, mouse, and zebra fish embryos indicate that RA functions as an antagonist of posterior *Fgf8* expression. In particular, RA functions to limit the anteroposterior extent of *Fgf8* expression and confines it to the primitive streak or tailbud. Whether this reflects a direct effect of RA on the *Fgf8* gene remains to be determined, but a conserved RARE is found in the promoter regions of human, mouse, and rat *Fgf8* genes. This function is consistent with the observation that *RARE–lacZ* expression in mouse embryos is high in tissues anterior to the primitive streak, then progressively much weaker in the primitive streak itself. Primitive neuroectodermal cells thus exist in a zone of high FGF8 and low RA signaling, but on exiting the primitive streak anteriorly, these cells enter a zone of high RA emanating from the directly adjacent somitic mesoderm, which downregulates *Fgf8* and stimulates neuronal differentiation.

RA also controls left–right patterning of somite pair formation, and this is proposed to function via antagonism of *Fgf8* expression in neuroectoderm. RA synthesized in presomitic mesoderm by *Raldh2* is required to maintain bilateral symmetry between the left and right somite columns; for instance, loss of RA can result in embryos displaying 11 somites on the left side but only nine somites on the right side. Presomitic mesoderm in mouse *Raldh2*$^{-/-}$ embryos displays left–right asymmetric expression of *Hes7* and *Lfng*, required for periodic mesodermal segmentation, suggesting that a loss of RA allows left–right asymmetry to occur in presomitic mesoderm, where it normally does not occur. On the other hand, lateral plate mesoderm (where left–right asymmetry normally occurs) still maintains left–right asymmetry of key genes, including *Nodal* and *Pitx2* in *Raldh2*$^{-/-}$ embryos. RA thus acts as a buffer to prevent left–right asymmetry from occurring in presomitic mesoderm. In the 'clock and wavefront' model of somitogenesis, a moving wavefront of *Fgf8* gene expression in the primitive streak regresses posteriorly as the body axis extends, and mesodermal segmentation occurs just anterior to the *Fgf8* expression domain. FGF8 signaling also plays a required role in the node, a structure located anteromedially in the primitive streak that produces

left–right asymmetry in the lateral plate mesoderm. As *Fgf8* mRNA is not detected in the node, nearby cells in the epiblast ectoderm that do express *Fgf8* mRNA evidently secrete FGF8 that travels to the node. A hypothesis for how RA functions to prevent somite left–right asymmetry states that by limiting the anterior extent of *Fgf8* expression in the ectoderm, RA ensures that FGF8 signaling in the node is not excessive.

RA signaling monitored with *RARE–lacZ* exhibits no left–right difference in presomitic or lateral plate mesoderm at early somite stages, and RA activity extends from the posterior neural plate into node ectoderm, where it meets the *Fgf8* expression domain in the epiblast (primitive ectoderm) and then fades out. Although RA activity exists in presomitic mesoderm, it is absent in node mesoderm. Thus, it appears that RA functions in node ectoderm during somite left–right patterning. *Raldh2*$^{-/-}$ embryos exhibit a bilateral anterior shift of *Fgf8* expression into node ectoderm and neural plate, where it normally is not expressed. It is important to note that a loss of RA does not result in an anterior advance of *Fgf8* expression in presomitic mesoderm (which normally expresses *Fgf8* at a lower level than that seen in primitive ectoderm), and no ectopic *Fgf8* expression is observed in node mesoderm. These findings have provided evidence that the site of RA action during control of left–right asymmetry is the node ectoderm and adjacent neural plate.

Somite left–right patterning defects in *Raldh2*$^{-/-}$ embryos can be rescued with low-dose maternal dietary RA supplementation. It is interesting that RA provided to only the six-somite stage rescues somite patterning defects until at least the 25-somite stage. Thus, RA is needed only during a short time frame early in somitogenesis. Studies on rescued *Raldh2*$^{-/-}$ embryos carrying *RARE–lacZ* have provided further evidence that the site of RA action during control of left–right asymmetry is the node ectoderm and adjacent neural plate. Examination of such embryos at somite stages 2–10 revealed no *RARE–lacZ* expression in somitic or presomitic mesoderm (normally the source of RA), but *RARE–lacZ* was expressed in posterior neural plate and node ectoderm. As mentioned earlier, selective action of RA in neuroectodermal tissues may be due to expression of *Crabp2* that functions to sequester RA.

The limited time frame needed for RA action to correct somite left–right asymmetry is likely due to a transient RA requirement in the node as the node regresses after the ten-somite stage. At the two-somite stage, *Raldh2* is expressed in posterior presomitic mesoderm lying lateral to the node, whereas at the ten-somite stage, *Raldh2* is no longer expressed in presomitic mesoderm but remains in somites. Consistent with this observation, *RARE–lacZ* expression is present in the posterior neuroectoderm and node ectoderm during early stages but later retracts anteriorly such that by the ten-somite stage, there is no longer RA activity in the posterior neuroectoderm adjacent to the presomitic mesoderm; RA activity remains in neuroectoderm adjacent to the somites that have formed. These findings provide evidence that after the node regresses, RA is no longer needed to limit *Fgf8* expression posteriorly.

RA Acts in a Paracrine Fashion to Regulate Posterior CNS Patterning

As *Raldh2* and *RARE–lacZ* are normally expressed in presomitic and somitic mesoderm, it was initially presumed that RA functions locally in these mesodermal tissues during somite formation as well as in the adjacent neuroectoderm, where *RARE–lacZ* expression is also observed. However, studies on RA-rescued *Raldh2*$^{-/-}$ embryos demonstrate that RA needs to function only in the adjacent node ectoderm and neural plate to regulate early somite patterning, plus in the neuroectoderm of the hindbrain and spinal cord to regulate posterior CNS patterning. Thus, RA generated by *Raldh2* in mesoderm may function solely as a paracrine signal as no autocrine function has been uncovered.

See also: Floor Plate Patterning of Ventral Cell Types: Ventral Patterning; Hox Gene Expression; Morphogens: History; Motor Neuron Specification in Vertebrates.

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Hox Genes Expression

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Introduction

What Are *Hox* Genes?

Hox genes encode a family of homeodomain-containing transcription factors required for patterning regional properties along the rostral–caudal (R/C) axis during embryogenesis. The *Hox* gene family is highly conserved in organisms ranging from *Drosophila* to humans. In most vertebrates, except ray-finned fishes, there are 39 members segregated into four tightly clustered gene arrays (A–D) on four separate chromosomes (**Figure 1**). The *Hox* complexes are believed to have arisen by tandem duplication of a single gene to create a cluster, followed by duplication and divergence of the ancestral cluster associated with large-scale genomewide duplications in vertebrate evolution. This gene family is defined by sequence conservation, their genomic organization, and the presence of a homeobox or homeodomain motif within the protein. This domain is a helix–turn–helix protein motif responsible for sequence-specific DNA binding. These genes were initially identified in fly mutant screens as genes important in regulating segmental identity. One of the most famous examples is a gain-of-function mutation of the *Antennapedia* (*Antp*) gene, which transforms fly antenna into legs (**Figure 2**). Further investigation led to the discovery of 8 Hox proteins in *Drosophila*, each with roles in patterning and R/C-restricted patterns of expression.

Hox Gene Expression

Initial experiments demonstrating dramatic phenotypes associated with *Hox* gene deficiencies stimulated exploration of their expression patterns in *Drosophila* and other organisms. Surprisingly, developmentally regulated, nested and ordered patterns of expression were observed during embryogenesis. This further elevated interest in this family as developmental regulators because their patterns of expression along the embryonic R/C axis correlated with their gene order along the chromosome, suggesting they might provide a molecular means of defining head from tail or different structures from a common tissue. In the mouse, *Hox* gene expression begins at approximately embryonic day 6 (E6), one-third of the way through mouse development, after gastrulation has begun and the R/C axis has extended and become patterned. During the middle third of mouse development, *Hox* genes are highly expressed in the developing neural tube, often in dynamic patterns. The pattern of expression of individual *Hox* genes generally correlates both temporally and spatially with the location of the gene within its complex, a phenomenon called collinearity. Genes at the 3′ end of the complexes (paralog group 1) are expressed earliest with the most rostral boundaries of expression, and these two characteristics progressively change in successive genes in the complex, such that the most 5′ genes (paralog group 13) are only expressed caudally and have a delayed onset of expression compared to 3′ genes (**Figures 1, 3** and **4**). Although the spatial/temporal aspects of expression patterns progressively change between adjacent paralog groups, within paralog groups expression characteristics and even protein functions are often similar. Although the focus of this article is on the role of *Hox* genes in R/C patterning, they are expressed and have important functions in many other tissues. *Hox* genes have roles in hematopoiesis; lung, thyroid, parathyroid, kidney, breast, and muscle development; and in development of the structure of the head and neck and in the gastrointestinal and urogenital systems, where they have at least partially collinear patterns of expression. A common feature of *Hox* genes is that they provide a means of specifying different regional properties along the axis of a tissue. Within these systems they can do so by regulating a broad range of cellular activities, such as proliferation, differentiation, migration, patterning, adhesion, and cell death. In fact, part of the excitement about *Hox* genes is that they provide insight into the cellular and molecular mechanisms that use common building blocks to create different tissue structures (leg vs. antenna, hindbrain vs. spinal cord, and rib vs. tail bone).

Hox Gene Function

Since their initial discovery and analysis in *Drosophila*, Hox proteins have been demonstrated to be crucial molecules in regulating R/C patterning and defining segmental identity in vertebrates as well. Targeted mutagenesis of *Hox* genes in the mouse or manipulation of *Hox* gene expression in chickens via *in ovo* electroporation often alter segmental identity in the hindbrain and spinal cord as demonstrated by changes in motor neuron differentiation and identity. For instance, when either *Hoxa1* or *Hoxb1* is deleted by gene targeting, it perturbs the development of rhombomere-specific motor neuron populations as evidenced by altered motor neuron migration and/or projection patterns. When either of these is ectopically expressed in rhombomere 1 (normally a region devoid of *Hox*

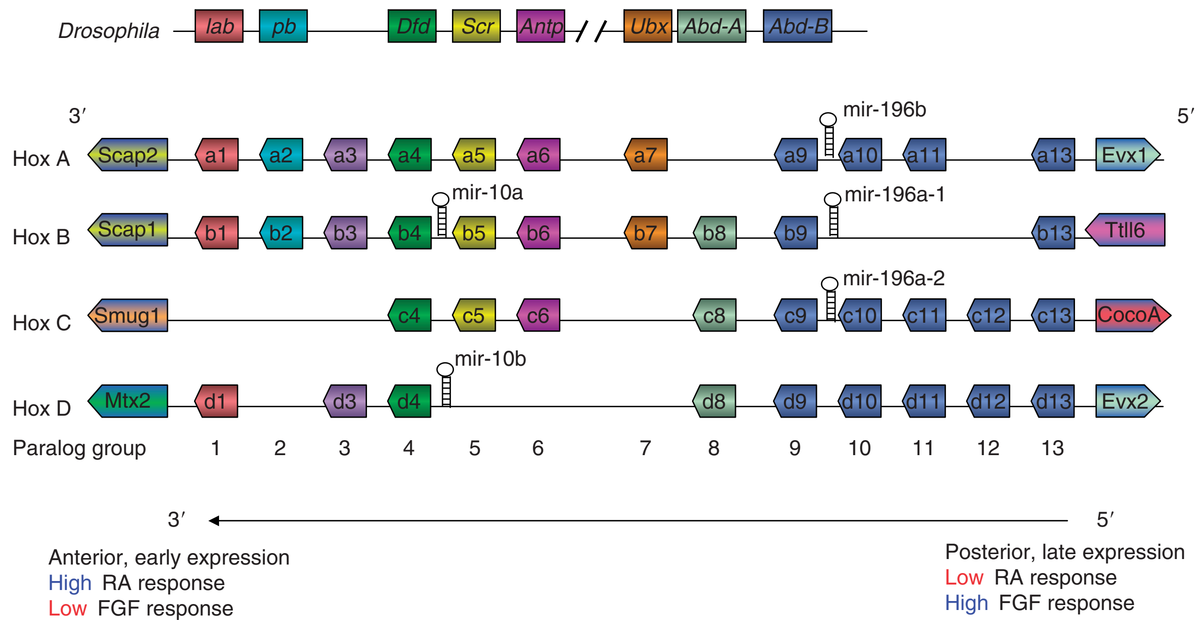

Figure 1 Schematic diagram of the split *Drosophila Hox* complex and its mammalian homologs. Homologs are indicated in the same color as their *Drosophila* homolog, listed in 3′ to 5′ orientation, and transcriptional direction is indicated by the pointed edge of each box. Paralog groups are indicated by numbers below each column. miRNAs embedded within clusters are indicated. Next adjacent genes 3′ and 5′ of each complex are also listed.

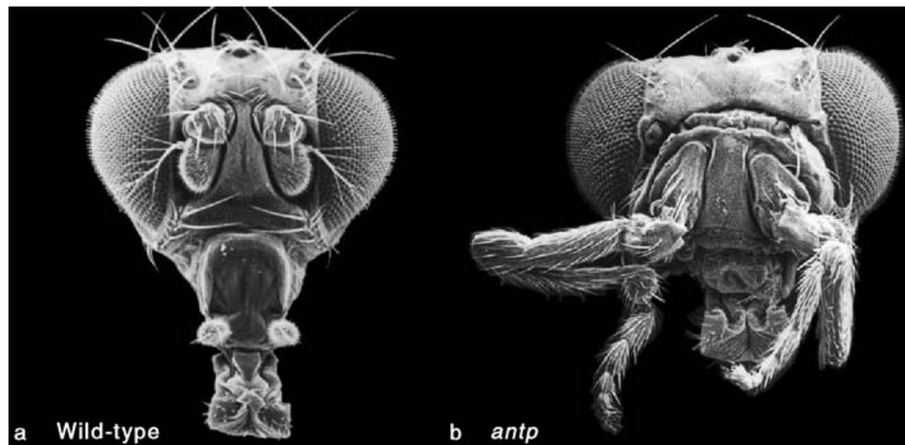

Figure 2 Scanning electron micrographs of wild-type (a) and *Antennapedia* (*antp*) gain-of-function mutant (b). Ectopic expression of *antp* in the antenna primordium transforms them into leglike structures. Courtesy of F Rudi Turner, Indiana University.

expression), it triggers ectopic development of motor neuron types typically found in more posterior segments. Similar experiments manipulating *Hox* gene expression in the developing chick spinal cord have shown that the products of *Hox* genes are the primary factors defining motor neuron pool subdivisions which innervate specific limb muscles. These studies emphasize that within the hindbrain and spinal cord, Hox proteins play a pivotal role in developmental patterning by defining segmental identity and controlling segment-specific structures and cell types. Pharmacological studies also support this role in that agents that perturb *Hox* regulatory pathways result in corresponding shifts in *Hox* expression profiles and regularly lead to alterations in segmental identity. Hence, detailed knowledge of the mechanisms of *Hox* gene regulation is fundamental to understanding how segmental identity is established and maintained. The following sections summarize the current understanding of basic mechanisms of *Hox* gene regulation within the hindbrain and spinal cord and provide specific examples to demonstrate key points.

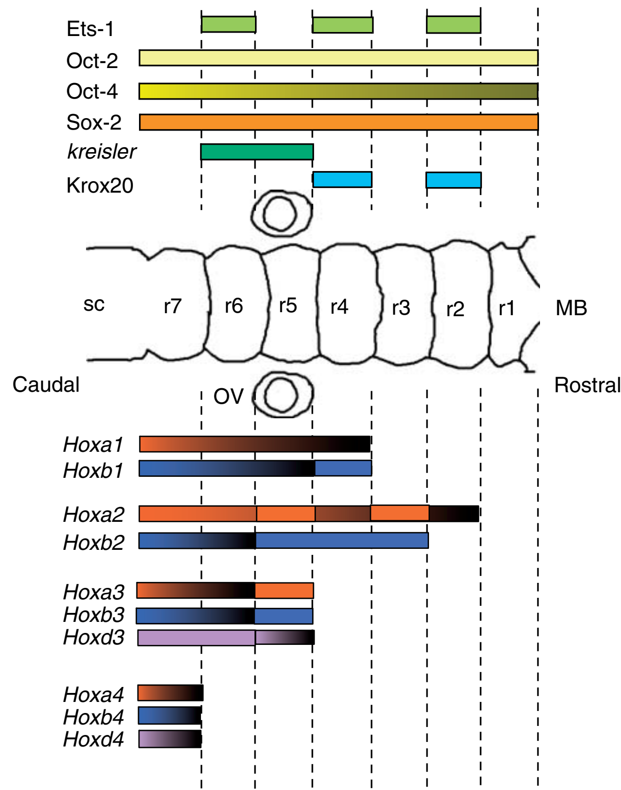

Figure 3 Expression patterns of *Hox* gene regulatory transcription factors and *Hox* genes are indicated by colored bars above and below the schematic of the developing mouse hindbrain at E9.5. Color gradients indicate relative expression levels or caudally regressing patterns of expression. r1–r7, rhombomeres 1–7; MB, midbrain; SC, spinal cord. Dashed lines indicate rhombomere boundaries.

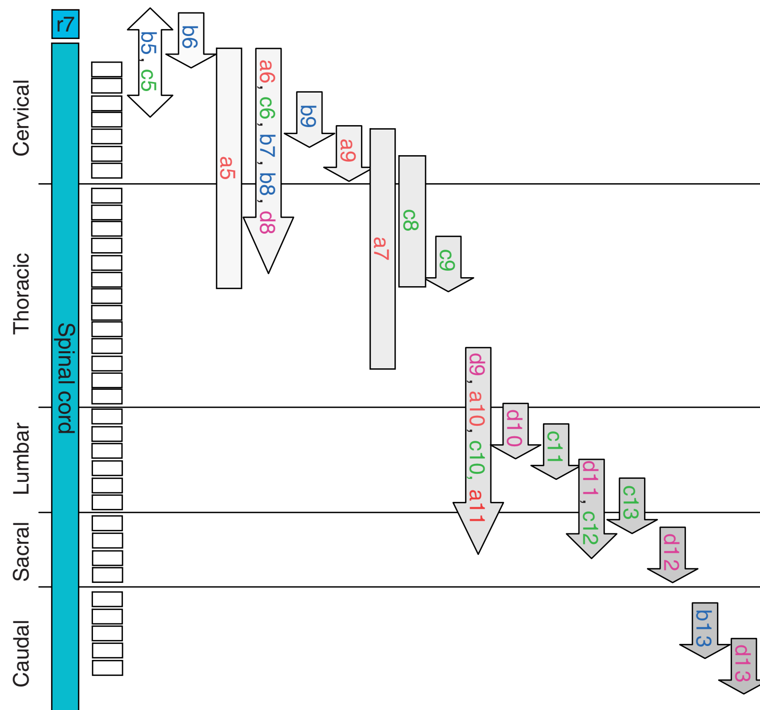

Figure 4 Expression patterns of caudally expressed *Hox* genes between E11.5 and E12.5. Spinal cord regions and associated segments are indicated on the left. The blunt ends of arrows indicate expression boundaries and the pointed ends signify that expression continues rostrally or caudally in the direction of the arrows. Expression patterns that have both anterior and posterior boundaries within the limits of the diagram are indicated as boxes.

Signaling Pathways Are Important Regulators of *Hox* Gene Expression

The retinoic acid (RA) and fibroblast grow factor (FGF) signaling pathways regulate significant aspects of *Hox* gene expression patterns. Manipulation of either of these pathways results in both alterations in *Hox* gene expression patterns and changes in R/C segmental identity. This has been demonstrated by adding exogenous RA or FGF, generating vitamin A-deficient (VAD) embryos, employing chemical agonists/antagonists, or expressing dominant negative FGF receptors. Additionally, mice engineered to be deficient in Cdx transcription factors, one of the integrators of FGF signaling, also display phenotypes which are strikingly similar to mice bearing homozygous *Hox* gene deficiencies. The teratogenic effects of RA exposure on human development and the similarity of the results to those of experiments done in mouse, chick, frog, and fish model systems corroborate the importance of *Hox* genes in segmental identity and the importance of RA in *Hox* gene regulation.

Regulation of *Hox* Genes by RA

RA, the active form of vitamin A, is produced by an enzymatic pathway that culminates in the conversion of retinaldehyde to RA by retinaldehyde dehydrogenase (RALDH). Lipophilic RA then diffuses or is transferred into surrounding tissues and cells so that it is able to bind to retinoic acid receptors (RARα, -β, and -γ and RXRα, -β, and -γ). These nuclear receptors exist as heterodimers that together bind retinoic acid response elements (RAREs) within the genome and modulate gene transcription. The binding of RA to these receptors converts these bound heterodimers to transcriptional activators via increasing their affinity for coactivators and decreasing their affinity for corepressors. RA signaling relevant for R/C patterning of the hindbrain and spinal cord is initially regulated by controlling RA production via *Raldh2* expression in somitic mesoderm. After RA has been produced, local concentrations are modulated by cytoplasmic retinoic acid binding proteins (CRABP1 and -2), clearance by the blood, isomerization from 9-*cis* to the less potent all-*trans* RA, and, finally, degradation via the Cyp26 family of enzymes. This provides a complex metabolic mechanism to ensure the levels of this important signal are very carefully modulated in a tissue-specific and temporally specific manner. A transgenic mouse line bearing an RA responsive marker gene shows that these mechanisms synergize to produce substantial RA signaling in the eye, telencephalon, and throughout the spinal cord and adjacent mesoderm/somites from the level of the caudal hindbrain throughout the majority of the trunk, with the lowest levels at the node and tailbud at later stages.

RA Response Specificity and Diversity

Many *Hox* genes have been shown to be RA responsive, and several functional RAREs have been identified near *Hox* genes demonstrating that they are direct targets of the signaling pathway. Multiple DR5 (direct repeat + 5-base pair spacer; i.e., AGGTC AnnnnnAGGTCA) and DR2 RAREs have been identified within the *Hox* clusters and associated with either activation or repression of one or more neighboring genes. For example, transcription of both *Hoxb3* and *Hoxb4* is activated by a single DR5 RARE located in the intergenic region between these two genes. *Hoxb1*, on the other hand, is associated with three separate RAREs, all of which have distinct influences on its expression. A DR5 RARE 5′ of *Hoxb1* mediates repression of *Hoxb1* in rhombomeres 3 and 5, whereas a DR2 RARE and a DR5 RARE 3′ of *Hoxb1* both activate expression of *Hoxb1* but in different spatial patterns (**Figure 5**). Several other 3′ *Hox* genes not mentioned here also have RAREs with similar organization and function as those mentioned previously. Thus, RA affects a broad range of *Hox* expression patterns, and although these responses can be regulated both directly and indirectly, it is clear that *Hox* genes are often direct targets of RA activity. Additional modes of regulation continue to be investigated.

FGF Regulates *Hox* Expression

FGFs are also important regulators of *Hox* gene expression. FGFs are an expanding family of secreted signaling molecules that includes more than 20 members. After secretion, these soluble proteins bind to transmembrane tyrosine kinase receptors, FGFR1–4. These ligand-bound receptors dimerize, autophosphorylate themselves, and transduce intracellular signals via mitogen-activated protein kinase, phosphatidylinositol 3-kinase, or protein kinase C/protein lipase C (PLCγ), among others. Activation of these pathways can result in a broad spectrum of responses depending on the context; however, activation of *Cdx1–4* homeobox gene expression is one of the primary modes of influencing *Hox* gene expression. Cdx homeodomain transcription factors, homologs of the *Drosophila* caudal protein, are capable of binding *Hox* gene enhancers and directly regulating *Hox* transcription. Hence, FGFs activate *Hox* gene expression indirectly via the *Cdx* family of genes. FGF8 expressed in the isthmus (a thin region that separates the midbrain from the hindbrain) and the node (a signaling center and region of posterior

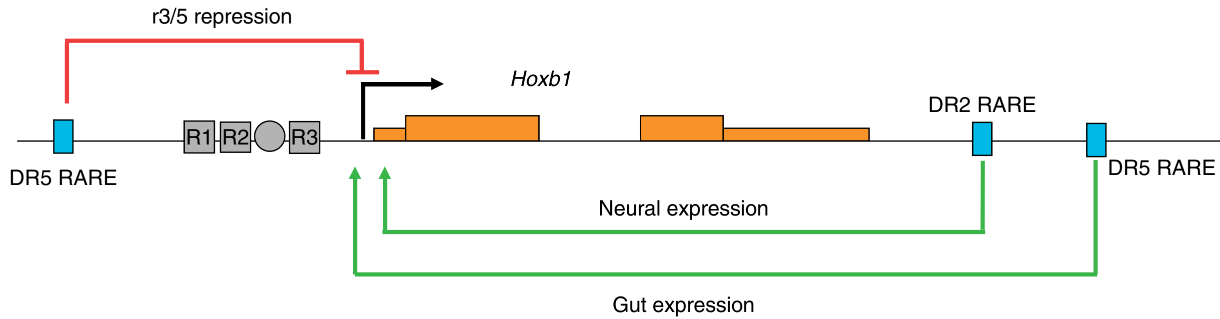

Figure 5 RA regulation of *Hoxb1*. *Hoxb1* has three retinoic acid response elements (RAREs) indicated by blue boxes. Two of these activate expression of *Hoxb1* (green arrows) and one mediates repression (red line). The *Hoxb1* autoregulatory *Hox* response elements are indicated as gray squares and circles. DR2 and -5, direct repeat 2- and 5-base pair spacers.

elongation) has significant patterning influences on the R/C axis. FGF8 from the node influences *Hox* gene expression throughout much of the spinal cord during elongation of the axis. Isthmic sources of FGF8 are critical for maintaining the identity of rhombomere 1, the first hindbrain segment, but its role in *Hox* gene repression in this uniquely Hox-free region is poorly understood.

FGF and RA Regulate Opposing Groups of *Hox* Genes

Although both RA and FGF exhibit dose-dependent activation of *Hox* gene expression, they display opposing preferences for which *Hox* genes are activated by each. RA preferentially activates *Hox* genes in the 3′ portion of the complex (*Hox1–5*) and has little influence on the expression of 5′ genes (*Hox6–13*). For instance, expression of a dominant negative RAR diminishes *Hoxb4* expression in the developing spinal cord but does not alter *Hoxb9*. Functional RAREs responsible for this preferential activation have been identified in the 3′ portions of the complexes. In contrast, FGF preferentially influences *Hox* genes in the 5′ portion of the complex (*Hox6–13*) and can expand their rostral boundaries of expression. FGF specificity appears to be determined, in part, by *Cdx* expression domains, which supports the hypothesis that Cdx proteins play an important role in integrating FGF signaling. FGFs are capable of activating expression of both 5′ and 3′ *Hox* genes and expanding their expression domains, but activation of 3′ *Hox* genes in the hindbrain is limited by the availability of Cdx. Whereas exogenous FGF can extend the expression of *Hoxb9* from its caudal boundary up to the level of the otic vesicle, *Hoxb4* expression that normally terminates just posterior to the otic vesicle cannot be expanded by additional FGF alone. Ectopic expression of a dominant active Cdx (XcadVP16) in the presence of exogenous FGF, however, can expand both *Hoxb4* and *Hoxb9* expression past the otic vesicle and into the hindbrain.

FGF and RA Generate Opposing Gradients

RA and FGF signaling oppose each other on multiple levels. They preferentially influence opposing groups of *Hox* genes and also positively and negatively cross-regulate the opposing pathway. For example, FGF can activate transcription of RARs, activate expression of *Cyp26* (the enzyme responsible for RA degradation), and block expression of *Raldh2* and thus attenuate RA production. Conversely, RA can activate *FGFR* expression, block expression of fgf8, block expression of *Cdx1*, and stimulate its own degradation by upregulation of *Cyp26*. These diverse mechanisms work together to produce opposing gradients of FGF and RA signaling activity, which allows a wide range of concentration-dependent, segmentally restricted responses to FGF and RA, including their regulation of *Hox* gene expression (**Figure 6**).

Additional Signaling Pathways

Although FGF and RA appear to be the most influential signaling pathways regulating *Hox* genes, other pathways do appear to impinge upon *Hox* gene expression to a lesser extent. Gdf11, a secreted transforming growth factor-β homolog, can influence *Hox* gene expression and sensitize neural tube progenitors to FGF8 emanating from the node. The Wnt signaling pathway is another critical signal that influences regional character. Wnts can modulate Cdx1 activity and have an indirect input on *Hox* expression via this route. This further illustrates the importance of understanding Cdx regulation because it appears to integrate RA, FGF, and Wnt signaling in the regulation of *Hox* gene expression in the developing central nervous system and axial skeleton.

Regulation of *Hox* Genes by Transcription Factors

Regulation by transcription factors serves to establish, stabilize, and define the boundaries of *Hox* gene

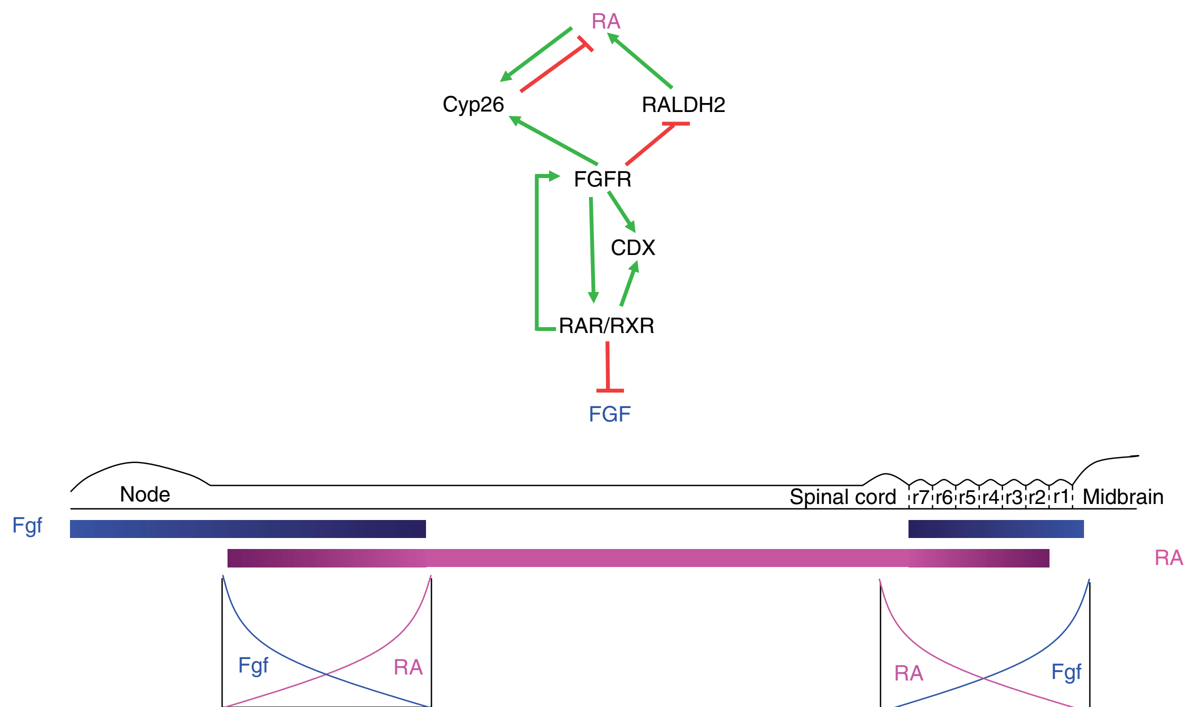

Figure 6 FGF and RA form opposing gradients through cross-regulation. (Top) Regulatory relationships between elements of the FGF and RA pathways. Activation is indicated by green arrows and repression by red lines. (Bottom) FGF (blue) and RA (purple) gradient formation along the rostral–caudal axis of the developing mouse embryo.

expression initiated by the previously mentioned signaling systems. Some of these upstream transcription factors activate *Hox* gene transcription in a defined way, limited by the boundaries of their own expression. Many of the transcription factors that regulate *Hox* gene expression, however, are ubiquitously expressed and presumably rely on association with additional proteins to achieve specificity. Exploring these cooperative mechanisms of specificity is an important area of current investigation.

Four spatially restricted and developmentally regulated transcription factors have been shown to be important regulators of *Hox* expression patterns: Krox 20, Kreisler (MafB), and Sox/Oct. *Krox 20* encodes a zinc finger transcription factor expressed in rhombomeres 3 and 5. Binding of Krox 20 to its target sequences upstream of *Hoxa2* and *Hoxb2* and between *Hoxb3* and *Hoxb4* activates expression of these genes in rhombomeres 3 and 5. *Kreisler,* encodes a bZIP leucine zipper containing transcription factor expressed in rhombomeres 5 and 6, is required to activate transcription of *Hoxa3* and *Hoxb3*. Two other transcription factors important in stem cell maintenance, Sox2 and Oct4, may form a complex and participate in regulating *Hoxb1* in rhombomere 4.

Polycomb group (PcG) proteins and trithorax group (TrxG) proteins are two classes of chromatin-modifying proteins that epigenetically regulate *Hox* gene expression. These groups form multiprotein chromatin remodeling complexes that either repress transcription (PcG) or activate transcription (TrxG). Although these complexes are ubiquitously expressed and regulate many genes and cellular behaviors, mutations in members of both of these groups can alter *Hox* gene expression patterns and compromise R/C patterning in ways similar to both *Hox* gene deficiencies and abnormal FGF or RA signaling. PcG regulation of *Hox* expression may be direct because the PcG protein Phc2 can bind directly to the *Hoxb8* promoter. Additional regulation of this system appears necessary, however, because Phc2 can be observed at the *Hoxb8* promoter in tissues with high *Hoxb8* expression and also in tissues with low *Hoxb8* expression levels. PcG mutations also alter *Cdx* expression, and thus may have additional indirect inputs into *Hox* regulation. Several other ubiquitous transcription factors have been shown to be capable of regulating *Hox* genes. Factors such as YY1, Sp1, USF, NFY, and AP2 can modulate *Hox* expression in some contexts. The mechanism by which these ubiquitous transcription factors regulate specific aspects of *Hox* gene expression remains unclear; however, it may be related to their association with other factors. Sp1 can interact with AP2 and indirectly cooperate with Hox proteins

themselves. USF and NFY can interact with each other, and YY1 and Plzf can both interact with PcG components, although the roles of these interactions *in vivo* remain poorly characterized.

Auto-/Cross-Regulation

In addition to regulation by numerous other transcription factors, *Hox* genes also exhibit extensive autoregulation. Hox proteins can bind to *Hox* responsive elements (HREs), which consist of a *Hox* target site and usually include an adjacent binding site for one or more cofactors, such as Pbx, Prep, or Meis. Among the numerous possibilities, HRE-mediated *Hox* regulation usually functions in three main ways to refine and maintain *Hox* expression: *Hox* genes may maintain their own expression (autoregulation), activate expression of other *Hox* genes expressed in the same segment (cross-activation), or have antagonistic interactions in which one gene represses the expression of another *Hox* gene (cross-repression). These are all important processes in constructing and maintaining the complement of *Hox* genes expressed at each axial level and in defining boundaries between adjacently expressed genes.

The most extensively studied HRE is that of *Hoxb1*, which contributes to its expression in rhombomere 4. This element contains multiple Hox/cofactor binding sites and exhibits both autoregulation by Hoxb1 and cross-activation by Hoxa1. RA signaling and RAREs are directly responsible for the initial activation of *Hoxb1* expression. However, at later stages of development the HRE assumes regulatory control of *Hoxb1* transcription and Hoxb1 maintains its own expression. Genetic studies in mouse reveal that both the *Hoxa1* and *Hoxb1* genes are required for normal expression of *Hoxb1* in rhombomere 4. HREs have been identified either functionally, by sequence comparison, or inferred by transcriptional responses to *Hox* overexpression and are associated with the regulation of at least one-fourth of all *Hox* genes. Auto- and cross-regulatory interactions between the *Hox* genes appear to be fundamental mechanisms for modulating Hox transcriptional activity. Although both autoregulatory and cross-activation functions of the *Hoxb1* HRE can be clearly demonstrated, these regulatory interactions become less distinguishable for *Hox* members more 5′ in each complex because of the increasing number of *Hox* genes expressed at more posterior levels and the potential for indirect relationships. For 5′ *Hox* genes, it is likely that HREs are utilized to produce defined boundaries between different *Hox* gene expression domains by cross-repression. Such relationships have been demonstrated for *Hoxc5*–*Hoxc8*, *Hoxc6*–*Hoxc9*, and *Hox4*–*Hoxa7* such that ectopic expression of one gene of the pair represses the expression of the second gene, producing precise boundaries between expression domains of the two genes. Many of these relationships have not been demonstrated to be direct; however, several of the *Hox* genes involved are associated with functional HREs (**Figure 7**).

Hox Cofactors

Although isolated Hox proteins show sequence-specific binding to a 4-base pair A/T-rich target site (ATTA or TAAT), their affinity for this site is usually low and the length of the site allows little specificity in target site selection. It is thought that associations with cofactors may confer specificity to the Hox proteins in target site selection. HREs that contain both a Hox binding site and cofactor target sites can dramatically increase affinity of Hox + cofactor complexes for the site, specificity of the response, and the ability of the complex to stimulate transcription. The primary Hox cofactors are of the Meinox/TALE (three amino acid loop extension) family of proteins which include Pbx1–3, related to Drosophila Extradenticle (Exd), and Prep1-4/Meis1-3, related to *Drosophila* Homothorax (Hth). Pbx proteins directly bind to Hox proteins via a hexapeptide motif, X (Y/F) (P/D) WM (K/R), N-terminal to the Hox homeodomain, whereas a C-terminal portion of the homeodomain participates in stabilizing this association. The interaction of Hox and Pbx expands the target site specificity to a consensus ATGATTNATNN, where the Hox protein binds to the TNATNN portion of the half site

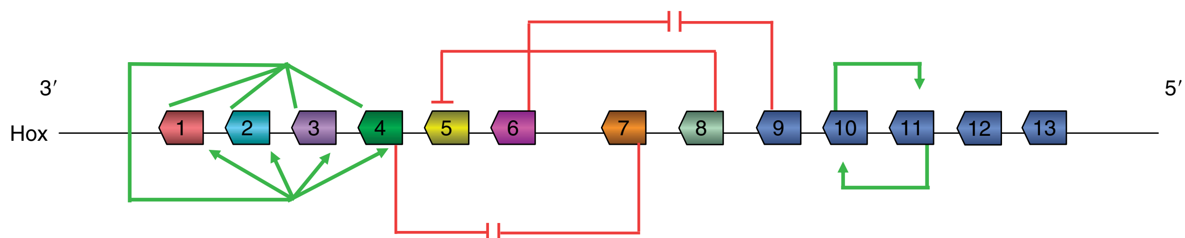

Figure 7 *Hox* gene auto-/cross-regulatory relationships. Regulatory relationships between paralog groups are indicated by green arrows, indicating positive loops, or red lines, indicating repressive relationships.

and Pbx binds to the ATGAT element. The binding selectivity of a Hox–Pbx target site is determined, in part, by site preferences conferred by the identity of the Hox protein present in the heterodimer. Prep/Meis cofactors initially bind to Pbx, which subsequently binds Hox proteins to form a ternary complex. These ternary complexes bind expanded target sites and activate transcription at a level higher than Hox/Pbx dimers and to a much greater extent than Hox proteins alone. Hox paralogs 1–10 can bind Pbx or Pbx–Prep/Meis heterodimers, paralogs 9 and 10 can bind Meis in the absence of Pbx, and groups 11–13 lack the Pbx interacting motif but can form complexes with Prep/Meis. Pbx/(Prep/Meis) heterodimers may also bind HREs in the absence of Hox and repress transcription. Both *in vitro* biochemical experiments and *in vivo* mouse deletions of these cofactors demonstrate their importance in *Hox* gene regulation and the function of HREs. Whereas the formation of Hox–Pbx heterodimeric complexes is DNA dependent, the formation of Pbx–Prep/Meis heterodimers is not. Pbx–Prep heterodimers can form in the cytosol and heterodimerization facilitates the nuclear localization of Pbx. Understanding these protein complexes and the events that modulate Hox/cofactor interactions is important in building a clear understanding of how Hox proteins find and act on their target sites *in vivo*.

Although the functional significance of Hox protein interactions is only beginning to be explored, several other developmentally important transcription factors have been shown to be capable of physically interacting with Hox proteins and these may also be relevant *in vivo* as cofactors or modulators. Modulation of Hox protein function may explain additional aspects of Hox protein target site specificity and switch Hox transcriptional activity between activation and repression. The transcription factors Sp1, Btg1, Smads, and members of the Maf family can interact with Hox proteins in some contexts. Although many of these interactions were investigated in artificial systems, these proteins were capable of interacting with Hox proteins and affecting their ability to transactivate reporter expression. Sp1 and Hoxa13, known regulators of BMP4, cooperate in activating transcription from the BMP4 promoter. Btg1 and-2 have been identified as Hoxb9 interacting proteins by the yeast two-hybrid assay and work together to activate transcription from a *Hoxb9* response element-driven reporter. Although Maf proteins have been shown to interact with several Hox proteins, these experiments did not reveal the function of these interactions. Kriesler, a member of this Maf family of proteins, has been shown to both regulate *Hox* gene expression and be involved in R/C patterning of the hindbrain; thus, it seems unlikely that these interactions would be without functional consequence during hindbrain development. These potential Hox cofactors participate in many different systems, but the importance of their interaction with Hox proteins remains unknown.

Initiation Versus Maintenance

Regulatory influences on *Hox* gene expression often result in expression patterns controlled in two separate phases, an early initiation phase and a later maintenance phase. Many of the 3′ *Hox* genes are regulated primarily by a combination of retinoic acid response elements and autoregulatory HREs. RA functions early in initiating expression in the absence of active *Hox* gene expression, and as Hox proteins accumulate within cells, the HREs may then assume regulatory influence over these genes. This mechanism is critical in maintaining *Hox* expression because expression patterns of *Cyp26* expand to encompass much of the hindbrain, reducing RA there and decreasing RARE-mediated *Hox* gene expression. Experiments in which the DR2 RARE 3′ of *Hoxb1* was deleted demonstrate this phenomenon very clearly. They show that the DR2 RARE 3′ of *Hoxb1* is required for correct temporal and spatial initiation of *Hoxb1* expression, and the remaining autoregulatory elements are capable of directing accurate *Hoxb1* spatial expression patterns at later time points.

Competition and Sharing

Unlike most other genes, *Hox* genes are organized into dense arrays/clusters. Nine to 11 genes are clustered within each ~130 kb genomic locus and are all transcribed off the same DNA strand. Mechanisms that actively isolate individual *Hox* genes from the influences of other nearby *Hox* gene enhancers have been found in the *Drosophila* HOM-C cluster; however, such elements do not appear to be present, as a general rule, in vertebrates. Additionally, in mouse, an entire *Hox* cluster is approximately the size of a single *Hox* gene, such as *Antp* in *Drosophila*. As a consequence of this close proximity, adjacent *Hox* gene promoters often appear to share or compete for enhancers. Transgenic mouse experiments exploring the regulatory landscape around *Hoxb4* and *Hoxb5* revealed that enhancer sharing, selectivity, and competition are all mechanisms regulating the expression of these two genes. Multiple elements between *Hoxb4* and *Hoxb5* participated in the regulation of both genes, which is largely responsible for overlapping *Hoxb4* and *Hoxb5* expression domains. One control element, equally capable of activating *Hoxb4* and *Hoxb5*, was preferentially recruited to

activate the *Hoxb4* promoter compared to that of *Hoxb5* when given a choice. Although few additional examples of promoter competition exist, either because of their rarity or because of their experimental in tractability, enhancer sharing appears to be common between neighboring *Hox* genes. In the case of *Hoxb3* and *Hoxb4*, at least two distinct elements in the intergenic region between these genes influence the expression of both genes. Similarly, shared elements have also been identified between *Hoxb5* and *Hoxb6*. The compactness of the *Hox* complexes and the absence of insulator elements blocking the influence of nearby regulatory elements suggest that enhancer sharing must be a common feature of *Hox* gene regulation.

Posttranscriptional Regulation of *Hox* Genes

Although mRNA levels and spatial/temporal localization are normally good indicators of protein distribution, it is becoming clear that they may be misleading in relation to Hox protein distribution. Because antibodies to Hox proteins have been particularly difficult to produce, expression analysis has often relied on *in situ* detection of mRNA. Several cases in which immunodetection of Hox protein has been achieved have demonstrated that protein expression domains often do not encompass the entire domain in which mRNA is expressed. Careful analysis of the regulation of the *Hoxb4* gene allowed the observation of a difference in reporter gene expression patterns dependent on whether the reporter utilized the endogenous *Hoxb4* 3′untranslated region (UTR) and polyadenylation signal or used an exogenous polyadenylation signal. Although the *Hoxb4* 3′ UTR was present in each of these reporter constructs, its inclusion into the mRNA transcript produced altered patterns of expression. This clearly demonstrated that *Hoxb4* was posttranscriptionally regulated and identified the domain responsible for this regulation. Similarly, mRNA expression and protein levels of some of the *Hoxc* genes in the spinal cord have been shown to be discordant, indicating that translational regulation may indeed be an additional mechanism modulating Hox expression.

Concurrent with these observations, microRNAs (miRNAs) (small regulatory RNAs that can mediate both pre- and posttranscriptional regulation through sequence-specific binding to target mRNAs) were being investigated as *Hox* regulatory molecules. Multiple miRNAs have been identified embedded within *Hox* complexes. mir-196a1/a2/b, which are located 5′ of the *Hox9* paralog in *Hox* B, C, and A clusters, respectively, are predicted to target *Hoxa7*, *Hoxb8*, *Hoxc8*, and *Hoxd8* transcripts for degradation. Verification of miRNA targets *in vivo* and assessment of their relevance has just begun; however, degradation products resulting from mir-196 targeting of *Hoxb8* have been isolated from mouse embryos, demonstrating that this process is functional *in vivo*. *Hox* genes have been identified as the targets of these and other miRNAs. Posttranscriptional regulation may be a consistent mechanism by which *Hox* genes are regulated; however, the importance of miRNAs in this process has not been demonstrated.

Global Regulatory Elements

By far the most elusive *Hox* gene regulatory process is the mechanism by which collinear expression of *Hox* genes is achieved. One hypothesis focuses on long-range elements outside of the complex (global regulatory elements) that mediate processive regulatory influences on *Hox* genes that travel along the DNA through the *Hox* complex. Experiments in which a *Hoxb1* reporter was inserted 5′ of *Hoxd13* showed that some aspects of the reporter expression were affected by the location of the reporter in the complex which were not seen when the reporter was inserted randomly, suggesting that location within the complex could influence expression pattern. Separate experiments showed both a sequential de-compaction of *Hox* complexes during development and partial collinearity of ‘active’ chromatin marks spreading through the *Hoxb* locus during differentiation, further supporting the global element plus spreading model. Although some elements capable of long-range influences on multiple *Hoxd* genes have been identified for the limb expression, global elements capable of long-distance, sequential regulation of entire complexes in most other sites of *Hox* expression have yet to be identified. In contrast, local elements capable of specific and ordered expression for many of the individual genes have been well characterized. Thus, it remains possible that many aspects of collinear expression could be achieved through the collective activity of locally positioned internal elements rather than through a single global modulator of each cluster. Clearly, if global control regions are a common feature regulating collinear *Hox* gene expression, they are not solely responsible for this phenomenon. Rather, it seems likely that diverse mechanisms are used to open the clusters to make them available for transcription that vary at different times and locations. A variety of locally recruited activators and repressors (including the Hox proteins) may work in concert with local and long-range chromatin remodeling complexes to impart the ordered domains of *Hox* expression.

Conclusions

Hox proteins play a fundamental role in the development of R/C identity in diverse tissues in addition to many less appreciated roles in organogenesis and cell differentiation. They participate in giving R/C segments of the hindbrain and spinal cord their unique identity through their nested expression patterns within these structures. The host of Hox proteins present at each axial level then conveys identity onto that segment observable by segment-specific gene expression patterns, neuronal phenotypes, and cell migratory behavior. Appreciation of Hox gene collinear expression patterns and their regulation are essential aspects of understanding mechanisms of R/C polarity within the hindbrain and spinal cord. *Hox* genes are regulated on multiple levels. Expression is initiated by transcription factors and secreted molecule signaling systems and is subsequently maintained via Hox-mediated auto- and cross-regulatory mechanisms. Experiments have begun to unveil an additional level of *Hox* gene regulation that functions posttranscriptionally; however, the full significance of this has yet to be determined.

A burgeoning area of exploration is the role of Hox cofactors and other interacting proteins. These experiments open the possibility of an additional level of modulation of Hox protein binding, activation/repression, and target site selection. Although much of these data have yet to be shown relevant *in vivo* during development, they could vastly expand our understanding of the role of Hox proteins and their mechanisms of action. More attention must be directed toward this avenue of investigation, but it promises to be an exciting future for Hox gene study.

Although it is clear that Hox proteins play a critical role in R/C patterning and identity, several questions remain about how this occurs. Altering *Hox* gene expression patterns within the hindbrain and spinal cord often results in dramatic alterations in cell identity or behavior; however, how Hox proteins regulate target genes to achieve these effects is largely unknown. A few downstream target genes activated or repressed by Hox proteins have been identified, but our understanding is too limited to create a picture of the pathways under Hox regulation that impose diverse regional characters. This is an exciting area for future study. Systems biology approaches and genomic analyses are all aimed at understanding how the alteration of one gene can impact on the outcome of cell behaviors to result in differences such as a leg versus an antenna. This is an elusive basis of morphogenesis, and understanding these mechanisms and their outcomes opens the possibility of a wealth of knowledge about the genetic programs that control normal development, how they are perturbed in human diseases, and how they might be involved in producing diversity. *Hox* genes have fundamental roles in these processes and provide an opportunity for exciting insight into the mechanisms responsible for building the basic body plan of animals.

See also: Anterior-Posterior Spinal Cord Patterning of the Motor Pool; Neural Patterning: Midbrain–Hindbrain Boundary; Retinoic Acid Signaling and Neural Patterning; Transcriptional Networks and the Spinal Cord; Wnt Pathway and Neural Patterning.

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Anterior–Posterior Spinal Cord Patterning of the Motor Pool

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Introduction

Locomotor behaviors in vertebrates depend on the formation of selective connections between motor neurons and their synaptic targets in the spinal cord and periphery. The precision in which spinal circuits are assembled is critical in controlling the precise temporal activation of muscle groups in the limbs. Many of the spinal circuits that control simple locomotor behaviors, such as the alternation of left and right limbs or the coordination extensor and flexor muscles during locomotion, are established during the early stages of embryonic development. Locomotor circuits appear to be shaped initially independent of sensory experience, suggesting a high degree of genetic determinism in their formation. A critical feature of all spinal locomotor circuits is the establishment of precise connections between motor axons and muscle targets in the limb.

Motor neurons share certain basic features that distinguish them from other classes of neurons in the spinal cord, and they also acquire specialized properties that allow them to make selective connections with target cells. For example, all spinal motor neurons possess axons that project outside the spinal cord and release acetylcholine as the primary form of neurotransmission. In many if not all other regards, motor neurons are a highly diverse class of neuron. This diversity is most apparent in the motor neuron subtypes that innervate skeletal muscles in the limb, where each muscle is innervated by dedicated groups of motor neurons called motor pools. The typical vertebrate limb contains more than 50 muscle groups, and each of these targets is innervated by a unique pool of motor neurons.

Anatomical Organization of Motor Neuron Cell Bodies and Their Axonal Projections

During development, motor neurons acquire subtype identities that define their position within the spinal cord and determine their ability to selectively innervate peripheral targets. Motor pool identities emerge over a series of sequential stages, and each step restricts the potential of motor axons to innervate alternate targets. An early step in the differentiation of motor neurons involves the segregation of their cell bodies into longitudinally arrayed columns, each column containing motor neurons that project their axons to a common peripheral target. Five major columnar groups of motor neurons are generated within the spinal cord, four of which localize to specific positions along the rostrocaudal axis (**Figure 1**). Of particular importance to the specification of motor pool fates is the generation of the lateral motor column (LMC) because the acquisition of an LMC identity directs motor axons toward the limb. Motor neurons within the LMC are generated selectively at brachial (forelimb) and lumbar (hindlimb) levels of the spinal cord and contain the motor pools that innervate specific limb muscles.

Motor neurons within the LMC can be further delineated on the basis of how their axons initially project into the developing limb bud. The cell bodies of motor neurons within the LMC that project dorsally or ventrally into the limb are segregated from one another and define two divisional identities within the LMC. Medially positioned LMC motor neurons (LMC_m) project ventrally within the limb bud mesenchyme while laterally positioned LMC neurons (LMC_l) project dorsally. These divisional subtypes define two coherent subgroups of motor neurons within the LMC and define an initial choice point for motor axons projecting into the limb.

Motor pools are organized within the columnar and divisional identities of motor neurons. A motor pool is defined as the group of motor neurons that project to a single muscle target in the limb. In many cases the cell bodies of motor neurons are clustered in discrete nuclei, although the physiological relevance to motor pool clustering is still uncertain. Anatomical studies of the position of motor pools in the spinal cord have revealed that each motor pool occupies a stereotypical position in the spinal cord. A motor pool within the LMC typically spans two to three segments of the spinal cord, and the number of motor neurons within a given pool is proportional to size of the muscle it innervates.

Classical Studies on Motor Neuron Development in the Chick

Many of the insights into the mechanisms controlling the synaptic specificity of motor neurons emerged from classical manipulations of the neural tube in chick embryos. The idea that motor neurons have intrinsic properties that allow them to selectively innervate specific muscle targets in the limb was supported by studies in which motor neurons were displaced from their normal position within the spinal cord. In one set of experiments, lumbar-level neural tube was rotated

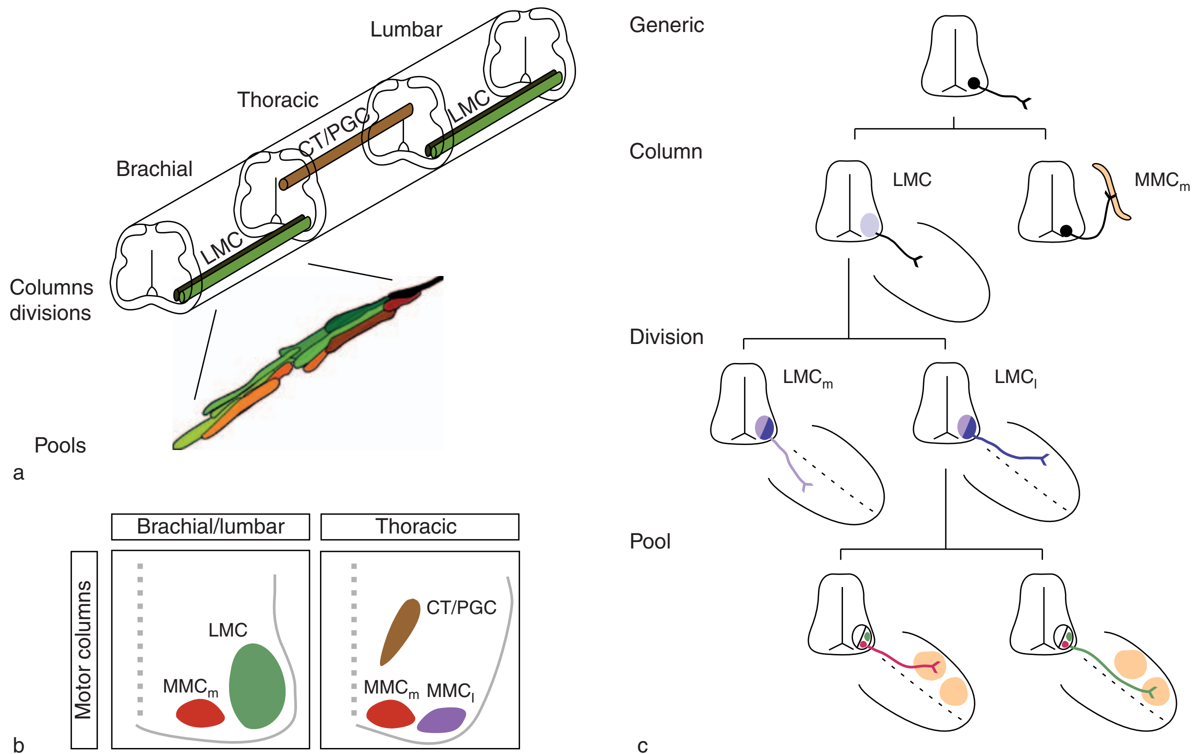

Figure 1 Motor neuron organization within the spinal cord and projection patterns of motor axon. (a) Motor columns and motor pools are generated at specific locations along the rostrocaudal axis. The cell bodies of motor neurons that send axons to the limb are contained within the lateral motor column (LMC) at brachial and lumbar levels of the spinal cord. Preganglionic motor neurons called the column of Terni (CT) in chick or preganglionic columns (PGC) in mouse are found at thoracic levels. The medial and lateral divisions of the LMC are depicted in different colors. Motor pools are generated at specific rostrocaudal positions within the LMC. (b) Schematic of a cross section of brachial/lumbar and thoracic regions of a chick spinal cord, showing the position of motor columns. LMC neurons are found at brachial and lumbar levels of the spinal cord. Motor neurons within the medial division of the medial motor column (MMC_m) are generated at all rostrocaudal levels of the spinal cord and extend axons to axial muscles. At thoracic levels, two segmentally restricted columns are generated, a set of lateral MMC (MMC_l) neurons that project their axons to body wall muscles and CT/PGC neurons that project axons to sympathetic neuronal targets. (c) Stages of motor neuron specification. Generic motor neurons share features common to all motor neurons, such as the projection of axons away from the spinal cord. Columns are sets of motor neurons that project to distinct regions in the periphery. Divisions are binary subdivisions of columns. Pools are subsets of motor neurons within the LMC that innervate a single muscle target in the limb. (a) Derived from Dasen JS, Tice BC, Brenner-Morton S, and Jessell TM (2005) A Hox regulatory network establishes motor neuron pool identity and target muscle connectivity. *Cell* 123: 477–491.

along the rostrocaudal axis, and the projection patterns of the displaced motor neurons were assessed using retrograde labeling assays. After inversion of the neuron tube along the rostrocaudal axis, motor neurons were still capable of finding their appropriate muscle targets even though they entered the limb from inappropriate positions. Thus, the position in which a motor axon enters the limb is not the primary determinant of its projection pattern or the selection of its synaptic target. In addition, these observations are consistent with the view that aspects of motor pool identity are specified prior to limb innervation.

The intrinsic properties of neurons within a pool presumably allow motor axons to differentially respond to guidance cues in the limb. In experiments in which mirror-image duplications of the limb musculature have been generated, motor axons within a single pool innervate both the normal muscle target and the duplicated muscle. These observations reinforce the idea that motor neurons have intrinsic properties that respond to positional cues and also underscore the importance of limb guidance cues in defining motor axon trajectories. A major challenge over the past 20 years has been to identify the molecules expressed within motor neurons and the limb mesenchyme that control motor axon projection patterns and synaptic specificity.

Positional Information and Cell Type Specification in the Spinal Cord

Motor neurons, like other cell types in the spinal cord, acquire their identities in response to positional cues acting along the dorsoventral and rostrocaudal axis of the neural tube. The pathways controlling

motor neuron subtype identity involve both extrinsic signals, typically in the form of secreted morphogens, and intrinsic signals, in the form of cell type-specific transcription factors. In the mechanisms that control motor pool specification, the intrinsic signals are particularly relevant because transcription factors are differentially expressed by motor neuron subtypes and presumably regulate the downstream genes involved in motor axon guidance decisions.

A major function of extrinsic signals is to establish unique patterns of transcription factor expression in naïve neuronal cell types. Depending on the relative position of neural progenitors from the source of a secreted signal, cells within the neural tube are exposed to different levels of morphogens. In the ventral neural tube, distinct classes of progenitors are specified in response to secreted signals originating from surrounding mesoderm, including sonic hedgehog from the notochord and floor plate and retinoic acid from the paraxial mesoderm. Graded sonic hedgehog and retinoic acid signaling induces the patterned expression of transcription factors in progenitor cells along the dorsoventral axis (**Figure 2**). These initial patterns of transcription factor expression are further refined through the selective cross-repressive interactions between pairs of transcription factors that act to sharpen the boundaries between progenitor domains and ensure that each progenitor expresses a unique transcription factor profile.

Progenitor cells expressing a specific pattern of transcription factors give rise to distinct classes of postmitotic neurons, including motor neurons. After leaving the cell cycle, all spinal motor neurons express transcription factors that are critical for generic features of their identity. Transcription factors expressed by early postmitotic motor neurons include the homeodomain proteins Hb9, Lhx3, Isl1, and Isl2. Genetic analysis of mice lacking these transcription factors has revealed they are required in each of the subsequent steps in motor neuron differentiation as mice lacking these transcription factors show defects in motor neuron columnar and pool specification. Some of the transcription factors required for early aspects of motor neuron specification are subsequently used in the further diversification of motor neuron subtypes. For example, the LIM homeodomain protein Lhx3 is initially required in all motor neurons and also has later function in the specification of the nonsegmentally restricted motor column that projects to axial muscle.

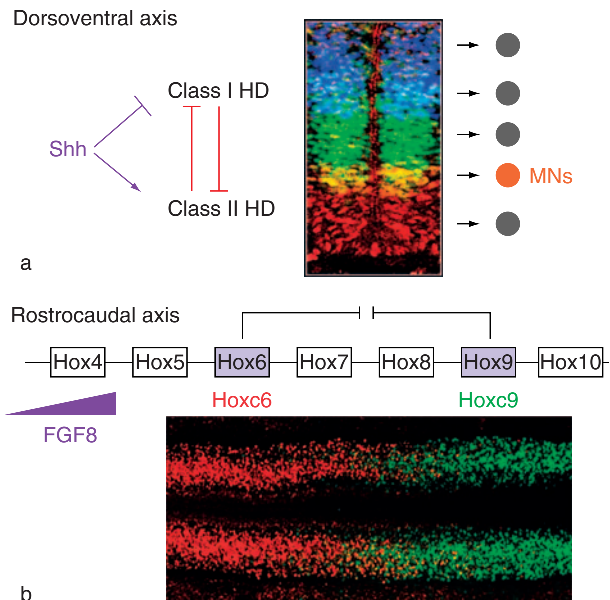

Figure 2 Specification of motor neuron (MN) subtypes along the dorsoventral and rostrocaudal axes. (a) Along the dorsoventral axis of the neural tube, motor neurons are generated in response to the graded activities of sonic hedgehog (Shh). Shh regulates the expression of transcription factors in progenitor cells. Class I transcription factors are induced by Shh, whereas class II transcription factors are repressed. Selective cross-repressive interactions between class I and class II transcription factors sharpens the boundaries between progenitor domains. Each of these progenitor domains gives rise to a distinct class of neuron, including motor neurons. Retinoic acid from the paraxial mesoderm also influences the pattern of transcription factors in neural tube progenitors (not shown). (b) Along the rostrocaudal axis, graded fibroblast growth factor (FGF) signaling induces the expression of chromosomally linked *Hox* genes in the neural tube. Genes located at one end of the cluster are expressed more rostrally, and genes at the opposite end are expressed caudally, in response to higher levels of FGF. The initial domains of Hox expression are further refined through selective cross-repressive interactions. Mutual exclusion of two Hox proteins is shown in top view of a longitudinal section through the spinal cord.

Establishing Patterns of *Hox* Gene Expression along the Rostrocaudal Axis

Although the transcriptional programs mediated by signaling along the dorsoventral axis of the neural tube define how motor neurons as a generic class are specified, additional signaling pathways are necessary for their further diversification into columnar and pool subtypes. Both the columnar and the pool identity of motor neurons requires that certain motor neuron subtypes are generated at specific rostrocaudal positions within the spinal cord. This allows motor neurons to be generated in proximity to their peripheral targets. For example, LMC neurons are generated selectively at limb levels of the spinal cord, while preganglionic column of Terni (CT) motor neurons are generated at thoracic levels in proximity to their synaptic targets in the autonomic nervous system. The columnar and pool identities of motor neurons have been typically defined by their cell body position within the spinal cord, their axonal trajectories, and late profiles of LIM homeodomain transcription

factor expression. However, the early signaling events that control motor neuron columnar and pool specification have only recently been explored.

How are motor neuron subtypes generated at distinct segmental levels of the spinal cord? One class of transcription factors known to be critical for establishing differences in cell identity along the rostrocaudal axis consists of members of the *Hox* gene family. In vertebrates, *Hox* genes encode a large family of homeodomain transcription factors consisting of 39 members organized into four chromosomal clusters. The expression patterns of individual *Hox* genes along the rostrocaudal axis of the spinal cord are closely related to the chromosomal position of a gene within a cluster – a principle called spatial colinearity (**Figure 2**). *Hox* genes at the 3′-end of a chromosomal cluster are expressed more rostrally in the embryo than genes at the 5′-end, which are expressed more caudally.

Similar to patterning events along the dorsoventral axis, *Hox* gene expression along the rostrocaudal axis is controlled by the actions of secreted signaling molecules which act in graded manner. Genes within a *Hox* cluster are sequentially activated in response to the activities of several signaling molecules, including fibroblast growth factors (FGFs), retinoids, and members of the transforming growth factor-β superfamily. Graded FGF signaling in particular appears to be important for the initial induction of *Hox* gene expression at brachial, thoracic, and lumbar levels of the spinal cord. An organizing region at the posterior end of the embryo, called Hensen's node in chick, is a source of FGF signaling, and as the node regresses caudally, more posterior regions of the spinal cord are exposed to FGF in higher concentration and over longer periods of time. Although FGF signaling appears to be essential in establishing the initial pattern of *Hox* gene expression in the neural tube, the final pattern observed in motor neurons is dictated largely by regulatory interactions between *Hox* genes.

Hox Proteins Function in the Specification of Segmentally Restricted Motor Columns

Several early studies have provided suggestive evidence that *Hox* genes are involved in specifying motor neuron subtype identities along the rostrocaudal axis. Experimental manipulation of mesodermally derived signals is known to affect the specification of segmentally restricted motor columns, such as LMC and CT motor neurons, and these changes in columnar fate are accompanied by alterations in the pattern of Hox protein expression. In addition, *Hox* genes are known to control cell type specification along the rostrocaudal axis of the hindbrain, and certain *Hox* mutants show defects in the projection of motor axons in the limb. However, one of the difficulties in relating the expression patterns of Hox proteins to motor neuron subtype identities was the lack of molecular markers that are specific for columnar subtypes.

The identification of genes expressed by segmentally restricted motor columns has permitted the analysis of the early steps in motor neuron diversification. LMC and CT motor neurons are generated at specific rostrocaudal levels and express unique molecular markers. LMC neurons can be defined by expression of retinaldehyde dehydrogenase-2 (RALDH2), an enzyme involved in retinoid synthesis, while CT motor neurons in chick selectively express bone morphogenetic protein-5, a member of the transforming growth factor-β superfamily. The expression of specific Hox proteins coincides with the position in which these molecularly defined columnar subtypes

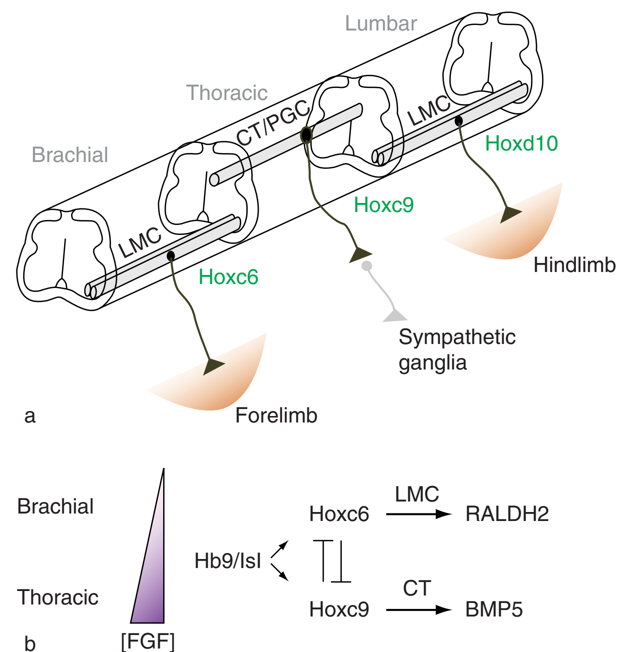

Figure 3 The role of Hox proteins in generating segmentally restricted motor neuron (MN) columnar subtypes. (a) Hoxc6, Hoxc9, and Hoxd10 are expressed in MNs (defined by Hb9 and ISL expression) at distinct rostrocaudal levels of the spinal cord. Each motor column has a distinct peripheral target. (b) Model indicating the roles of fibroblast growth factor (FGF) signaling and Hox expression in the specification of MN columnar identity. FGF establishes an initial pattern of Hox expression in the spinal cord. Cross-repressive interactions between Hoxc6 and Hoxc9 proteins refine the distinct Hox profiles of lateral motor column (LMC) and column of Terni (CT) neurons. Hoxc6 activity in brachial MNs directs expression of retinaldehyde dehydrogenase-2 (RALDH2; a marker for LMC neurons) and late features of LMC identity, whereas Hoxc9 activity in thoracic MNs directs bone morphogenetic protein-5 (BMP5) expression (a marker for CT neurons). Derived from Dasen Js, Liu J-P, and Jessel TM (2003) Motor neuron columnar fate imposed by sequential phases of Hox-c activity. *Nature* 425: 926–933.

are generated. Hoxc6 is expressed by brachial LMC neurons, Hoxc9 by thoracic CT neurons, and Hoxd10 by lumbar LMC neurons (**Figure 3**). The correlation in the pattern of Hox expression with LMC and CT columnar subtypes suggests that the same signals that control Hox expression may also specify columnar fates and that Hox proteins may function in the specification of columnar identities.

Consistent with a model in which *Hox* gene expression is controlled by graded FGF signaling, elevation of FGF levels at brachial levels of the spinal cord *in vivo* induces a pattern of Hox expression characteristic of thoracic levels. This switch in Hox expression patterns is accompanied by a conversion of brachial LMC neurons to a CT cell fate, as the CT marker bone morphogenetic protein-5 is ectopically expressed by brachial-level motor neurons. These effects of FGF on the columnar identity of motor neurons appear to be directly mediated by changes in *Hox* expression. Misexpression of Hoxc9 at brachial levels is sufficient to convert LMC neurons to CT neurons, while expression of Hoxc6 or Hoxd10 at thoracic levels can convert CT neurons to LMC neurons. In addition to these changes in motor neuron identity based on the expression of molecular markers, switching the pattern of Hox expression leads to alterations in the peripheral pattern of motor axon connectivity. For example, conversion of LMC to CT neurons forces limb-level motor neurons to project to sympathetic chain ganglia.

Hox proteins can transcriptionally cross-repress each other's expression, ensuring that columnar subtypes are generated only at specific segmental levels (**Figure 3**). Thus some of the mechanistic principles that govern dorsoventral patterning of the neural tube are shared in patterning along the rostrocaudal axis. One important difference is that while transcriptional cross-repression along the dorsoventral axis occurs in neural progenitors, transcriptional cross-repressive interactions between different Hox proteins occur predominantly in postmitotic cells. Nevertheless, these findings reinforce the view that cross-repressive interactions have critical roles in generating cellular diversity in the central nervous system.

Hox Transcription Factors and Motor Pool Identity

Studies on the columnar identity of motor neurons have provided evidence that Hox proteins contribute to neuronal specification along the rostrocaudal axis of the spinal cord. Many additional Hox proteins are expressed by motor neurons in patterns that do not coincide with the rostrocaudal positional boundaries of motor columns, suggesting additional roles in motor neuron diversification. The requirement for more than 50 motor pools to innervate each of the muscles in a vertebrate limb suggests a significant number of Hox proteins would need to be expressed by LMC neurons. Accordingly, of the 39 *Hox* genes, 21 are expressed in discrete subpopulations of motor neurons at brachial, thoracic, and lumbar levels of the spinal cord in a manner consistent with a role in motor pool specification.

In trying to understand the developmental programs that control motor pool specification, two organizational features of motor pools are particularly relevant (**Figure 4**). First, each motor pool occupies a stereotypic rostrocaudal position within the spinal cord. Thus some aspects of motor pool organization parallel the pattern of motor neuron columnar organization. Second, within a single segmental level, multiple motor pools can be present, and therefore some aspects of motor pool differentiation appear to emerge independent of the early signals that confer rostrocaudal positional information.

Identification of Motor Pools at Early Stages by Transcription Factor Expression

One of the difficulties in trying to define the pathways that control motor pool identity has been relating their anatomical organization with the patterns of Hox protein expression at the time that pools are specified. Motor pools have been defined classically by means of retrograde labeling assays performed after motor axons have reached their muscle targets, yet pool identities appear to be specified several days earlier in development. In addition, the pattern of Hox expression by motor neurons is complex; of the 11 Hox proteins expressed by brachial LMC neurons, a single motor neuron may express up to four different ones. To explore a role for Hox proteins in motor pool identity, what was needed was a set of molecular markers that are expressed by individual pools early in development.

Analysis of transcription factor expression has revealed that brachial and lumbar LMC neurons express an assortment of pool-specific transcription factors. Within the brachial LMC, anatomically defined motor pools can be molecularly defined by expression of the ETS transcription factor Pea3, the runt-related protein Runx1, and the POU-domain factor Scip (Pou3f1). Expression of these transcription factors defines motor pools that occupy stereotypic positions along the rostrocaudal axis and within a given segment. The expression of these pool-specific transcription factors has been used to ascertain the potential contribution of Hox proteins to the anatomical positioning and peripheral connectivity of motor pools.

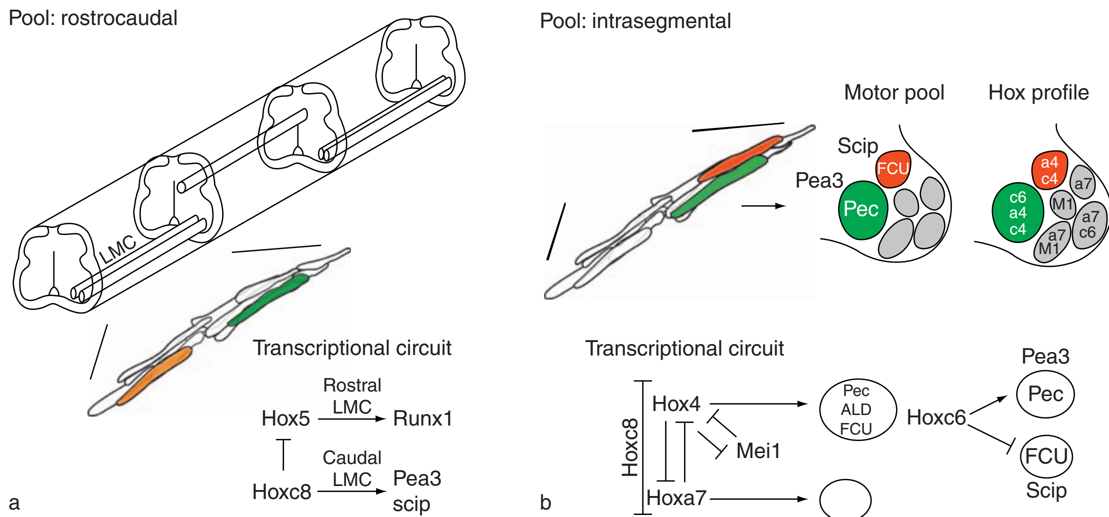

Figure 4 Regulatory networks of Hox proteins control motor pool identity and connectivity. (a) Hox proteins determine the rostrocaudal position of motor pools within the lateral motor column (LMC). At brachial levels of the spinal cord, cross-repressive interactions between Hox5 proteins and Hoxc8 establish the boundary between molecularly defined motor pools. Hox5 proteins are required to generate the motor pool that expresses the transcription factor Runx1 in rostral LMC neurons. Hoxc8 is required in caudal LMC neurons to generate the motor pools that express the transcription factors Pea3 and Scip. (b) Intrasegmental specification of motor pool identity. At a single axial level of the spinal cord, approximately six to ten pools are generated. Motor pools projecting to the pectoralis (Pec) and flexor carpi ulnaris (FCU) can be molecularly defined by expression of the transcription factors Pea3 and Scip, respectively. Both Pec and FCU pools express unique profiles of Hox expression. For simplicity, Hoxa4 and Hoxc4 are shown as Hox4, Hoxa5, and Hoxc5 are shown as Hox5; Mei1 is abbreviated as 'M1', Hoxc6 as 'c6', etc. The patterns of Hox expression in the Pec and FCU pools are established through a transcriptional circuit driven by Hox repressive interactions. Derived from Dasen JS, Tice BC, Brenner-Morton S, and Jessell TM (2005) A Hox regulatory network establishes motor neuron pool identity and target muscle connectivity. *Cell* 123: 477–491.

Hox Proteins and Motor Pool Rostrocaudal Positional Specification

Like motor columns, each motor pool occupies a stereotypic rostrocaudal position within the spinal cord. The position at which motor pools are specified appears to be determined by the pattern of Hox protein expression along the rostrocaudal axis. Brachial LMC neurons, defined by Hoxc6 and RALDH2 expression, can be further subdivided along the rostrocaudal axis by differential expression of Hox3, Hox4, Hox5, Hox7, and Hox8 proteins. For example, motor neurons in the rostral half of the brachial LMC express Hox5 proteins (Hoxa5 and Hoxc5) while motor neurons in the caudal half express Hoxc8. At the boundary between these two regions, expression of Hox5 and Hoxc8 are mutually exclusive.

The exclusive domains of Hox5 and Hoxc8 protein expression within the brachial LMC define a positional boundary between certain motor pools (**Figure 4**). The motor pool defined by expression of Runx1 is generated within the domain of Hox5 expression, whereas the pools expressing Pea3 and Scip are generated within the domain of Hoxc8 expression. Altering the pattern of Hox expression in these territories leads to changes in motor pool identity, defined by a switch in the molecular profile of pool-specific transcription factors. In addition, changing the pattern of Hox expression alters the peripheral connectivity of motor axons. For example, misexpression of Hoxc8 in the domain of Hox5 expression induces expression of Pea3 in rostral LMC neurons, and these ectopically generated Pea3 motor neurons project to the normal muscle target of this motor pool.

The mechanisms by which the rostrocaudal boundaries of motor pools are established by Hox proteins largely follow the mechanisms of motor neuron columnar identity; motor pool boundaries are established through selective cross-repressive interactions between Hox proteins. Rostral misexpression of Hoxc8 extinguishes expression of Hox5 proteins in a cell-autonomous manner; removing Hoxc8 expression from caudal LMC neurons leads to an expansion in the domain of Hox5 expression. In addition to Hox5 and Hoxc8, several other Hox proteins have rostrocaudal positional boundaries within the brachial LMC, and these boundaries likely correspond to the rostral and caudal limits of other motor pools.

Hox Proteins and the Intrasegmental Diversification of Motor Pool Identities

Multiple motor pools are generated at a given segmental level of the spinal cord, and this intrasegmental diversification appears to be established independent of the early patterning signals acting along the

rostrocaudal axis of the neural tube. Lineage analysis of motor neurons generated from a single rostrocaudal position has revealed that the fate of progenitors is not fixed because progenitors can give rise to motor neurons that occupy several different motor neuron subtypes. In addition, motor neurons that occupy a pool are initially dispersed within the spinal cord, and only relatively late in development do they cluster into discrete nuclei. Together, these observations suggest that there is no prepattern to motor pool specification at intrasegmental levels but that each motor neuron acquires a specific pool identity on a cell-by-cell basis.

Hox proteins appear to contribute to the intrasegmental diversification of motor pool identities. At late stages of development, the pattern of Hox protein expression becomes progressively restricted to distinct motor pools that occupy a given segment. The expression of the transcription factors Pea3 and Scip in brachial LMC neurons has been used to explore the function of Hox proteins in the intrasegmental diversification of motor pools because pools expressing these transcription factors are generated in overlapping segmental positions. The late patterns of Hox protein expression in pools defined by Pea and Scip expression suggest a model in which the intrasegmental diversification is driven by cross-repressive interactions between Hox4, Hox6, and Hox7 proteins (**Figure 4**). Experimental manipulation of the intrasegmental pattern of Hox expression alters the pattern of Pea3 and Scip expression by motor pools and the connectivity of their motor axons to their peripheral muscle targets.

The mechanisms that define the pattern of Hox expression at intrasegmental levels of the spinal cord appear to be distinct from those that control the pattern of Hox expression along the rostrocaudal axis. Along the rostrocaudal axis, cross-repressive interactions between Hox proteins are apparent shortly after motor neurons leave the cell cycle. In contrast, at intrasegmental levels, individual LMC neurons appear to express an initial cohort of Hox proteins based on their position along the rostrocaudal axis. Through continuous cross-repressive interactions that may be biased for the expression of one Hox protein over another, motor pools eventually express specific combinations of Hox proteins.

One corollary of this model is that biases in the efficacies of Hox cross-repression may explain differences in motor pool size. The number of motor neurons allocated to a specific pool is proportional to the size of the muscle it innervates. These differences in pool size emerge independent of trophic signals from the limb and thus appear to be intrinsically determined during an early phase in motor pool specification. Understanding the mechanisms controlling the differences in motor pool size may be relevant in understanding the allocation of cell numbers to specific neuronal fates in other regions of the nervous system.

Conclusions

Together, the studies described above provide evidence that members of the *Hox* gene family have important roles in the specification of motor pool subtypes. Yet many questions remain as to how Hox proteins contribute to the intrinsic programs that determine the specificity of motor neuron connectivity. Hox proteins function in many contexts throughout the embryo, and within the nervous system the same Hox factor can be expressed by multiple classes of neurons. These observations raise the question of how Hox proteins control gene expression in individual neuronal subtypes. Studies in *Drosophila* and other model systems have provided evidence that the specificity of Hox function is determined through interactions with other DNA-binding proteins. It remains to be determined whether Hox target specificity in motor neurons is controlled through interactions with additional motor neuron-restricted transcription factors.

Although these studies of motor pool specification have helped define some of the transcriptional networks that determine motor neuron identity, the pathways downstream of Hox proteins are less clear. The studies described earlier suggest that the combinatorial expression of Hox proteins in motor pools controls the expression of pool-specific transcription factors. Many of these pool-specific transcription factors may in turn control the expression of receptors which guide motor axons to specific muscle targets. Alternatively, Hox proteins themselves may directly regulate the expression of guidance molecules that are involved in intermediate choice points for motor axons projecting along the major axes of the limb.

Hox factors are expressed by several classes of neurons in addition to motor neurons, including sensory neurons and interneurons. One of simplest circuits in the spinal cord is the monosynaptic stretch reflex circuit, which in its most basic form consists of a motor neuron, a sensory neuron, and a muscle target. One possibility is that the precision of connections in this circuit arises from matching profiles of gene expression in motor neurons and sensory neurons. The expression of Hox factors in muscle sensory neurons parallels the segment-specific Hox patterns in motor neurons, raising the intriguing possibility that Hox factors are involved in the formation of sensorimotor circuits. The analysis of Hox function in sensorimotor connectivity provides a starting point for exploring more-complex levels of spinal circuitry, such as the local networks of interneurons and motor

neurons that give rise to rhythmic patterns of activity in the spinal cord and form the central pattern generators required for coordinate locomotor behaviors.

See also: Hox Gene Expression; Motor Neuron Specification in Vertebrates; Transcriptional Networks and the Spinal Cord.

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Neural Patterning: Midbrain–Hindbrain Boundary

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Neural Induction and Posteriorization

The nervous system arises from the neural plate, which is induced during gastrulation (neural induction). Initially, the whole neural plate is of anterior character, and later it acquires posterior values leading to the formation of forebrain, midbrain, hindbrain, and spinal cord (posteriorization). Neural identity is induced by the interplay of antagonists of the bone morphogenetic proteins (BMPs) and the direct inducing activity of fibroblast growth factors (FGFs). In addition to FGFs, other signaling molecules, such as Wnts, retinoic acid (RA), and Nodals, are among the signaling molecules that have been proposed to elicit caudalizing activity. In frog and fish, high levels of BMP activity suppress anterior neural development and conversely, abrogation of BMP activity can promote neural specification. However, evidence from chick suggests that suppression of BMP activity is not sufficient to induce neural identity and that earlier signals, most likely FGFs, promote a 'prospective' state which is followed by maintenance of neural identity by BMP antagonists. Data suggest a model in which inhibition of BMP signaling promotes induction of anterior neural ectoderm, whereas in addition to its function in posteriorization, FGF signaling is necessary for specification of posterior neural ectoderm independent of BMP activity. This model links the two concepts of 'neural induction' and 'posteriorization' of neural tissue closely together by the combinatorial role of FGF signaling.

Positioning of the Brain Primordia

Identification of posteriorizing factors has been difficult because the various signaling pathways that modulate early from anterior to posterior (AP) pattern are often involved in many other events by influencing each other. Wnt molecules have been studied extensively and have been found to be good candidates for involvement in AP regionalization. Neural ectoderm is first induced with anterior character, and the most rostral cells retain this. The cells of the anterior border of the neural plate (ANB) in fish or anterior neural ridge (ANR) in chicken and mouse, located at the rostralmost tip of the neural ectoderm, have been identified as a signaling source promoting anterior forebrain gene expression. In fish, one of the secreted proteins responsible for the activity of the ANB is Tlc, a member of the secreted Frizzled related protein (sFRP) family generally considered as Wnt antagonists. Implanted Tlc-releasing cells are able to restore anterior forebrain identity in embryos lacking endogenous ANB cells. In mouse, Fgf8 has been shown to be required in the ANR for induction of Foxg1 expression and influences cell survival, and in zebra fish Fgf8 is required for patterning anterior commissural territories derived from the anterior neural plate. In general, anterior neural identity is reflected by the ubiquitous expression of *otx2*, a later marker of the forebrain and midbrain primordia. Following the induction of anterior neural tissue, Wnt signaling has been shown to directly repress the anterior expression domain of *otx2* and induces the expression of the posterior marker *gbx1* (the functional homologue of *Gbx2* in fish) without Fgf or Nodal signaling from the adjacent germ layers – respectively the endoderm and the mesendoderm. The signal is Wnt8 emanating from the posterior end of the neural plate, the margin of the embryo. Regionalized expression of the posterior neural markers *gbx1* and *gbx2* positions the boundary between midbrain and hindbrain by mutual repression of *otx2*. Following the establishment of the Otx2–Gbx interface, an MHB-specific cassette of markers is induced around the MHB: Pax2 and Wnt1 anterior to the MHB in the Otx2-positive midbrain domain, Fgf8 posterior to the MHB in the Gbx-positive hindbrain domain, and Engrailed 1 and 2 spanning the whole midbrain–hindbrain territory (**Figure 1**).

Similar to the interface between *Otx2* and *Gbx* at the MHB, a mechanism for the formation of the prospective ZLI has been proposed during mid-somitogenesis. The interface between the anterior Six3 expression domain and a posterior Irx3 domain should determine the future position of the ZLI. Chick explant culture experiments have suggested that the position of this interface is also controlled by Wnt signaling. Wnt3a induces expression of Irx3 and represses Six3. In addition, in *Six3-/-* mice, the prosencephalon is severely truncated, and ectopic expression of Six3 in chick and fish embryos shows that Six3 is a direct negative regulator of Wnt1 expression. Nevertheless, the role of Six3 in positioning the ZLI is still debatable due to the subsequent rapid retreat of its expression domain to the most anterior part of the forebrain prior to *shh* expression in the ZLI. In addition, there are no *in vivo* data supporting this mechanism, and genetic loss-of-function phenotypes have yet to prove this

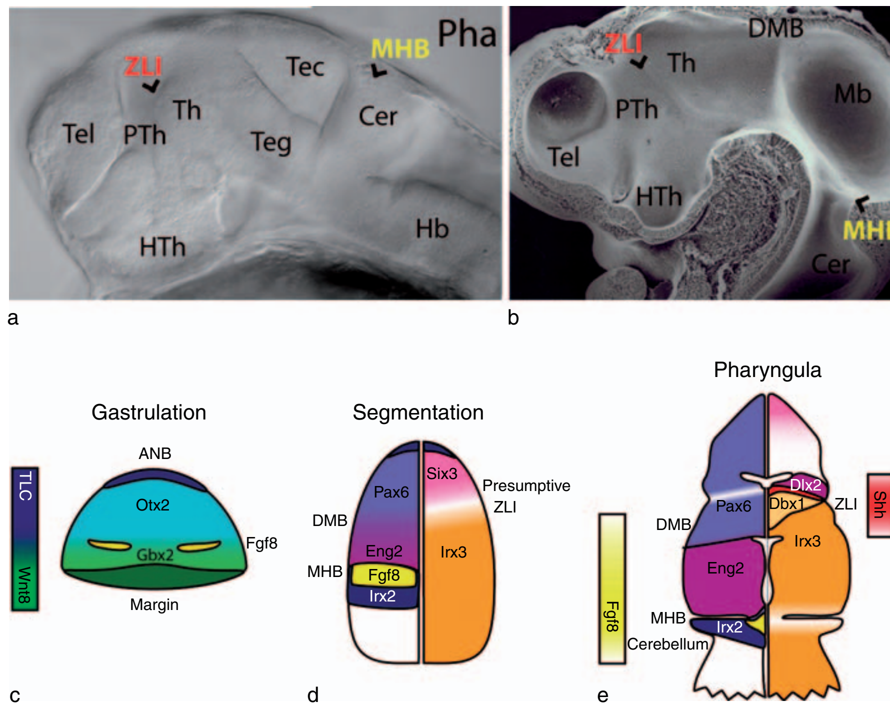

Figure 1 Positioning of organizing centers during development of the neural tube. (a and b) Comparative brain anatomy of a fish embryo (32hpf) and a chicken embryo (HH26) at the pharyngula stage. The two organizing centers, the ZLI (red) and the MHB (yellow), are located at morphological outstanding places (arrows). At gastrulation stages, mutual repression of Otx and Gbx positions the principal signal of the MHB organizer, Fgf8 (yellow wings in c). During the segmentation period (d), Anteriorly to the MHB, Fgf8 pattern the midbrain and posteriorly Fgf8 induces cerebellar fate via activation of Irx2 (shown in the left half of the embryo). At the same time, the forebrain organizer is positioned anteriorly to Irx3 in chick or Irx1 in mouse and fish. At the pharyngula stage (e), Fgf8 maintains the characteristics of the midbrain and cerebellum and sets the anterior border of the midbrain via repression of pax6. At the same time (shown in the right half of the embryo), Hh signaling from the ZLI induces the proneural genes in the forebrain (Dlx2 and Dbx1a). The range of the principal signals is shown in the columns located to the left and right of the schematic neural tube. Cer, cerebellum; Hb, hindbrain; HTh, hypothalamus; MB, midbrain; MHB midbrain–hindbrain boundary; PTh, prethalamus; Tec, tectum; Teg, Tectum; Tel, telencephalon; Th, thalamus; ZLI, zona limitans intrathalamica.

concept. Evidence from work done in mouse and fish suggests a rather different mechanism: The transcription factors Fez/Fezl in the prethalamic anlage form an interface with Otx1/2, which is expressed in the anlagen of the ZLI and thalamus. This interface coincides exactly with the anterior boundary of the presumptive ZLI. Subsequently, the expression of the thalamic Irx genes, such as Irx1b in fish, represses the ZLI territory and therefore sets the posterior boundary and gives the ZLI its characteristic tapering shape.

In contrast to the induction of Fgf8 at the MHB, sonic hedgehog (Shh) expression in the ZLI is gradually recruited from ventral to dorsal. Cell movement provides a limited contribution in the dorsal extension of *shh* expression as the ZLI matures. Our understanding is limited with regard to the mechanisms leading to this dorsoventral induction of Shh expression in the ZLI. Induction via ventral Hh signaling or via AP signaling takes center stage in these arguments. In a chick explant approach, it was suggested that Shh from the basal plate is required for the formation of the ZLI. However, induction of Hh expression is independent of Hh signaling, or indeed any ventral signaling, as shown by *in vivo* studies in fish. By taking advantage of zebra fish mutants that lack the entire basal plate of the neural tube and therefore lack ventral *Shh* expression in the midline, namely the one-eyed pinhead and cyclops mutants, establishment of the ZLI was observed as an independent process. Furthermore, all Hh signaling is dispensable for the formation of the ZLI, as shown by the slow muscle omitted mutant embryo (*smu*), which carries a mutation in the coreceptor Smoothened, lacking any Hh signaling. Although the ZLI appears narrower in *smu* mutant embryos compared with wild-type siblings, grafting of wild-type cells in an *smu* mutant background shows that Hh signaling from the ZLI still has the ability to regionalize the territory appropriately. One possible explanation for the differences between the results observed in fish and chick is that in the latter, experimentally induced reduction of ventral Hh signaling causes the ZLI to mature more

slowly. Alternatively, the positive feedback autoregulatory mechanism for *hh* expression, a plausible mechanism in chick, is less evident in fish. The persistence of *shh* expression in the ZLI in *smu* mutant embryos indicates that a positive feedback autoregulation is indeed of little importance in zebra fish.

Function of Local Organizers

As mentioned previously, posteriorization of the neural tube sets the rough position of the brain primordia. To build such a complex structure as the brain, further regionalization processes are required. Indeed, following the coarse AP organization set up during gastrulation, local organizers refine the neural plate. The concept of 'local organizers' describes specific groups of cells that can direct development of the surrounding tissue by signaling molecules. To define a cell population as an 'organizer,' the tissue has to fulfill defined characteristics:

1. The activity resides in a defined population of cells.
2. Removal of these leads to the lack of specific structures.
3. Translocation of an organizer population to an ectopic competent location such as by grafting causes the induction of an ectopic structure.
4. Organizers refine simple spatial organizations into a more elaborate pattern of subregions – a complex spatial diversification of fields. Solely ectopic provision of its organizing or so-called principal signal is able to mimic the function of an organizer population.
5. The efficiency range of organizers depends on the timing of their appearance, how their effective signaling molecules spread, and how signals are translated into specific values at the target sites.

The isthmic organizer forms at the boundary between midbrain and hindbrain and is the best understood local signaling center in the developing central nervous system. Its inductive properties were first described in chicken by grafting isthmic tissue into ectopic locations in the neural tube, resulting in the respecification of the neural tissue to ectopic midbrain and/or cerebellum. Implanted beads soaked with Fgf8 protein can mimic these effects and show that it is therefore the principal signaling molecule secreted by the MHB organizer. A requirement for Fgf8 in MHB development was demonstrated in zebra fish and mouse. The global knockout of Fgf8 in mouse fails to gastrulate and dies very early. In contrast, the zebra fish mutant *acerebellar* (*ace*) overcomes the gastrulation defect and allows the study of specific late functions of Fgf8, supported by antisense studies in fish and conditional knockout in mice. The lack of functional Fgf8 results in loss of the isthmic organizer and subsequently in a loss of the cerebellum. Fgf8 is required to maintain marker gene expression in the midbrain and isthmus but not to induce midbrain, which has already been set up during gastrulation stages. Moreover, the analysis of the midbrain in *ace* mutant fish shows that the MHB is required for AP polarization of the tectum, including the graded expression of ephrin ligands in the midbrain neuroepithelium, and for proper retinotectal map formation. In fish and mouse, Fgf8, together with Engrailed 2, is also necessary to maintain the position of the boundary between the diencephalon and the mesencephalon (DMB) by repressing the expression of *pax6*, a key regulator of forebrain development. Fgf8 secreted from the MHB organizer is also involved in patterning the anterior hindbrain. The most anterior hindbrain segment, namely rhombomere 1, which lies closest to the MHB, does not express any *Hox* genes. However, after transplantation to an ectopic location, within the hindbrain, rhombomere 1 tissue starts to express *Hox* genes. Consistent with this observation, it has been shown that both isthmic tissue and Fgf8 can inhibit expression of *Hox* genes in chick. It has also been shown that alternative splicing of Fgf8 accounts for the higher receptor-binding affinity of Fgf8b relative to Fgf8a, and for the unique ability of Fgf8b to transform midbrain into cerebellum. Taken together, these observations suggest that Fgf signaling from the MHB is required for repression of diencephalic fate, correct midbrain patterning, cerebellum induction, and anterior hindbrain patterning.

Initially, another secreted factor, Wnt1, is broadly expressed throughout the midbrain until its expression becomes restricted to the dorsal midline and a narrow band anterior to the MHB, abutting the expression domain of Fgf8 anteriorly. Wnt1 mutant mice show severe midbrain defects. In fish, Wnt1, Wnt10b, and Wnt3a are partially redundant in their capacity to regulate gene expression at the MHB, and they are required to maintain *pax2* and *fgf8*. In addition, Wnt1 is dependent on proper expression of Fgf8 and Lmx1b as well as Pax2 and Engrailed function during mid-somitogenesis and serves therefore as an example of cross-regulation of different signaling pathways in fish and chicken. However, ectopic application of Wnt1 protein does not show inductive effects comparable to those of Fgfs, suggesting a permissive role for Wnt signaling in pattern formation at the MHB and an important role in cell survival and proliferation.

The function of a further local organizer, the ZLI, has been studied in various species. Similar to the

MHB organizer, the ZLI is located at a boundary between the prethalamus and the thalamus, and members of various signaling families such as Fgfs, Wnts, and Hh are expressed at these structures. This organizing center has been characterized and it was found that ZLI exerts its function by releasing signaling molecules from the hedgehog family, sonic hedgehog-a (Shh-a), and additionally in fish Shh-b (formerly known as tiggy-winkle hedgehog). Hh signaling from the ZLI directly regulates the acquisition of the cellular identity in the diencephalon, namely the acquisition of prethalamic neuronal fate anterior to the ZLI and thalamic fate posterior to the ZLI. Lack of Hh signaling results in a loss of the expression domains and therefore in the loss of cellular identity.

As described previously, a definitive characteristic of an organizer is its ability to induce ectopic cell fate in host tissue following heterotopic transplantation. This capability has been shown for the MHB organizer, which can induce ectopic tectal and/or cerebellar structures according to the place where the organizer is grafted provided it is competent. The ZLI has yet to be confirmed as a true organizer in this way.

Boundaries and Lineage Restriction

The position of organizers often correlates with the position of constrictions in the neural plate. These constrictions are mainly manifestations of gene expression boundaries. To regulate organizer activity, a mechanism must exist that not only ensures the position of the organizer but also defines the actual number and position of cells contributing to the signaling center. One control mechanism is to restrict cell movement across boundaries, which subsequently results in the polyclonal formation of lineage restriction compartments.

A study that followed the fate of hundreds of cells in the developing MHB territory in fish showed a cell lineage restriction boundary between midbrain and anterior hindbrain that corresponds to the Otx–Gbx boundary. A study using an inducible transgenic marker in mice also indicates restricted mixing behavior between the midbrain, isthmic area, and the forming cerebellum. Therefore, the isthmus might show the characteristics of a compartment rather than a single boundary, at least on the dorsal aspects of the neural tube. Eventually, the dorsal part of the midbrain–isthmus boundary seems to allow a small number of cells to cross, although the mouse study could not exclude initiation of the lineage marker prior to the establishment of lineage restriction. Late morphological cell movement at the MHB has also been addressed in chicken. Cells from the dorsal isthmus populate the dorsal midline of the midbrain as well as the cerebellum, and application of Fgf8 protein triggers cell movement and expression of roof plate marker ectopically in, for example, the diencephalon. In summary, there is evidence for restricted cell movement at the MHB in the ventricular zone, and the isthmus may therefore form a separate compartment, but lineage restriction is not absolute and allows cell movement under certain conditions and at specific stages, such as matured neurons in the mantel zone.

Compartmentalization in the forebrain territory has been analyzed for the past 15 years mostly in chick. Various models have been proposed, correlating morphological data and gene expression domains. Analysis of boundaries in the forebrain revealed that there are only three true lineage restriction boundaries: the boundary between pallium and subpallium within the telencephalon, the boundary between the forebrain and midbrain, and the interface between the presumptive prethalamus and the thalamus – the ZLI. Interestingly, the boundary between the prethalamus and the ZLI as well as the boundary between the ZLI and the thalamus are lineage restrictive and, thus, the ZLI is a true compartment. In chick, the presumptive ZLI territory is flanked by the expression domain of lunatic-fringe (Lfng), a glycosyl transferase that modulates the activity of the Notch receptor. The wedge-shaped Lfng-free territory later narrows to a band marking the definitive ZLI territory. Whether horizontal intercalation of cells or differential growth of the adjacent territories causes this phenomenon is unclear.

First Step in Competence: Integration of the Signal by the Surrounding Tissue

The ability of a tissue to respond to signals from an organizer is called competence. With regard to signaling molecules, competence is first defined by availability of their receptors and connected downstream pathway. For the MHB organizer, Fgf receptors (FgfRs) are expressed in the presumptive territory of the midbrain and hindbrain, and ligand activation triggers the intracellular signal transduction cascade of the MAP kinase (MAPK). High levels of expression of FgfR1 can be detected directly around the boundary. Consequently, knockdown of the FgfR1 and downstream players of the MAPK pathway such as the MAPK and Erk2 causes defects in gene expression at the boundary and a loss of the MHB structure. In parallel, Fgf signaling activates a number of repressors that act in a negative feedback loop, such as the coreceptor Sef and the intracellular inhibitors Spry and MKP3. Functional studies in fish have shown that membrane-bound Sef acts as an inhibitor *in vivo*. This repressive activity is elicited by direct binding

to FgfR1 *in vitro*, resulting in prevention of autophosphorylation of the receptor, which is necessary for signal transduction. Sprouty was originally described in *Drosophila* as an inhibitor of growth factor signaling, and experiments in vertebrates confirmed this function with regard to Fgf signaling. Misexpression of Spry4 inhibits the MAPK pathway and consequently leads to the loss of the MHB territory – a phenotype comparable to the Fgf8 mutant *acerebellar*. At the level of the nucleus, competence to respond to Fgf8 signaling is specifically regulated in the neuroectoderm by the zebra fish *spiel-ohne-grenzen* gene encoding the zebra fish oct4 homologue.

For the Hh family of signaling molecules, expressed at the ZLI, the situation is more complex. The ligand-binding component of the receptor of the morphogen is Patched (Ptc), which, being induced by Hh, is a bona fide target gene of Hh signaling. After binding of Hh to Ptc, the signal-transducing component Smoothened mediates the Hh response, presumably via activation of the Gli genes. Loss of Hh signaling can be achieved by interfering with different players of the signaling pathway: by misexpression of a dominant negative form of Patched construct, lacking an extracellular binding site for Hh; analysis of the *smu* mutant embryos; or analysis of embryos treated by the Hh pathway inhibitor cyclopamine. In fish and chick, these approaches revealed a direct dependency of prethalamic as well as thalamic development on functional Hh signaling from the ZLI. Dlx2-positive neuronal precursors in the prethalamic territory as well as Dbx1a- and Gbx2-positive neuronal progenitors in the thalamus do not appear in the absence of Hh signaling, suggesting a definitive requirement for the signal.

A Further Aspect of Competence: Predetermination of Cellular Fate

The definitive fate of the competent tissue is defined less by the principal signal than by the set of transcription factors exerting this function upon activation by the signal. The homeobox transcription factor Pou2/Oct4 is expressed in the early MHB anlage and is disrupted in the zebra fish mutant *spiel-ohne-grenzen*. Besides an early role for regulation of MHB genes, Pou2/Oct4 has a permissive role in mediating hindbrain competence to respond to FGF signaling and therefore restricts influence of the organizer posterior to the MHB.

For Hox gene-mediated positional information, it has been shown that Pbx proteins are necessary and sufficient, and a further facet of its role as competence factor has been revealed: Engrailed, together with Pbx, is required for proper establishment of the midbrain territory. Similar to the Hox genes, Pbx binds to Engrailed and its absence leads to comparable phenotype with the Engrailed knockdown: downregulation of Fgf8 and caudal shift of the posterior forebrain into the presumptive midbrain territory (**Figure 2**).

Interestingly, a family of molecules that are able to modulate the response to signaling molecules and are asymmetrically expressed at boundaries has been identified: the Iroquois genes, which are involved in establishing neuronal prepattern in *Drosophila*. Irx2 is expressed posterior to the MHB in the presumptive hindbrain before the onset of Fgf8 expression and has been shown to mediate the competence of this region to form the cerebellum in response to Fgf signaling. Fgf8 via MAPK signaling is required to convert Irx2 from a transcriptional repressor to an activator: Thus, an activated form of Irx2 can convert presumptive tectum into cerebellum in the absence of FGF signaling. Irx2 fused to the engrailed repressive domain has the opposite effect when electroporated into the hindbrain: The entire cerebellum is transformed into

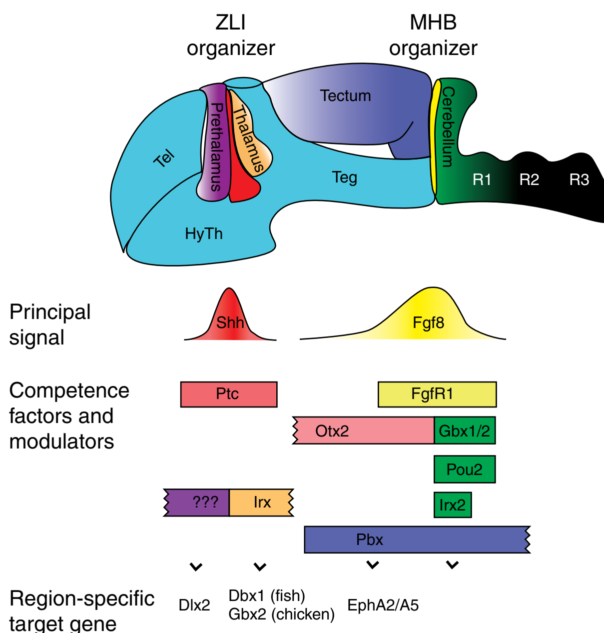

Figure 2 Function of the organizing centers. The lateral view of a schematic fish brain shows the influencing range and function of the two organizers: the ZLI organizer releases Shh (red), which binds to its receptor Ptc (light red; competence). In addition, the tissue around the organizer is already prepatterned (prethalamus, purple; thalamus, beige) and awaits the activation by Hh signaling to switch on the prethalamic program, induction of Dex2 expression, or thalamic program, induction of Dbx1a or Gbx2. Similarly, the tissue around the Fgf8-releasing center (yellow) is prepatterned and competent by the expression of the Fgf receptor 1. Anteriorly, in the tectum Fgf8 induces EphA2/A5, whereas posteriorly Irx2 and Pou2 (green) are needed to induce cerebellar fate.

midbrain tectum. This suggests that the midbrain/rhombomere 1 region has a tectal default state. The Irx2-related gene Irx3 is expressed exclusively posterior to the ZLI in the presumptive thalamus, and its ectopic misexpression anteriorly endows the prethalamus with thalamus-specific expression. This indicates that the Iroquois genes mediate positional competence on either side of signaling centers such as the MHB organizer and the ZLI organizer. Interestingly, the expression pattern of the Iroquois genes is independently induced of the principal signal and is more likely directly established by the early Wnt gradient in the neural plate. Further confirmation of this model derives from the finding that in fish, when wild-type cells are transplanted into a *smu* mutant embryo, they start to activate autonomously the proper gene expression with regard to the position of the ZLI, suggesting a correct prepattern independent of Hh signaling. Thus, principal signals from the organizers trigger the timing of the initiation of the downstream cascade rather than determination of the definitive fate of the surrounding tissue.

Functional Range of Organizers Is Determined by the Receiving Field

Apart from accurately controlling expression of the signaling molecule, a further way to control the functional range of signaling molecules has been identified. The receiving tissue actively regulates the range and the steepness of gradients from molecules released from source tissue. These mechanisms are the facilitation of signal spreading by modulation of extracellular matrix components, uptake of the signal molecule and direct degradation by 'restrictive clearance,' or uptake and re-release of signaling molecules by 'planar transcytosis.'

For signaling molecules in general, it has been shown that components of the extracellular matrix, such as the heparan sulfate proteoglycans (HSPGs), influence signaling range. For Fgf signaling, HSPGs function as coreceptors in the receptor complex and facilitate spreading of Fgf molecules through the extracellular space (ECS) in *Drosophila*. Consistently, enzymes involved in the biogenesis of heparan sulfate chains were found to be important for FGF signaling in flies and in mice. In mouse, a conditional knockout of Ext1, a glycosyltransferase involved in chain elongation of HSPGs, causes defects in the inferior colliculi and the cerebellum, a phenotype reminiscent of a loss of Fgf-dependent MHB function. A mutation in the heparan sulfate copolymerase *tout velu* and a mutation in the cell surface HSPG *dally* cause a decrease of movement of Hh molecules from their site of production. Furthermore, digestion of HSPGs by heparinase treatment strongly reduces the level of Shh signaling range in mice.

Advances have been made in understanding how signaling factors spread dynamically in embryos on a subcellular level. Identification of cellular components involved in signaling will yield a full understanding of the inductive mechanisms involved. The direct observation of tagged versions of signaling molecules has been correlated with the indirect evidence of the induction of gene expression. For Fgf8, an increase in internalization through activating Rab5-dependent endocytosis leads to a shortened functional range of the signaling factor. Conversely, reduced Fgf8 uptake by downregulation of Rab5 activity causes a wider signaling range of Fgf8 and subsequently a broader induction of target genes, such as Spry4 or the Ets transcription factors Erm and Pea3, a mechanism referred to as 'restrictive clearance' (**Figure 3**).

In the *Drosophila* wing, similar observations were made for wingless, for which different internalization rates operate posterior and anterior to the signaling center, which leads to different signaling ranges and creates an asymmetric response. By analogy, endocytosis might regulate the difference between

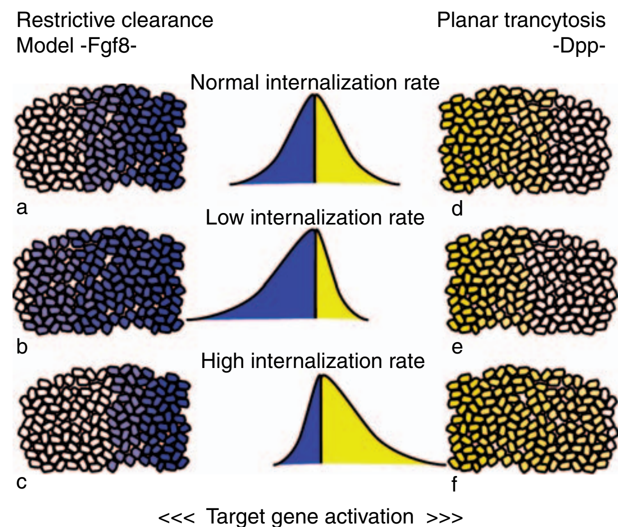

Figure 3 Influence of the competent tissue on the range of a signaling center. During gastrulation, in zebra fish, the range of Fgf8 is directly influenced by the capacity of internalization: Low internalization leads to a broader activation of the target gene (blue), whereas enhanced uptake leads to a restriction of target gene activation (c). Internalization is linked to degradation and restrictive clearance of the principal signal. In *Drosophila*, in wing imaginal discs, the signaling factor decapentapledgic (Dpp) behaves exactly the opposite: Low uptake restricts very strongly the induction of the target gene shown in yellow (d and e), whereas a higher level of internalization leads to a broad activation of target genes (f). Internalization is therefore linked to the propagation of the signaling factor and called planar transcytosis.

long-range activity of Fgf8 anterior to the MHB and short-range function in cerebellar induction and Hox gene repression. A further concept for signal spreading is provided by studies of the signaling molecule Dpp in the *Drosophila* wing imaginal disk. A higher rate of internalization led to a further distribution due to the fact that Dpp is only partially internalized for degradation, but a significant amount of the factor is released again. Thus, a planar transcytosis model allows the possibility that tight control of signaling range by the balance between degradation and release is determined by the receiving tissue as well as by the level of signal. It will be interesting to determine whether planar transcytosis exists in vertebrate embryos and whether this mechanism is employed in organizer signaling.

In this article, we described the formation and the function of two organizing centers in the early neural plate, the MHB organizer and the ZLI organizer. Apparently independent of organizer activity, the neural tube shows an initial expression pattern of modulating and determining factors, anticipating the actual fate of the regions. The organizers define the exact time points when these fates are going to be realized. Subsequently, the surrounding tissue reacts to the signal and initiates the downstream program. Furthermore, the surrounding tissue can influence the signaling range directly. The elaborate interplay between modulating factors and organizer activity sets the stage for the next level of complexity during brain development. It is obvious that this is only a next step and that many more have to follow to build as complex a structure as the human brain. Nevertheless, the prime importance of the exact timing of organizer activity is manifest, and alteration in these early events leads to gross malformation of the brain.

See also: Forebrain Development: Holoprosencephaly (HPE); Forebrain Development: Prosomere Model; Forebrain: Early Development; Midbrain Patterning; Sonic Hedgehog and Neural Patterning.

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Midbrain Patterning

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The midbrain, or mesencephalon, is defined by embryology. It is the middle of the three primary cerebral vesicles of the vertebrate neural tube, lying between the forebrain and hindbrain vesicles. The neural tube constriction formed at the junction of the midbrain and hindbrain vesicles is called the isthmus. This tissue harbors a major signaling center that patterns the adjoining midbrain and hindbrain. No comparable anterior signaling center has yet been identified at the junction between the midbrain and forebrain vesicles. By gene expression, however, the transition between dorsal midbrain and caudal forebrain is very abrupt, suggesting the presence of an important developmental boundary. Studies of cell lineage have provided evidence that both the isthmus and the dorsal midbrain–forebrain junction form barriers to cell mixing. Whether the midbrain vesicle constitutes a true developmental compartment, however, remains unresolved.

Pattern of the Adult Midbrain

The adult midbrain comprises a dorsal tectum (Latin for 'roof') and a ventral tegmentum (from 'tegmen,' which is Latin for 'cover'). In mammals, the cerebral peduncle, a fiber bundle of descending neocortical axons, is found beneath the tegmentum at the base of the midbrain. The tectum is dominated by a single structure, the optic tectum, or, in mammals, the superior colliculus. The optic tectum is a sensorimotor structure whose roles include a 'sentinel' function of orienting the head and eyes toward salient stimuli. It is organized into layers, with superficial layers receiving sensory input and deep layers containing premotor cells controlling eye and head movements. These sensory and motor layers contain sensory and motor maps that are in register across the layers and can be viewed as forming a coordinated representation of eye-centered external space when projected onto the tectal surface. The dominant sensory input to the optic tectum is from the retina. Retinal terminations form a coherent retinotopic map across the contralateral optic tectum, with the retinal temporal–nasal axis aligned with the tectal anterior–posterior axis and the retinal ventral–dorsal axis corresponding to the tectal medial–lateral axis. In addition to its intrinsic role in sensorimotor processing, the optic tectum serves as a relay for visual information traveling to the forebrain. The dorsal midbrain also contains an ascending relay for auditory and lateral line sensory information. This posterior midbrain structure is called the inferior colliculus in mammals, the mesencephalicus lateralis dorsalis nucleus in birds, and the torus semicircularis in other vertebrates ('torus' is Latin for 'bulge').

The ventral midbrain contains a complex constellation of nuclei, many of which are involved with motor system function. Among these are the red nucleus, which is part of the cerebellar motor system, and the nigral complex, a basal ganglia component that includes the dopamine neurons of the nigrostriatal and mesolimbic systems. The ventral midbrain also contains the most anterior motor neurons of the vertebrate nervous system. These are the somatic motor neurons of the oculomotor nucleus, which innervate extraocular muscles, and the visceral motor neurons of the Edinger–Westphal nucleus, which supply the ciliary parasympathetic ganglion. The oculomotor nucleus and the Edinger–Westphal nucleus are together called the oculomotor complex. Their motor axons travel to the eye's orbit in the third cranial nerve.

Early Midbrain Patterning: Boundaries and Signals

The regionalization of the brain into forebrain, midbrain, and hindbrain occurs early in gastrulation through the action of axial signals that organize the embryonic body plan. Once regionalized, the brain vesicles are patterned largely independently by local organizing centers. In the midbrain, the presence of two such organizing centers, the isthmic organizer (IsO) at the midbrain–hindbrain boundary and the rostral floor plate (rFP) of the ventral midbrain midline, is well established. The roof plate (RP), which runs along the dorsal midline of the midbrain, is also enriched in known developmental signaling molecules and is likely to serve as a midbrain signaling center.

Establishment of the Isthmic Organizer

Midbrain patterning depends critically on the establishment of the IsO. The IsO is initially formed anterior to the physical constriction of the midbrain–hindbrain junction. By gene expression, the IsO falls precisely at the posterior expression boundary of *OTX2*, a vertebrate homolog of the *Drosophila orthodenticle* gene, and the anterior limit of a second homeobox gene, *GBX2*, a vertebrate homolog of the *Drosophila* gene *unplugged* (**Figure 1(a)**). Gain- and loss-of-function

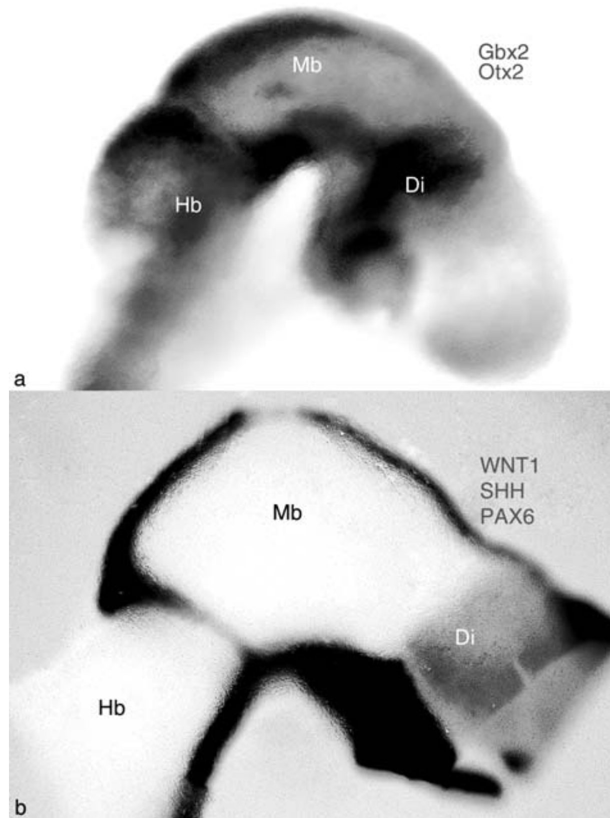

Figure 1 Identification of the major regional boundaries of the embryonic midbrain by gene expression for transcription factors and signaling molecules. (a) Plane of apposition of *Otx2* and *Gbx2* homeobox gene expression identifies the midbrain–hindbrain junction and the location of the isthmic organizer. Side view of an embryonic day 9 mouse prepared for whole mount *in situ* hybridization. Anterior is to the right. *Otx2* (blue) is expressed throughout midbrain and into forebrain, whereas *Gbx2* (brown) labeling is in hindbrain. (b) Polygon formed by gene expression markers of midbrain boundaries outlines the right midbrain of a chick embryo. Whole mount *in situ* hybridization of dissected stage 17 chick brain oriented with anterior to the right and probed for *WNT1*, *SHH*, and *PAX6* gene expression. The diencephalon–midbrain boundary is identified by *PAX6* expression in the diencephalon, the ventral midbrain midline is marked by *SHH* labeling, and the isthmic organizer and the roof plate are demonstrated with *WNT1* gene expression. Di, diencephalon; Hb, hindbrain; Mb, midbrain.

experiments suggest that mutual repression between *OTX2* and *GBX2* is critical for the positioning and maintenance of the IsO.

A number of transcription factors are expressed within the midbrain and at the IsO and are required for the specification of the IsO and the midbrain. These include the paired box genes *PAX2/5/8*, the engrailed homeobox genes *EN1/2*, and the LIM homeobox gene *LMX1B*. In addition, WNT and FGF8 signaling molecules (WNT1 and FGF8/17/18) are expressed by the IsO (**Figure 1(b)**). The initial induction of these IsO genes is thought to depend on the mesendodermal tissues lying subjacent to the neural tube. Subsequently, within the neural tube, cross-regulation among *EN1/2*, *PAX2/5/8*, *WNT1*, and *FGF8* is required to establish and maintain IsO identity. Many of these genes are at first broadly expressed and progressively become restricted to partially overlapping but distinct domains of the isthmus, the posterior midbrain, and rhombomere 1 of the hindbrain. In mice, the loss of *Pax2/5/8*, *En1/2*, *Wnt1*, or *Lmx1b* function results in variable phenotypes depending on the genetic background. The most severe of these phenotypes is a partial to complete deletion of the IsO and the adjacent midbrain and anterior hindbrain.

Midbrain Patterning by FGF8

FGF8 at the IsO is expressed in a band around the anteriormost hindbrain immediately behind a ring of WNT1 expression. Transgenic mouse analyses, quail–chick chimeras, and FGF8 delivery by plasmid electroporation or ligand-soaked bead have demonstrated that FGF8 signaling is critical to midbrain patterning. In midbrain, FGF8 delivery shifts the isthmus forward and converts midbrain into hindbrain. In caudal diencephalon, FGF8 delivery induces an ectopic IsO (*EN1/2+*, *WNT1+*, and *FGF8+*) and the ectopic IsO converts the surrounding diencephalon into midbrain tissue (**Figure 2(b)**). This transformation can be quite dramatic, producing a full midbrain duplication in extreme cases. Unlike the native midbrain, the duplicated midbrain has its IsO at its anterior end, resulting in anterior–posterior patterning that is mirror image to that of the native midbrain. This induction of an ectopic midbrain cannot be produced throughout forebrain; it can only be elicited caudal to the zona limitans intrathalamica (zli), a signaling center that bisects the diencephalon. Evidence suggests that the transition from permissive (post-zli) to nonpermissive (pre-zli) tissue may be controlled by the expression domains of the iroquois homeobox gene *IRX3*, expressed posterior to the zli, and the sine oculis homeobox gene *SIX3*, expressed anterior to the zli.

The Forebrain–Midbrain Junction

Genetic fate mapping studies have shown that a lineage restriction boundary preventing the mixing of midbrain and forebrain cells is established at the diencephalon–midbrain boundary (DMB) by embryonic day (E) 9.5 in the mouse. Interestingly, this boundary may fall precisely along the midbrain–forebrain junction only dorsally. Ventrally it is shifted anteriorly into the subthalamic region. Interactions between the midbrain–hindbrain junction genes *EN/PAX2/FGF8* and the paired box homeobox gene

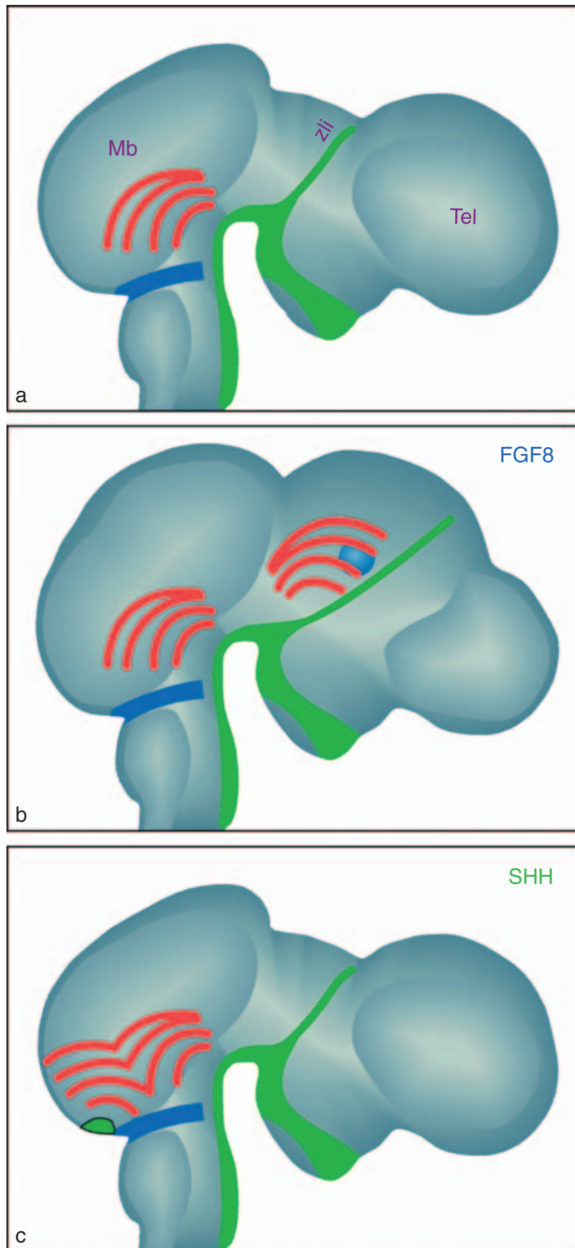

Figure 2 Regulation of midbrain pattern formation by FGF8 and Shh. (a) Chick brain at embryonic day 6, illustrating FGF8 expression at the midbrain–hindbrain boundary (blue) and Shh expression at the ventral midline and extending up the zona limitans intrathalamica (zli) in diencephalon (green). The anterior–posterior polarity of the midbrain is illustrated for ventral midbrain by a midbrain arcs schematic (red). (b) Exogenous FGF8 delivered to the diencephalon induces an ectopic isthmic organizer and a duplicate midbrain with a polarity mirror image to that of the native midbrain. (c) Ectopic Shh can ventralize dorsal midbrain and induce a duplicate set of midbrain arcs. Mb, midbrain; Tel, telencephalon.

PAX6, which is expressed in the diencephalon, have been shown to regulate DMB positioning in birds and fish. Thus, the establishment of the DMB critically depends on the prior specification of the IsO.

Induction of the Midbrain Floor Plate

The rFP and the underlying axial mesoderm (notochord and prechordal plate) express the signaling molecule Sonic hedgehog (Shh), a principal architect of ventral midbrain patterning. The origin of the floor plate and its mode of induction have long been a matter of controversy. A dual origin for spinal cord floor plate is indicated by zebra fish mutant analyses and quail–chick chimera experiments which show that the medial floor plate is derived from the node, expresses Shh, and helps induce lateral floor plate in adjoining neural tube. In the zebra fish, the specification of the medial floor plate depends on nodal signaling. Shh signaling is required for the maintenance but not the induction of the medial floor plate.

The rFP of the midbrain eventually forms a complex, wedge-shaped structure – one much broader than the floor plate of spinal cord and hindbrain. The rFP is likely to be induced by nodal and Shh signaling derived from a transient contact between the prechordal plate and the midbrain anlage. Subsequently, the notochord is briefly apposed to the ventral midbrain, but its rapid retraction strongly suggests that, unlike the arrangement in spinal cord in which notochord and floor plate collaborate on cell type specification, the rFP alone provides patterning signals for rFP expansion and the specification of ventral midbrain cell types. Fate mapping studies have shown that the rFP contains a mixed population of cells that at least in the chick incorporates a unique set of cells from a region anterior to the node called area a.

Roof Plate

The midbrain RP expresses the rich collections of WNT and BMP signaling molecules and ZIC and LMX transcription factors that are found in roof plate at other axial levels of the nervous system and that are thought to contribute to dorsal neural tube patterning. In midbrain, loss-of-function experiments for these molecules have only presented extreme phenotypes, either midbrain deletions or exencephaly, or no apparent patterning effects, possibly reflecting signaling redundancy. Consequently, the contributions of the midbrain RP to midbrain patterning remain obscure.

Interactions among Midbrain Organizers

Despite the complete orthogonality of the IsO and the rFP signaling centers, there is clear evidence for cross-regulation in their construction and maintenance. When Shh signaling is perturbed, the midbrain–hindbrain junction is disrupted, allowing mixing of midbrain and hindbrain cells. Moreover, in the mouse, loss of Shh

leads by E12.5 to a severe loss of *Wnt1* and a complete loss of *Fgf8* in the IsO. Interactions between the IsO and the RP include a direct contribution of midbrain–hindbrain boundary cells to the developing midbrain roof plate.

Ventral Midbrain Patterning

Medial Longitudinal Fasciculus

The first neurons to be born in ventral midbrain are part of a cohort of cells that straddle the midbrain–forebrain junction and contribute to the interstitial nucleus of Cajal and the nucleus of Darkschewitsch of the adult. The axons of these early born neurons travel posteriorly in a paramedian position and pioneer a major fiber tract that corresponds to the medial longitudinal fasciculus of the mature brain stem.

Midbrain Arcs

The embryonic midbrain is organized into a series of longitudinal territories arrayed bilateral to the ventral midline. These territories were first identified in the mantle layer of the chick embryonic midbrain with acetylcholinesterase histochemistry, which identifies postmitotic neurons in embryonic brain. Because these longitudinal territories constitute acetylcholinesterase-rich columns and have arcuate shapes, which are accentuated by the cephalic flexure, they are called midbrain arcs. The acetylcholinesterase-poor columns between the arcs also contain neurons and are referred to as interarcs. The arcs are numbered 1–5, from medial to lateral, and the interarcs are identified by their flanking arcs. Each arc and interarc expresses a unique signature of transcription factors and neurotransmitter-specific genes. For example, arcs 2 and 3 are enriched in the GABA synthetic enzyme *GAD2* and the zinc finger transcription factor *GATA2*, whereas the two-thirds interarcs expresses the transcription factor gene *PAX6* (**Figure 3**). Combined molecular and tract-tracing studies have shown a clear relationship between the first arc and nuclei of the adult midbrain. The first arc contains primordia for the oculomotor complex and the red nucleus. These primordia are separated within the first arc along the ventricular–pial axis, with the primordium of the oculomotor complex next to the ventricular layer and that of the red nucleus more pial. The possible nuclear fates of the lateral arcs and interarcs are not clear, in part because of the absence of selective markers for many adult ventral midbrain structures. Resolution of this important question will likely require genetic fate-mapping experiments.

The ventricular zone of ventral midbrain progenitor cells also contains arcuate territories. These territories are readily demonstrated as arcuate periodicities in the expression patterns of signaling molecules, including

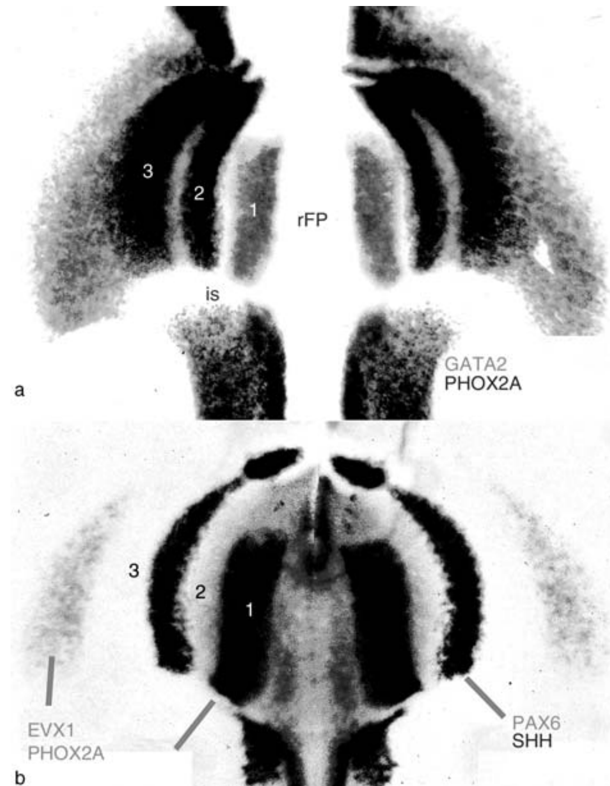

Figure 3 Arcuate organization of the embryonic midbrain tegmentum demonstrated by transcription factor gene expression. Illustrated are dissected flat mounts of embryonic day 5 chick brain stem prepared for *in situ* hybridization. The tissue documentation is arranged with the ventral midline oriented vertically, the midbrain arcs 1–3 identified on the left side only, and the isthmus (is) and hindbrain (hb) shown at the bottom of the panel. (a) *PHOX2A* labeling (brown) identifies the oculomotor complex of the most medial, or first, arc, whereas *GATA2* message enrichment (blue) distinguishes arcs 2 and 3. (b) Panel of three homeobox genes indicates regularly spaced arcuate territories (blue) corresponding to arc 1 (*PHOX2A*), interarc 2/3 (*PAX6*), and interarc 3/4 and arc 4 (*EVX1*). Gene expression for *SHH* (brown) illustrates the balloon-like expansion of the *SHH*-rich floor plate during midbrain development. rFP, rostral floor plate.

the WNT ligands WNT5A and WNT7A and ligands of the NOTCH receptor. The ventricular zone periodicities and the mantle layer arcs lie in radial registration, suggesting that midbrain arc patterning is set up in the ventricular zone and that arcs are populated by direct radial migration. Intracellular dye labeling studies demonstrating that radial clusters of coupled cells extend into the ventral midbrain mantle layer support this model.

Shh misexpression experiments have shown that the entire midbrain arc pattern, including the ventricular zone periodicities, can be generated by an ectopic source of Shh (**Figure 2(c)**). These ectopic arcs have precisely the same relative spatial positioning and the same molecular identity as the native arcs. The size

and shape of the ectopic arcs, however, are controlled by the size and shape of the ectopic Shh source. This type of pattern regulation is predicted by morphogen-based positional signaling and provides support for an 'action-at-a-distance' mode of Shh signaling in midbrain development. A specific requirement for Shh in the generation of the midbrain arcs and the specification of their constituent cell types has been demonstrated in loss-of-function experiments in chick and mouse.

At a morphological level, the midbrain arcs are the midbrain continuation of an embryonic columnar organization that runs from the spinal cord through brain stem to the subthalamic region of the caudal diencephalon. At a molecular level, however, the midbrain arc patterning mechanism shares only some features with those of the caudal central nervous system, and there is no coherent mapping that carries the molecular signature of the spinal cord domains V0–V1–V2–MN–V3 forward to the arc and interarc organization of the midbrain.

Oculomotor Complex

The midbrain oculomotor complex (OMC) is a derivative of the first arc. The earliest specific marker for these cells is the paired-like homeobox gene *PHOX2A*, which can be detected in still dividing ventricular zone precursor cells. In mice null for *Phox2a* function and in the zebra fish *phox2a* mutant *soulless*, the oculomotor nucleus and the nearby trochlear nucleus of anterior hindbrain are not generated. OMC neurons also express PHOX2B, which shares an identical homeodomain with PHOX2A, and the LIM homeobox gene *ISL1*, a pan-motor neuron marker, but they do not express the homeobox gene *HLXB9*, which is found apparently in all somatic motor neurons posterior to the trochlear nucleus. In this regard, the midbrain OMC is more akin to the branchial and visceral motor neurons of hindbrain than the somatic oculomotor neurons of the hindbrain abducens nucleus. Phox2b is required for the development of the hindbrain branchiovisceral motor neurons but not for the generation of OMC. Experiments in mouse in which *Phox2a* coding sequence is replaced by that of *Phox2b* demonstrate only a partial rescue, suggesting that the nonhomeodomain sequence differences between *Phox2a* and *Phox2b* are important for OMC development.

The human midbrain oculomotor system and its peripheral targeting are affected in many congenital cranial dysinnervation disorders. In one of these, CFEOM2 (congenital fibrosis of the extraocular muscle 2), patients present a bilateral ptosis (drooping eyelids) with eyes primarily in an exotropic (outward-looking) position. This presentation indicates that third cranial nerve motor function, which in humans includes innervation of the levator palpebrae superioris (upper eyelid muscle), is lost. Gene mapping studies have identified three distinct *PHOX2A* mutations in CFEOM2 patient families, at least one of which appears to be a null.

Red Nucleus

The red nucleus primordium is identified in the first arc by its specific expression of the POU homeobox gene *POU4F1/BRN3A*. Gene targeting studies have demonstrated that the absence of *Pou4f1* gene function leads to loss of the red nucleus by birth. In mice mutant for the homeobox gene *Emx2*, the *Pou4f1*-positive neurons of the red nucleus are also generated, but they are lost earlier – by midgestation.

Midbrain Dopamine Neurons

Midbrain dopamine neurons are born in ventromedial midbrain within the Shh-expressing ventricular zone of the rFP. Specific sites of origin likely include the ventral midline and the ventricular zone overlying the first arc. Some dopamine neurons remain medially to form the A10 complex of the ventral tegmental area. The A9 and A8 dopamine cell groups of the substantia nigra and retrorubral area are populated by a reelin-dependent lateral migration. Studies of transcription factors involved in midbrain dopamine development have identified a large number of genes that regulate distinct aspects of dopamine neuron production, differentiation, and survival. These factors include a suite of homeodomain transcription factors, En1/2 and Lmx1a/b, that are also expressed in the IsO or rFP, and the nuclear receptor Nr4a2/Nurr1, which is required for expression of the dopamine synthetic enzyme tyrosine hydroxylase. Particularly notable among these factors is the paired-like homeobox gene *PITX3*, which is a uniquely specific marker of apparently all midbrain dopamine neurons. In mice lacking *Pitx3* gene function, there is a severe reduction in midbrain dopamine cell generation that particularly affects the A9 dopamine cell group.

Nuclei Not of Midbrain Origin

Classical and modern embryology have shown that some nuclei found at the midbrain–hindbrain junction in the adult brain are clearly of hindbrain origin. These include the trochlear nucleus of the fourth cranial nerve and the serotonin cells of the raphe system. The cholinergic isthmic nucleus of birds, which maintains reciprocal connections with the optic tectum, is also generated in hindbrain. A hindbrain origin is therefore possible for its predicted mammalian homolog, the parabigeminal nucleus.

Dorsal Midbrain Patterning

The tectal–tegmental boundary is readily demonstrated by many developmental control genes that mark dorsal midbrain selectively, including the paired box homeobox genes *PAX3*/*7*, the homeobox gene *DBX1*, and the nuclear receptor *NR2E1*. The possibility that this sharp tectal–tegmental boundary may form a cell lineage boundary has not been addressed, but tissue culture experiments have shown that ventral cells progressively lose their capacity to intermix with dorsal cells. As is true for the dorsal spinal cord, dorsal midbrain identity can be switched to a ventral character by ectopic expression of Shh. Unlike the dorsal spinal cord, however, dorsal midbrain growth and specification are dependent on ventral midbrain Shh signaling. In mice lacking *Shh* or the Shh effector *Smo*, the dorsal midbrain is severely reduced in size and an early molecular marker of dorsal midbrain identity, *Dbx1* gene expression, is lost. The molecular mechanism for this Shh-dependent effect is unknown, but because of the distances involved, it seems likely to involve intermediary signals. One candidate intermediary is *Fgf15*, which is expressed in embryonic dorsal midbrain and is largely lost from this tissue in the *Shh* mutant mouse.

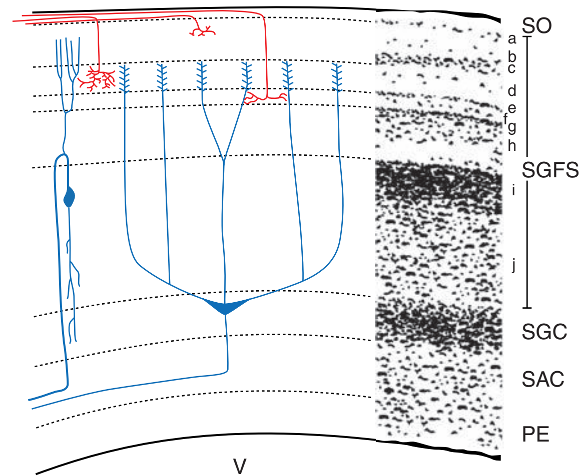

Figure 4 Cellular organization of the chick optic tectum. Cytoarchitects distinguish up to 16 tectal layers between the pial surface (top) and the midbrain ventricle (V). The layering of the optic tectum serves to organize the cell bodies, the dendritic arborizations, and the inputs of the tectal neurons. Illustrated in red are three examples of retinotectal axons that target specific lamina within the retinorecipient layers a–f. Illustrated in blue are two examples of tectal projection cells – one in stratum griseum centrale (SGC) that has 'bottlebrush' dendritic endings in layer d and issues ascending projections to the thalamus, and a second in layer i that projects to the isthmic nuclear complex. PE, the periventricular and ependymal layers; SAC, stratum album centrale; SGFS, stratum griseum and fibrosum superficialis; SO, stratum opticum.

Mesencephalic Trigeminal Nucleus

The mesencephalic trigeminal nucleus is found in all jawed vertebrates and contains primary sensory neurons for jaw muscle proprioception. Its constituent neurons are the first to differentiate in the midbrain and issue descending axons that on leaving midbrain pioneer the lateral longitudinal fasciculus of the hindbrain. Whether these primary sensory neurons are generated directly in brain or are returnees of neural crest origin has been difficult to resolve with experimental embryological methods. Molecular studies using neural crest markers and genetic fate mapping support a central origin for these cells.

Optic Tectum

Proper tectal histogenesis entails the generation of a layered structure that shares many features with cortex, including layer-specific cell types with apically oriented dendrites and topographically organized inputs that target particular layers (**Figure 4**). The cellular mechanisms for constructing the optic tectum, however, parallel only in part those found in cortex. The deepest layers of the optic tectum, including the output neurons of the stratum griseum centrale, are generated first. The next born neurons, however, do not follow the simple neocortical 'inside-out' pattern but, rather, are produced in a much more complex manner. The cells of the prospective stratum griseum and fibrosum superficialis layers, which are designated the tectal plate in development, are born in two major waves, with superficial layers a–g produced first and the deeper layers h–j born later. In addition, within both waves, there are further temporal gradients, with layers a–g following an inside-out pattern of birthing and layers h–j observing an 'outside-in' sequence. Retroviral lineage analysis has shown that the tectal plate is principally populated by clonally related cells traveling from the ventricular zone along radial glia. However, there are also early and late tangential migrations. Some of these tangential migrations give rise to both neurons and glia.

One role of tectal lamination is to segregate inputs to particular dendritic territories. Focusing on the retinal input layers a–f, anatomical mapping studies have identified a large number of potential cell surface cues that are expressed in specific retinorecipient laminae and could contribute to retinal axon layer targeting. These include adhesion molecules of the cadherin family and the extracellular matrix molecule versican, a member of the aggrecan family of chondroitin sulfate proteoglycans. Function-blocking experiments employing an antibody to N-cadherin or a lectin that binds to versican have demonstrated lamina-specific defects in retinal axon arborization. Inappropriate laminar targeting by incoming retinal

fibers has also been found following misexpression of the Groucho family corepressor TLE4 in the embryonic chick tectum. The TLE4 defect is likely secondary to a disruption of tectal cell-type specification which also causes lamina formation defects.

The most studied of the tectal input topographies is that of the retina. It now appears almost certain that retinotectal topography is set up by multiple independent batteries of guidance molecules acting along orthogonal axes. These guidance ligands are found in increasing gradients across the ventral-to-dorsal and anterior-to-posterior axes of the tectum, and their receptors are found in gradients across the retina. Current models of map formation are that each ligand has attractive and repulsive actions and that retinal axons distribute themselves across the tectal axes based on the attractive–repulsive set point conferred by their retinal position-dependent receptor status. The retinotectal ligand–receptor combinations identified to date are: (1) ephrin-As and EphA receptor tyrosine kinases, (2) RGM and neogenin receptors for the tectal anterior–posterior axis, (3) ephrin-Bs and EphB receptors, and (4) Wnt3 and the WNT receptors Ryk and Frizzled-5 for the tectal dorsal–ventral axis. In addition to these specific guidance molecules, direct axon competition for tectal target space is thought to regulate retinotopic map development, although the specific molecular mechanisms involved in this process remain obscure. Finally, neuronal spiking activity is needed for refinement of the retinal topographic map. Activity-dependent mechanisms are also likely to mediate sensory map registration across the tectal layers, integrating input from the isthmic nuclear complex and from nonvisual sensory structures.

The axon guidance gradients found in the tectal anterior–posterior axis are set up by the IsO. The 'naive' model is that the IsO releases FGF8, which forms an instructive, posterior high concentration gradient that is converted into a cellularized expression gradient of transcription factors (such as En1/2) and target ligands (such as eprhin-A2 and ephrin-A5). A striking modification of this model derives from the finding that soluble En2 homeodomain protein can act directly to guide retinal axons, repelling temporal axons and attracting nasal ones, which accords precisely with the posterior high gradient in EN2 expression. For the tectal dorsal–ventral gradients, it is anticipated that the RP plays a major instructive role, and there is direct evidence that rFP contributes to retinotectal dorsal–ventral patterning as well.

Midbrain Patterning and Invertebrates

Midbrain is a feature of vertebrates, and its possible status in invertebrates is uncertain. In the cephalochordate *Branchiostoma*, for example, even the presence of a tectum-like structure is disputed and the best guide for a possible midbrain is the positioning of the anterior-most motor neurons. Modern molecular developmental biology, however, has identified regional markers of anterior–posterior identity that allow informative comparisons to be made within and across phyla. For assessing the origins of midbrain, a potentially informative marker is the paired-like homeobox gene *DMBX1* (diencephalons/mesencephalon homeobox gene). *DMBX1* expression onset precedes the establishment of the IsO. It is first detected during gastrulation as a crescent in the anterior neural plate and then accumulates at the level of the prospective midbrain and, with the appearance of definitive brain vesicles, is strongly expressed in the embryonic midbrain and adjoining posterior diencephalon. Unfortunately, the functional importance of DMBX1 to midbrain development is unclear; whereas *dmbx1* zebra fish morphants show reduced tectal growth, *Dmbx1*-deficient mice present no gross abnormalities in brain development. Moreover, as development proceeds, *DMBX1* is found in other central nervous system sites including the hindbrain. Despite these handicaps, DMBX1 is the single best molecular marker of embryonic midbrain tissue.

A *DMBX1* ortholog has not been described in protostomes. However, comparative genomics has readily identified *DMBX* orthologs throughout invertebrate deuterostomes, including cephalochordates, urochordates, echinoderms, and hemichordates. The finding of one *Hydra* and six *Nematostella* DMBX sequences indicates that this gene was present in the cnidarian–bilateralian ancestor. Studies of *dmbx* gene expression in the hemichordate acorn worm *Saccoglossus* provide strong support for the conclusion that midbrain-like regional gene expression and isthmus-like organizer activity were present in at least the deuterostome ancestor. The midlevel structures of the acorn worm, the mesosome and the anterior metasome to the level of the first gill slit, express makers characteristic of midbrain and caudal forebrain, including *dmbx*, *dbx*, *otx*, *engrailed*, and *pax2*. In addition, the posterior end of this region is marked by a *fgf8*- and *wnt1*-rich signaling center. By contrast, transcription factor markers characteristic of anterior and ventral forebrain are found anteriorly, in the prosome, and *hox* genes are expressed only posterior to the first gill slit. This finding of a conserved core of midbrain patterning circuitry does not mean that the shared ancestor of vertebrates and hemichordates had a midbrain. The predicted nervous system of the deuterostome ancestor, like that of the acorn worm, is an epidermal nerve net.

See also: Forebrain Development: Holoprosencephaly (HPE); Forebrain Development: Prosomere Model; Forebrain: Early Development; Neural Patterning: Midbrain–Hindbrain Boundary; Wnt Pathway and Neural Patterning.

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Forebrain Development: Prosomere Model

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Definition

The prosomeric model is a segmental structural model of the brain of vertebrates that explicitly holds that the brain is formed by an uninterrupted series of transverse subunits of the neural tube, generally called neuromeres. Among such subunits is a large rostral forebrain unit – the secondary prosencephalon – that encompasses hypothalamus, eyes, and telencephalon, followed by three caudal forebrain or diencephalic neuromeres (i.e., prosomeres), which are regarded as being serially homologous with more-caudal neuromeres, namely, a single midbrain mesomere, 11 hindbrain rhombomeres, and the spinal myelomeres. It is important to note that for descriptive purposes, the model postulates in all vertebrates a morphogenetic bending of the longitudinal axis of the tubular neural primordium, most marked at the cephalic flexure, whose incurvation causes wedge-shaped deformation of the topologically transverse cylindrical neuromeric sectors of the neural tube. These units share a set of fundamental longitudinal zones (due to common dorsoventral (DV) patterning processes) and therefore represent segments, that is, metameric developmental units (anteroposterior (AP) patterning). The common causal background of the longitudinal zones establishes the property of metamery (i.e., serial homology) across all neuromeres, irrespective of their differential molecular identities and individual prospective adult fates and of the variable border properties of the cells found at the interneuromeric boundaries. Therefore, the prosomeric model visualizes all vertebrate brains as segmented structures constructed along the same Bauplan (same set of DV and AP developmental units). Orthogonal intersection of DV and AP boundaries in the neural tube wall defines a checkerboard pattern of domains (histogenetic areas) in which specific properties and finer regionalization phenomena appear (shared or not among vertebrates). This makes the model useful for systematic descriptive neuroembryology, comparative neuroanatomy, and causal analysis of conserved or variant brain morphogenesis.

Characteristics

Why We Use Models

Structural neurobiology studies the form and functional inner structure of brains. This needs a conceptual model in which all sorts of detailed data on form and structure down to cellular aspects can be systematically accumulated, organized, compared, and differentiated one from another in their mutual relationships. The basic model of the vertebrate brain is the concept of the closed neural tube, from which adult brains emerge via differential morpho- and histogenesis. Since we cannot know all from the beginning, morphological models essentially are reasonable and useful conjectures about how many parts there are and how they are patched together. Such models are periodically perfected over time, becoming in the case of brains increasingly complex operational scaffolds based on accumulated data and a number of assumptions. There is always the possibility of constructing better (or worse) models.

A good model in essence should be parsimonious; that is, it should identify a minimal set of characteristic parts or landmarks in the modeled system, which can be generally recognized and seem to encompass, or be able to explain, most if not all available structural data. A good model also may delimit various 'unfilled' conceptual domains, where new data should fit in (as the periodic table of chemical elements did when it was first formulated). Such predictive aspects of models are highly useful because they implicitly indicate which new questions might be meaningful or how best to pose and answer them in practice. Simultaneously, models are instrumental in providing possible significance to any new, unexpected observation. Scientists in principle believe in and use a particular model as long as it seems to accommodate established knowledge, inspire significant research, and allow satisfactory incorporation of emerging sets of new data. Historical periods in which technological improvements produce radically novel sorts of data are particularly critical for the survival of a model.

Models widely shared among a scientific community represent a scientific paradigm. In contrast to hypotheses and theories, paradigms are not meant to be tested, since one must believe in one of them and use it as if it represented the truth, in the very process of testing a hypothesis experimentally. A paradigm comes dangerously close to becoming a dogma, a belief that wholly escapes criticism or doubt and is considered ascientific. Several models may coexist historically, sometimes because each one is perceived to have different advantages, but usually due to lack of awareness that one of them is distinctly better than the others, compounded with the human tendency to persist irrationally in long-held beliefs. Nevertheless, models and paradigms eventually may be perceived as

obsolete and be discarded by newer, less committed generations of scientists, particularly when they are manifestly unable to account for some data and efforts to apply them lead to highly unparsimonious, complicated lines of thought. Continued use of an obsolete model, or mixed-up joint use of elements of different models, tends to obstruct the progress of science.

Some neuroscientists wrongly think that they do not use a neural model. This means they simply are unaware of the model they are using. Frequently, some aspects they mistakenly regard as facts actually are conjectures. Dogmatic conscious or unconscious belief in models is a condition that is prone to poor thinking and poor science. Due to the great complexity of the studied organ, brain science is a field where such interpretive malfunctioning is not uncommon.

Neuromeric Models

The earliest morphological models of the brain were based on the adult form of the human and animal brains. This approach provided over time a rich set of neuroanatomical terms and conjectural meanings, many of which are now obsolete, although some old terms and concepts still persist in textbooks. During the late nineteenth century, and as a result of various important advances such as microscopy, evolutionary theory, and cell theory, comparative anatomical and embryological knowledge of brains advanced enough to allow the initial formulation of developmental brain models generally valid for all vertebrates. Developmental models also appeared for the entire body. The first generally accepted developmental structural paradigm for brains was a segmental model of the neural tube, which appeared hand in hand with a segmental model of the body and head of vertebrates. The axial skeleton was conceived as being segmented into metameric vertebrae (with a number of units fused together in the sacrum and in the cranial basis). The branchial apparatus also seemed segmented into serial branchial arches and slits. The brain and the set of spinal and cranial nerves were postulated to consist of a number of segmental units correlated one to one with the vertebrae and/or branchial arches.

The term 'neuromere' that was soon applied to these transverse neural units was coined by the American scientist Orr, who very ably characterized histologically in lizard embryos the relevant hindbrain, midbrain, and forebrain neuromeric units. He also provided a clear-headed morphological analysis of longitudinal zonation and axial bending of the brain, largely consistent with the present-day prosomeric model (**Figure 1**). Orr's study is the historic root of the prosomeric model, though previous and subsequent writings by von Kupffer, Hill, His, Neal, Palmgren, Rendahl, Tello, and Vaage, among others, contain less explicit antecedents. A large-scale review of shared neuromeric structural data collected for all vertebrate lineages from agnatha to mammals was published by von Kupffer at the turn of the twentieth century.

Wilhelm His produced an alternative very influential neural model, though he certainly must have known the neuromeric views of von Kupffer and other contemporaries well. His defined the floor, basal, alar, and roof plates, the alar-basal boundary (sulcus limitans of His), the concept of isthmus, and the idea of neural tube morphogenetic deformation due to axial bending. This model was very influential because it underpinned the first Nomina Anatomica in 1895, whose committee was presided over by His. The model was not explicitly neuromeric, though His' concepts of axial bending and longitudinal zonation and most of his transverse boundaries, including those of the isthmus, clearly were consistent with neuromeric models (**Figure 2**).

Columnar Models

At the height of the prestige of neuromeric brain models, an important unrelated breakthrough resulted from the analysis of functional components in the cranial and spinal nerves. It was discovered that each nerve component (motor or sensory fibers) either originates from or projects on a distinct columnar domain of the hindbrain or spinal cord. Separate columns could be assigned to visceral and somatic nerve components. Afferent fibers usually bifurcate into ascending and descending branches that distribute widely within the corresponding column. In so doing, they do not respect the neuromeric boundaries. These data were widely perceived as important, and they threw doubt on the neuromeric models, at least for application to advanced embryos and adults, since the basic functional organization of the hindbrain and spinal cord seemed to be columnar and not segmental, irrespective of the separate, more or less periodic nerve roots, and the peripheral dermatomes and myotomes. It was increasingly thought that maybe neuromeres were transient early embryonic phenomena without impact in the mature brain, in which a columnar arrangement of functions emerges. While Europe immersed itself in World Wars I and II, a new school of US neuroanatomists bloomed, led by JB Johnston and CJ Herrick, and its members proceeded to explore these new columnar ideas. Already in 1910, Herrick postulated columnar subdivisions in the diencephalon, which he initially thought might be continuous caudally with brain stem columns and extend rostrally into telencephalic ones. This work

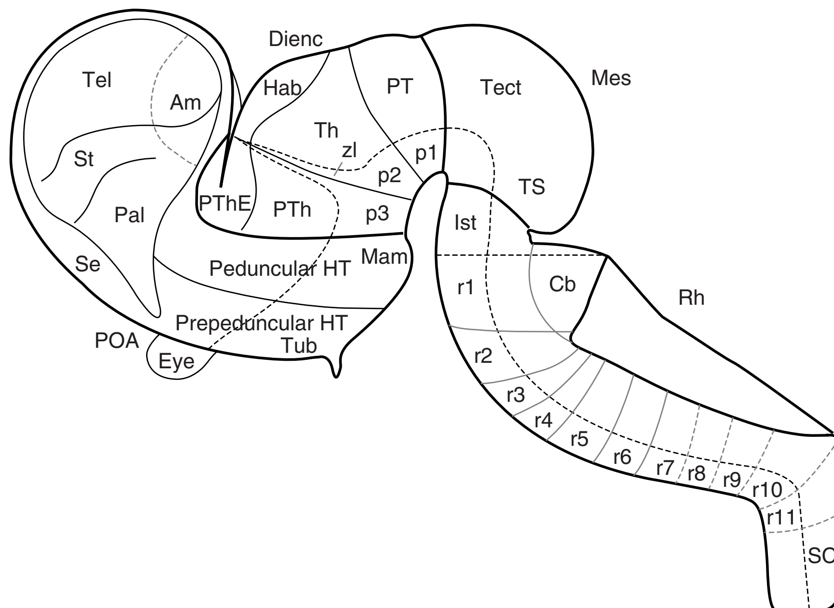

Figure 1 Schema of the prosomeric model of Puelles and Rubenstein (2003). The forebrain lies to the left. Note axial bending at cephalic flexure. The longitudinal alarbasal boundary is present throughout the lateral wall of the neural tube, symbolizing all longitudinal components (floor and roof plates not represented); a singularity known as zona limitans (zl) is a transversal spike-like deviation of the general alar-basal boundary. The secondary prosencephalon (Sec.Pros.) is the rostralmost and most complex unit, consisting of telencephalon (Tel), eye and hypothalamus (HT: divided in two parts). Septum (Se), striatum (St), pallidum (Pal), preoptic area (POA), and amygdala (Am) regions are identified within the telencephalon; the pallium lies under the label Tel. Tuberal (Tub) and mammillary (Mam) subregions of the hypothalamus are marked. The caudal forebrain or diencephalon consists of three prosomeres (p1–p3), whose alar regions include the pretectum (PT), the thalamus and habenula (Th–Hab), and the prethalamus and prethalamic eminence (PTh, PThE); a specific tegmental domain corresponds to each of them (under p1–p3 labels). A simplified view of the large mesencephalic alar plate (Mes) divides it into superior colliculus or tectum (Tect) and inferior colliculus or torus semicircularis (TS); 'colliculi' are mammalian terms. The hindbrain or rhombencephalon (Rh) contains 12 neuromeric units, from the isthmus (Ist) and ehombomere 1 (rl) down to rhombomere 11 (r11), which limits with the spinal cord (SC). Note the cerebellum (Cb) forms mainly across isthmus and r1.

originated the prevalent present-day dogma of the structural division of the diencephalon into epithalamus, dorsal thalamus, ventral thalamus, and hypothalamus, considered to be longitudinal columns of the forebrain. This emphasis was accompanied by negation of the cephalic flexure (or simply, not attaching morphological meaning to it).

Soon afterward it was recognized that the postulated diencephalic columns are not continuous with the brain stem and telencephalic ones. As a consequence, each of these sets had to be conceived of as forming independent partial models of the respective brain parts. This left in between three vaguely defined, unmodeled, and badly understood transition areas: isthmus, pretectum, and telencephalic stalk. Efforts to extrapolate to diencephalon and telencephalon the columnar 'functions' of the brain stem and spinal cord (i.e., visceral-somatic sensory and motor functional correlations) were also unproductive. Paradoxically, though the promise of the columnar model stumbled on the forebrain, this did not lead to any doubts about its potency as a paradigm or usefulness as a forebrain model, because by that time it had become a neuroanatomical dogma. For a long time, dogmatic transmission of the columnar Herrick model in research and classroom pushed the alternative neuromeric models nearly to oblivion. Most neuroscientists to this day have been made to believe that the supposedly dorsoventral columnar series of epithalamus–dorsal thalamus–ventral thalamus–hypothalamus is a fact, not a risky conjecture of a hundred years ago.

The fundamental failure of the columnar forebrain model was that it redefined the observable forebrain axis, negating its observable curvature and substituting an arbitrary ideal straight axis which is not supported by any specific data. The columnar straight brain axis crosses from the pontine brain stem into the the 'caudal' hypothalamus, then traverses the hypothalamus and preoptic area 'longitudinally,' to enter the telencephalon and end in the olfactory bulb (this last part is obviously inconsistent with the paired paramedian nature of the olfactory bulbs and telencephalic hemispheres). Herrick curtly explained such pragmatic axial redefinition as "controversial... but convenient."

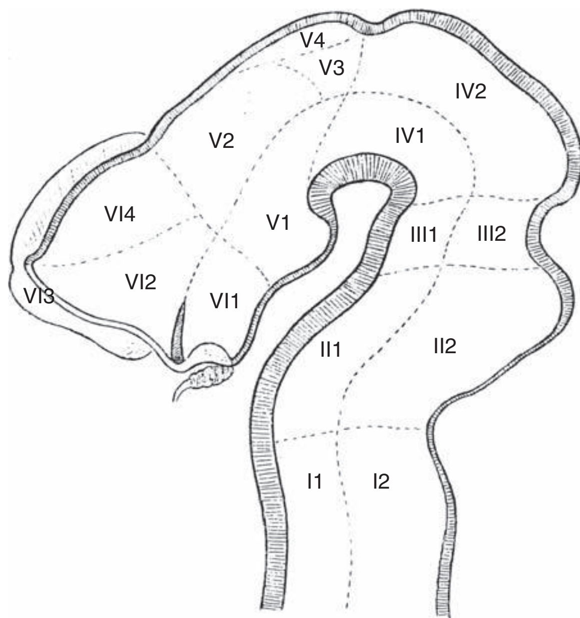

Figure 2 Model of W His (1895). Six transversal units (I–VI) are recognized along the bent neural tube. The alar-basal boundary appears through the entire lateral wall, parallel to the floor and roof plates. Domains I and II correspond to the myelencephalon and metencephalon, respectively. Domain III is the isthmus. Domain IV is the midbrain, and domain V is the 'diencephalon proper.' Domain VI contains part of hypothalamus, the eye, and the telencephalon. Basal plate domains are identified as I (II–VII) and alar plate domains are marked as 2 (I2–VI2). An additional domain VI3 represents the olfactory bulb, and the V3 and V4 domains refer to the metathalamus and habenula, respectively.

In the subsequent era of spectacular experimental neuroanatomical advances (axonal degeneration, axonal transport, electron microscopy, chemical anatomy), which extended up to the recent 1980s, Herrick's columnar model seemed to encompass without problems the accumulating hodological and chemoarchitectonic data on the forebrain. Stereotaxic topographic references for lesions and tracer injections worked well with the idea of a straight axis of the entire brain, which could be naively thought to be reproduced by the length axis of the stereotaxic apparatus. Only isolated embryologists (and then only those who looked at whole mounts and sagittal sections, procedures that curiously fell into disuse) insisted now and then that the brain axis is always curved and therefore topologic transversal and longitudinal dimensions had to be defined in accordance with the specific part of the neural tube considered.

The Rebirth of Neuromeric Models

A fully new set of neuroanatomical developmental data started to accrue during the 1980s and 1990s. These data included observations on the expression domains of neural developmental genes, possible thanks to the new *in situ* hybridization protocol for transcribed messenger RNA (and other correlative molecular biology and genomic advances). This procedure renders visible the cells that are in the process of reading out piecewise the information coded in the genome. Since many of these genes are causally determinant of the structural and histogenetic patterning of the neural tube wall, their expression patterns and the boundaries defined by them are highly relevant for brain models. It was soon discovered that some genes show longitudinal patterns of expression and others show transversal patterns (actually, both aspects usually appear in combination). All efforts to encompass these patterns within the forebrain columnar model have failed or have led to unparsimonious and highly convoluted *ad hoc* interpretations. On the other hand, the hindbrain and spinal cord columnar model does agree significantly with longitudinal gene patterns but highlights at the same time that observed transversal patterns relate specifically to the old neuromeric models. It turns out that neuromeric and columnar patterns coexist in the hindbrain and spinal cord, as predicted long ago by defenders of the segmental approach.

Similar analysis of forebrain gene expression data in the context of a forebrain neuromeric model (using the original bent axis) showed the capacity of this model to encompass and give morphologic significance (developmental function) to the new set of molecular causal data. The apparent rebirth of a neuromeric paradigm in the forebrain and hindbrain (where neuromeres are best visible) pointed the way to the possibility of conceiving a general segmental model of the entire central nervous system, in which longitudinal zones (columns, but different ones in the forebrain than those postulated by Herrick and his followers) and transverse neuromeres combine to interpret and predict the nature of causal phenomena operating in the construction of the brain. This sort of ultimate or synthetic brain model was called the prosomeric model, as developed in several reports and reviews by Puelles and Rubenstein.

See also: Forebrain Development: Holoprosencephaly (HPE); Forebrain: Early Development.

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Forebrain: Early Development

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Key Features of Early Forebrain Development

The forebrain is specified within the anterior neural plate during gastrulation, and by the end of somitogenesis, this territory has established ventrally the hypothalamus and floor plate and dorsally the telencephalon, eye, and diencephalon, which itself is further subdivided in prethalamus, zona limitans intrathalamica (ZLI), thalamus, and pretectum (**Figure 1**). All forebrain structures arise from a simple sheet of neuroepithelial cells, which undergoes regionalization and dramatic morphogenesis during early developmental stages. The morphology and function of various forebrain structures may be different among vertebrates, but the general organization and early patterning steps are likely conserved.

The initial formation of the forebrain is linked to the specification of neural identity in the embryonic ectoderm. Neural character is induced by gastrula stages through a combination of fibroblast growth factor (FGF) activity and extracellular antagonists of bone morphogenetic proteins (BMPs). The induced neural plate initially has anterior character, as evidenced by expression of genes that later become restricted to prospective forebrain and midbrain regions. Subsequently, the activity of various signaling molecules (including FGFs, Wnts, and retinoic acid) emanating from posterior regions of the embryo leads to caudalization of the neural tissue. These two events, neural induction and caudalization, eventually result in the generation of a nascent central nervous system (CNS) with graded anteroposterior (AP) character.

To acquire and maintain anterior character, the rostral neural plate must avoid exposure to caudalizing factors. Several mechanisms ensure this happens. These vary between species, but there are some common themes. For instance, gastrulation movements shift the anterior neural plate away from the sources of caudalizing signals. In addition, locally secreted signals from a variety of rostral tissues antagonize the activity of the caudalizing factors, protecting the anterior neural plate and ensuring it retains anterior character.

The Wnt/β-catenin pathway is perhaps the most crucial of the signaling cascades critical for early regional AP patterning of the rostral neural plate. Embryos carrying mutations that lead to enhanced Wnt activity in the anterior neural plate lack the telencephalon, rostral hypothalamus, eyes, and prethalamic territories. Thus, specification of the rostral forebrain requires low levels of Wnt/β-catenin activity. Conversely, local suppression of Wnt signaling can lead to the expansion of the rostral at the expense of more caudal forebrain domains.

The gradual regionalization of the neural plate as a consequence of the activity of Wnts and other signals leads to the subdivision of the anterior neural plate (or prospective forebrain) into discrete territories defined by the restricted expression of various transcription factors of the Emx, Irx, Pax, Six, and other families. Some of these transcription factors are likely to be direct effectors of the signals that regionalize the neural plate. For instance, Six family genes are repressed by Wnt signals and consequently expressed only in rostral regions. In turn, Six family proteins suppress Wnt activity, thus establishing a feedback loop that ensures tightly regulated levels of Wnt activity in the prospective forebrain.

One consequence of the initial regionalization of the neural plate is the establishment of groups of cells that function as local signaling centers, orchestrating subsequent neural development at different positions of the neuroepithelium (also called secondary organizers). These organizers influence cellular fate and growth of the adjacent tissues, further refining the initial pattern of the neural plate.

Along the rostrocaudal axis of the forming rostral CNS, cell populations with organizer properties are found at the anterior neural border or anterior neural ridge (ANB or ANR), at the ZLI, and at the midbrain–hindbrain boundary (MHB). The ANB/ANR is a source of signals including Wnt antagonists and FGFs that promote telencephalic fate; the ZLI is established later than the ANB/ANR and the MHB and is a source of sonic hedgehog (Shh) which influences the patterning and growth of surrounding prethalamic and thalamic structures; and the MHB is a source of FGF and Wnt signals that pattern the midbrain and rostral hindbrain and contribute to defining the caudal boundary of the forebrain.

Along the dorsoventral axis, the most prominent cells with signaling properties are ventral midline cells of the floor plate and hypothalamus. These cells produce Hh, Nodal, and other signals and are important for regionalization and neuronal patterning of the ventral forebrain and for splitting of the initially coherent eye field into left and right eyes. Cells along the dorsal midline of the forebrain, including the derivatives of the ANB/ANR and more caudal roof plate tissue, are also the source of various secreted signals, including FGFs, Wnts, and BMPs. The roles for such signals are

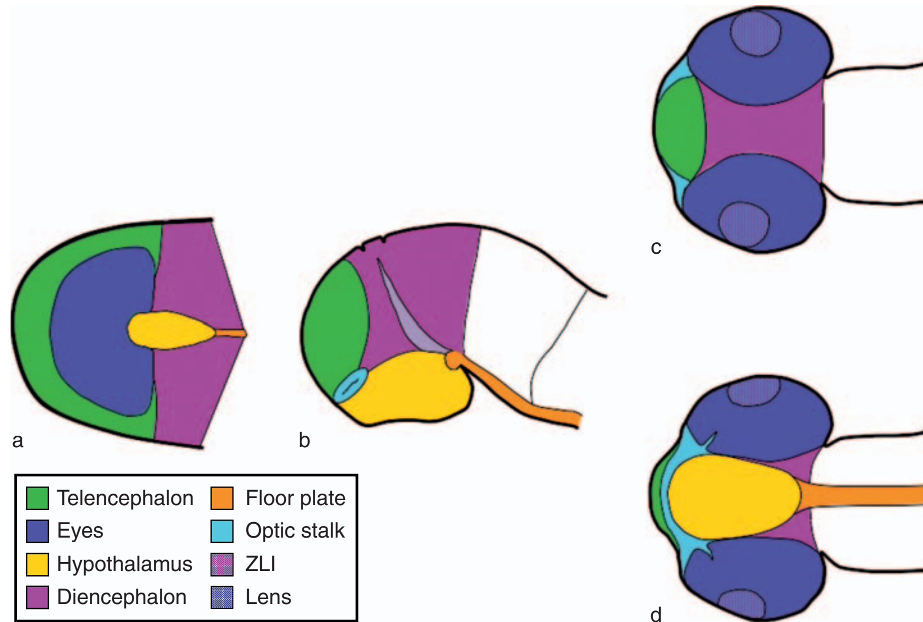

Figure 1 (a) Organization of the forebrain territories at neural plate stage. The groups of cells giving rise to each territory are shown in different colors. (b–d) The same color code is used to show the relative positions of the different territories in embryonic brains at a later stage of development, from lateral (b), dorsal (c), and ventral (d) views. ZLI, zona limitans intrathalamica.

perhaps best understood with respect to the regionalization of the mammalian dorsal telencephalon.

The morphogenesis that occurs during transition from the simple sheet of cells that constitutes the anterior neural plate to the complex structures of the mature forebrain and eyes (see **Figure 1**) is the least understood aspect of early forebrain development. This area is likely to see considerable research activity in the next few years.

Specification and Early Development of Various Forebrain Domains

Hypothalamus

The hypothalamus is the master regulator of neuroendocrine functions in the brain. It is a derivative of the most ventral/rostral regions of the prospective forebrain, and medial (ventral) regions of the hypothalamus are in continuity with floor plate tissue that runs the entire length of the ventral CNS.

During gastrula stages, prospective midline neural plate cells that will form floor plate and hypothalamus are located near the organizer from which they will extend rostrally and caudally along the CNS as convergence and extension movements shape the AP axis of the embryo. Initially, all prospective neural midline may be specified with floor plate character. However, as rostral midline neural tissue progressively extends within the rostral neural plate, it escapes the caudalizing influence of signals from caudal mesendodermal and neural tissues and begins to acquire rostral, hypothalamic identity. Thus by the end of gastrulation, ventral midline tissue of the neural plate is overtly divided into rostral, hypothalamic, and more caudal floor plate domains (**Figure 1**). The prospective hypothalamic domain is subsequently further subdivided along the AP axis and differentiates as specific territories with different identities and different signaling properties.

The Nodal and Hedgehog (Hh) signaling pathways are implicated in the induction of both hypothalamus and floor plate although their relative contributions to this process vary between classes of vertebrates. In fish, Nodal signaling is essential for induction of medial regions of the floor plate and caudomedial regions of the hypothalamus. Hh signaling is not essential for formation of these domains but is critical for the induction and patterning of ventral tissues adjacent to the most medial floor plate and lateral and rostral to the caudal hypothalamus. Similar distinctions between medial and lateral floor plate and hypothalamic domains probably exist in all other vertebrate classes, but at least in mammals, the Hh pathway plays a more prominent role in the formation of all midline structures.

As with more dorsal regions of the CNS, the level of Wnt/β-catenin signaling influences the rostrocaudal

character of the ventral midline and adjacent tissue. Hypothalamic fate specification requires low levels of Wnt/β-catenin activity, and if Wnt signaling is enhanced, cells with more caudal floor plate character extend further rostrally into the territory normally occupied by hypothalamus. Within the hypothalamus itself, raised Wnt signaling can promote caudal at the expense of rostral hypothalamic identity, suggesting that a low level of Wnt signaling may cooperate with Nodal signals to confer posterior hypothalamic identity. In addition to Nodals and Wnts, BMP and Hh signals are also important in conferring regional hypothalamic character to neurons generated in this portion of the ventral forebrain. For instance, Shh together with BMP7 is sufficient to confer hypothalamic dopaminergic identity to neural progenitor cells *in vitro*.

The various signals that influence hypothalamic development are thought to induce transcription factors that further refine signaling activity and confer regional character and specific neuronal fates on ventral CNS cells. For instance, *six3* expression is suppressed by Wnt signaling and in turn represses Wnt activity whereas, conversely, *irx3* expression is induced by Wnt signaling and itself promotes Wnt signaling. Mutual repressive interactions between Six and Irx family homeodomain proteins therefore contribute to the division of rostral neural tissue into rostral *six3*-expressing and caudal *irx3*-expressing domains. Several of the transcription factors that function downstream of these early regional patterning events to promote the generation of hypothalamic neurons have been characterized and include homeodomain proteins of the Nk2 family and neurogenin subfamily of basic helix–loop–helix proteins.

Telencephalon

The adult telencephalon is the most structurally diverse region of the vertebrate forebrain, but at early stages of development, patterning mechanisms are likely to be conserved across all vertebrates. At these early stages, the telencephalon has two major subdivisions: a dorsal pallial region that gives rise to the neocortex in mammals and analogous structures in other vertebrate classes and a ventral, or subpallial, region that gives rise to basal ganglia or striatopallidal structures.

At the neurula stage, the prospective telencephalon constitutes a band of cells located in the most anterior neural plate, rostral and lateral to the eye field (**Figure 1**). For the telencephalon to form, Wnt/β-catenin signaling must be suppressed, and several genetic mutations in fish which lead to enhanced Wnt activity in the anterior neural plate lead to transformation of the telencephalon (and eyes) to more caudal forebrain fates. Wnt signaling is suppressed by a variety of antagonists secreted from cells within the prospective telencephalon itself and from adjacent tissues, including underlying mesendoderm. For instance, in fish, cells located in the ANB/ANR express a Wnt antagonist of the secreted frizzled-related protein family, which when overexpressed expands telencephalic territories. Secreted frizzled-related proteins are believed to function by binding Wnt proteins and thus preventing Wnt ligands from interacting with their receptors. Although high levels of Wnt activity at early stages of development can suppress all telencephalic development, graded levels of Wnt activity can also influence regional patterning within the nascent telencephalon, promoting dorsal, pallial fates and suppressing ventral, subpallial identity.

In addition to Wnt antagonists, the ANB/ANR expresses FGF family members, and these signaling proteins play a variety of crucial roles in telencephalic development. It is unclear whether FGF signaling is required for induction of the telencephalon, but subsequent to induction, FGFs regulate regional patterning. Together with Shh, FGF signaling is required for induction of ventral, subpallial telencephalic character. Studies in mouse have revealed that within the dorsal, pallial telencephalon, FGF signaling is a critical regulator of arealization of the cortex. This polarization is mediated by transcription factors that appear to have graded activity across the pallium. For instance, antagonistic interplay between Pax6 and Emx2 influences the caudomedial and rostrolateral character of pallial territories. Thus, Emx2 mutant mice display expanded rostrolateral pallial character at the expense of caudomedial territories, whereas Pax6 mutants display the opposite phenotype.

Eye

The neural retina, pigment epithelium, and optic stalk or nerve are all derivatives of the anterior CNS. Within the neural plate, precursors for these eye structures initially form a coherent field of cells, termed the eye field, which spans the midline. Morphogenetic movements in combination with signals derived from midline tissues subsequently result in cells from the eye field moving laterally and evaginating to form the left and right optic vesicles. Distal cells within the vesicles form the neural and pigmented retina while more proximal and medial cells contribute to the optic stalks or nerves.

During gastrulation, the eye field is defined by the combinatorial expression of various transcription factors including Pax6, Six3, and Rx. Mutations in these genes result in eye malformations in all organisms analyzed, from humans to mice and fish. All these genes, generically known as eye specification genes, are part

of a complex cross-regulatory network essential for eye development, and their functions during this process are highly conserved throughout the animal kingdom. It is unclear how the expression of these genes is first induced, but expression is at least modulated by the same signals that regionalize other domains of the anterior neural plate. Thus, for example, Pax6, Rx3, and Six3 are restricted to anterior regions of the zebra fish neural plate by the activity of the canonical Wnt/β-catenin pathway, and excess Wnt activity can suppress both their expression and subsequent eye formation. The eye field is therefore induced in anterior regions of the prospective forebrain, where high levels of Wnt/β-catenin activity are absent. At least in fish, the eye field is further protected from canonical Wnt signaling by the activation, within the prospective eye field, of a noncanonical Wnt pathway that antagonizes canonical Wnt signaling, thereby promoting the expression of *rx3* and *six3* in this domain.

Both telencephalon and eye field are induced in regions of low or absent Wnt signaling. Although the mechanisms that distinguish prospective telencephalon from eye field are unclear, it is likely that graded BMP signaling is involved in the segregation of these two territories. At least in fish, high levels of BMP signaling encroaching from the margins of the neural plate suppress expression of eye field genes, limiting the lateral extent of eye field specification. Thus, the activation of the eye specification genes is promoted in the region sandwiched between prospective telencephalic and dorsal diencephalic domains that receive high levels of BMP signaling and more caudal diencephalic domains that receive high levels of Wnt signaling. The Rx3 transcription factor is likely to be an effector of the fate choice between telencephalon and eye (in addition to a role in eye morphogenesis), as telencephalic markers are ectopically expressed in the eye field of zebra fish rx3 mutants. It is unclear whether the eye specification genes are under the direct transcriptional control of these signaling pathways; to date, only *six3* has been shown to be transcriptionally regulated by the Wnt signaling pathway.

The eye primordium is specified as a single domain straddling the midline, but two optic vesicles, evaginating from the lateral walls of the neural tube, are established from this single domain. This is accomplished by morphogenetic processes that split the territory in two and lead to lateral evagination of the optic primordia. Do the eye specification genes instruct a specific program of morphogenesis for the eyes, or does this process depend on other, yet to be described, mechanisms? The answer is probably a combination of both. For example, *rx3* mutants in zebra fish and medaka show defects in the evagination of the optic vesicles, but early markers of eye field formation are initially expressed normally, suggesting that *rx3* regulates morphogenesis of the forming optic vesicles. However, the genes and proteins that are the direct effectors of the morphogenetic movements are currently unknown.

Nodal and Hh signals emanating from ventral midline tissues are also involved in the separation of the retinae. Disruption of either of these signals leads to phenotypes in which the retinae remain fused across the midline of the brain and the optic stalks are absent. In these conditions, the hypothalamus also fails to form, and it has been proposed that the absence of this structure, which normally would move anteriorly between the eyes, contributes to the failure of eye field separation. Other mutant conditions affecting the rostral movement of the hypothalamus, such as *silberblick/wnt11* mutant in zebra fish, similarly show varying degrees of cyclopia. Ongoing studies using advanced imaging and transgenic approaches are allowing much better documentation of the morphogenetic processes that accompany eye formation in normal development and in conditions that lead to cyclopia, and new insights into these processes will soon be forthcoming.

Diencephalon

According to the prosomeric model of forebrain organization, its most caudal part is subdivided into prethalamus (previously ventral thalamus), thalamus (previously dorsal thalamus), and pretectum, with the ZLI being located between prethalamus and thalamus (**Figure 1**(**b**)). Of all the major subdivisions of the forebrain, these domains are the most poorly understood in terms of their early development. For instance, it remains uncertain whether these three subdivisions are already specified at neural plate stages of development or whether they resolve at later stages. The ZLI is considered to be the boundary between prechordal and chordal domains of the brain, and one would expect this position to be defined very early, but as yet no sufficiently detailed fate maps exist to indicate where within the neural plate the cells that constitute the ZLI originate.

Despite this uncertainty, it is generally thought that the ZLI will form at a position defined on the neural plate by the boundary of expression between *six3* in the rostral forebrain and *irx3* in the posterior diencephalon–midbrain at neural plate stages. As discussed in the section titled 'Hypothalamus,' the expression of these genes is regulated by Wnt/β-catenin activity, which activates *irx3* and suppresses *six3*.

This implies that a specific level of Wnt/β-catenin activity defines the position at which the ZLI forms and thus the relative size of prospective prethalamus and thalamus.

Later in development, when the embryo has gone through neurulation, the ZLI forms a compartment between the prethalamus and the thalamus. The ZLI expresses Shh and is therefore potentially a secondary organizer in the forebrain. In support of this, studies in mice, chick, and zebra fish suggest that Hh proteins secreted from the ZLI regulate regional patterning and growth of the prethalamus and the thalamus. It is likely that further roles will be found for the ZLI in the near future.

The boundary between the caudalmost forebrain and the midbrain corresponds to the interface between *pax6* expression in the forebrain and *pax2* and *engrailed* expression in the midbrain. Similar to the case with Six3 and Irx3, corepressive interactions between these genes are thought to define the position of the forebrain–midbrain boundary. Midbrain development is promoted by FGF8, and overexpression of *fgf8* is able to expand *en2*, inhibit *pax6* expression, and therefore shift the forebrain–midbrain boundary rostrally.

The dorsal part of the diencephalon is unusual in exhibiting marked asymmetries between left and right sides of the brain. These asymmetries are likely present in all vertebrates but are more prominent in some species than others. Studies in zebra fish are unraveling the mechanisms that establish this asymmetry and determine the directionality and laterality of the asymmetric nuclei.

See also: Forebrain Development: Holoprosencephaly (HPE); Forebrain Development: Prosomere Model; Retinoic Acid Signaling and Neural Patterning; Wnt Pathway and Neural Patterning.

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Forebrain Development: Holoprosencephaly (HPE)

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Introduction and Scope

Holoprosencephaly (HPE) has long fascinated clinicians, basic scientists, and the public alike. This fascination results largely from its striking phenotype, which includes features such as a single midline eye (cyclopia) and a single forebrain holosphere rather than two hemispheres (**Figure 1(a)**). HPE also represents the most common congenital birth defect of the human forebrain (1 in 10 000 live births, 1 in 250 conceptions). Although previously considered to result from defective cleavage (i.e., splitting of the eye and forebrain fields), it is now well appreciated that the forebrain malformation results from primary defects in midline induction. Correspondingly, when factors needed to adopt midline fates are deficient, HPE is commonly observed. HPE, therefore, serves as a central paradigm for understanding how forebrain midline fates are specified.

The focus of this article is to describe forebrain midline development as revealed by studies on the cells and molecules implicated in HPE. After a brief description of HPE, we describe the following as they relate to forebrain midline development: (1) the organizers (signaling centers), (2) the genes and signaling pathways, and (3) the interactions among signaling pathways. Among the highlights are a tidy categorization of causal HPE genes into four well-known signaling pathways and the recent delineation of interactions among these pathways that help to explain long-standing conundrums about HPE phenotypes.

Definition of Holoprosencephaly

Primary midline failure in HPE has myriad secondary effects on forebrain development, which often lead to confusion and error regarding the HPE designation. Morphologically, the brunt of HPE neuropathology occurs at the midline rather than in more lateral structures. This includes marked reduction to total absence of midline tissues and, correspondingly, the failed separation of adjacent forebrain structures that are normally bilateral (**Figure 1(b)**). (Although often referred to as fusion, the term failed separation is more accurate.) At the molecular level, these morphological defects equate to absence of midline marker expression and aberrant continuity of lateral markers across the midline. This definition helps to exclude disorders such as microencephaly (small brain size), absence of the corpus callosum, hydrocephalus and absence of the septum pellucidum (which give the appearance of fused ventricles), and aprosencephaly/atelencephaly (absence of forebrain/telencephalon).

HPE can be divided into two categories with qualitatively different midline phenotypes: (1) classic and (2) middle interhemispheric (MIH) (**Figure 1(a)**). Classic HPE is further divided into alobar, semilobar, and lobar subtypes based on the degree of severity, with alobar being the most severe. The feature common to classic HPE subtypes is ventral predominance – that is, the neuropathology is most severe ventrally and then extends variably and in graded fashion to the rostral, dorsal, and posterior domains. In contrast, the MIH form (also known as syntelencephaly) exhibits midline failure that is restricted to the dorsal forebrain, generally affecting the posterior frontal and parietal lobes in humans (**Figure 1(a)**). Notably, MIH HPE lacks significant pathology away from the dorsal region – that is, the rostral and ventral domains, including the eyes and face, are relatively unaffected.

Organizers in Forebrain Midline Induction

Like other tissues, the development of the forebrain midline is governed by morphogenetic movements and organizers (signaling centers), which are localized groups of cells that produce morphogens. Morphogens are molecules that promote different cell fates at different concentrations. The key signals identified in forebrain midline induction are secreted proteins that are well-known morphogens in development.

Conceptually, induction of the forebrain midline occurs after neural induction, anterior–posterior (AP) patterning, and forebrain specification. Despite this useful conceptual distinction, however, there is significant overlap among these processes in terms of timing and the factors that govern them. For example, many of the signaling centers that govern neural induction, AP patterning, and forebrain specification are located at the embryonic midline, and these same signaling centers regulate forebrain midline induction. Thus, to provide an appropriate and comprehensive framework, we first summarize the earliest stages of forebrain development.

Nonneural Organizers of the Neural Plate

In the early embryo, forebrain development is regulated by organizers that lie outside the developing nervous system (i.e., in nonneural tissues). These

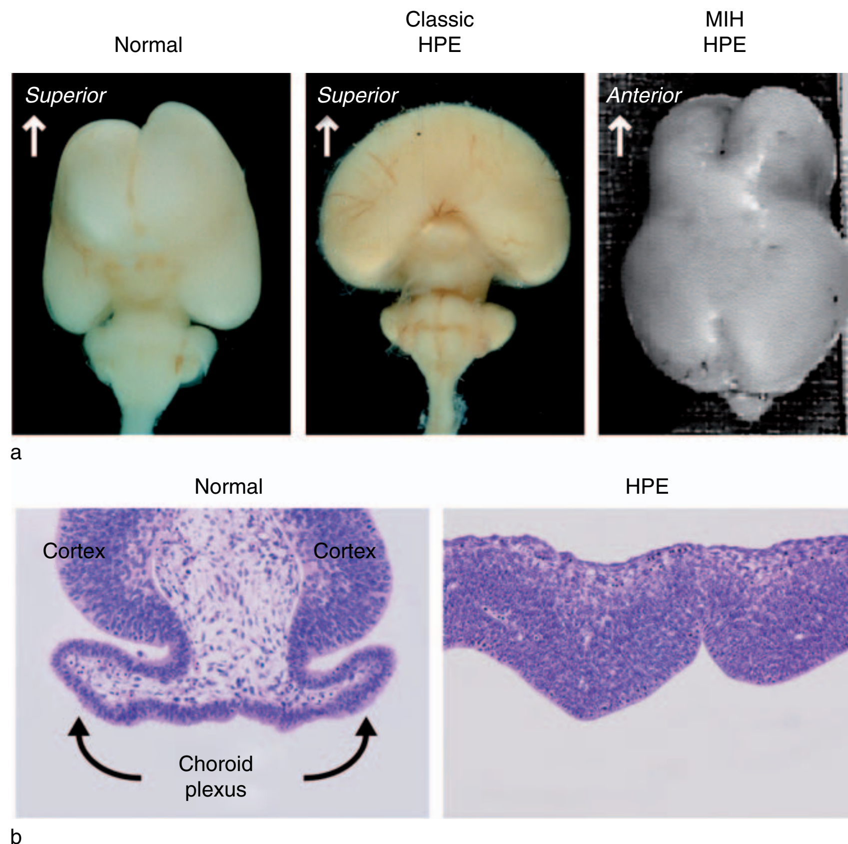

Figure 1 Holoprosencephaly (HPE): (a) normal forebrain compared to classic and middle interhemispheric (MIH) HPE in human fetuses; (b) HPE histology. In (a), the normal forebrain is composed of two hemispheres separated by an interhemispheric fissure. In severe (alobar) classic HPE, hemispheric separation is undetectable throughout the forebrain. Classic HPE neuropathology is always most severe ventrally, with variable extension to the rostral and dorsal domains. In MIH HPE, the lack of hemispheric separation is restricted to the dorsal regions, with no apparent extension to rostral or ventral domains. In (b), central to all types of HPE is failed midline induction, which results in absent midline structures and the continuity of lateral tissues across the midline. In this example of MIH HPE (following roof plate ablation), mutant mice lack choroid plexus and have a continuous cortex across the dorsal midline. Ventral tissues in (b) removed for visual clarity. (a, right panel) Reproduced from Marcorelles P, Loget P, Fallet-Bianco C, et al. (2002) Unusual varient of holoprosencephaly in monosomy 13q. In: *Pediatric and Developmental Pathology*, vol. 5, figure 2a, p. 174, with permission from Springer Science and Business Media. (a (left, middle), b) Reproduced with permission from the Journal of Neuropathology and Experimental Neurology.

nonneural organizers act before and during gastrulation to induce neural fate and AP patterning and then induce organizers within the forebrain tissue itself. Induction of these local neural organizers is often homeogenetic in nature – the nonneural cells instruct their neural neighbors to adopt a fate similar to its own – with the homeogenetic signals being the morphogens themselves. Important nonneural organizers of the forebrain include the node, anterior visceral endoderm (AVE), epidermal ectoderm, and prechordal plate.

The Node and Anterior Visceral Endoderm: Morphogen Antagonists and Forebrain Specification

Neural induction The node and AVE have primary importance in neural induction in mammals (**Figure 2**). Despite lingering questions and apparent differences among species, antagonism of bone morphogenetic protein (Bmp) signaling consistently takes center stage in the process of neural induction, and both the node and AVE are rich sources of Bmp antagonists, such as Noggin and Chordin. In some species, Bmp antagonism suffices for neural induction, whereas additional competence signal(s) – most likely fibroblast growth factors (Fgfs) – seem to be required in others.

Forebrain specification The AVE, which eventually underlies the future forebrain after cell movements and rotation (**Figure 2(a)**), has been shown in mice and chicks to regulate AP patterning of the neural plate. In addition, the AVE is necessary and sufficient for forebrain specification. Multiple AVE-derived

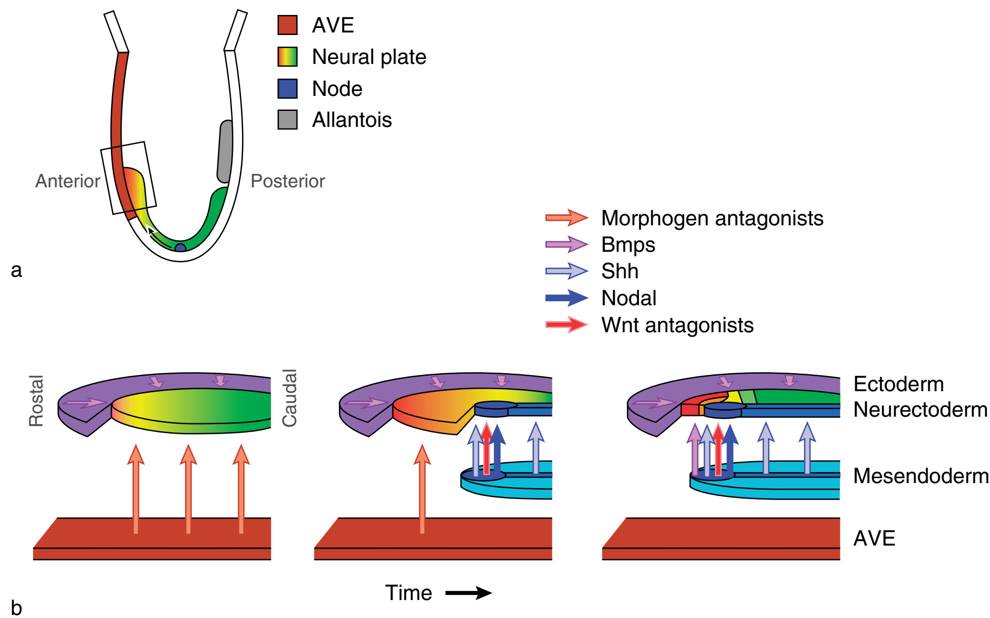

Figure 2 Nonneural organizers of the forebrain: (a) schematic cross section of early embryo; (b) patterning of the anterior neural plate by nonneural organizers (tissue layers separated for visual clarity). In (a), the extraembryonic anterior visceral endoderm (AVE) has rotated to underlie the future forebrain while cells in and around the embryonic node intercalate and migrate rostrally between the ectoderm and endoderm to form the prechordal plate and notochord. In (b), before prechordal plate arrival, the forebrain anlage is adjacent to the AVE (brown) and nonneural ectoderm (purple). AVE-derived morphogen antagonists are involved in AP patterning and forebrain specification. Antagonism of Bmp signaling is central to the earlier process of neural induction, but the roles of ectoderm-derived Bmps at the neural plate stage remain largely undefined. The prechordal plate and associated mesendoderm (blue) intercalate between the AVE and neural plate (neurectoderm). The prechordal plate produces multiple signals (Nodal, Shh, Bmp7) that influence rostral morphogenesis and induction of midline neural tissue. AP, anterior–posterior; Bmp, bone morphogenetic protein; Shh, Sonic hedgehog; Wnt, Wingless-int. (b) Adapted from *Developmental Cell*, vol. 6, Wilson SW and Houart C, Early steps in the development of the forebrain, p. 171, 2004, with permission from Elsevier.

signals mediate these processes, including cerebrus and dickkopf, which antagonize Bmp, Wingless-int (Wnt), and Nodal signaling. Antagonism of multiple morphogens, many of which caudalize neural tissue, is central to AVE function. Thus, AVE-dependent forebrain specification is not instructive *per se* but, instead, involves protecting the forebrain from factors that impart caudal character, such as Wnts, Fgfs, and retinoic acid. This has contributed to the so-called default model in which forebrain fate represents the default state of neural tissue unexposed to patterning signals. Eventually, AVE signaling to neural tissue is altered by the intercalation of mesendoderm during gastrulation (**Figure 2**(**b**)).

Role in anterior neural border specification The AVE has been implicated in specifying the anterior neural border (ANB), the local neural organizer at the edge of the rostal neural plate. Removal of the AVE or inactivation of its morphogen antagonists results in the absence of the ANB and telencephalon. In addition to cerebrus and dickkopf, the AVE produces secreted Frizzled-related proteins (sFRPs), which are (**Figure 3**), Wnt antagonists, and Wnt antagonism is known to be required for ANB formation.

Epidermal Ectoderm: Bmps and Roof Plate Induction

Bmp antagonism in neural induction Continuous with the neural plate is nonneural epidermal ectoderm, the future skin. Prior to and during neural induction, the ectoderm possesses high Bmp signaling levels, and antagonism of this high-level signaling is central to neural induction. Ultimately, high Bmp signaling confers epidermal fate. Epidermal ectoderm-derived Bmps are likely to mediate multiple processes at the rostral and lateral margins of forebrain neural plate, although these functions remain undefined.

Role in roof plate induction In the chick and mouse spinal cord, Bmps from epidermal ectoderm induce the formation of the roof plate (RP), the dorsal midline organizer of the neural tube (**Figure 3**(**a**)). (Note that the lateral margins of neural plate become the dorsal midline of neural tube after neurulation.) Because the RP continues to produce Bmps, epidermal ectoderm-dependent RP formation serves as a classic example of homeogenetic induction, and it appears likely that homeogenetic RP induction occurs at all levels of the developing neural tube.

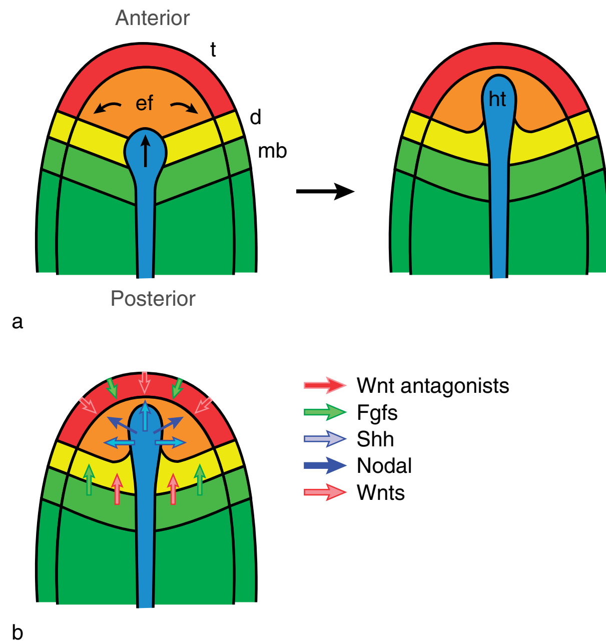

Figure 3 Local neural organizers of the neural plate: (a) rostral morphogenesis of the hypothalamic anlage; (b) signals produced by local neural organizers. In (a), cells of the hypothalamus (ht) originate at or near the node and then move rostrally under the influence of the prechordal plate. This morphogenesis results in the lateral displacement of cells in more rostral fields, particularly the eye field (ef). In (b), Wnt antagonists and Fgfs (most notably Fgf8) are produced at the rostral margin of the neural plate (the anterior neural border). The hypothalamic anlage produces Nodal and Shh, whereas Fgfs and Wnts are produced caudally. d, diencephalon; Fgf, fibroblast growth factor; mb, midbrain; t, telencephalon; Adapted from Wilson SW and Houart C (2004) Early steps in the development of the forebrain. *Developmental Cell* 6: 168, figure 1, with permission from Elsevier.

Prechordal Plate: Nodal, Shh, Bmp, Ventral Midline Induction, and Role in Holoprosencephaly

Prechordal plate formation During gastrulation, mesendodermal cells from the node migrate rostrally along the midline between the ectoderm and endoderm layers. These cells form the tight notochord, which underlies the neural tube up to the midbrain–forebrain boundary and the prechordal plate, a loose cell collection that underlies the emerging forebrain (**Figure 2**(**b**)). The prechordal plate shares certain similarities with the notochord, in particular the production of the Sonic hedgehog (Shh) morphogen. Unlike the notochord, however, the prechordal plate also produces several other signaling proteins, including Nodal, Bmp7, and Wnt antagonists.

Regulation of hypothalamic morphogenesis and induction of ventral midline organizers The prechordal plate regulates three different aspects of hypothalamic (HT) development: (1) the rostral migration of future HT cells, (2) HT induction, and (3) HT patterning. The hypothalamus then serves as a local neural organizer caudomedial to the eye field. Prechordal plate signals also induce local organizer function in the ventral telencephalic midline (VTM; preoptic and entopeduncular regions, and then later in the medial ganglionic eminence). Nodal and Shh signals account for these prechordal plate-dependent functions. The prechordal plate has also been implicated in ANB development because Shh is needed to maintain Fgf8 expression in the ANB.

Role in classic holoprosencephaly Among nonneural organizers, only the prechordal plate has been directly implicated in HPE induction, and prechordal plate defects generate classic HPE phenotypes. Manual prechordal plate removal results in a single eye field, failed HT induction, and molecular defects characteristic of classic HPE. In addition, genetic analyses of the Nodal, Shh, and Bmp signaling pathways point to the primary involvement of the prechordal plate in classic HPE induction.

Local Neural Organizers at the Forebrain Midline

Under direction of nonneural organizers, local organizers are established within forebrain tissue itself (**Figure 4**). This occurs during the period spanning the end of gastrulation and the beginning of neurulation (neural tube formation). Local midline organizers include the hypothalamus and ventral telencephalic midline (VTM) ventrally, the ANB rostrally, and the RP dorsally. Induction of ventral and rostral organizers occurs before that of the RP, which is completed after neurulation. Among these, the RP has been directly implicated in MIH HPE, whereas molecular genetic and transplant studies also suggest direct ANB involvement in classical HPE.

Hypothalamus and Ventral Telencephalic Midline: Nodal, Shh, and Ventral Patterning

Hypothalamic morphogenesis and induction In fish and chicks, HT cells originate near the node, where they are intermingled with floor plate cells. HT cells then migrate rostrally toward the telencephalon, which results in a lateral displacement of forebrain tissue, most notably the eye field (**Figure 3**(**a**)). This morphogenesis lags slightly behind that of the prechordal plate (**Figure 2**(**b**)), and the prechordal plate regulates HT morphogenesis via Nodal signals. Although less well appreciated than the signaling functions, defects in rostral HT morphogenesis are easy to envision as the culprits of cyclopia and HPE. Prechordal plate signaling via Nodal and Shh signals is then responsible for inducing HT and VTM fates and signaling functions.

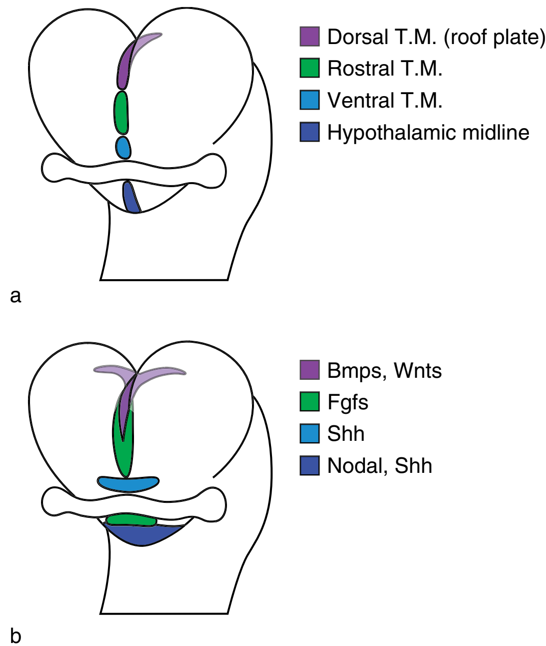

Figure 4 Local neural organizers of the rostral neural tube: (a) midline organizers; (b) morphogen expression. In (a), specialized signaling centers are aligned at the midline following neurulation, including at the closed rostral end of the neural tube. In (b), the midline organizers serve as central sites of morphogen production, which then extends beyond the midline to involve additional sites. Dorsally, Bmps and Wnts are produced at the midline and then secondarily at the dorsomedial edges of telencephalon (choroid plexus and cortical hem). Rostral Fgf8 expression extends to involve a domain caudal to the eye field. Conversely, Shh production extends rostrally to involve the ventral telencephalic midline rostral to the eye field. TM, telencephalic midline.

Nodal and Shh expression Following induction, the HT continues to produce Nodal and Shh (**Figure 3**(**b**)). Nodal and Shh signals from the prechordal plate and/ or produced locally then regulate HT patterning. The VTM is induced to express Shh, but not Nodal, and Shh expression from the VTM eventually extends into the medial ganglionic eminence (MGE) (**Figure 4**). These ventral Shh sources are probably responsible for inducing and patterning the septum, MGE, and lateral ganglionic eminence (LGE) in the ventral telencephalon.

Roles in patterning and holoprosencephaly pathogenesis Distinguishing the roles of ventral neural organizers from those of the prechordal plate has been difficult due to their common expression of morphogens. Nonetheless, both the hypothalamus and VTM seem certain to be involved in HPE pathogenesis, if not its induction. Based on proximity and timing, the VTM may be directly responsible for the basal ganglia defects (e.g., basal ganglionic fusion) that are frequently seen in classic HPE.

Anterior Neural Border: Wnt Antagonists, Fibroblast Growth Factors, and Telencephalic Specification and Patterning

Anterior neural border induction In addition to the AVE, the prechordal plate and epidermal ectoderm are well positioned to participate in ANB specification. Shh signaling, most likely from prechordal plate, is needed to maintain Fgf8 expression in the ANB, although initial Fgf8 induction can occur in the absence of Shh.

Expression of Wnt antagonists and Fibroblast growth factors Following induction, the ANB expresses two different classes of secreted products: (1) Tlc, a Wnt antagonist of the sFRP family and (2) a number of Fgfs, which vary among species (e.g., Fgf3/8 in fish and Fgf8/ 15/17/18 in mice) (**Figure 3**(**b**)). Fgf8 is particularly important for ANB function. Fgf8 expression does not remain restricted to the ANB, however; it extends caudally into the optic region and then splits. Low-level Fgf8 expression is also detectable in dorsal telencephalic and diencephalic midline regions after neurulation, which may represent continued Fgf8 expression that originated at the lateral margins of neural plate.

Roles in telencephalic specification and patterning Ablation and transplant studies suggest a central role for the ANB in telencephalic specification and patterning. These two functions are mediated by the two different classes of ANB products. Tlc mediates ANB-dependent telencephalic specification by antagonizing caudal Wnt sources. The Fgfs (particularly Fgf8) mediate ANB-dependent telencephalic patterning rather than its initial specification, promoting rostral telencephalic fates over caudal ones.

Potential roles in midline patterning and holoprosencephaly induction ANB tissue rescues midline eye-field fates in Nodal mutants, implicating the ANB in the eye defect of classic HPE. Although Fgf8 expression extends beyond the ANB, genetic analyses suggest a prominent role for the ANB in Fgf8 mutant HPE phenotypes.

Roof Plate: Bmps, Wnts, Dorsal Midline Induction, and Role in Middle Interhemispheric Holoprosencephaly

Roof-plate induction Based on analogy to the spinal cord, nonneural (epidermal) ectoderm probably has a central role in inducing forebrain RP, although this remains unproven.

Expression of Bmps and Wnts Following its formation and induction during neurulation, the RP expresses several Bmps (2/4/5/6/7/12), as well as multiple Wnts.

Bmp4, which is the best-studied Bmp in forebrain development, is expressed in the RP and abuts the Fgf8 domain in the rostral midline (**Figure 4(b)**).

Roles in dorsal midline induction In addition to minor lineage contributions, the RP governs induction of dorsal fates near the telencephalic midline, most notably the telencephalic choroid plexus epithelium (CPe) and cortical hem (the small CPe–cortex junctional region). The RP also patterns the adjacent dorsal cortical primordium. Based on rescue and gain of function studies, many of these RP-dependent functions are mediated by Bmps. These Bmp-dependent RP functions at the dorsal telencephalic midline parallel those in the dorsal spinal cord and cerebellum.

Role in middle interhemispheric holoprosencephaly In addition to failed dorsal midline induction, genetic RP ablation in mice results in MIH HPE. The ablation phenotype also accurately predicts CPe and hippocampal defects in MIH HPE patients, thus supporting a causal role for the RP in human MIH HPE.

Molecules and Pathways in Forebrain Midline Induction and Holoprosencephaly

With a cellular framework now established, we turn our attention to the individual genes and signaling proteins involved. Although selective molecular expression allows for functional assignment to specific organizers in some cases, individual signals and genes are most often expressed beyond the confines of single organizers (e.g., after homeogenetic induction; **Figure 4**). However, whether they inform organizer function or not, molecular analyses have illuminated a great deal about midline patterning and HPE. We start by grouping causal HPE genes into defined signaling pathways, then discuss each pathway in turn.

Genes Implicated in Holoprosencephaly

Several HPE genes have been identified from studies in fish, mice, and humans. In humans, nine genes have been identified, with a similar number of genes awaiting identification. Most of the identified genes have known functions in specific morphogen pathways. Some encode transcription factors (e.g., Six3, Zic2, and Tgif) with less certain relationships to individual pathways or organizers, although recent studies have begun to clarify their roles as well (**Table 1**).

Table 1 Holoprosencephaly genes and signaling pathways[a]

NODAL	*SHH*	*FGF8*	*BMP*
Nodal	SHH	Fgf8	Chordin
Cyclops	PTCH	SIX3	Noggin
Squint	DHCR7		Twsg1
One-eyed pinhead	GLI2		ZIC2
TDGF1/CRIPTO	Smo		
FAST1	Cdo		
	DispA		
	SIX3		

[a]Genes from humans and experimental organisms linked to definitive holoprosencephaly (HPE) phenotypes, grouped by signaling pathway. Human genes are shown in upper case. TGIF may be involved in both Nodal, Bmp, or retinoic acid signaling in the forebrain, although this remains undefined. Megalin may be involved in either Shh or Bmp signaling. SIX3 and ZIC2 may be involved in additional signaling pathways.

Signaling Pathways in Forebrain Midline Induction and Holoprosencephaly

Nodal signaling at the ventral midline

Genetic associations with holoprosencephaly. Defects in several Nodal signaling components lead to HPE phenotypes in fish, mice, and humans. These include Nodal ligands, a receptor subunit (ActRIIA), extracellular cofactors (EGF-CFC family), a transcriptional transducer (Smad2), and a transcriptional cofactor (Tgif). Genetic loss of function yields predictable phenotypes based on the known positive or negative regulation of Nodal signaling by these genes, with the exception of Tgif. (HPE is associated with reduced Nodal signaling and Tgif loss of function, but, as a negative regulator, Tgif loss of function should lead to increased Nodal signaling.)

Nodal sites and functions: Confined expression to ventral tissues. Among its many developmental functions, Nodal signaling is required for the initial specification of the prechordal plate, where Nodal ligands are expressed. Prechordal plate-derived Nodal signals then regulate HT morphogenesis and induction (**Figure 2(b)**). Nodal signaling is the only pathway known to regulate rostral HT morphogenesis. Later, Nodal is expressed in the hypothalamus itself (**Figure 3(b)**), but does not extend beyond the ventral midline during this developmental period.

Nodal mutant holoprosencephaly phenotypes: Severe classic holoprosencephaly extending to the dorsal midline. Despite the restriction of Nodal expression to ventral tissues, Nodal mutants have a particularly severe HPE phenotype that extends to involve the rostral and dorsal midlines. This severe classic HPE phenotype is reminiscent of those associated with defects in Shh signaling.

Shh signaling at the ventral midline

Genetic associations with holoprosencephaly. Like Nodal, studies from different organisms implicate several Shh signaling components in ventral midline induction of the forebrain. These include the ligand, its cholesterol-based posttranslational modifications,

Shh release (DispA), receptors (Ptch, Smo), and the coreceptor or trafficking molecule (megalin). By and large, genetic analyses of individual components have led to midline and HPE phenotypes consistent with their known positive or negative effects on Shh signaling.

Shh sites and functions: Confined expression to ventral tissues. Shh is co-expressed in prechordal plate with Nodals (**Figure 2(b)**). Prechordal plate Shh induces the overlying hypothalamus and VTM (preoptic and anterior entopeduncular regions), which leads to activated Shh expression in these tissues (**Figures 3(b)** and **4(b)**). Although the relative contributions of Shh from prechordal plate, the hypothalamus, and the VTM remain difficult to dissociate, ventral Shh signaling is clearly essential for midline eye-field specification and for excluding laterally expressed genes from the midline. The midline eye-field defects in Shh mutants do not result from aberrant HT morphogenesis, unlike Nodal mutants, and are therefore more clearly due to failed inductive signaling. At later stages, Shh production extends into more lateral regions of the ventral telencephalon (medial ganglionic eminence) (**Figure 4(b)**). Like Nodal, all Shh sources remain confined to ventral tissues during the HPE inductive period.

Shh mutant holoprosencephaly phenotypes: Severe classic holoprosencephaly extending to the dorsal midline. Like Nodal, Shh signaling defects in mice and humans lead to ventral predominant classical HPE, which typically extend to involve the rostral and dorsal midlines. For example, human HPE phenotypes due to Shh mutations include absence of the interhemispheric fissure, absence of corpus callosum, and failed cortical separation in rostral and dorsal telencephalic domains. At the dorsal midline, Shh null mice exhibit no telencephalic CPe and aberrant expression of lateral cortical markers across the midline. Notably, the absence of CPe and abnormal midline expression of lateral cortical markers mimics the dorsal midline phenotype induced by RP ablation.

Fgf8 signaling at the rostral midline

Genetic association with holoprosencephaly. Although not yet implicated in humans, Fgf signaling from the ANB has been implicated in midline patterning defects in fish (Fgf8 and Fgf3) and mice (Fgf8). Significantly, Fgf8 mutations have recently been shown to result in definitive HPE phenotypes in mice. Other dedicated Fgf signaling components have yet to be implicated in HPE, although Six3 has a critical role in Fgf8 induction.

Fgf8 sites and functions: Anterior neural border with ventral and dorsal extensions. Fgfs are not expressed in nonneural tissues near the forebrain but are, instead, limited to local neural organizers. In the forebrain neural plate, the expression of Fgf8 and other Fgfs (which differ among species) initiates in the ANB at the margin of the telencephalic field (**Figure 3(b)**). Fgf8 expression eventually extends caudally into the midline eye field and then splits bilaterally (**Figure 4 (b)**). After neurulation, high-level Fgf8 expression continues at the rostral telencephalic midline, although lower levels also become detectable in the dorsal telencephalic and diencephalic midline regions. Caudally, Fgfs are also expressed in the isthmus organizer, the local neural organizer at the midbrain–hindbrain boundary.

Fgf8 studies and mutant. holoprosencephaly phenotypes: Variable, extending to the dorsal midline Fgf8 mutant phenotypes are variable but include those corresponding to classic HPE. Defects in HT and VTM induction correlate with Fgf8 activity level, based on analysis of hypomorphic Fgf8 mouse mutants. Moreover, ectopic Fgf8 studies in chicks indicate the positive regulation of midline fate (including induction of an ectopic sulcus reminiscent of the interhemispheric fissure). Notably, midline induction failure in Fgf8-dependent HPE can also extend to the dorsal midline. Unlike its consistently positive regulation of ventral midline fates, however, Fgf8 effects on dorsal midline development are nonlinear. Recent studies in the chick midbrain provide a plausible explanation – namely that Fgf8 both promotes RP competence (positive regulation) and inhibits RP differentiation (negative regulation).

Bmp signaling at the ventral and dorsal midlines

Genetic association with holoprosencephaly. Genetic studies on the Bmp antagonists Noggin and Chordin in mice have directly implicated Bmp signaling in HPE. Other dedicated Bmp signaling components have yet to be associated, although a major site of Zic2 action appears to be upstream of the Bmp-producing RP. Tgif (a Smad corepressor) may also act in the Bmp pathway, and Bmp signaling has been implicated in many of the midline defects associated with RP ablation in mice.

Bmp sites and functions: Ventral and dorsal midline tissues. Prior to gastrulation, multiple Bmps are expressed in ectoderm, where the antagonism of Bmp signaling induces neural fate. Following neural induction, Bmps continue to be expressed at high levels in nonneural (epidermal) ectoderm contiguous with the neural plate. Noggin and Chordin are expressed at highest levels in the prechordal plate and notochord from early stages, whereas Bmp7 expression in the prechordal plate begins later (**Figure 2(b)**). Following neurulation, the RP becomes an epicenter for Bmp expression (**Figure 4**), although many Bmps maintain expression domains in the overlying ectoderm,

mesenchyme, and elsewhere. Bmp expression is maintained at later stages in two dorsal telencephalic midline tissues derived or induced by the RP: CPe and cortical hem, which is the CPe–cerebral cortex junctional region that serves as a Wnt production epicenter.

Bmp mutant holoprosencephaly phenotypes: Classic and middle interhemispheric forms. The Noggin/Chordin mutant HPE phenotype falls into the ventral-predominant classic HPE category. This correlates with their major site of expression in the forebrain region, the prechordal plate. Bmp signaling has been shown to suppress Shh expression, which probably accounts for the need to antagonize Bmp signaling in the prechordal plate (**Figure 2(b)**). Bmp-antagonist studies provide additional evidence for the primary role of prechordal plate in classic HPE induction. On the other hand, RP ablation induces MIH HPE, including the absence of CPe and aberrant midline expression of cortical markers (**Figure 1(b)**). Bmp signaling has been directly implicated in CPe induction and suppression of cortical markers at the midline, and multiple midline defects caused by RP ablation can be rescued by exogenous Bmp4. Thus, Bmp signaling appears to be centrally involved in RP-dependent midline and HPE induction.

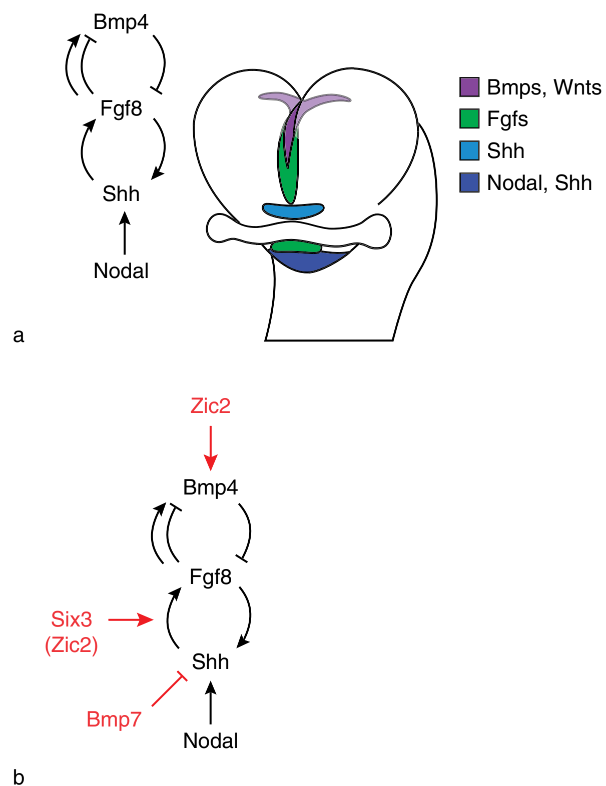

Figure 5 Genetic network in forebrain midline induction: (a) the core morphogen interaction network; (b) additional network components. In (a), The genetic network starts with ventral Nodal, which positively regulates ventral Shh production. Shh then engages in a positive feedback circuit with Fgf8 in the ventral and rostral domains. Fgf8 has both positive and negative influences on the dorsal midline, serving initially as a competence factor for RP induction. Later, Fgf8 acts as an inhibitor of RP maturation and participates in a negative feedback loop with dorsal Bmp4 and other Bmp signals. This core network explains how defects in morphogens expressed exclusively in ventral domains (Nodal and Shh) can spread to involve the rostral and dorsal midlines, which is characteristic of classical HPE. Conversely, this network also explains why primary RP defects that cause MIH HPE do not spread rostrally or ventrally. In (b), Bmp7 produced by the prechordal plate antagonizes Shh production, which accounts for the classic HPE phenotype due to Noggin and Chordin loss. Six3 mutations also cause classic HPE, which is consistent with Six3 involvement in multiple circuits that all serve to promote the Shh → Fgf8 positive feedback loop. Although the mechanism by which Zic2 mutations lead to classic HPE remains uncertain, Zic2 mutation can also result in MIH HPE due to its role in RP induction. HPE, holoprosencephaly; MIH, middle interhemispheric; RP, roof plate.

Interactions among the Signaling Pathways

Several insights into midline patterning and HPE have come from recent *in vivo* and *in vitro* studies that have determined how the various signaling pathways interact. In addition to these interactions, we discuss how the transcription factors implicated in HPE impact these signaling pathways (**Figure 5**).

Nodal → Shh Pathway in the Ventral Midline

As previously discussed, Nodal and Shh share not only common expression sites but also similarity in mutant phenotypes. Genetic epistasis experiments provide significant evidence for Nodal being upstream of Shh in the pathway leading to HT induction. For example, Nodal null mutants lack prechordal plate and Shh expression, and exogenous Shh rescues HT and ventral telencephalic Nodal defects. Shh appears sufficient to mediate many, but not all, Nodal-dependent functions (e.g., diencephalic defects in Nodal mutants are not rescued by Shh, suggesting a separate Shh-independent Nodal pathway). Subsequent HT patterning involves parallel pathways for Nodal and Shh (as well as Bmp7), with the two morphogens biasing toward opposing HT fates.

Shh → Fgf8 Positive Feedback Loop in the Ventral and Rostral Midlines

Evidence for a positive feedback loop Fgf8 expression is almost entirely lost in Shh mutant mice, but Fgf8 expression is dramatically upregulated in mouse mutants with increased Shh signaling (Gli3 mutants), indicating that Shh signaling positively regulates Fgf8 expression. The critical Shh sources at the ventral

midline (whether the prechordal plate, hypothalamus, or VTM) remain to be determined. Interestingly, Shh is not required for Fgf8 induction (based on rescued Fgf8 expression in Shh/Gli3 double null mutants) but, rather, for its maintenance once induced. Conversely, Fgf8 loss in fish and mice leads to decreased Shh expression in the hypothalamus and VTM. Consistent with a positive feedback loop, Fgf8 and Shh expression are consistently co-regulated whenever Bmp signaling is altered, either genetically or with exogenous proteins in chicks and mice. The positive feedback loop between Shh and Fgf8 is similar to that described in limb development.

Convergence of Shh and Fgf8 signaling in the eye field In addition to the positive feedback loop, Shh and Fgf8 signaling cooperatively induce optic stalk fate in the eye field. The eye field is well placed for such convergence, where nearby Shh and Fgf8 expression domains are closely apposed (**Figure 4(b)**). In cyclopic Shh mutant eyes, exogenous Fgfs or ANB transplants are sufficient to rescue optic stalk fate. In addition, the absence of ventral telencephalic fates in Shh −/− mice is suppressed by Gli3 inactivation, implying the presence of another ventralizing signal. This most likely includes Fgf8, which is dramatically upregulated in Shh/Gli3 compound null mice.

Positive Regulation of the Shh → Fgf8 Loop by Six3

Although Six3 does not fit neatly into a single signaling pathway, it is embedded in two defined positive feedback loops that serve to promote the Shh → Fgf8 loop. Six3 is expressed in the anterior neural plate and ANB, where it is required for forebrain and eye specification. These Six3 functions involve a feedback loop between Six3 and Wnt repression. Six3 is also required for ANB and Fgf8 induction. Following Fgf8 induction, Six3 serves as a competence factor for Fgf8-dependent telencephalic gene expression, resulting in a Six3 → Fgf8 positive feedback loop in the telencephalon. Six3 is also strongly expressed in the eye field and hypothalamus, where it confers competence for Shh-dependent HT fate. Thus, all defined Six3-dependent loops and pathways act to promote the Shh → Fgf8 positive feedback loop.

Negative Regulation of the Shh → Fgf8 Loop by Bmps

As already discussed, several experimental manipulations that alter Bmp signaling (e.g., Bmp and Noggin bead placements in chicks; RP ablations, Chordin/Noggin double knockouts, Noggin electroporations, and exogenous Bmp applications in mice) lead to the same conclusion – namely that Bmp signaling negatively regulates both Fgf8 and Shh expression. As discussed earlier, Bmps in the prechordal plate (**Figure 2(b)**) probably account for the HPE phenotype seen in Chordin/Noggin compound null mice. Bmps produced in the dorsal midline region may also negatively regulate Fgf8 and Shh because Fgf8 and Shh expression are well maintained (and Fgf8 possibly upregulated) following genetic RP ablation in mice.

Fgf8 → Roof Plate Pathway: Positive and Negative Regulation by Fgf8

Studies in fish and mice indicate that reduced Fgf8 expression can lead to dorsal midline defects and HPE. This indicates that Fgf8 can positively regulate dorsal midline induction. However, until recently, most gain-of-function studies have provided evidence for negative Fgf8 regulation of dorsal midline development. Significantly, analyses of hypomorphic Fgf8 mouse mutants have provided evidence for nonlinear Fgf8 effects (i.e., reduced Fgf8 signaling can have both positive and negative effects on the dorsal midline), and recent studies in chick midbrain provide an explanation for this nonlinearity. These studies identified two different roles for Fgf8 in RP development. Fgf8 first acts as a competence factor for RP induction (e.g., Fgf8 beads can induce an ectopic RP *in vivo*). At later stages, Fgf8 inhibits RP maturation, probably by antagonizing transforming growth factor (TGF)β signals, including Bmps, which positively regulate RP differentiation. Earlier studies in the chick forebrain suggest similar Fgf8 functions in the forebrain, although these remain unproven. The ability of Fgf8 to confer RP competence provides, for the first time, a mechanism accounting for failed dorsal midline development following Fgf8 reduction.

Zic2 → Roof Plate Pathway in the Dorsal Midline

Studies in mice demonstrate Zic2 expression prior to neurulation, followed by preferential Zic2 expression in the dorsal forebrain. Zic2 knockdown in mice leads to defective neurulation kinetics and absence of RP induction, which leads to a dorsal HPE phenotype. Notably, among known HPE genes, only *ZIC2* has been specifically linked to human MIH HPE (single case predicting a hypomorphic 12-amino-acid in-frame deletion), although most human *ZIC2* mutations lead to classic HPE. The classic phenotype could be related to Zic2 expression in ventral midline tissues. Nonetheless, the mouse knockdown studies and hypomorphic human allele suggest that dorsal midline development – and RP induction in particular – are particularly sensitive to reduced Zic2 level. Zic2 expression is well maintained following RP ablation in

mice and is insensitive to exogenous Bmps in explants and dissociated cells, which argues against positive feedback from the RP back to Zic2.

Sufficiency of Morphogen Interactions to Account for Classic and Middle Interhemispheric Holoprosencephaly Phenotypes

As mentioned earlier, how pure ventral signaling defects caused by Shh and Nodal mutations can spread to involve the dorsal midline has been a conundrum that lacked explanation. Studies of the developing spinal cord, which so often serve as the precedent for the developing forebrain, do not help in this regard. For example, Shh loss in the spinal cord leads to ventral expansion of Bmp-regulated genes (as occurs in the forebrain), but it does not lead to RP failure or other dorsal midline defects.

Recent studies, particularly those involving Fgf8 and the RP, provide for a coherent genetic regulatory network that resolves this apparent conundrum and is sufficient to account for both classic and MIH HPE phenotypes (**Figure 5**). In this network, Fgf8 serves as the key intermediary between ventral Nodal/Shh and dorsal Bmp signaling (via the ANB and rostral midline, which distinguish the anterior neural tube from more caudal regions). The role of Fgf8 as an intermediary results from both its involvement in positive feedback with Shh and its positive regulation of dorsal midline development by providing competence for RP (and dorsal Bmp) induction. Via Fgf8, defects in Nodal and Shh signaling then lead plausibly to failed dorsal midline development, as seen commonly in classic HPE.

Conversely, the RP ablation and Bmp genetic studies help to explain why MIH HPE defects do not spread ventrally. Based on a relatively large number of studies, Bmp signaling acts uniformly to negatively regulate both Fgf8 and Shh expression (**Figure 5**). Reduction of dorsal Bmp signaling (e.g., due to RP failure), therefore, serves to maintain or upregulate the pathways responsible for patterning the rostral and ventral midlines, thus preserving these domains in MIH HPE. The unidirectionality of spread in HPE (i.e., ventral to dorsal in classic HPE but not vice versa in MIH HPE) is also consistent with the temporal order of differentation, with the induction of ventral and rostral midline signaling centers occurring prior to neurulation and RP induction.

Interestingly, in virtually all cases examined, positive and negative regulation of the involved signaling pathways occurs at the level of individual morphogen mRNAs. Conceivably, regulation of signaling pathways could occur at myriad levels (e.g., extracellular antagonists, extracellular diffusivity, cell surface receptors, intracellular transducers, or degradation), and regulation at multiple levels is likely at the forebrain midline, as it is elsewhere. Thus, it is perhaps remarkable that steady-state mRNA levels of the morphogens alone are sufficient to account for the spectrum of midline patterning defects seen in HPE. Although unproven, we might then predict that regulation at other levels of signaling should occur predominantly in the same positive or negative direction as those for the individual morphogen mRNAs.

Future Directions

Several unanswered questions remain about the pathways involved in forebrain midline induction and HPE. The extent to which the simple morphogen interaction network models the HPE phenotypes requires further testing. In addition, notably absent from most of this article is a detailed discussion of Wnt signaling. Wnts have well-defined roles in AP patterning, including telencephalic specification, but their precise roles in midline patterning remain undefined. Because Six3 and Wnt suppression make up a positive feedback loop central to telencephalic induction, Wnt suppression is at least indirectly involved in promoting competence (via Six3) for Shh- and Fgf8-induced ventral midline fates. Rostral Fgf8 signaling has also been implicated as a negative regulator of dorsal Wnt signaling, although the role of this pathway in HPE pathogenesis is unclear. Another conceivable participant is retinoic acid (RA). RA has been implicated as one of the caudalizing signals whose antagonism is required for forebrain fate specification. Excessive RA signaling can lead to an HPE-like phenotype in mice, which is consistent with its ability to downregulate Shh expression.

The role of Tgif also remains somewhat mysterious. A significant number of human *TGIF* mutations (mostly predicting loss of function) have now been associated with HPE. As a repressor of Nodal and RA signaling, Tgif does not yet fit well into the Nodal–HPE pathway (Tgif loss-of-function mutations should lead to increased rather than decreased Nodal signaling), but it fits with RA (the same mutations mimic excessive RA). However, total Tgif loss in mice does not lead to midline induction failure or HPE. In addition, Tgif expression after neurulation appears preferentially in dorsal domains and is higher in the cortex than the dorsal midline. Maintained Tgif expression is dependent on an intact RP and is positively regulated by exogenous Bmps in culture, which is consistent with a role for Tgif in HPE pathogenesis but not in induction *per se*. Additional basic studies on Tgif function in early forebrain development are needed to clarify its placement in the genetic regulatory network that underlies forebrain midline and HPE induction.

See also: Bone Morphogenetic Protein (BMP) Signaling in the Neuroectoderm; Forebrain Development: Prosomere Model; Forebrain: Early Development; Midbrain Patterning; Sonic Hedgehog and Neural Patterning.

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Neural Patterning: Arealization of the Cortex

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Introduction

The cerebral cortex is the largest and most complex component of the brain, and more so than any other brain component has been affected by evolutionary processes resulting in a tremendous increase in size and complexity, exhibited by primates, including humans, by cetaceans (e.g., dolphins, whales), and by elephants, which have the largest brains among all species. The neocortex is the largest region of the cerebral cortex; it exhibits the most substantial increase in size and complexity in more advanced species, disproportionately so compared to the midbrain and hindbrain, and also exhibits arguably the most significant phylogenetically distinct specializations.

Early in embryonic development of mammals, the neural plate differentiates into the neural tube, and during this process the rostral end of the neural tube develops three vesicles, the forebrain, midbrain, and hindbrain, that ultimately give rise to the brain. Soon thereafter, the dorsolateral aspects of the forebrain evaginate to generate the telencephalic vesicles, and the remainder of the forebrain becomes the diencephalon. The expression of individual transcription factors (TFs) or combinations of TFs correlates with morphologic boundaries within the telencepahlon and has important roles in telencepahlon patterning into two major subdivisions – the ventral telencephalon, which gives rise to the striatum and basal ganglia, and the dorsal telencephalon, which gives rise to the cerebral cortex. The diencephalon differentiates into several components, the major ones including the dorsally positioned epithalamus (habenula) and dorsal thalamus, which reciprocally connect with the neocortex, and the ventrally positioned hypothalamus.

Putting the Neocortex in Its Place

Although the focus here is on mechanisms that control area patterning of the neocortex, it is important to place the neocortex into some context. Within the cerebral cortex, the neocortex is sandwiched between what have classically been considered the more primitive archicortex (including entorhinal cortex, retrosplenial cortex, subiculum, and hippocampus) and paleocortex (olfactory piriform cortex). The neocortex is the most highly differentiated and intricately organized region of the cerebral cortex, and its laminar patterning is distinct from that of the other cortical regions, having the largest number of layers: six major, radially stacked, stratified layers, each containing a heterogeneous population of neurons that are morphologically, connectionally, and functionally distinct from those of other layers.

In its tangential dimension, the neocortex is organized into subdivisions referred to as cortical 'fields' by some authors, but more commonly as cortical 'areas.' Areas are distinguished from one another by major differences in their cytoarchitecture and chemoarchitecture, input and output connections, and patterns of gene expression. In the adult, the transition from one neocortical area to another is typically, but not always, abrupt, with borders that can be sharply defined by area differences in cytoarchitecture and chemoarchitecture, and in some instances by the distributions of projection neurons, input projections, or gene expression patterns. These attributes form a specific combination of properties that is unique for each area, and together with unique combinations of gene expression, determine the functional specializations that characterize and distinguish areas in the adult.

The neocortex has four 'primary' areas. In addition, scores of higher-order areas are positioned between the primary areas, serving as higher-order processing centers focused on a particular modality – for example, particularly features of vision, movement, or attention related to the visual field; many higher-order areas are multimodal. Three of the primary areas are sensory: the primary visual (V1), somatosensory (S1), and auditory (A1), which process primary information received from the eye/retina (vision), body (somatosensation), and inner ear/cochlea (audition), respectively. The only major sense not processed by the neocortex is smell. Distinct orders are sensed by olfactory and vomeronasal receptors in the nose and related peripheral organs and are processed in the olfactory piriform cortex, positioned within the cerebral cortex, usually ventrolateral to more rostral parts of neocortex. The fourth primary area of the neocortex is the motor (M1) area, which controls voluntary movement of body parts.

These primary sensory and motor areas are conserved among all mammals, as is the general spatial relationship between them: V1 is positioned caudally; M1 is positioned rostrally, and S1 is located between them; A1 is located caudolaterally to S1. However, some animals with unusual or large and atypical peripheral appendages/sense organs (e.g., the platypus' bill or the echo-location system in bats) have modifications on this general geometrical scheme of area patterning.

The relationships between a primary cortical area and nuclei in dorsal thalamus are critical both for

adult function and for development and differentiation of areas. Dorsal thalamus has four principal thalamic nuclei that functionally and connectionally parallel the four primary cortical areas. Each primary cortical area sends outputs from layer 6 neurons to a principal thalamic nucleus, and receives, from the same nucleus, thalamocortical afferent (TCA) inputs that terminate primarily in layer 4, thereby generating the reciprocal area- and nuclei-specific connections between cortex and thalamus. Like cortical areas, individual nuclei are responsible for processing modality-specific sensory information. The four principal thalamic nuclei receive modality-specific sensory information from peripheral sense organs or receptors and form reciprocal projections with their related primary cortical areas. A simplified wiring circuit has the ventroposterior (VP) nucleus of dorsal thalamus reciprocally connecting with S1, the dorsal lateral geniculate nucleus connecting with V1, the ventral part of the medial geniculate nucleus connecting with A1, and the ventrolateral nucleus connecting with M1. Thus, the modality of a primary cortical area is determined by the modality of the principal thalamic nucleus with which it is reciprocally connected. Higher-order areas are often multimodal and their modalities are determined largely by the modality of the inputs that they receive from other areas – primary as well as higher-order areas, through intracortical axonal projections.

Corticogenesis

Areas of the neocortex differentiate within an earlier, more or less uniform structure composed of postmitotic neurons, termed the cortical plate (CP) (**Figure 1**). Most neocortical neurons, including all projection neurons and glutamatergic neurons, are generated in the ventricular zone (VZ) of the dorsal aspect of the lateral ventricle, or, at later stages, in a second germinal zone, the subventricular zone (SVZ). The VZ generates deeper layer neurons, including the subplate (SP) and the major classes of layer 6 and layer 5 projection neurons, whereas the SVZ is the primary source of superficial layer neurons. In primates, the SVZ is substantially larger than in other mammals (e.g., mice). In addition, the primate SVZ shows substantial differences in mitotic activity in caudal cortex (and possibly elsewhere); these differences may underlie the major increase in the numbers of neurons in the upper layers in V1 compared to adjacent higher-order visual areas (e.g., V2), which in turn likely contributes to the substantial differences in cytoarchitecture between V1 and V2.

The first postmitotic neurons accumulate on the top of the VZ, forming the preplate (PP), positioned just beneath the pial surface. PP neurons are derived not only from the VZ, but also from the cortical hem or other sources external to the neocortex. Neurons subsequently generated in the VZ migrate along the processes of radial glia, which are actually the VZ progenitor cells, then aggregate within the PP, thereby forming the CP, which splits the PP into a superficial marginal zone (MZ) (future layer 1) and a deep SP. The CP gradually differentiates in a deep to superficial pattern, and layers 6 through 2 emerge. The MZ contains Cajal–Retzius neurons that express reelin, a large, secreted protein required for the proper radial migration of CP neurons and their formation of layers. Cajal–Retzius neurons are generated in multiple sites external to the cortical VZ, primarily within the cortical hem. SP neurons have been proposed to serve a number of critical roles in cortical development, including the pioneering of the internal capsule, the path of the major input and output projections between the cortex and the rest of the central nervous system (CNS).

A majority of cortical interneurons, which are γ-aminobutyric acid (GABA)ergic and also typically express a distinct neuropeptide (e.g., somatostatin, substance P, or neuropeptide Y), are generated primarily within the medial and caudal ganglionic eminences. These interneurons migrate along multiple pathways to reach the cortex, and migrate within the cortex along tangentially aligned pathways, being directed by many molecules, including members of the slit, semaphorin, and neuregulin families. Once interneurons reach an appropriate position, they migrate perpendicular to their original migratory plane and migrate radially into the CP.

Areas Differentiate within a ‘Uniform’ Cortical Plate Characterized by Exuberant Distribution of Projection Neurons and a Lack of Other Area-Specific Features Except Area-Specific Thalamocortical Afferent Input

It has been assumed that the specification and differentiation processes of neocortical areas are controlled by interplay between intrinsic mechanisms (i.e., genetic mechanisms that operate within the cortex) and extrinsic mechanisms (TCA input or information relayed by it, for example). However, only in the present millennium has compelling evidence emerged for the genetic regulation of arealization, including

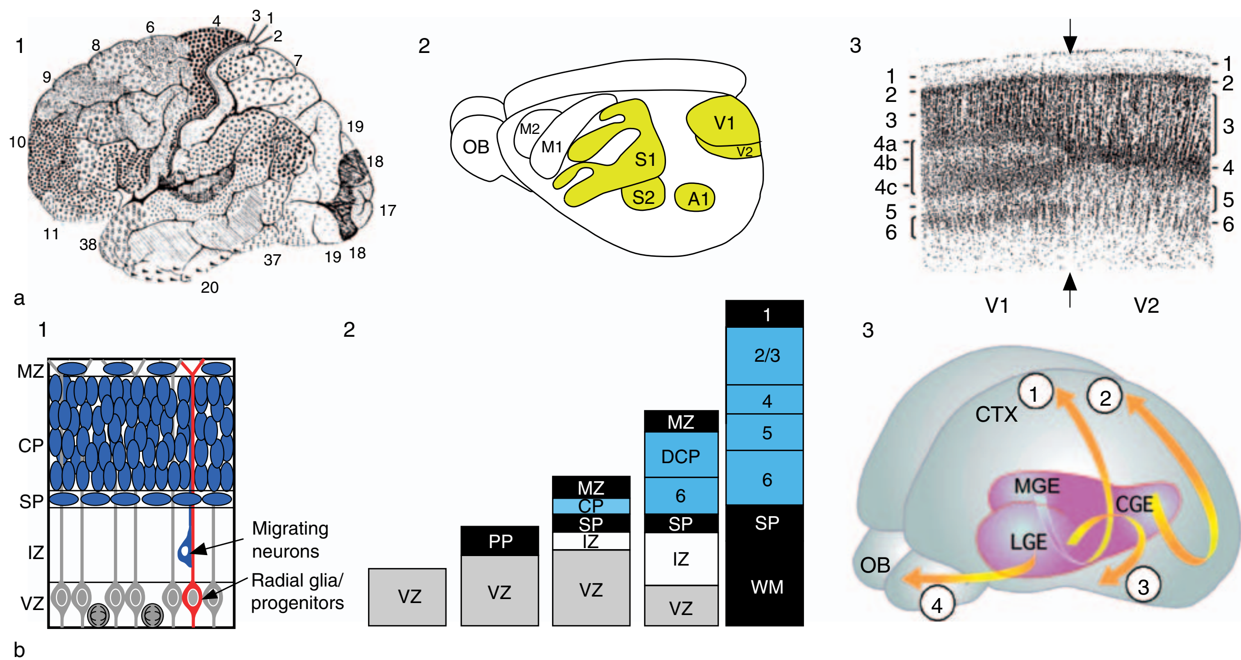

Figure 1 Basics of corticogenesis. (a) Organization of the neocortex into areas. (1) Areas of the human cerebral cortex as defined by Brodmann. View of the lateral surface of the human cerebral hemisphere shows a number of cytoarchitectonically distinct areas, such as the primary visual area (area 17, also termed V1) at the caudal pole, the primary somatosensory area (area 3, also termed S1) in the middle, and, just rostral to it, the primary motor area (area 3, also termed M1). (2) Selected areas of the rat neocortex from Kaas and colleagues showing sensory areas (V1, primary visual; V2, secondary visual; S1, primary somatosensory; S2, secondary somatosensory; A1, primary auditory) as well as the primary and secondary motor areas (M1, M2) and the olfactory bulb (OB). (3) An example of abrupt borders between areas (from an 8-month human fetus), showing the V1–V2 cytoarchitectonic border, revealed using a Nissl stain. Each area has six primary layers, but their architecture and internal sublayering differ. (b) Generation, migration, and lamination of neocortical neurons. (1) Most neocortical neurons, including all glutamatergic and projection neurons, are generated in the neocortical ventricular zone (VZ) and later in the subventricular zone (not shown). Most cortical plate (CP) neurons (SP, subplate) migrate radially on radial glial fibers from the VZ, through the intermediate zone (IZ), and aggregate in the CP. The radial glia are also the progenitors in the VZ that give rise to cortical neurons. (2) Layer development of the rodent neocortex. The first neurons generated in the VZ, as well as neurons that migrate tangentially into the marginal zone (MZ) from external germinal zones, such as the cortical hem, aggregate on top of the VZ and form the preplate (PP), which is later split into MZ and SP by the later generated CP neurons (WM, white matter). (3) Most neocortical interneurons (i.e., GABAergic interneurons of various peptide phenotypes) are generated in the medial ganglionic eminence (MGE, pathway 1) and caudal ganglionic eminence (CGE, pathway 2) and migrate tangentially through the intermediate zone and marginal zone to distribute across the neocortex, and then turn perpendicular to their tangential path and migrate radially into the cortical plate (CTX, cortex). The lateral ganglionic eminence (LGE) is the source of GABAergic interneurons that migrate into the piriform cortex (pathway 3) and to the olfactory bulb (pathway 4). Cortical plate neurons are generated in an inside-out fashion, and layers differentiate from the cortical plate in the same pattern: earlier born neurons form the deeper layers, while later born neurons migrate past them and form the more superficial layers. Interestingly, interneurons generated external to the cortex also become distributed in an inside-out gradient dependent upon their birth date. (b3) Adapted from Corbin JG, Nery S, and Fishell G (2001) Telencephallic cells take a tangent: Non-radial migration in the mammalian forebrain. *Nature Neuroscience* 4(supplement): 1177–1182.

the first demonstration of regulatory genes that control area specification and evidence for patterning centers and signaling molecules that establish the initial patterning of these genes.

Cytoarchitecture and Exuberant Projection Neurons

The CP lacks the many features that distinguish areas in the adult cortex, even after all CP neurons have been generated and layers begin to differentiate within it. The sharp cytoarchitectonic borders evident between areas in the adult cortex are lacking; indeed, other than differences in thickness, CP cytoarchitecture is uniform across its tangential extent. Also absent are the restricted, area-specific distributions of distinct types of projection neurons characteristic of the functional specializations of different cortical areas in adults. Instead, cortical projection neurons have widespread distributions early on that include parts of areas, and even entire areas, in which they are not found in the adult cortex, and their restricted adult cortical distributions come about by the elimination of functionally inappropriate axon segments and branches. This mechanism is used to generate the characteristic patterning of callosal, intracortical, and subcortical projections of the mammalian neocortex. Although the area-specific

adult distributions of projection neurons are sculpted primarily through selective axon elimination, areal differences in the initial distribution of projection neurons have been reported. For example, the adult M1 has a higher density of layer 5 corticospinal neurons than does S1; prior to the phase of axon elimination this difference is present but not to the same degree. Therefore, even in this situation, selective axon elimination contributes to the generation of the distribution and density of projection neurons found in adult brains.

Area-Specific Thalamocortical Afferent Input and Potential Roles in Area Patterning

To sum, all classes of projection neurons initially exhibit an 'exuberant' distribution far broader than that found in the adult brain. In contrast to this lack of area specificity in the early distribution of projection neurons, the area-specific projection of TCAs originating in the principal sensory nuclei of dorsal thalamus is evident even at the early stages in their development. Progress has been made in defining mechanisms of TCA pathfinding, particularly subcortically from dorsal thalamus to the neocortex, but the molecular control of TCA targeting of specific areas remains poorly defined relative to mapping in other systems, such as retinal projections. As in the visual system, area-specific TCA targeting is likely primarily controlled intracortically by guidance molecules, and influenced by neural activity. SP neurons and their axons have also been implicated in the development of area-specific TCA targeting, but their role and the molecular basis are vague.

TCAs form the major input to the neocortex, and relay visual, auditory, and somatic sensations to the primary sensory areas of the neocortex in an area-specific manner. Since TCAs are the sole source of modality-specific sensory information to the neocortex, the functional specializations of the primary sensory areas are defined by, and dependent upon, TCA input. The differentiation of anatomical features that distinguish cortical areas, including architecture and distributions of output projection neurons, depends to a large extent upon TCA input. Consistent with this role, the TCA projection exhibits area specificity throughout its development, and the gradual differentiation of areas within the CP parallels the elaboration of the TCA projection within it. In addition, a variety of manipulations, including peripheral manipulations and transplantation experiments, have demonstrated that the CP exhibits considerable plasticity in the development of area-specific features, and that diverse parts of the CP initially have similar potentials to develop features unique to a specific area. Again, TCA input has been implicated as a major influence controlling this plasticity in the differentiation of area-specific features.

The role of TCAs in shaping cortical architecture is not limited to these later events in the differentiating CP. *In vitro* experiments suggest that TCAs release a diffusible mitogenic activity that promotes the production of both glia and neurons by explants of the cortical VZ. If a similar mechanism operates *in vivo*, such an early influence of TCAs on corticogenesis could contribute to reported areal differences in neuronal production in the SVZ in occipital areas (V1 vs. V2), and therefore, as previously described, to the cytoarchitectural differences between areas that become evident later in development.

Indirect Evidence for Intrinsic Genetic Regulation of Area Identity

Evidence for the genetic control of arealization is recent and limited, but compelling. The initial evidence was indirect and based upon the differential expression patterns of numerous genes, ranging from TFs, cell adhesion molecules, and axon guidance receptors and ligands, within cortical progenitors in the VZ or their progeny in the CP. These patterns, which were typically graded across the anteroposterior (AP) and dorsoventral (DV) cortical axes, were shown to develop independently of TCA input by analyses of mutant mice with targeted deletion of TFs (Gbx2 or Mash1, neither of which is expressed in the cortex but which are required for TCAs to reach the cortex). However, the most dramatic areal changes in gene expression, and changes that match expression patterns to cortical areas or their borders, occur postnatally, coincident with the development of patterned TCA input to the CP. Thus, although the early differential expression of many genes may be independent of TCA input, TCAs may later influence the differentiation of more complex patterns.

Roles for Morphogens and Transcription Factors in Control of Area Identity

Two different major forms of gene products influence the intrinsic generation of positional or area identity in the developing neocortex. These two forms of proteins are TFs, which are typically localized to the nucleus of the producing cell, and secreted morphogens, which are secreted by the producing cells. TFs are expressed by neocortical progenitors and directly regulate the positional or area identity that is inherited by their progeny, whether these progeny are additional progenitors or are neurons that form the CP and SP. Secreted morphogens are produced in discrete signaling centers and are believed to be secreted and to diffuse from their source, forming gradients across the dorsal telencephalon (neocortical) VZ that are highly

concentrated at the site of production and progressively diminish in concentration distal to their source. Here we first consider TFs that impart area identities to cortical progenitors, then in the subsequent sections consider roles for morphogens in area patterning.

Intrinsic Control of Area Identity by Differential Expression of Transcription Factors in Cortical Progenitors

The key criteria for a gene that specifies area identity in cortical progenitors is straightforward: it must be expressed by progenitors in the VZ and/or SVZ and must function in a differential manner across the cortical axes, either through differential expression or expression of cofactors or other mechanisms that differentially influence its function, and must impart areal properties. Functionally, genes that regulate arealization, in principle, could have a range of effects – for example, from conferring the complete set of properties that comprise the area identity of a cortical neuron, to a subset of these properties, to regulating the expression of axon guidance molecules that control the area-specific targeting of TCAs. Scores of genes meet the differential expression criteria to be the source of TFs that regulate area patterning, but as of this writing, only four TFs have been shown to function in regulating arealization: Emx2, Pax6, COUP-TFI, and Sp8. Emx2 is a homeodomain TF related to the *Drosophila* gene *empty spiracles* (*ems*), Pax6 is a paired box domain TF, COUP-TFI is an orphan nuclear receptor, and Sp8 is a zinc-finger TF related to the *Drosophila* gene *buttonhead* (*btd*).

Within the cortex, all TFs but COUP-TFI have their expression almost exclusively limited to progenitors in the embryonic VZ. COUP-TFI is expressed in a high caudolateral to low rostromedial gradient both in progenitors and in their CP progeny. Emx2 is expressed in a low rostrolateral to high caudomedial gradient, and Pax6 is expressed in an opposing pattern, a high rostrolateral to low caudomedial gradient across the VZ of embryonic cortex. All three of these TFs are expressed in progenitors throughout embryonic cortical neurogenesis. In contrast, Sp8 is expressed only during the early phase of neurogenesis, and is expressed in a high rostromedial to low caudolateral gradient. Loss-of-function studies have been done in mice for each of the four genes, and gain-of-function studies have been reported for all but the gene encoding COUP-TFI.

Emx2 To date, of the four TFs that function in regulating arealization, Emx2 has been most extensively studied. Genetic manipulations that change the levels of Emx2 expression in cortical progenitors result in disproportionate changes in the sizes of the primary sensory and motor cortical areas. For example, increasing Emx2 expression in neocortical progenitors has no effect on overall cortical size, but results in a significant decrease in the sizes of rostral cortical areas, including S1 and motor areas, and a significant increase in caudal areas, including V1. Decreasing levels of Emx2 expression has the opposing effect on area sizes. Changes in the levels of Emx2 in cortical progenitors appear to result in a complete change in the area identity of their neuronal progeny, to match the contracted or expanded areas.

COUP-TFI Cortical deletion of COUP-TFI results in a massive expansion of anterior areas, including motor, to occupy most of parietal and occipital (sensory) cortex, paralleled by substantial reduction in the sizes of the three primary sensory areas that become compressed and aligned mediolaterally along the caudal pole of the cortical hemisphere. Thus, COUP-TFI is required to balance the patterning of neocortex into motor and sensory areas, and appears to operate through a novel genetic pathway to repress the identities of rostral cortical areas, such as motor, allowing for appropriate specification of the sensory cortical areas. COUP-TFI might also have a role in specifying the identities of sensory areas.

Pax6 Roles for Pax6 in area patterning are presently vague. The initial studies that implicated Pax6 in area patterning depended upon marker analyses of the small eye mutant mouse; these mice are deficient for functional Pax6 protein and die at birth, before cortical areas differentiate, and in addition lack TCA input. Nonetheless, these marker analyses implicated Pax6 in specifying anterior area identities associated with motor areas, consistent with its highest expression in progenitors that give rise to anterior areas. However, a recent gain-of-function study of Pax6 that used a yak transgenic approach to overexpress Pax6 severalfold in cortical progenitors reports minimal or no changes in area patterning. This discrepancy could be explained in several ways. One appealing way is that another gene, for example, that for COUP-TFI, normally represses, in the cortical fields, Pax6 function that would give rise to sensory areas, and therefore represses the effect of Pax6 overexpression. In any case, additional work must be done to properly define the role for Pax6 in the regulation of area patterning.

Sp8 Sp8 has been recently shown to have multiple distinct roles in area patterning, one by its influence on the expression of the morphogen fibroblast growth factor 8 (FGF8) within the commissural plate (CoP), and the other through its expression directly

in cortical progenitors. Sp8 is expressed in a high to low, anteromedial to posterolateral, gradient across the VZ, and is also transiently expressed in the CoP coincident with the expression domain of FGF8, a morphogen implicated in area patterning of the neocortex. Sp8 and FGF8 exhibit reciprocal induction in the embryonic telencephalon, but ectopic expression of Sp8 and FGF8 in anterior dorsal telencepahlon around the normal FGF8 expression domain in the CoP has opposing effects on area patterning, with ectopic expression of FGF8 inducing a posterior shift of cortical areas and ectopic expression of Sp8 inducing an anterior shift – suggesting that in parallel to regulating FGF8 expression, Sp8 also activates a distinct signaling pathway for cortical area patterning. Further, deletion of Sp8 from cortical progenitors results in a reduction of anterior cortical areas, such as motor areas.

Interactions between regulatory genes in controlling area patterning Other TFs are likely involved as primary regulators of area patterning and cooperate with Emx2, Pax6, COUP-TFI, and Sp8. In addition, Emx2, Pax6, COUP-TFI, and Sp8 have inductive or repressive effects upon one another that affect their influence, their level of expression, and even their domain of expression. For example, Sp8 is a direct transcriptional activator of FGF8, and Sp8 induction of FGF8 is repressed by Emx2, which binds Sp8. This provides a mechanism to limit FGF8 expression to the CoP. In addition, many of these TFs influence the expression of one another. For example, Emx2 and COUP-TFI appear to repress Pax6, and Pax6 appears to repress Emx2. Further, as described in the following sections, FGF8 influences the expression of many of these TFs. In summary, gradients of TFs expressed within cortical progenitors in the VZ/SVZ impart area identities, and cooperate with one another, and with as yet unidentified TFs, to activate signaling pathways that control area patterning of the neocortex.

Morphogens/Signaling Molecules Establish Graded Patterns of Transcription Factor Expression in Cortical Progenitors

Current evidence indicates that morphogens secreted by signaling centers located at the perimeter of the developing dorsal telencephalon (dTel) establish gradients of TFs across the dTel VZ, which in turn determine the area fate of cortical progenitors and their progeny. These morphogens include FGF8 expressed by the anterior neural ridge (ANR), which later becomes the CoP, located at the anterior midline of the dTel, and also members of the wingless-int (Wnt) and bone morphogenetic protein (BMP) families, expressed by the cortical hem and choroid plexus epithelium (CPe), located at the dorsal midline of more posterior dTel (**Figure 2**).

Recent studies have begun to better define these patterning centers and, early in development, the action of their signaling molecules to establish and maintain across the neocortical VZ the graded expression of TFs that directly impart area identity to cortical progenitors. The initial domain of FGF8 expression in the ANR/CoP is located at the rostral edge of the prospective neocortex, and later this expression domain extends caudally along the dorsal midline toward diencephalon. Bone morphogenetic proteins (BMP2, BMP4, BMP5, BMP6, and BMP7) and Wnts (Wnt2b, Wnt3a, and Wnt5a) are expressed in the cortical hem, a midline structure adjacent to the hippocampal anlage.

FGF8 FGF8 is the intensively studied member of the FGF family that is expressed in the ANR/CoP. At the neural tube stage, approximately embryonic day 8.5 (E8.5) to E9.5 in mouse, the FGF8-expressing domain is positioned in a discrete dorsomedial domain. After invagination of the neural tube (~E10.5), the FGF8 expression domain is positioned in a small domain in the medial wall of the two cortical hemispheres called the ANR, with the highest expression of FGF8 localized in the anterior region of the cortex. FGF8 expression continues robustly through E12.5 in mouse and then tapers off significantly. Experimental evidence shows that the high rostromedial expression of FGF8 establishes, at least in part, the graded expression of Emx2 and COUP-TFI through repression. Further, changes in levels of FGF8 in embryonic mice result in area shifts that mimic those expected by decreasing or increasing the graded expression of Emx2 across the neocortical VZ, and are likely due to such an effect on Emx2 levels (**Figure 3**).

Wnts and BMPs A second signaling center is the cortical hem that expresses members of the Wnt and BMP families of ligands. These factors begin to be expressed slightly later than FGF8 (~E9.5) and are expressed immediately lateral to the FGF8 domain during the neural tube stage. After invagination of the neural tube, Wnts and BMPs are localized within a domain called the cortical hem, which is positioned just immediately dorsal to the FGF8 expression domain. Along the anteroposterior axis of the dTel, the cortical hem shares a similar anterior boundary with FGF8 but extends significantly further caudally within the medial cortical wall. Expression of signaling molecules from the cortical hem appears to be active until slightly later in development, as expression of Wnts and BMPs remains

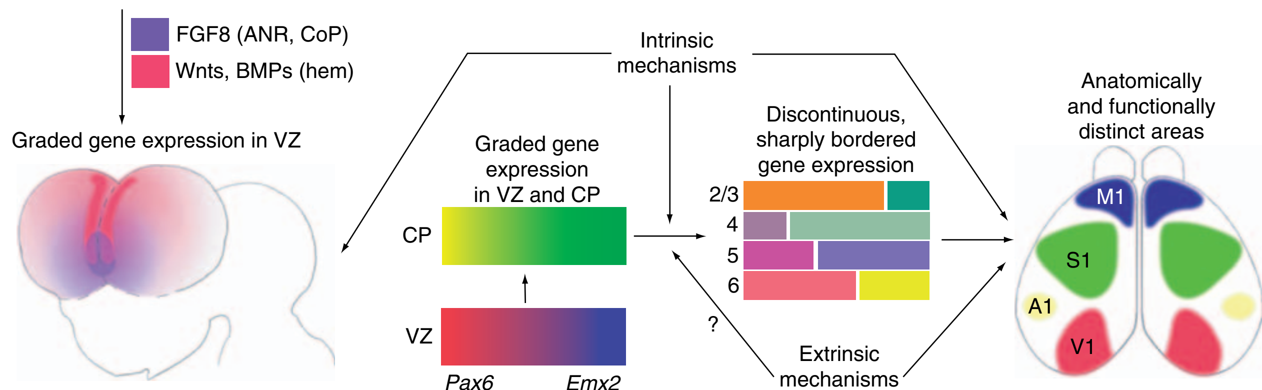

Figure 2 Mechanisms of specification and differentiation of neocortical areas. The initial, tangential gradients of transcription factors in the ventricular zone (VZ) are likely established by signaling molecules/morphogens secreted from patterning centers, such as fibroblast growth factor 8 (FGF8) from anterior neural ridge (ANR), which later becomes the commissural plate (CoP), and Wnts and bone morphogenetic proteins (BMPs) from the cortical hem. The graded expression of certain transcription factors, such as Pax6, Emx2, COUP-TFI, and Sp8, imparts positional or area identities to cortical progenitors, which is imparted to their neuronal progeny that form the cortical plate (CP). The CP also initially exhibits gradients of gene expression that are gradually converted to distinct patterns with sharp borders. Coincident with this process, distinct cortical layers (2–6), and the anatomically and functionally distinct areas seen in the adult (M1, motor; S1, somatosensory; A1, auditory; V1, visual), differentiate from the CP. Genes that are differentially expressed across the cortex are often expressed in different patterns in different layers, suggesting that area-specific regulation of such genes is modulated by layer-specific properties, raising questions about the definition of area identity. Although the initial establishment of the graded gene expression in the embryonic CP is controlled by mechanisms intrinsic to the telencephalon, the much more complex differentiation patterns established postnatally, often both spatially and temporally in parallel with the development of thalamocortical afferent input, suggest that these later events might well be regulated influences that arise extrinsic to the cortex, such as thalamocortical afferents. Future studies are required to determine and clarify these mechanisms. Adapted from O'Leary DDM and Nakagawa Y (2002) Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. *Current Opinion in Neurobiology* 12: 14–25.

strong until approximately E13.5, after which the cortical hem shrinks in size, develops into the fimbria (an axonal tract of the hippocampal formation), and slowly downregulates its expression of these signaling molecules. Although Wnts have been shown to enhance proliferation of cortical progenitors, little evidence implicates Wnts and BMPs in regulating area patterning.

Anteroposterior Patterning of the Cerebral Cortex: Are Arealization and Regionalization Related?

For TFs that exhibit low to high AP graded expression patterns in the neocortex, their graded expression patterns often continue beyond the posterior extent of the neocortex and into other regions of the cerebral hemisphere and through the hippocampal fields. Examples of TFs that function in neocortical arealization and exhibit this form of continuing graded expression include Emx2 and COUP-TFI. These continuous, graded expression patterns suggest a relationship between arealization of the neocortex and arealization of other regions of the cerebral cortex, as well as a relationship between arealization and regionalization of the cerebral cortex. This observation warrants further investigation into these potential relationships.

Translation of Graded Expression of TFs by Cortical Progenitors and Their CP Progeny into Sharp Borders Exhibited by Cortical Areas

The graded expression of TFs expressed by cortical progenitors in the VZ/SVZ, and early on by their neuronal progeny in the CP, eventually transforms into sharply bordered patterns of gene expression that often relate to borders of cortical areas. Studies in *Drosophila* embryos have suggested mechanisms that might operate in the mammalian cortex to accomplish this change. For example, the graded distribution of dorsal, a regulatory protein, generates (through concentration-dependent differences in binding efficacy to promoter and repressor elements) expression patterns of downstream genes with sharp borders that align with the boundaries of different embryonic tissues and related patterns of gene expression. A different example is the development of the sharply patterned expression of the *even-skipped* gene through the combined

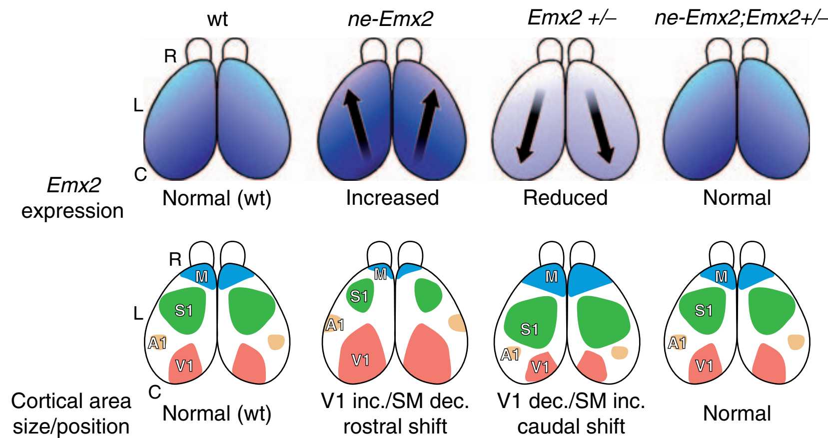

Figure 3 The *Emx2* gene imparts area identities to cortical progenitors and their progeny: levels of *Emx2* expression control sizes of cortical areas. Top panel shows relative levels of graded *Emx2* expression in cortical progenitors in four genotypes: wild type (wt), with normal levels of *Emx2*; *ne-Emx2* transgenic mice, in which an *Emx2* transgene is driven by a nestin promoter in cortical progenitors and results in about a 50% increase in overall *Emx2* levels; *Emx2* heterozygous mice (*Emx2*$^{+/-}$) with only one wt *Emx2* allele, resulting in about a 50% decrease in overall *Emx2* levels; and *ne*-Emx2; Emx2$^{+/-}$ mice with overall levels of *Emx2* being essentially normal due to the combination of endogenous *Emx2* and the *Emx2* transgene. The arrows show the predicted and observed shifts in area patterning. The bottom panel shows area patterning using a schematic of the four primary areas, motor (M), somatosensory (S1), auditory (A1), and visual (V1). The *ne-Emx2* transgenic mice exhibit decreased sizes of S1 and M, an increase in V1, and a rostral shift in all areas. The *Emx2*$^{+/-}$ mice exhibit increased sizes of S1 and M and decreased size of V1. The sizes of the primary cortical areas in the *ne-Emx2*; *Emx2*$^{+/-}$ mice revert toward their normal sizes observed in wt. Therefore, the sizes of the areas depend on the overall level of graded *Emx2* expression, and changes in area sizes due to increases or decreases in *Emx2* can be rescued by restoring *Emx2* to wild-type levels in cortical progenitors. Adapted from Hamasaki T, Leingartner A, Ringstedt T, et al. (2004) EMX2 regulates sizes and positioning of the primary sensory and motor areas in neocortex by direct specification of cortical progenitors. *Neuron* 43: 359–372; and from Leingartner A, Thuret S, Kroll TT, et al. (2007) Sizes of somatosensory and motor cortical areas determine proficiency at tactile and motor behaviors. *Proceedings of the National Academy of Sciences of the United States of America* 104: 4153–4158, Copyright (2007) National Academy of Sciences, USA.

action of multiple activators and repressors of its transcription; *even-skipped* is expressed in stripes perpendicular to the AP axis of the *Drosophila* axis where expression of repressors is subthreshold and that of activators is suprathreshold.

Similar mechanisms appear to operate in the developing spinal cord, where sonic hedgehog (shh), a gene product secreted by the notocord and floor-plate, represses or induces the expression of different classes of TFs in the VZ of ventral spinal cord. Initially, these TFs have graded, overlapping expression patterns which progressively sharpen through mutual repression, to generate sharply bordered patterns of expression. This mechanism generates genetically distinct domains of progenitors defined by their expression of unique subsets of TFs, which in turn generate unique classes of spinal interneurons and motor neurons.

Similar mechanisms likely operate in the neocortex to generate areas, but important differences are evident. For example, at no time during neocortical neurogenesis are sharply bordered patterns of regulatory genes observed in the neocortical VZ; all have graded expression patterns. Initially, even expression in the CP is graded, but at later stages of development many genes acquire expression patterns with an abrupt border that, in some instances, matches the border between areas.

What Is ‘Area Identity’?

The findings and issues discussed in the preceding sections lead directly to a major issue that remains unsettled: the extent to which areas are genetically distinct. Area-specific genes *per se* either do not exist or are very rare at the developmental stages when area identities are being genetically specified, and perhaps even later, when the anatomical features that distinguish areas begin to differentiate within the CP. Thus, in terms of gene expression, a neocortical area is not defined by the expression of a specific set of genes restricted to that area. Instead, a neocortical area is defined by the expression of a unique subset of genes, each of which is also expressed in other areas. However, the actual scenario is even more complex, since each layer has a unique profile of gene expression. Each gene differentially expressed in the neocortex, and expressed in more than one layer, has different expression patterns in each layer. An excellent example is *MDGA1*: this gene encodes a protein that is an immunoglobulin cell adhesion molecule. It is expressed in layers 2/3 through the neocortex,

>and is also expressed in layers 4 and 6 within S1. Thus, it has both layer- and area-specific patterns of expression, and can be used to define an area, S1, but its expression is not limited to that area. Thus, the term 'area identity' in the strictest sense might not truly exist for neurons across layers, and uniquely marks neurons with the specific identity of a given area.

See also: Cerebral Cortex: Symmetric vs. Asymmetric Cell Division; Motor Neuron Specification in Vertebrates.

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Neural Patterning: Eye Fields

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Introduction

Patterning of the central nervous system (CNS) has a crucial role in shaping functional organization. Owing to its external position in the embryo, the eye is often used as a model to study CNS patterning. Establishment of the anteroposterior (A-P) and dorsoventral (D-V) axes constitutes a fundamental step in retinal development. Several morphogens and transcription factors expressed asymmetrically in the early retina play key roles in the establishment of the A-P and D-V axes of the retina. The neural retina is a highly organized sensory organ that receives, integrates, and transmits visual information. Light is captured by the photoreceptor cells, and its information is converted into chemical signals to be sent through a series of interneurons to the projection neurons of the retina, the retinal ganglion cells (RGCs).

During embryonic development, the axons of RGCs grow from the eye to the contralateral optic tectum (OT), the primary visual center in lower vertebrates, or the superior colliculus (SC) in mammals and form a topographic map of visual space. RGC axons emanating from the anterior (nasal) retina project to the posterior OT/SC, while those from the posterior (temporal) retina project to the anterior OT/SC. Likewise, axons from the dorsal retina project to the lateral (L, or ventral) OT/SC, while those from the ventral retina project to the medial (M, or dorsal) OT/SC. For the formation of topographic maps, molecular gradients in origins and targets are essential, as predicted by Sperry in the classical chemoaffinity hypothesis. The family of Eph receptor tyrosine kinases and their ligands, the ephrins, have been shown to fulfill these criteria and control topographic map development.

Members of the EphA family expressed with a temporal high-nasal low gradient in the retina and of the ephrinA family expressed with a posterior high-anterior low gradient in the OT/SC determine the retinotectal projection along the A-P axis. The receptors of the EphB family expressed with a ventral high-dorsal low gradient in the retina, and the ephrinB ligands expressed with a medial high-lateral low gradient in the OT/SC control the mapping of retinal axons along the M-L axis. Recent advances in the study of the molecular mechanisms for retinal patterning are discussed below, and the molecular cascade controlling the events in the chick are highlighted. It is not yet clear whether the patterning mechanism revealed in the chick retina is wholly valid for mammals.

Retinal Patterning along the A-P Axis

When is the A-P axis determined in the developing retina? Fate mappings of the avian anterior forebrain have revealed that there is a distinct area for the presumptive retina in the neural plate, and the A-P axis of the presumptive territory in the neural plate corresponds to the A-P axis of the retina. In the A-P subdivision during the brain's development, boundaries are established at the zona limitans intrathalamica and between the forebrain and midbrain and the midbrain and hindbrain by mutual repression between *Six3* and *Irx3*, *Pax6* and *Pax2*/*En1*, and *Otx2* and *Gbx2*, respectively. Fibroblast growth factor-8 (*FGF-8*) is expressed at the anterior neural ridge and the midbrain–hindbrain boundary and helps to further subdivide the brain. The homeobox genes modulate the response to FGF-8 in their territories – within the *Six3* territory in the anterior forebrain, FGF-8 can induce the expression of *FoxG1*, a winged-helix/forkhead (Fox) transcription factor, whereas in the *Irx3* domain, FGF-8 in the midbrain–hindbrain boundary activates *En1*.

The forebrain is further subdivided by the mutual repression between *FoxG1* (also referred to as Brain Factor 1, *BF1*) and the related gene *FoxD1* (Brain Factor 2, *BF2*). These two *Fox* transcription factors are expressed in adjacent domains in the prospective eye region at the time the optic vesicle evaginates. It is thus considered that the establishment of the retinal polarity along the A-P axis is triggered by FGF signaling. In the chick embryo, misexpression of *FGF-8* upregulates the expression of *FoxG1* and downregulates that of *FoxD1* in the optic vesicle. After the formation of the optic cup, *FoxG1* is expressed in the nasal retina and *FoxD1* is expressed in the temporal retina in a complementary pattern by counteracting each other. Because surgical manipulations of the chick optic vesicle begin to induce severe disturbances to the visual projection map from Hamburger–Hamilton (HH) stage 11/12 (on E1.5), retinotopic specification along the A-P axis of the retina is considered to be determined at or prior to the optic vesicle stage. Ectopic misexpression of *FoxG1* or *FoxD1* in the chick retina reversed the topographic map in the retinotectal projection along the A-P axis. These factors are thus supposed to play a key role in

the initiation of retinal regional specification along the A-P axis.

Subsequently, two homeobox transcription factors, *GH6* and *SOHo1*, are known to be expressed specifically in the nasal region of the developing chick retina. Both *GH6* and *SOHo1* are downstream targets of the Fox genes. Misexpression of *FoxG1* induces the expression of *GH6* and *SOHo1* in the temporal retina. In contrast, *FoxD1* inhibits the expression of these two homeobox genes in the retina. Misexpression of *GH6* or *SOHo1* in the retina results in errors in the projection of retinal axons to the OT along the A-P axis, due to the repression of *EphA3* expression in the temporal retina.

In contrast to *EphA3*, four EphA receptors (*EphA4*, *EphA5*, *EphA6*, and *EphA7*) are uniformly expressed in the chick retina. On the other hand, *ephrinA2* (see below) and *ephrinA5* are also expressed with a nasal high-temporal low gradient in the retina. Overexpression of ephrinAs in temporal axons leads to errors in the topographic targeting of temporal axons, suggesting a role for retinal ephrinAs in the formation of topographic projections. Thus, EphAs uniformly expressed in the retina are also thought to be involved in the topographic projection along the A-P axis, with the help of ephrinAs expressed with a gradient in the retina.

It has been revealed that *FoxG1* controls the expression of all the molecules distributed asymmetrically along the A-P axis through multiple mechanisms in the chick. *FoxG1* is known to act as a transcriptional repressor. The expression of downstream targets of *FoxG1* in the retina is affected by the misexpression of a chimeric protein that consists of a *Drosophila* even-skipped repression domain and a winged-helix (WH) DNA-binding domain of FoxG1. The repressing construct of FoxG1 regulates the expression of *SOHo1*, *GH6*, *EphA3*, *FoxD1*, and *ephrinA5*, similar to the wild type, but not the expression of *ephrinA2*. On the other hand, mutant FoxG1 deficient in DNA-binding ability exerts similar effects on the expression of *SOHo1*, *GH6*, *EphA3*, *FoxD1*, and *ephrinA2* as the wild-type FoxG1, but not on the expression of *ephrinA5*. These results suggest that *FoxG1* controls *ephrinA5* by a DNA-binding-dependent mechanism, *ephrinA2* by a DNA-binding-independent mechanism, and *FoxD1*, *SOHo1*, *GH6*, and EphA3 by dual mechanisms. It is important to note that FoxG1 interferes in bone morphogenetic protein 2 (BMP2) signaling and thereby induces oblique-gradient expression of ephrinA2. The BMP2 signal resultantly plays a pivotal role in the topographic projection along both axes (see below).

What would be the point of using two distinct mechanisms, DNA binding-dependent and DNA binding-independent, to regulate the expression of topographic molecules? One answer is for security to maintain regulation of the EphA/ephrinA system. For instance, if the DNA binding-dependent mechanism is lost because of a mutation in the WH domain, FoxG1 can still regulate the asymmetrical distribution of *EphA3* and *ephrinA2* through the remaining DNA binding-independent mechanism. On the other hand, if the DNA binding-independent mechanism is lost, FoxG1 can still regulate the asymmetrical distribution of *EphA3* and *ephrinA5* through the DNA binding-dependent mechanism. Gene knockout of *ephrinA2* and *ephrinA5* in mice suggests the importance of this redundancy: in the single knockout mice, a substantial proportion of the retinal axons projected normally onto the SC. Since the total EphA/ephrinA system plays an essential role in the formation of the retinotectal map, this dual regulatory system might have evolved during the evolution of the visual system.

In *FoxD1*-deficient mice, ephrinA(s) is highly expressed in both halves of the retina. Misexpression of *FoxD1* inhibits the expression of *ephrinA5* in the chick nasal retina. These results suggest that *FoxD1* is essential for the establishment of the temporal regional specificity in the retina. To reveal the function of *FoxD1* in detail, further experiments are needed. In addition, there is the possibility that yet unknown factors, which are expressed specifically in the temporal retina as the counterpart of *SOHo1* and *GH6*, regulate the regional specification of the temporal retina. *FoxG1* and *FoxD1* are thus thought to be located at the top of the gene cascade for the regional specification along the A-P axis and determine the nasal or temporal specificity in the retina through multiple mechanisms. The cascade of control genes for the formation of the A-P axis of the chick retina is shown in **Figure 1**.

Mouse *FoxD1* plays a role in the specification of the ventrotemporal (VT) retina. In the chick, ipsilateral projection of retinal axons is not observed. In contrast, in mammals, ipsilaterally projecting axons arise from the VT region. The decision of RGCs from the VT retina to project ipsilaterally at the optic chiasma is controlled by the zinc-finger transcription factor *Zic2*. The VT axons are positive for EphB1 and repelled by ephrinB2 expressed in the midline glial cells at the optic chiasma. Conversely, an LIM homeodomain transcription factor, *Islet2*, is expressed in a pattern complementary to *Zic2*, repressing *Zic2* expression in the retina. In the *FoxD1*-deficient mice, the VT axons aberrantly project contralaterally because the expression of *Zic2* and *EphB1* is missing. It is not clear whether *FoxD1* also regulates the ipsilateral projection in other species such as *Xenopus*, in which ipsilateral projections develop after metamorphosis.

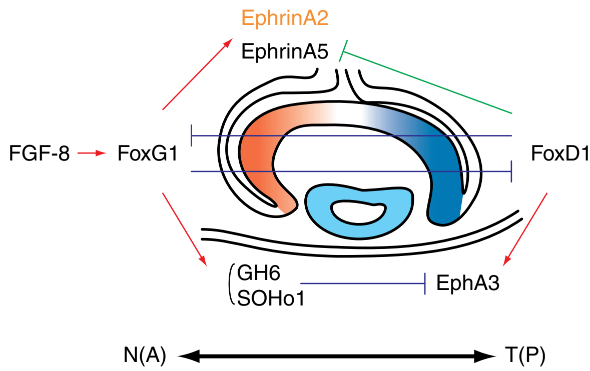

Figure 1 Molecular control of anteroposterior (A-P) patterning. FGF-8 derived from the anterior neural ridge induces *FoxG1* expression in the retina. The counteraction between *FoxG1* on the nasal side and *FoxD1* on the temporal side governs the retinal patterning along the A-P axis early on in development. Subsequently, the transcription factors GH6 and SOHo1 in the nasal retina begin to be expressed under the control of *FoxG1*. *GH6* and *SOHo1* inhibit the expression of *EphA3*. *FoxG1* and *FoxD1* also counteract each other to determine the expression pattern of *ephrinA5*. *FoxG1* induces the expression of *ephrinA2*. Arrows and T-bars indicate positive and negative effects, respectively. These effects are not necessarily induced by direct action. Nasal (N; anterior, A) is left, and temporal (T; posterior, P) is right.

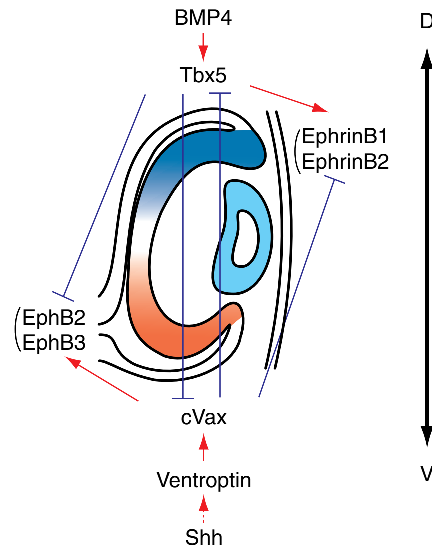

Figure 2 Molecular control of dorsoventral (D-V) patterning early in development. The counteraction between *BMP4* on the dorsal side and *Ventroptin* on the ventral side governs the retinal patterning along the D-V axis early in the developmental process. Subsequently, transcription factors Tbx5 in the dorsal retina and cVax in the ventral retina begin to be expressed under the control of the BMP4 signal. *Tbx5* and *cVax* counteract each other to determine the expression pattern of EphB receptors and their ligands, ephrinBs. Sonic hedgehog (Shh) derived from the ventral midline presumably induces expression of *Ventroptin* in the retina. Arrows and T-bars indicate positive and negative effects, respectively. These effects are not necessarily induced by direct action. Dorsal (D) is upwards, and ventral (V) is downwards.

Retinal Patterning along the D-V Axis at the Early Stages

Soon after A-P polarity is determined, initial D-V patterning also develops through the actions of morphogens and transcription factors. **Figure 2** shows the schema for the establishment of the D-V axis of the chick retina early on. BMP signaling is required for the D-V patterning of the CNS in vertebrates. The retinal patterning along the D-V axis also appears to be controlled by a gradient of BMP4 signaling, emanating from the dorsal retina. Ventroptin, a BMP antagonist, in the ventral retina counteracts BMP4. Overexpression of *BMP4* in the chick retina upregulates a dorsally expressed T-box transcription factor, *Tbx5*, and downregulates a ventrally expressed *Emx* homeobox gene, *cVax* and *Ventroptin*. In the *BMP4* knockout mouse, the expression of *Tbx5* is lost. Overexpression of *Ventroptin* in the chick retina leads to a downregulation of *BMP4* and *Tbx5* expression and to upregulation of *cVax* expression. The counteraction between *BMP4* and *Ventroptin* thus governs the regional specification along the D-V axis in the retina by controlling the expression of the downstream transcription factors, *Tbx5* and *cVax*.

Sonic hedgehog (*Shh*) is a ventralizing morphogen in many areas of the CNS. Shh expression in the ventral neural tube and the underlying axial mesendoderm extends to the neural and mesodermal tissue located between the two eyes. Therefore, it has been suggested that Shh acts as a long-range morphogen and controls the D-V patterning in the retina, although it is not expressed in the retina. Consistent with this view, misexpression of *Shh* in the chick and *Xenopus* retina suppresses the expression of *BMP4* and *Tbx5* and enhances the expression of *cVax*/*Vax2*. Because *Ventroptin* also controls the expression of these molecules, Shh supposedly ventralizes the retina through induction of *Ventroptin* expression.

Tbx5 begins to be expressed in the dorsal retina at the early optic cup stages. Misexpression of *Tbx5* in the chick retina enhances the dorsal expression of *ephrinB1* and *ephrinB2* and represses the ventral expression of *EphB2*, *EphB3*, and *cVax*. Additionally, ectopic expression of *Tbx5* causes ventral retinal axons to exhibit defects in topographic mapping. As the optic cup invaginates, *cVax* expression commences in the ventral regions of the retina. Overexpression of *cVax* downregulates the expression of the dorsalizing factors, *BMP4* and *Tbx5*, and upregulates that of the ventral markers, *EphB2* and *EphB3*. When *cVax* is misexpressed in the chick retina, the dorsal axons do not project to their correct target zones, whereas ventral axonal projections appear to be normal.

Thus, *cVax* is sufficient for retinal ventralization. *Tbx5* and *cVax* counteract each other similar to the relation between *BMP4* and *Ventroptin*. Although they are located downstream of *BMP4* and *Ventroptin*, their expression domains have a gap of significant width, in which neither of them is expressed. Two other members of the T-box gene family, *Tbx2* and *Tbx3*, are expressed with a dorsal high-ventral low gradient the same as *Tbx5*, but they cover the gap region. They are also involved in the D-V patterning of the retina. Therefore, a combination of these transcription factors may control the retinal patterning and retinotectal projection along the D-V axis.

Retinoic acid (RA) is also asymmetrically distributed along the D-V axis in the developing retina. The presence of dorsal and ventral zones of RA activity in the retina separated by an RA-free middle zone is conserved among vertebrate species such as zebra fish, chick, and mouse. The two zones of RA activity are sculptured by the expression of two different RA-synthesizing enzymes, RALDH1 in the dorsal retina and RALDH3 in the ventral retina. The presence of a mid-zone free of RA is due to the expression of multiple members of the Cyp26 family of RA-degrading enzymes. RA has been proposed to control the D-V patterning in the retina. In support of this hypothesis, mice deficient in some retinoid receptors show marked defects in development of the ventral eye. Addition of RA to zebra fish embryos during the eye's development results in ventral retinal duplication, while addition of an inhibitor of the ventral RA-synthetic enzyme causes deletion of the ventral retina. These results indicate that RA is essential for proper development of the ventral eye. When analyzed by gene expression, RA treatment of *Xenopus* embryos causes a reduction of *Tbx5* expression and expansion of *Vax2* expression, suggesting that RA has ventralizing activity. However, inhibition of RA signaling in chick or mouse embryos by overexpression of dominant-negative RA receptors or a double knockout of *RALDH1* and *RALDH3* does not significantly alter the expression of *Tbx5* and *cVax/Vax2*, indicating that RA is not involved in the early D-V patterning of the retina in these species. The role of RA in retinal D-V patterning should be clarified by further studies.

Retinal Patterning along the D-V Axis at the Later Stages

The counteraction between *BMP4* on the dorsal side and *Ventroptin* on the ventral side governs the regional specification in the chick retina along the D-V axis early on in the developmental process (HH stage 11 to E5), as described above. At later stages (from E5 onward), in proportion to the disappearance of *BMP4* expression from the dorsal retina, *Ventroptin* begins to be expressed with both a nasal high-temporal low and a ventral high-dorsal low gradient (oblique gradient) in the chick retina, or V/N-high pattern. At the same time, *BMP2* begins to be expressed with an oblique-gradient pattern complementary to that of *Ventroptin* along the two axes, or D/T-high pattern. Thus, the counteraction between *BMP4* and *Ventroptin* is relieved by that between *BMP2* and *Ventroptin*. *FoxG1* represses *BMP2* expression through inhibition of the BMP signaling, and thereby the oblique-gradient expression patterns of *BMP2* and *Ventroptin* are induced. Expectedly, misexpression of *Ventroptin* in the developing chick retina alters the retinotectal projection not only along the M-L axis but also along the A-P axis, owing to the induction of *ephrinA2* expression. It has been long believed that the A-P and D-V axes in the developing retina are determined independently, and also that the retinotectal projection along the two axes is controlled independently. However, these findings indicate that the retinal patterning and topographic mapping along both the A-P and D-V axes are determined in a coordinated manner.

BMP2 knockdown and *BMP2* misexpression in the chick retina at later stages alter the expression of the topographic molecules so far reported to have a gradient only along the D-V axis and that of *ephrinA2* so far only along the A-P axis. Furthermore, these molecules are expressed with an oblique gradient at the later stages, similarly to *BMP2* and *Ventroptin*. Although the axis along the fissure is defined as the D-V axis early on in development, these findings indicate that the D-V axis is tilted to the posterior side at around E5 through the switch from *BMP4* to *BMP2*, along with the corresponding change in the expression pattern of the downstream topographic molecules: the revised oblique D-V axis (D/T-V/N axis) is no longer perpendicular to the A-P axis from E6 onward, when retinal axons begin to project to the tectum in chick embryos. The polarity along the D-V axis was assumed to be determined between HH stage 8 and 14 in the chick retina based on transplant experiments. However, our findings indicate that the plasticity of the regional specificity along the first (orthogonal) and second (tilted) D-V axes is long maintained by BMP signaling and that *BMP2* is responsible for the maintenance of the second D-V axis at the later stages in the retina.

The presence of oblique-gradient molecules in the retina was not recognized until the discovery of Ventroptin. EphrinA2 has long been believed to be a topographic molecule only along the A-P axis. Presumably, this was because previous studies of retinal patterning and retinotectal projection focused on

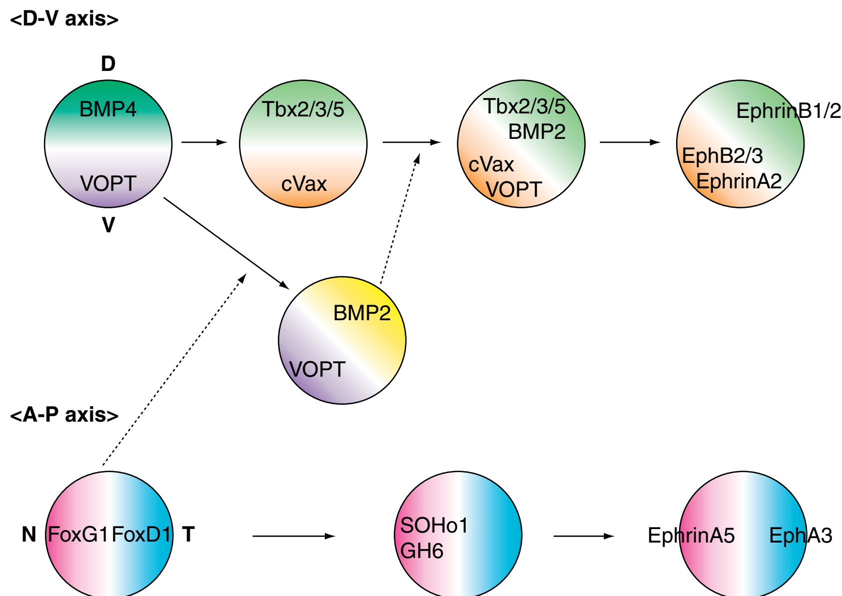

Figure 3 Developmental expression of topographic molecules in the chick retina. The counteraction between *FoxG1* and *FoxD1* determines the regional specificity along the anteroposterior (A-P) axis in the retina. *FoxG1* controls *ephrinA5* by a DNA binding-dependent mechanism, *ephrinA2* by a DNA binding-independent mechanism, and *FoxD1*, *SOHo1*, *GH6*, and *EphA3* by dual mechanisms. The counteraction between bone morphogenetic protein 4 (*BMP4*) and *Ventroptin* (VOPT) governs the regional specification in the retina along the dorsoventral (D-V) axis early in development (Hamburger–Hamilton stage 11 to E5). At around E5, BMP4 expression in the dorsal retina rapidly begins to disappear, and Ventroptin comes to be expressed in an oblique-gradient manner from E6 onward (in a ventral/nasal-high) gradient). Concomitantly, *BMP2* begins to be expressed in a pattern complementary to that of *Ventroptin* (in a dorsal/temporal-high gradient). The inhibitory action of FoxG1 on BMP signaling is attributable to the change in the expression of *Ventroptin* and *BMP2* to the oblique-gradient pattern. The counteraction between *Ventroptin* and *BMP2* determines the expression of the formerly dorsoventrally asymmetric molecules and *ephrinA2* in an oblique-gradient fashion and resultantly governs the retinal patterning and retinotectal projection along both the A-P and D-V axes. *EphrinA2* begins to be expressed much later than does *ephrinA5* from E6 in an oblique-gradient manner from the beginning under the control of the BMP2 signal. Development proceeds from left to right. Reproduced from Sakuta H, Takahashi H, Shintani T, et al. (2006) Role of BMP2 in retinal patterning and retinotectal projection. *Journal of Neuroscience* 26: 10868–10878.

only the A-P or the D-V axis individually. However, the oblique-gradient expression appears not to be specific to the chick, because *Vax2* is also reported to be expressed with an oblique-gradient (V/N-high) pattern in the mouse retina. Phenotypic defects in topographic mapping along the M-L axis are often accompanied by those along the A-P axis, and vice versa. That the revised D-V axis is not perpendicular to the A-P axis should explain why misexpression or knockdown of the topographic molecules along the revised D-V axis induces changes in the topographic mapping along the two axes in the OT/SC. In support of this view, *ephrinA2* and *Ventroptin* misexpression and *BMP2* knockdown in the developing chick retina all induce phenotypic defects in the topographic mapping along both axes. Moreover, overexpression of *BMP2* in the mouse eye apparently leads to topographic errors among ventral axons along the A-P axis in addition to the M-L axis in the SC. Thus, all the topographic molecules with the oblique-gradient expression probably affect the topographic mapping along both axes. The gene cascades of topographic molecules for the retinal patterning and retinotectal projection in the chick are shown in **Figure 3**.

Other Aspects of Retinal Patterning

The establishment of retinal polarity is the basis for both the topographic expression of the axon guidance molecules and the formation of the retinotectal map as described above. In addition, many retinal cells are asymmetrically organized within the retina. For instance, chick RGCs and rod photoreceptor cells show an asymmetric distribution along the D-V and A-P axes. Moreover, melanopsin-containing RGCs involved in the circadian photoentrainment show a D/T-concentrated distribution in the rat retina. The distribution of these cells in the retina is also thought to be determined by the topographic transcription factors. This view is supported by the observation that the V/N-concentrated distribution of rod photoreceptor

cells is severely disturbed by the ectopic expression of *cVax* and a dominant-negative form of *Tbx5*.

The distribution of photoreceptor cells is organized differently in the retina among the vertebrate species. Cone cells are asymmetrically distributed along both the D-V and A-P axes in the mouse retina, but only along the D-V axis in the rabbit retina. This appears to be the result of adaptations to the life style of each species during evolution and must be precisely controlled by a set of genes during development. The degree of inclination of the D-V axis may vary between species, leading to a species-specific distribution of particular retinal neuronal types.

See also: Bone Morphogenetic Protein (BMP) Signaling in the Neuroectoderm; Floor Plate Patterning of Ventral Cell Types: Ventral Patterning; Morphogens: History; Sonic Hedgehog and Neural Patterning.

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Notch Pathway: Lateral Inhibition

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The Notch Signaling Pathway

Briefly, at the core of the Notch signaling pathway is the Notch receptor, a single-pass transmembrane protein (**Figure 1**). The mature form of the receptor expressed at the cell surface is a heterodimer of an extracellular fragment (Notch^{EC}) and a membrane-spanning fragment (Notch^{TM}), generated during synthesis in the Golgi by proteolytic cleavage of the Notch protein at the S1 site by a furin convertase (**Figure 1**). Interaction of Notch with the Delta, Serrate, Lag-2 (DSL) family of ligands expressed on the surface of neighboring cells helps complete a sequence of three proteolytic cleavages that result in the release of the intracellular Notch domain (Notch^{IC}) from the plasma membrane (**Figure 1**). Activation of the Notch receptor requires that the ligands undergo endocytosis, an event that involves ubiquitination by RING E3 ligases such as Mindbomb and Neuralized (**Figure 2**). Internalization of the DSL ligand–Notch^{EC} complex by the endocytic machinery is likely to provide a force that separates the Notch^{EC} and Notch^{TM} fragments, facilitating subsequent cleavage of Notch that releases the transcriptionally active Notch^{IC} fragment into the cell (**Figure 2**). While interaction of Notch and a DSL ligand in a neighboring cell (in *trans*) facilitates activation of the Notch receptor, interaction of Notch with Delta in the same cell (in *cis*) prevents effective function of both Delta and Notch (**Figure 1**). The Notch^{IC} fragment translocates to the nucleus, where, together with a member of the DNA-binding CBF1–RBP-Jk, Suppressor of Hairless, Lag-1 (CSL) family of transcription factors and cofactor Mastermind, it drives expression of target genes with regulatory DNA sequence recognized by the CSL protein DNA-binding domain (**Figure 2**). In the absence of Notch^{IC}, the CSL protein is part of a repressor complex that maintains basal repression of target genes recognized by this DNA-binding factor (**Figure 2**).

Lateral Inhibition and Selection of a Cell within a Proneural Cluster

Notch signaling is best known for its role in mediating interactions that ensure adjacent cells acquire distinct fates (**Figure 2** and **Figure 3**). During lateral inhibition it ensures that only a single cell of a larger group of cells with the potential to adopt the same fate is selected to adopt a particular fate. This role is best illustrated by the function of Notch in neurogenesis.

During *Drosophila* neurogenesis, early patterning mechanisms initiate expression of 'proneural' genes in a small group of cells called a proneural cluster (PNC). These genes, including *achaete*, *scute*, *lethal* of *scute*, and *asense* in the Achaete–Scute complex or another family of genes related to *atonal*, encode basic helix–loop–helix (bHLH) transcription factors that give the cells in the PNC the potential to adopt a neural fate. The proneural factors can drive their own expression, and this positive autoregulation can drive progressively higher levels of proneural gene expression. If sufficiently high expression is achieved, it can become self-sustaining, independent of initiating factors, and the proneural factors are able to successfully initiate expression of additional genes that facilitate subsequent adoption of a neural fate. However, positive autoregulation can be regulated by additional factors that make it more or less effective, eventually determining whether critical levels of proneural factor expression can be established in a particular cell and hence whether that cell will actually adopt a particular neural fate.

The proneural factors drive expression or facilitate the function of the Notch ligand Delta (**Figure 2**). So as cells express higher levels of proneural factor, they become progressively better at activating Notch in neighboring cells. When Delta activates Notch in a neighboring cell within the PNC, it initiates the expression of genes in the Enhancer of Split (E(spl)) complex. These genes interfere with proneural factor expression, making it harder for the neighbor to acquire high levels of proneural expression. Like the proneural factors encoded by genes in the Achaete–Scute complex, some genes in the E(spl) complex encode bHLH transcriptional factors; however, they are part of a larger evolutionarily conserved family of Hairy E(spl)-related factors that are characterized by a C-terminal WRPW domain, which allows them to recruit corepressor Groucho and function as repressors to prevent expression of proneural genes. By promoting function of the Notch ligand Delta and activating Notch-mediated E(spl) transcription in neighboring cells, proneural factors indirectly inhibit proneural gene expression in neighboring cells, reducing their ability to adopt a neural fate. As a consequence of this process of lateral inhibition, only a single cell eventually acquires high enough levels of proneural gene expression to adopt a neural fate within a PNC. Adjacent cells in the PNC, characterized by relatively high levels of Notch activity, are prevented from adopting a neural fate, and they

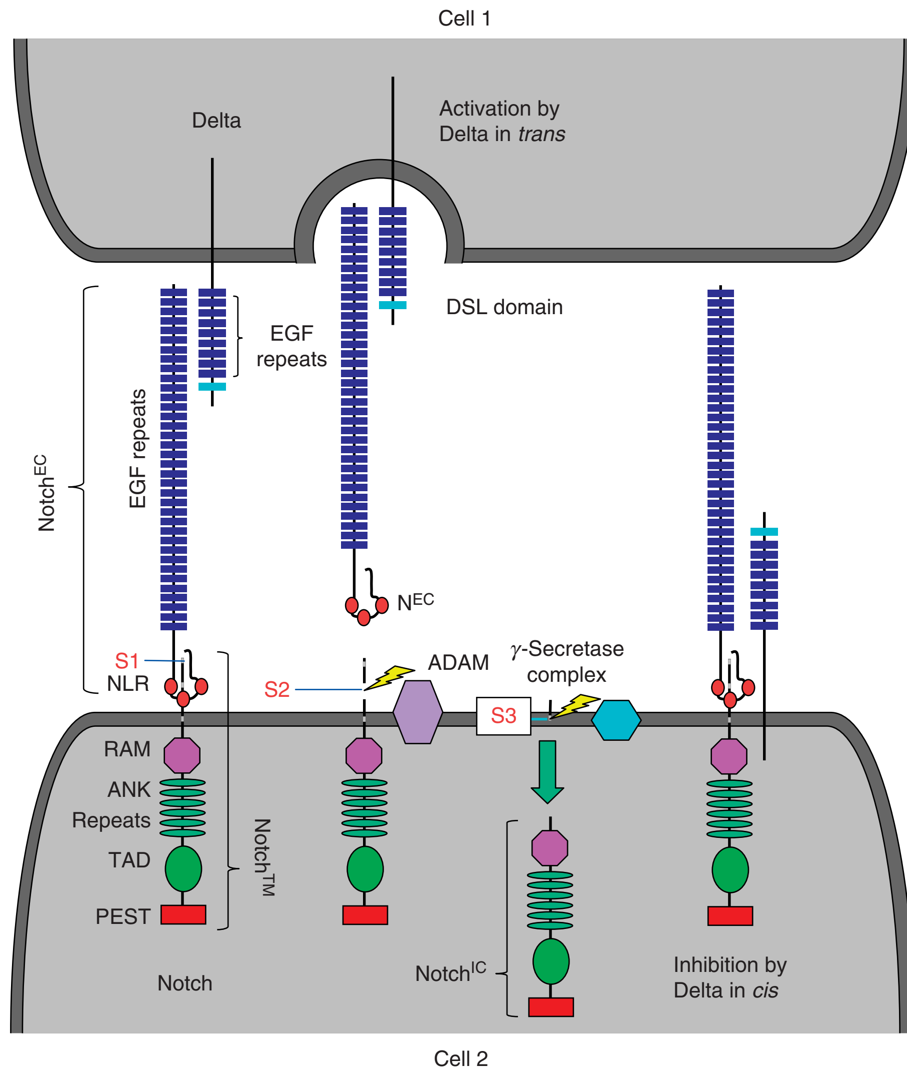

Figure 1 Schematic of Notch and Delta showing functional domains and proteolytic cleavage. The mature Notch receptor is a heterodimer containing a Notch^{EC} fragment and a Notch^{TM} fragment made by furin-based cleavage of the full length Notch protein at the S1 site. The Notch–Lin12 Repeats (NLR) in the Notch^{EC} fragment prevent access to the S2 site in the Notch^{TM} fragment. Endocytosis of Delta following interaction with Notch^{EC} in an adjacent cell (in *trans)* facilitates removal of the Notch^{EC} fragment and allows an ADAM protease access to the S2 cleavage site. S2 cleavage is followed by γ secretase-dependent cleavage at an S3 site, which releases the Notch^{IC} fragment into the cell. The Notch^{IC} fragment contains a RAM domain, ankyrin (ANK) repeats, and a transactivation domain (TAD), which allows it to function in transcriptional activation complex with Mastermind and a CSL protein like Su(H) in the nucleus. The PEST sequence allows rapid degradation of the Notch^{IC} fragment in the nucleus. Interaction with Delta in the same cell (*cis*) interferes with function of Notch. Notch^{EC}, extracellular fragment of Notch receptor; Notch^{IC}, intracellular fragment; Notch^{TM}, membrane-spanning fragment.

acquire the ability to adopt an alternate fate that is dependent on relatively high levels of Notch signaling. Failure of lateral inhibition mediated by Notch signaling results in a 'neurogenic' fate as too many cells within the PNC adopt a neural fate. This is accompanied by a deficit in the number of cells with the alternate fate that is dependent on relatively high levels of Notch signaling. Based on the neurogenic phenotype produced by loss of Notch signaling, many of the genes originally identified in the Notch signaling pathway were referred to as 'neurogenic' genes.

The genetic regulatory network described above, first discovered in *Drosophila* and subsequently in many other metazoan animals, describes the manner in which Delta–Notch signaling mediates lateral inhibition in a PNC to select a single cell that will adopt a neural fate. Though the details differ, this is how cells are selected for differentiation with distinct fates during neurogenesis in a wide range of circumstances in the central nervous system (CNS) and peripheral nervous system (PNS). In *Drosophila* during early neurogenesis, cells from PNCs in the neuroectoderm are

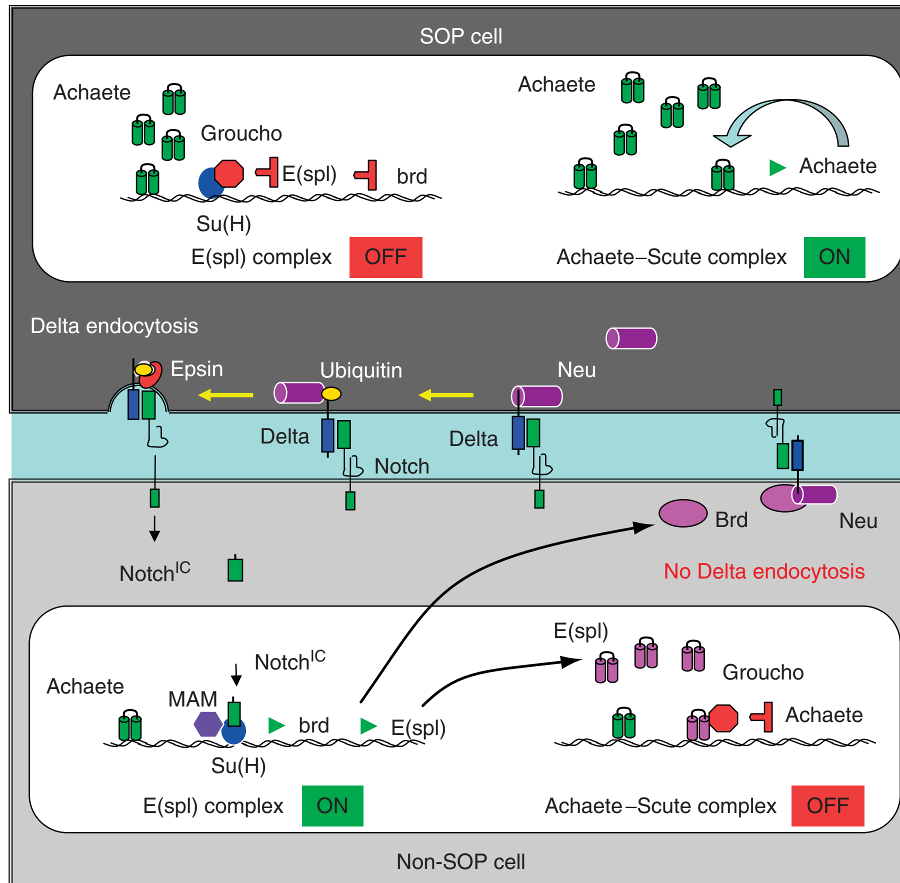

Figure 2 The dual role of Su(H) in activation and repression ensures complementary expression of genes in the Achaete–Scute and Enhancer of Split (E(spl)) complexes in the sensory organ precursor (SOP) and non-SOP cells. Relatively low levels of Notch activation in the prospective SOP cell permits effective self-activation by genes in the Achaete–Scute complex, which results in high levels of *achaete* expression. Despite presence of achaete-binding sites in the E(spl)-C enhancers, high levels of achaete proteins do not drive *E(spl)* expression in the SOP cell because, in the absence of Notch^{IC}, Su(H) maintains basal repression of genes in E(spl)-C. Suppression of E (spl) bHLH genes prevents repression of genes in the Achaete–Scute complex, and repression of *Bearded* (*brd*) genes prevents inhibition of Neuralized function in the prospective SOP. Hence the prospective SOP effectively activates Notch in the surrounding non-SOP cells, where Notch^{IC} makes Su(H) function as part of an activator complex and drives expression of genes in the E(spl)-C. In these non-SOP cells, bHLH E(spl) proteins repress *achaete* expression, while *brd* proteins prevent effective Neuralized function. E(spl), Enhancer of Split; Neu, Neuralized; Notch^{IC}, intracellular fragment of Notch receptor.

selected to become neuroblasts during formation of the CNS. Cells selected to become neuroblasts delaminate deep to the neuroectoderm and aggregate to form the neural anlage, while cells that are not selected to become neuroblasts remain superficial and eventually contribute to the formation of the epidermis. In the PNS, sensory organ precursors (SOPs) are selected from PNCs in imaginal discs. Cells in the PNC that are not selected to become an SOP also contribute to the epidermis, while the SOP cell undergoes a sequence of stereotypical divisions to produce cells of the sensory organ. Lateral inhibition also selects the R8 photoreceptors within PNCs at the advancing edge of the morphogenetic wave in the developing eye. In this context the selected cell does not undergo additional divisions; however, specification of R8 cells is critical because the R8 cell initiates the specification of the surrounding photoreceptors, and they in turn help specify additional cells of the ommatidium.

Cis-Regulatory Logic of E(Spl) Expression in a PNC

E(spl) and proneural genes are eventually expressed in complementary patterns in the PNC, with proneural expression restricted to the SOP, and E(spl) gene expression restricted to surrounding non-SOP cells. Analysis of enhancer elements that direct expression of E(spl) genes in PNCs of the wing imaginal disc reveal how the dual role of the CSL protein Su (H) in either transcriptional repression or Notch-mediated transcriptional activation establishes complementary expression of E(spl) and proneural genes in the PNC (**Figure 2**). The enhancer that determines Notch-mediated expression of E(spl) genes in the

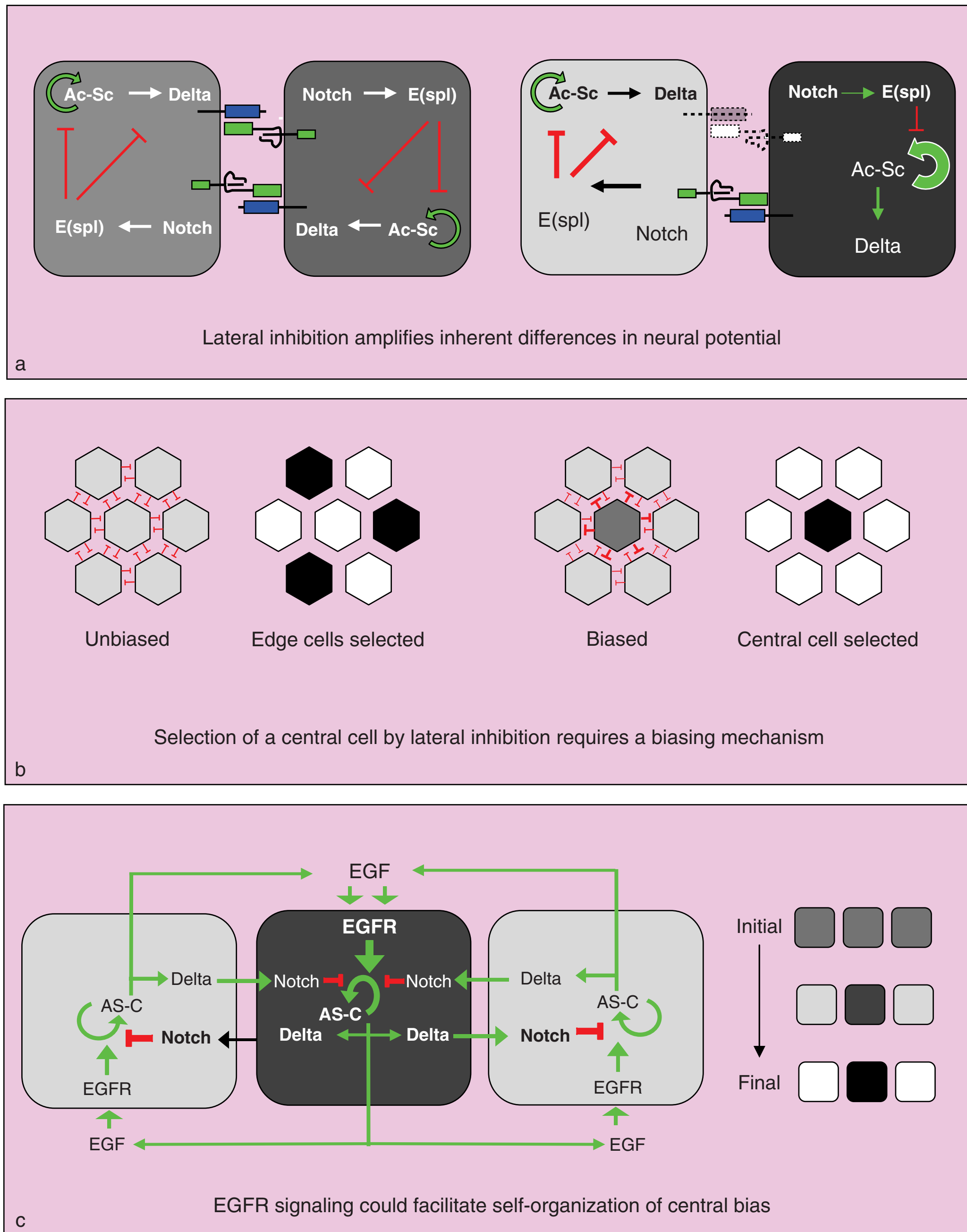

Figure 3 Lateral Inhibition selects only central cells when coupled with a central biasing mechanism. (a) In lateral inhibition, Notch signaling is part of negative feedback loop that amplifies differences in neural potential in adjacent cells. (b) If proneural genes were equally expressed in a proneural cluster (PNC), lateral inhibition would favor selection of cells at the edge of the cluster because edge cells do not receive inhibition from cells outside the PNC; in contrast, central cells receive inhibition from all surrounding neighbors. However, lateral inhibition could successfully select a central cell when coupled to a mechanism that ensures a central bias. Red bars represent lateral inhibition delivered to neighboring cells. (c) EGFR signaling promotes positive autoregulation by proneural genes. If each cell in the PNC were to be a source of a diffusible EGFR ligand, central cells would be exposed to the highest levels of the ligand and would have most efficient autoregulation of proneural genes. This defines a potential mechanism that facilitates self-organization of a central bias in proneural function that can then be amplified by Notch signaling leading to selection of a central cell. Ac-Sc, Achaete–Scute complex; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; E(spl), Enhancer of Split.

wing imaginal disc PNCs includes high-affinity sites for Su(H) binding, which allows E(spl) expression promoted by Notch^{IC}, Mastermind, and Su(H). However, the E(spl) enhancer also includes high-affinity sites for binding of proneural proteins Achaete and Scute. This seems paradoxical because the domain within the PNC where E(spl) genes are expressed is exactly where proneural expression is eventually

excluded; however, the combination of Su(H) and proneural binding sites in the enhancer ensures that Notch activation directs expression of E(spl) only within the PNC, where proneural expression has already been initiated. Though the E(spl) enhancer includes high-affinity proneural binding sites, high levels of proneural protein expression in the prospective SOP cannot independently drive E(spl) gene expression because in the absence of Notch^{IC}, Su(H) functions as part of a repressor complex that ensures genes in the E(spl) complex are not expressed. In surrounding non-SOP cells of the PNC, where Notch is activated by the SOP cell, Notch^{IC} makes Su(H) function as an activator to drive E(spl) gene expression.

The Complementary Role of Bearded Family Genes in the E(Spl) Complex

The E(spl) complex also includes non-bHLH genes of the *Bearded* (*Brd*) family. Their expression is also regulated by Notch activation; however, unlike the E(spl) bHLH genes, they do not inhibit proneural gene expression. Instead, they interfere with Delta function by inhibiting Neuralized mediated endocytosis of Delta, a step that is essential for effective activation of Notch in the neighboring cell (**Figure 2**). By inhibiting Neuralized function, *Brd* genes play a complementary role to bHLH E(spl) genes in making cells with Notch activation less likely to be selected as SOPs. Brd protein makes cells less effective at delivering Delta-mediated lateral inhibition to their neighbors and hence unlikely to win the competition to become an SOP. In the absence of Notch activation, Su(H) maintains repression of *Brd* genes in the prospective SOP cell, facilitating Neuralized function and making it more effective at delivering lateral inhibition to neighboring non-SOP cells.

Notch Signaling in the Progeny of Cells Selected by Lateral Inhibition

Though lateral inhibition typically refers to interactions between cells in the PNC that select a single cell, Notch signaling also has a critical role in determining distinct fates for the progeny of cells initially selected by lateral inhibition. For example, when a neuroblast divides, the daughter with less Notch activation becomes a ganglion mother cell (GMC), and the sibling with higher Notch activation becomes another neuroblast. When the GMC divides, the daughter with more Notch activity becomes a glial cell, and its sibling becomes a neuron. This lineage illustrates how the cell with less Notch activity is consistently permitted to adopt a fate that brings the cell closer to differentiating as a neuron, while the cell with greater Notch activity either remains as an undifferentiated progenitor or adopts a 'nonneural' fate. A similar pattern, discussed in more detail below, is seen in the lineage of the SOP.

Selecting a Central Cell within a PNC

The *Drosophila* epidermis develops more than 1000 bristles and other types sensory organs during development. Some of these bristles, called macrochaetes, are conspicuously large and located in stereotyped positions on the head and notum. Each macrochaete is derived from an SOP, previously also called a sense organ mother cell. In the wing imaginal disc, each SOP is, on average, selected from a cluster of 20–30 cells, called a PNC. All the cells in the PNC initially acquire the potential to become an SOP by their expression of proneural genes like *achaete* and *scute* that are part of the Achaete–Scute complex. However, competitive interactions mediated by Delta–Notch interactions eventually restrict high levels of proneural expression and SOP fate to a single cell or sometimes two cells within the PNC. High levels of proneural expression are first restricted to a smaller subset of cells within the PNC, called a proneural field. Then, within this field, the cell that attains the highest level of *achaete–scute* expression begins expressing *asense*, another proneural gene in the Achaete–Scute complex, and it acquires an SOP fate. The initial expression of proneural genes in stereotypical clusters is determined by the combinatorial function of heterogeneous 'prepattern' genes; however, the sustained high expression of proneural genes in a subset of cells depends on positive autoregulation by the proneural genes.

Biasing the Outcome of Notch Signaling

What determines which cell is eventually selected in a cluster and how is it reliably selected in a particular location within a large PNC? In the absence of additional biasing mechanisms, a central cell is least likely to be selected by lateral inhibition, and cells at the edge of a PNC are expected to have a competitive advantage (**Figure 3**). While cells at the edge do not receive lateral inhibition from neighbors outside the PNC, neighbors delivering lateral inhibition surround central cells.

Despite the inherent disadvantage of central cells with respect to lateral inhibition, it is often the case that they are the ones selected by lateral inhibition. Analysis of enhancer elements of the Achaete–Scute complex and epidermal growth factor receptor (EGFR) signaling suggests that interactions between EGFR and proneural factors might contribute to establishment of a central biasing mechanism during

selection of the SOP in the PNC for specific macrochaetes. The analysis of Achaete–Scute complex enhancers shows that one set of regulatory elements in a 'PNC enhancer' are responsible for initiating Achaete–Scute complex gene expression within a PNC in response to regulation by prepatterning genes, while another set of regulatory elements in a distinct 'SOP enhancer' mediate positive autoregulation by the proneural genes. The SOP autoregulatory enhancer is responsible for high levels of Achaete–Scute complex proneural expression in the cell that is eventually selected to become the SOP within the PNC. Proneural expression driven by proneural self-stimulation in the SOP enhancer is facilitated by EGFR signaling, and EGFR signaling is itself promoted by the proneural factors. As all the cells within the PNC secrete diffusible EGFR signals, central cells within a PNC are exposed to the highest levels of EGFR signaling. This results in particularly effective positive autoregulation of Achaete–Scute complex genes within central cells, biasing them for selection by lateral inhibition. Since diffusible EGFR ligands promote proneural expression by acting via the autoregulatory enhancer, their effect is limited to cells in the original PNC, where proneural expression was initiated by early patterning mechanisms, so EGFR signaling is unlikely to initiate ectopic proneural expression in cells outside the PNC.

Variations of the scheme described above play a role in biasing the outcome of lateral inhibition in various contexts. Selection of the progenitor for the *Drosophila* chordotonal organ, for example, is determined by the expression of another proneural gene, *atonal*, and it also involves an antagonistic relationship between EGFR and Notch signaling; however, in this context the initial progenitor selected by lateral inhibition continues to be the source of inductive EGFR signals, and it eventually recruits many more progenitors so that, unlike the SOP for external sensory bristles, the chordotonal organs are not isolated structures but organized to form small clusters.

Biasing Binary Cell Fate Decisions in the SOP Lineage

Competitive interactions mediated by Notch signaling continue to determine distinct cell fates in the progeny of cells initially selected by lateral inhibition. Here, the outcome of the competitive interactions is biased by the asymmetric inheritance of factors that can influence the efficacy of Notch signaling by progeny of the dividing cells. SOPs or the pI cells on the dorsal surface of the fly thorax (notum) undergo a series of stereotyped asymmetric divisions to generate cells that make cells of adult mechanosensory organs (**Figure 4**). The first of the asymmetric divisions is along the mediolateral axis and generates an anterior pIIb cell and a posterior pIIa cell. The pIIa cell divides once more to form a socket and a shaft cell, which are the external cells of mechanosensory organ. The pIIb cell divides twice to produce the internal cells of the sensory organ: the first division produces a glial cell and the pIIIb cell, and the pIIIb cell divides to form a sensory neuron and sheath cell. pIIa fate is dependent on high Notch activation, while pIIb fate is dependent on low Notch activation. Each sibling expresses both Delta and Notch and, in principle, could compete to acquire a dominant role in either delivering or receiving a Notch signal. However, asymmetric distribution of factors like Numb, Neuralized, and Sanpodo, determined by planar polarity signaling mechanisms, ensure that the anterior pIIb cell becomes more effective at delivering the Delta signal and the posterior pIIa cell more effective at having its Notch receptors activated. At pro-metaphase, planar polarity mechanisms segregate Partner of Inscrutable (Pins) to the anterior cortex of the pI cell, opposite to components of Par complex, including Bazooka (D-Par3), DaPKC, and DmPar6, which localize on the posterior cortex. Pins restricts localization of Baz to the posterior cortex of the dividing pI cell, and segregation of Baz along with DaPKC and DmPar6 at the posterior cortex restricts another factor, Lethal Giant Larvae, to the opposite anterior cortex. Lethal Giant Larvae in turn recruits Partner of Numb and Neuralized to the anterior cortex. As a consequence, following mitosis, Numb and Neuralized are segregated to the anterior pIIb cell. Numb prevents membrane localization of Sanpodo, a requirement for Notch function, and Numb also inhibits Notch in the pIIb cell by promoting its degradation. In addition, segregation of Neuralized to the pIIb cell allows this cell to internalize Delta in a manner that makes it effective in activating Notch in its neighbor. In contrast, absence of Numb and plasma membrane localization of Sanpodo make Notch effective in the pIIa cell, while absence of Neuralized prevents Delta in this cell from effectively activating Notch in its adjacent pIIb sibling. In this manner planar polarity mechanisms ensure that for the pII progeny of all the SOPs on the developing notum, the anterior sibling acquires a pIIb fate, and the posterior sibling acquires a pIIa fate. The coordinated fate of individual SOP progeny eventually ensures the coordinated orientation of all the sensory bristles in the notum.

A mechanism acting in parallel establishes an asymmetry in Delta recycling in the pIIa and pIIb cells (**Figure 4**). Shortly after division of the SOP, a Rab11-dependent recycling center is established around the centrosome in the pIIb cell. Establishment of this center is dependent on accumulation

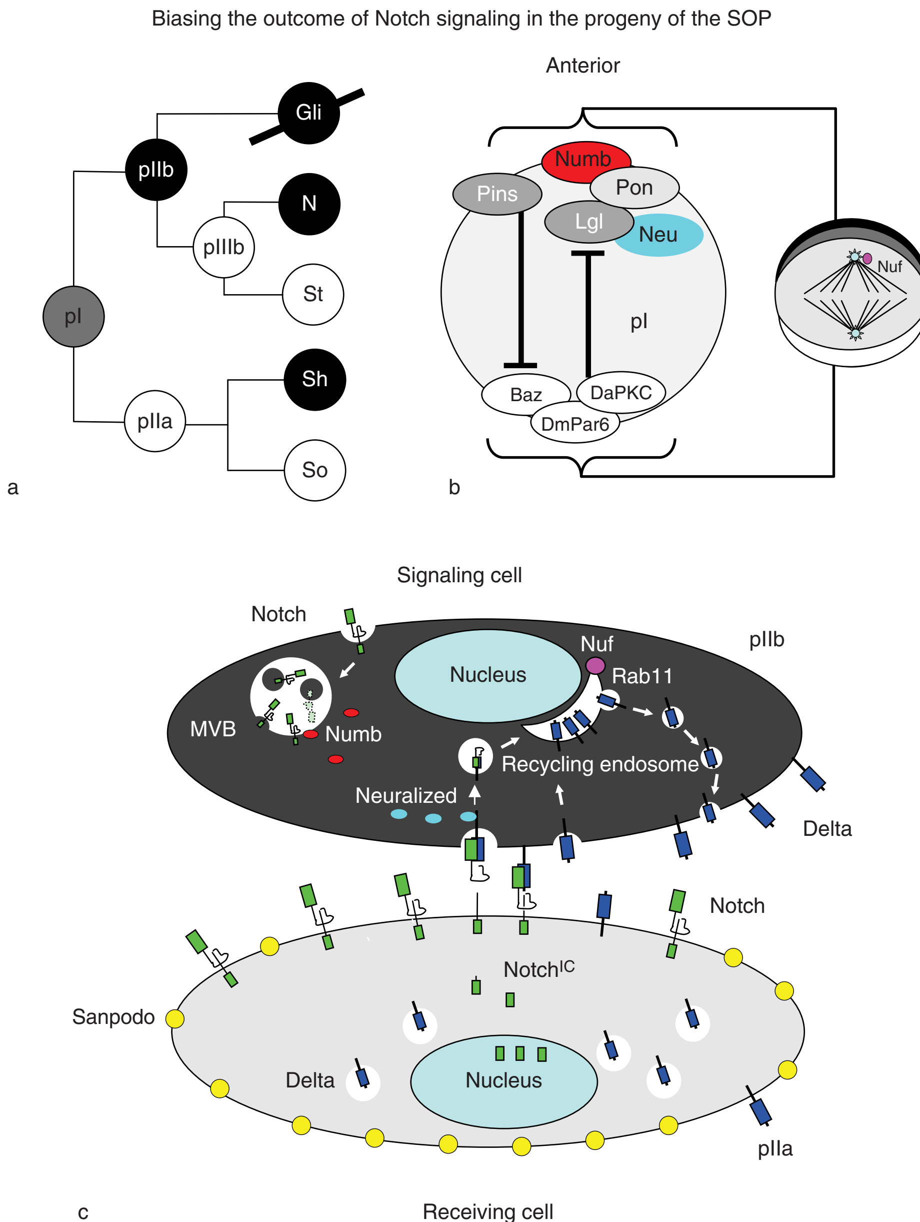

Figure 4 Mechanisms linked to planar polarity bias the outcome of Notch signaling in the progeny of the sensory organ precursor (SOP). (a) The SOP (pI) divides to form an anterior pIIb and posterior pIIa cell. The pIIa cell divides to form the Shaft (Sh) and Socket (So) cells. The pIIb cell divides to form a Glial (Gli) cell and a pIIIb cell. The pIIIb cell divides to form a Neuron (N) and a Sheath (St) cell. Cells with relatively low Notch signaling adopt the fate represented by the dark cells in the lineage. (b) At pro-metaphase, planar polarity mechanisms segregate Pins (Partner of Inscrutable) to the anterior cortex of the pI cell. Pins restricts localization of Baz to the posterior cortex of the dividing pI cell, and segregation of Baz along with DaPKC and DmPar6 at the posterior cortex restricts another factor, Lethal Giant Larvae (Lgl), to the anterior cortex. Lgl in turn recruits Partner of Numb (Pon) and Neuralized (Neu) to the anterior cortex. (c) Numb and Neuralized are segregated to the anterior pIIb cell. In addition, the Rab11 binding protein, Nuclear Fallout (Nuf), is permitted to associate with the centrosome in the pIIb cell. Numb prevents membrane localization of Sanpodo, a requirement for Notch function, and Numb also inhibits Notch in the pIIb cell by promoting its degradation in the MVB. Nuf facilitates organization of a Rab11-dependent recycling center in the pIIb cell, which makes Delta available at the cell surface. Neuralized allows the pIIb cell to internalize Delta and activate Notch in its neighbor. In contrast, absence of Numb and plasma membrane localization of Sanpodo makes Notch effective in the pIIa cell, while inhibition of Nuf association with the centrosome and absence of Neuralized makes Delta less effective at delivering a signal to the adjacent pIIb cell. MVB, multivesicular body; Notch^{IC}, intracellular fragment of Notch receptor.

of the Rab11-binding protein Nuclear Fallout, the *Drosophila* homolog of vertebrate Arfophilin, on the centrosome. Association of Nuclear Fallout with the centrosome is inhibited in the pIIa but allowed in the pIIb cell. As a consequence, soon after division, the pIIb cell begins recycling Delta internalized before or during mitosis, quickly establishing itself as the dominant signaling cell.

The fate of siblings following asymmetric division of the neuroblasts in the *Drosophila* CNS is also biased by asymmetric segregation of cell fate determinants like Numb. In this context, however, the asymmetric localization of cell fate determinants is not determined by planar polarity mechanisms but by asymmetries associated with apical–basal polarity in an epithelial cell. Division of the neuroblast, perpendicular to the apical–basal axis, creates a smaller basal sibling that inherits Numb. Numb inhibits Notch function and permits this cell to adopt a GMC fate. In contrast, higher Notch activation in the larger apical sibling allows that cell to retain a neuroblast fate.

Notch Signaling in Vertebrate Neurogenesis

Homologs of genes involved in Notch signaling have now been identified in almost all metazoans, and analysis of their function in vertebrate model systems including chick, zebra fish, mouse, and rat has demonstrated that Notch has a conserved role in vertebrate neurogenesis. Similarities have been shown in many contexts; two examples, from *Xenopus* and zebra fish, illustrate some of the similarities.

Early differentiating neurons, also called primary neurons in *Xenopus* and zebra fish, are distributed in three bilateral longitudinal columns in the neuroepithelium of the caudal neural plate. Within each longitudinal column, only a subset of cells become early neurons. Exaggerated Notch signaling reduces the number of neurons. In contrast, loss of Notch signaling in this context results in too many early differentiating neurons, a loss of many of the late differentiating 'secondary' neurons, and loss of the progenitor population that would normally permit neurogenesis to continue during development. In the case of primary sensory neurons, called Rohon Beard cells, Notch signaling helps neuronal progenitors choose between a neuronal fate and a neural crest fate. Loss of Notch signaling allows too many cells to become sensory neurons and not enough progenitors adopt a neural crest fate. In homozygous zebra fish Mindbomb^{ta52b} alleles, severe loss of neural crest cells results in a loss of pigment cells in the tail, which is why this allele was originally called 'white tail.' These observations in *Xenopus* and zebra fish illustrate how Delta–Notch-mediated lateral inhibition drives selection of a subset of cells that become neurons. Like neuroblasts in *Drosophila* that delaminate from the neuroectoderm, cells that are selected to become neurons release their apical or ventricular attachments, delaminate from the neuroepithelium, and eventually differentiate under the pial surface of the neural tube. Subsequent studies have shown that expression of an *atonal*-related gene, *neurogenin* (*ngn1*), helps define the proneuronal domains and gives cells the potential to become neurons. Notch signaling drives expression of Hairy–E(spl)-related genes (called ESR genes in *Xenopus*, HER genes in zebra fish, and HES genes in most other vertebrates), and Ngn1 expression is prevented by Notch signaling. If cells express high enough levels of Ngn1, they can initiate the expression of additional genes that facilitate stable acquisition of neuronal fate. For example, Ngn1 drives expression of *neuroD*, a bHLH proneuronal gene whose function is not as easily inhibited by Notch signaling. Ngn1 also initiates MyT1 expression, a factor that makes selected cells less sensitive to Notch signaling. In addition, P27^{xic1} stabilizes Ngn1 and facilitates exit from the cell cycle. Despite the obvious similarities, there are important differences in the early roles described for Delta–Notch signaling in neurogenesis: In *Drosophila*, Notch signaling selects neuroblasts that delaminate and come together to form the neural anlage, whereas in *Xenopus* and zebra fish, Notch signaling plays a role in the selection of cells that become the earliest differentiating neurons within the neural anlage (neural plate).

There are four Delta homologs in zebra fish (*deltaA*, *deltaB*, *deltaC*, and *deltaD*). Of these, *deltaA* and *deltaD* are expressed in proneuronal domains during early neurogenesis. As cells are selected to become neurons, they express higher levels of *deltaA*, and expression becomes less in surrounding cells, reflecting dynamic regulation that is consistent with the process of lateral inhibition. Though Delta–Notch signaling is likely to play a role in lateral inhibition and cells are selected in a salt-and-pepper pattern from longitudinal columns of *ngn1*-expressing cells, it is not clear whether the cells form small contiguous *ngn1*-expressing clusters from which individual cells are selected or whether early patterning mechanisms simply define a long proneuronal domain from which cells are selected in salt-and-pepper pattern by lateral inhibition. Lineage analysis shows that Notch signaling clearly regulates binary cell fate decisions and that Notch signaling has a recurring role in regulating both early and late neurogenesis. However, the precise role of individual Delta and Notch homologs in lateral inhibition and binary cell fate decisions is only beginning to be defined, and it remains unclear what mechanisms bias selection by lateral inhibition.

In the zebra fish, Delta–Notch signaling also helps select sensory hair cells in the ear and in neuromasts. Neuromasts, initially deposited by the caudally migrating posterior lateral line promordium (pllp), contain a central hair cell that is surrounded by

supporting cells. Analysis of Notch–Delta function shows that it helps select a central hair cell within a proneural cluster defined by *atonal homolog1* expression. The migrating pllp contains three or four nascent neuromasts at various stages of maturation. The least mature are at the leading edge of the pllp, where *notch3* is diffusely expressed within a cluster of cells representing the future neuromast. As the neuromast matures, *atonal homolog1*and *deltaA* expression becomes prominent in a central cell, and *notch3* expression gets restricted to surrounding cells. Following deposition, neuromasts mature to produce a cluster of hair cells, following a pattern of development reminiscent of *Drosophila* chordotonal organs. Further analysis of neuromast development is likely to provide details about how the proneural cluster is defined, how biasing mechanisms lead to selection of a central cell in this context, and how additional hair cells are added to the cluster. It will be interesting to compare how a mechanism that operates in neuromasts compares with those defined for PNCs where SOPs are selected.

Additional Roles for Notch Signaling

Examples from *Drosophila* development illustrate how Notch signaling feeds into genetic networks that determine cell fate and how the location of a cell and interactions with additional signaling mechanisms can influence the outcome of lateral inhibition. These examples provide a useful framework for understanding how similar cell fate decisions are made during neurogenesis in other metazoan systems. Mechanisms that regulate protein trafficking and cell biology have a critical role in Notch signaling.

While the role of Notch signaling in lateral inhibition during neurogenesis has been emphasized, it should be kept in mind that Notch has diverse roles in all tissues. In addition to its role in lateral inhibition and in binary cell fate decisions, where it mediates competitive interactions and prevents adjacent cells from adopting the same fate, Notch signaling is also involved in inductive interactions where expression of Notch ligand in one cell is required to activate Notch and induce a specific fate in an adjacent cell. In ddition, instead of participating in a negative feedback loop, Notch activation can be part of a positive feedback loop in which mutual activation of Notch in adjacent cells helps maintain high Notch signaling and determines similar fate in adjacent cells. At the wing margin in *Drosophila* and at rhombomere boundaries in the zebra fish hindbrain, sustained Notch signaling is linked to expression of Wingless/Wnt and helps establish signaling centers at tissue compartment boundaries. During somitogenesis, delay between transcriptional regulation of gene expression and feedback inhibition by the encoded proteins results in oscillating gene expression; furthermore, since cell–cell communication contributes to the mechanism, the oscillations are synchronized between cells, and the synchronized oscillations are used to establish the periodic pattern of somites. Finally, while Notch can influence tissue patterning through its influence on cell fate at a transcriptional level, it may simultaneously influence the organization of factors that determine cell morphology. Understanding the diverse yet conserved roles of Notch in cell fate and tissue morphogenesis remains an important and exciting challenge for the future.

See also: Helix–Loop–Helix (bHLH) Proteins: Hes Family; Helix–Loop–Helix (bHLH) Proteins: Proneural; Notch Signal Transduction: Molecular and Cellular Mechanisms.

Further Reading

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Notch Signal Transduction: Molecular and Cellular Mechanisms

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Pathway Overview

The Notch signaling pathway is one of a handful of signaling systems known to regulate the myriad of developmental decisions required for correct embryonic patterning and morphogenesis in metazoans. Consistent with this, Notch signaling affects the determination and differentiation of many different neural cell types in addition to regulating cell survival, adhesion, proliferation, and apoptosis. The activation of Notch by membrane-bound DSL (Delta, Serrate, LAG-2) ligands serves to limit signaling to cells in direct contact. Ligand binding leads to proteolytic release of the Notch intracellular domain (NICD) from the membrane, facilitating its role as a downstream signal transducer. The majority of signaling induced by Notch involves trafficking of NICD to the nucleus, where it interacts with a DNA-binding protein CSL (CBF1 in mammals, Su(H) in *Drosophila*, and LAG-1 in *Caenorhabditis elegans*) to recruit coactivators and turn on expression of target genes such as Hes-1 (hairy and enhancer of split-1) (**Figure 1**).

This mode of activation necessarily means that only one signal can be sent from each receptor molecule, and significantly, once a receptor is activated it cannot be reactivated. Given the direct nature of the signaling pathway, the core components are refreshingly few in number: ligand, receptor, and a DNA-binding downstream effector. However, this simplicity is balanced by a large, and still growing, number of requisite modulators that regulate this pathway.

Notch Receptors and DSL Ligands

The prototypic Notch receptor, *Drosophila* Notch (dNotch), is a type 1 transmembrane, cell surface protein with an ectodomain composed mainly of tandemly arrayed motifs related to epidermal growth factor (EGF-like repeats) (**Figure 2(a)**). Structure–function analyses indicate that the extracellular domain functions in ligand binding and repression of the signaling activity that is intrinsic to the intracellular domain. The Notch-related *Caenorhabditis elegans* proteins, GLP-1 and LIN-12, and the four mammalian Notch proteins, Notch1–4, all exhibit the same overall structure. Notch-related genes have been identified from a number of metazoans from sea urchin to human, but they are not present in plant genomes.

The DSL ligands that bind and activate the Notch/LIN-12/GLP-1 receptors comprise a family of proteins that are either Delta-like or Serrate-like (also known as Jagged) based on the structure of the two *Drosophila* ligands, Delta and Serrate (**Figure 2(b)**). In mammals, three Delta-like (Dll) genes have been identified (Dll-1, -3, and -4). Dll-2 has only been identified in *Xenopus* and four Dll genes have been reported for zebra fish, DeltaA–D, of which DeltaD is most similar to Dll-1. Only two Serrate-like subtypes (Serrate1/Jagged-1 and Serrate2/Jagged-2) have been isolated from humans, rats, mice, chickens, and frogs. There are three zebra fish Jagged genes (Jagged-1a, Jagged-1b, and Jagged-2).

Like the Notch receptors, the DSL ligands are single-pass cell surface proteins containing multiple EGF-like repeats; however, these ligands contain a signature motif dubbed DSL, which together with N-terminal (NT) sequences constitutes the ligand-binding domain. Although membrane attachment is thought to be important for activation of Notch, soluble DSL ligands have been found in worms, flies, and mammals. In fact, a search of the complete worm genome identified 10 DSL-containing ligands, with only five sequences predicting a transmembrane domain. In *Drosophila*, no soluble splice variants of Delta or Serrate have been reported; however, soluble forms of Delta, produced through cell surface proteolytic shedding, have been identified from embryos and cultured cells. The function of cleaved soluble DSL ligands in *Drosophila* is unclear, although when engineered soluble forms of Delta or Serrate are expressed in *Drosophila*, they produce phenotypes indicative of a loss in Notch signaling, suggesting that soluble ligands inhibit rather than activate Notch. Both Delta-like (Delta, Dll-1, and Dll-4) and Serrate-like (Jagged-1 and Jagged-2) ligands can be proteolytically processed by metalloproteases to effect extracellular domain shedding. The ability of soluble DSL ligands to either activate or inhibit signaling depends on their multimeric state; therefore, it is possible that the activity of soluble ligands may be regulated through interactions with the extracellular matrix as found for soluble growth factors. Alternatively, ligand shedding could represent a mechanism for downregulating activating ligand.

DSL ligands can also function outside of their role as activators of Notch signaling through interactions with the cytoskeleton via binding to PDZ-containing scaffolding proteins. PDZ-interaction motifs located in the C-terminus of DSL ligands can mediate interactions with MAGUK (membrane-associated guanylate

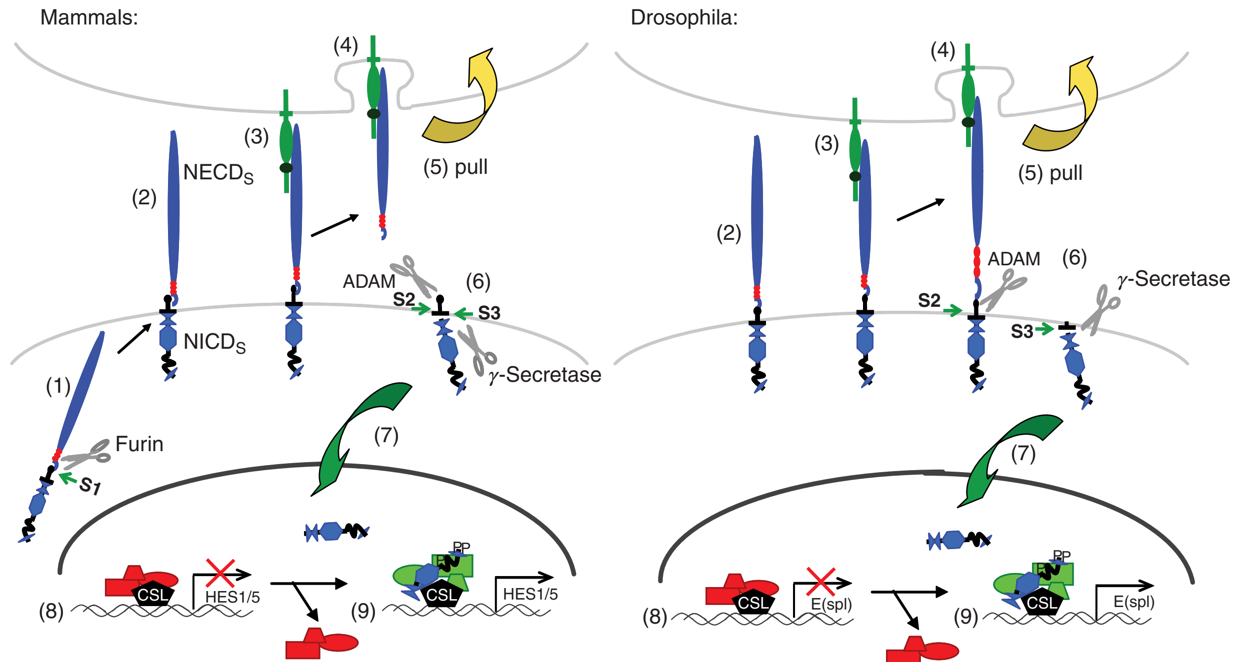

Figure 1 Notch signaling. In mammals (left), during trafficking to the cell surface mNotch is proteolytically processed by furin at the S1 site (1) and exists at the cell surface in a heterodimeric form (2). After ligand binding (3), DSL endocytosis of bound mNotch (4) is proposed to generate a 'pull' (arrow) that results in heterodimer dissociation (5) allowing removal of inhibitory sequences, including the LNR (red circles), that prevent ADAM cleavage. Following uptake of NECD$_s$ by the DSL cell, the remaining NICD$_s$ would then be exposed to activating proteolysis (6), first by ADAM to generate the S2 fragment that is subsequently cleaved by γ-secretase to produce the soluble NICD S3 fragment that translocates to the nucleus (7). Note that although mNotch S2 and S3 cleavages are shown here at the cell surface, it is also possible that either or both of these cleavages occur in an endosomal compartment. In the absence of Notch signaling, CSL recruits corepressors to inhibit gene transcription (8). When NICD is present, it displaces the corepressors and recruits coactivators to promote transcription of target genes such as Hes-1 or Hes-5 (9). In flies (right), there is evidence that cell surface dNotch is uncleaved (2). After ligand binding (3), the pull induced by DSL endocytosis (4 and 5) induces a conformational change that is thought to unmask the S2 site and allow for ADAM cleavage (6). This suggests that, unlike mammals, ligand-induced NECD release may be enzymatic, and not mechanical, in nature. Subsequent activating cleavage by γ-secretase (6) produces NICD that acts similarly to mammalian NICD in the nucleus to activate target genes such as Enhancer of split (E(spl)) (7–9).

kinase) family proteins. Mutation or deletion of the PDZ-binding domain in DeltaD, Dll-1, or Jagged-1 does not perturb Notch signaling, but rather produces phenotypes consistent with cytoskeletal involvement, such as aberrant cell migration or cell shape changes.

Regulation of Ligand-Induced Notch Signaling

Regulation of Ligand Expression

Cells take on distinct fates because Notch signaling is consistently activated in only one of the two interacting cells, highlighting the importance of establishing and maintaining signaling polarity. Studies in flies and worms have identified positive and negative transcriptional feedback mechanisms that amplify small differences in Notch and DSL ligand expression that bias which cells send or receive signals. Notch signaling can regulate gene expression of both the receptor and the ligand, and signaling from certain growth factor receptors (transforming growth factor-β (TGF-β) and vascular endothelial growth factor) also enhances DSL ligand expression. Other signaling pathways shown to potentiate DSL ligand expression include EGFR/MAP kinase, hormones, and NF-κB activation. Underscoring the importance of Notch and DSL ligand expression levels is the sensitivity to gene dosage reported for Notch-dependent processes, where both losses and gains in Notch activity produce mutant phenotypes. In humans, haploinsufficiency of either Jagged1 or Notch2 is associated with Alagille syndrome, whereas Notch1 haploinsufficiency is implicated in a subtype of inherited aortic disease.

Regulation of DSL Ligand Activity

The ability of cells to signal to Notch on adjacent cells is also regulated by endocytosis and membrane trafficking, consistent with the long-appreciated genetic interactions between Notch and shibire, which is the *Drosophila* homolog of dynamin, a key regulator of

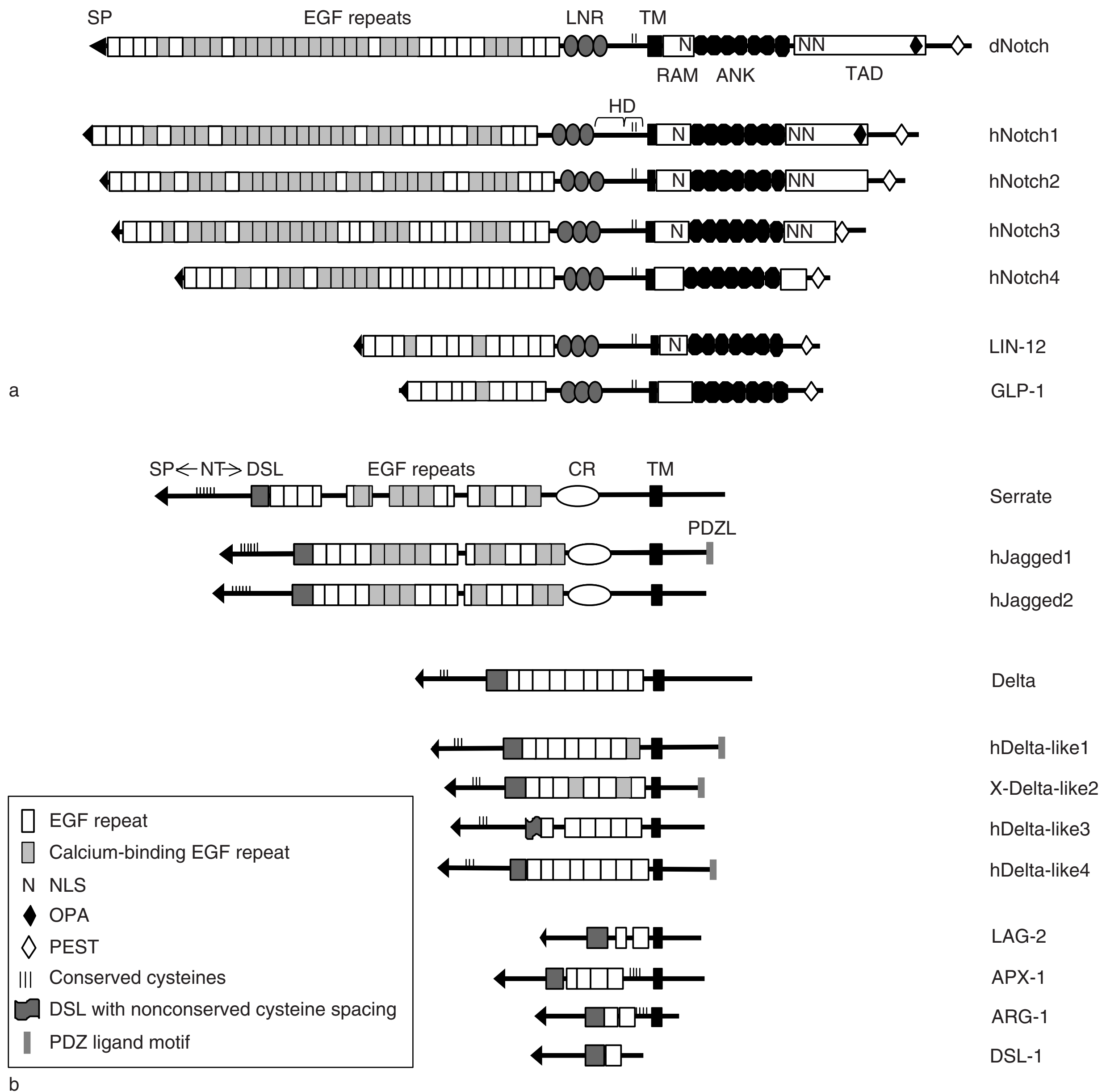

Figure 2 Notch receptors and DSL ligands. (a) The schematic is based on the following protein sequences from GenBank: *Drosophila* Notch, P07207; human Notch1, P46531; human Notch2, Q04721; human Notch3, Q9UM47; human Notch4, Q99466; *C. elegans* LIN-12, P14585; *C. elegans* GLP-1, P13508. (b) Structures are based on the following protein sequences from GenBank: *Drosophila* Serrate, P18168; human Jagged1, XP056118; human Jagged2, Q9Y219; *Drosophila* Delta, P10041; human Delta1, O00548; *Xenopus* X-Delta-2, AAB37131; human Delta3, Q9NYJ7; human Delta4, Q9NR61; *C. elegans* LAG-2, P45442; *C. elegans* APX-1, P41990; *C. elegans* ARG-1, T16213; *C. elegans* DSL-1, AAC04450. The drawings are approximately to scale. ANK, ankyrin-like repeats; CR, cysteine-rich region; DSL, Delta/Serrate/LAG-2 domain; EGF, epidermal growth factor-like; HD, heterodimerization domain; LNR, LIN-12/Notch related repeat; NLS, nuclear localization sequence; NT, N-terminal domain; OPA, glutamine-rich region; PDZL; PDZ ligand motif; PEST, motif rich in proline, glutamine, serine, and threonine residues that is important in protein turnover; RAM, CSL interaction domain; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain.

endocytosis. Dynamin is required in both signal-sending and signal-receiving cells; however, the exact functions for endocytosis in productive DSL-induced Notch signaling are unclear. Identification of additional endocytosis and membrane trafficking proteins that function in Notch signaling (**Table 1**) has provided some insight.

DSL endocytosis is required for Notch activation Interestingly, DSL ligands defective in endocytosis accumulate at the cell surface yet are unable to activate Notch, suggesting that cell surface ligand alone is not sufficient to initiate signaling. This is consistent with the dynamin requirement in signal-sending cells, and other endocytic proteins are required for DSL

Table 1 Endocytosis and trafficking regulation of Notch signaling

Drosophila	*C. elegans*	*Mammals*	*Role in Notch signaling*
In the signal-sending cell			
E3 ubiquitin ligases			
Neuralized	F10D7.5[a]	NEURL, NEURL2	RING-type E3 ligase that binds and ubiquitinates DSL ligands and promotes endocytosis
Mind bomb (dMib)		MIB1, 2	RING-type E3 ligase that binds and ubiquitinates DSL ligands and promotes endocytosis
Clathrin-mediated endocytosis			
Liquid facets (lqf)	Epn-1	Epsin 1, 2	Clathrin-associated sorting protein required for Delta signaling activity
Shibire	dyn-1	Dynamin1, 2	Endocytosis of ligands from PM; required for NECD *trans*-endocytosis and possible recycling
Clathrin heavy	Chc-1	CLTC	Coat protein of endocytic pits and vesicles; enhances Lqf phenotype
Auxilin	Dnj-25	Auxilin,GAK	J-domain containing protein proposed to function in CCV formation and/or uncoating; enhances Lqf
Eps-15	Ehs-1	Eps15	Required for *trans*-endocytosis and Notch activation
RalA	Ral-1	RalA	GTPase functioning in endocytosis, recycling, and actin organization; enhances Lqf phenotype
Endosomal recycling			
Rab 11	Rab-11.1	Rab 11A	Asymmetrically distributed in signal-sending cell that may promote DSL recycling
Sec 15	C28G1.3[a]	Sec15	Component of the exocyst that may function in Delta recycling
In the signal-receiving cell			
E3 ubiquitin ligases			
Su(dx)	WWP1	Itch (Mm)/AIP4 (Hs), WWP 1, 2	Nedd-type E3 ligase that binds and ubiquitinates Notch; endosomal sorting and degradation of Notch, Deltex
Dnedd4	Y92H12A.2[a]	NEDD4-1,-2 (Nedd4l)	Nedd-type E3 ligase that binds and ubiquitinates Notch; endosomal sorting and degradation of Notch
D-Smurf	Ce33003[a]	hSmurf1,2	Nedd-type E3 ligase with partial genetic redundancy with Su(dx) in *Drosophila*
Cbl	sli-1	Cbl	E3 ligase that interacts with Notch; Notch lysosomal degradation
Deltex		DTX1-4	RING-type E3 ligase involved in endosomal sorting of Notch; ubiquitination(?) and proteosomal degradation of Notch involving Kurtz; effector of ligand- and CSL-independent signaling(?); positive modulator of Notch in *Drosophila*
Endocytosis/early endosome sorting			
Shibire	dyn-1	dynamin 1, 2	Endocytosis of Notch from the PM; required for S3 cleavage of Notch to produce NICD
Merlin/Expanded		NF2	FERM protein that clears Notch from the PM; negative modulator of Notch signaling
Avalanche		syntaxin7, 12	Early endosomal SNARE protein involved in Notch degradation and endocytosis; no genetic interaction with Notch
Rab5	rab-5	rab5A,C	Early endosome trafficking protein involved in Notch degradation and endocytosis; no genetic interaction with Notch
Hrs	hgrs-1	Hrs (HGS)	Directs ubiquitnylated cargo to ESCRT machinery; no genetic interaction with Notch except in ligand-independent Notch activity induced by LOF Lethal giant discs
Multivesicular body formation			
Erupted	C09G12.9[a]	tsg 101	ESCRT-I protein that restricts ligand-independent Notch activation; MVB sorting of Notch for degradation
Vps25	WO2A11.2[a]	Vps25	ESCRT-II protein that restricts ligand-independent Notch activation; MVB sorting of Notch for degradation
Lethal giant discs	Y37H9A.3[a]	CC2D1A,B	Restricts ligand-independent Notch activation; functions in endosomal sorting after Hrs
Other proteins with roles in endosomal sorting			
Kurtz	F53H8.2[a]	ARRB1, 2	Nonvisual β-arrestin that binds Deltex; proteosomal degradation of Notch that involves Deltex

[a]Homology by protein sequence only.

Where no gene is listed, no ortholog has been identified. CCV, clathrin-coated vesicle; CSL, CBF1/Suppressor of Hairless/LAG-1 Notch downstream effector; Eps, epidermal growth factor receptor pathway substrate; ESCRT, endosomal sorting complexes required for transport; FERM, 4.1 protein/ezrin/radixin/moesin; hrs, hepatocyte growth factor receptor tryosine kinase substrate; Hs, human; LOF, loss of function; Mm, mouse; MVB, multivesicular body; NECD, Notch extracellular domain; Nedd, neural precursor cell expressed, developmentally downregulated; NICD, Notch intracellular domain; PM, plasma membrane; RING, really interesting new gene; Su, Suppressor; tsg, tumor susceptibility gene; Vps, vascuolar protein sorting.

activity (**Table 1**). For example, the E3 ubiquitin ligases Neuralized and Mind bomb ubiquitinate the intracellular domains of invertebrate and vertebrate DSL ligands and promote their endocytosis. DSL ligands must be ubiquitinated to signal, and only ubiquitinated DSL ligands in the presence of Liquid facets, the sole *Drosophila* epsin-related clathrin-accessory protein, are competent to activate Notch on adjacent cells. Signal-sending cells also require additional proteins that function in clathrin-mediated endocytosis, such as clathrin, dynamin, auxilin, and Eps15, for DSL ligands to signal effectively. Together, these findings highlight the importance of DSL endocytosis in Notch activation, but the exact function is unclear and remains controversial.

DSL endocytosis functions in recycling The requirement for epsin-dependent endocytosis in the signal-sending cell has been suggested to reflect a role for epsin in promoting DSL recycling to produce an 'active' ligand through clustering, posttranslational modification, or maintaining high levels at the cell surface; however, direct demonstration that DSL ligands actually recycle is lacking.

DSL ligand recycling could produce strong signal-sending cells through asymmetric localization of Neuralized that potentiates ligand activity by promoting ubiquitin–epsin-dependent endocytosis and trafficking of DSL ligand. In addition, asymmetric partitioning of the recycling endosome has also been proposed to regulate Delta levels or activity by recycling Delta to the cell surface where it can activate Notch. The differential recycling of Delta has suggested a mechanism by which ligand could be concentrated in signal-sending cells. Consistent with a role for DSL recycling in Notch activation, losses in Sec15, which functions with Rab11 in trafficking proteins from the recycling endosome to the plasma membrane, also produce cell fate transformations indicative of defects in Notch signaling. Interestingly, the mechanism responsible for the apparent polarized distribution of recycling endosomes is different from that required for asymmetric segregation of Neuralized, indicating that there are multiple mechanisms operating to establish and maintain signal-sending potential.

That only some Notch-dependent processes require Sec15 activity is inconsistent with DSL recycling producing an 'active' ligand because all Notch signaling should require this. Nonetheless, Dll1 recycling defects in bone marrow stromal cells suppress Notch-dependent phenotypic changes in lymphoid progenitors, suggesting that DSL recycling is necessary to produce an active ligand. However, the reported accumulation of Dll1 to recycling endosomes could decrease Dll1 at the cell surface, thereby accounting for the losses in Notch signaling. In this case, the need for Dll1 to pass through the recycling endosome could reflect a mechanism to replenish cell surface ligand, especially given that Dll1 density determines the lymphoid fate generated from hematopoietic stem cells.

DSL endocytosis functions in proteolytic activation of Notch DSL endocytosis could function beyond presentation of cell surface ligand for Notch engagement, such as proteolytic activation of Notch to generate the NICD that functions as the downstream signal transducer (**Figure 1**). DSL binding induces a series of proteolytic cleavages in Notch, first at the extracellular ADAM site followed by γ-secretase cleavage within the membrane to release the NICD required for signaling. Although DSL is required to produce NICD from full-length Notch, it is unclear how ligand binding promotes ADAM cleavage, a necessary step in activating γ-secretase proteolysis. Studies of flies have suggested that endocytosis of DSL bound to Notch on adjacent cells (designated transendocytosis) might produce a molecular strain in Notch leading to conformational changes that facilitate ADAM cleavage. Since efficient γ-secretase cleavage depends on removal of the Notch extracellular domain (NECD), DSL endocytosis could also promote Notch signaling by clearing the ADAM-shed NECD through uptake by ligand cells.

DSL endocytosis promotes physical dissociation of Notch Findings with mammalian cells support a role for NECD transendocytosis by DSL ligand cells in Notch signaling but indicate that NECD is not proteolytically released. Rather, NECD transendocytosis by DSL ligand cells in the presence of ADAM inhibitors suggests that NECD is physically removed from Notch present on the surface of interacting cells. To understand this result, it is important to note that most of the mammalian Notch (mNotch) at the plasma membrane is an intramolecular heterodimer, formed through furin-proteolytic processing during transport to the cell surface (**Figure 1**). The NECD and membrane-bound NICD heterodimeric subunits remain associated following furin cleavage through noncovalent interactions that keep Notch intact and inactive. Although mNotch activation relies on heterodimeric formation, the significance of this processing event has remained controversial given that *Drosophila* Notch (dNotch) does not appear to require furin processing to signal. However, an unprocessed mNotch receptor is neither dissociated nor activated by DSL cells. Since DSL binding in the absence of endocytosis does not promote heterodimer dissociation, forces created during ligand endocytosis are proposed to remove

the NECD subunit ($NECD_s$) and expose the NICD subunit ($NICD_s$) to activating proteolysis (**Figure 1**). In support of this idea, a majority of activating Notch mutations in T cell acute lymphocytic leukemia are missense mutations that alter heterodimer stability and effect dissociation independent of ligand.

Interestingly, amino acid insertions near the ADAM cleavage site do not alter heterodimer stability but are nonetheless constitutively active. The inserted residues are proposed to expose the ADAM cleavage site within the intact heterodimer, which may be similar to how DSL binding activates dNotch. Specifically, there may be a requirement to remove or distance the inhibitory LNR domain of Notch, located just upstream of the HD (**Figure 2**), for S2 cleavage to occur. In fact, the LNR domain has been shown to be necessary for inhibiting S2 cleavage but not for maintaining the heterodimeric structure of Notch1. Together, these data suggest that DSL binding followed by endocytosis activates mNotch through heterodimer dissociation, whereas conformational changes that facilitate ADAM proteolysis of unprocessed dNotch may underlie its activation.

DSL epsin-dependent endocytosis generates a pulling force Since only ligands internalized by epsin appear competent to signal, what role could epsin play in Notch activation? It is possible that Notch binding to DSL could induce ligand clustering, which would amass ubiquitin binding sites for epsin via its ubiquitin interacting motif (UIM) repeats. By assembling multiple low-affinity UIM–mono-ubiquitin interactions, strong epsin–ubiquitinated DSL interactions could be generated, which may be necessary to overcome any resistance to DSL internalization when it is bound to Notch. In fact, replacement of the Delta intracellular domain with a single ubiquitin motif that can be further ubiquitinated allows internalization and signaling activity in zebra fish. However, a nonextendable ubiquitin only weakly signals in flies even though it promotes endocytosis, supporting the idea that multiple ubiquitin interaction sites are required for DSL to activate Notch.

Epsin is a multidomain protein that in addition to binding ubiquitinated cargo interacts with membrane phospholipids, clathrin, and other clathrin adaptors as well as Cdc42 GTPase-activating proteins that regulate actin dynamics. Together with reports that the actin cytoskeleton and dynamin produce membrane constriction and tension during the process of endocytosis, it is tempting to speculate that epsin is required for ligand activity to create an endocytic vesicle endowed with sufficient force to induce conformational changes that either physically pull the mNotch heterodimer apart or promote ADAM cleavage of intact dNotch. This may explain why ubiquitinated ligands internalized in cells lacking epsin are unable to signal and why only DSL ligands internalized in an epsin-dependent manner are competent to signal.

Mechanotransduction in DSL-Induced Notch Signaling

That endocytosis, rather than proteolysis, drives mNotch dissociation suggests a mechanism of activation that involves mechanotransduction to allow activating proteolysis to occur, rather than relying completely on proteolytic cleavage as proposed for dNotch. However, if an intact dNotch can be activated, why is heterodimeric formation required for activation of mNotch? Activation of a heterodimeric receptor through physical dissociation might allow for additional mechanisms of Notch activation. In fact, noncanonical ligands have been reported, and in particular the extracellular matrix proteins MAGP1 and-2 (microfibril-associated glycoprotein) dissociate mNotch independent of ADAM cleavage and DSL binding that leads to proteolytic activation of Notch. Since MAGP binds and dissociates Notch cells autonomously, it might allow Notch to function as a mechanosensor to detect changes in shear forces produced by blood flow, which, like Notch signaling, is implicated in arterial–venous fate determinations.

How do soluble DSL ligands activate Notch? If DSL endocytosis of Notch triggers proteolytic activation, how can soluble ligands activate signaling? Although soluble DSL ligands activate Notch signaling, they are less active than cell-associated ligands and require clustering or attachment to surfaces to efficiently activate signaling and induce biological responses. In fact, prefixed Delta-expressing cells that are presumably endocytosis defective activate Notch target genes. Perhaps soluble ligands attached to the extracellular matrix or cell surface bind to Notch, generating pulling forces through cell detachment and/or Notch endocytosis. This mechanism may account for the signaling activity of naturally occurring soluble DSL ligands identified for *C. elegans*, in which the Notch-related GLP-1 is also heterodimeric.

Endocytosis and Trafficking Regulate Notch Levels and Activity

Signal-receiving cells regulate basal levels and activity of cell surface Notch through ubiquitin-dependent endocytosis, sorting, and trafficking. Removal and replenishment of cell surface Notch independent of ligand might influence signaling through availability

of receptor for activation. However, the requirement for dynamin in signal-receiving cells suggests a role for Notch endocytosis, in concert with DSL endocytosis, to effect conformational changes and/or receptor dissociation through pulling forces exerted on ligand–receptor complexes. In addition, endocytosis and trafficking of Notch could promote interactions with activating proteases since both ADAMs and γ-secretase are integral membrane proteins. Alternatively, endocytosis could function to release NICD from the membrane following γ-secretase cleavage at the cell surface.

Where Does DSL-Induced Proteolytic Activation of Notch Occur in Cells?

Whereas S2 and S3 cleavages of Notch are usually depicted to occur at the cell surface, ADAMs are found at the plasma membrane, early endosomes, and lysosome, so theoretically, cleavage could occur at any of these sites. Although evidence for intracellular cleavage of mNotch by ADAMs is lacking, γ-secretase proteolysis may occur inside cells since both monoubiquitination and clathrin-mediated endocytosis of Notch are required for signaling. Ubiquitination could allow endocytosis and/or trafficking that directs the ADAM/S2 cleavage fragment to an intracellular γ-secretase-rich compartment for S3 cleavage. However, the cellular site for γ-secretase cleavage of Notch is controversial and data support cleavage at the cell surface as well as within the cell. Nonetheless, γ-secretase activity is present in lysosomes and is enhanced by low pH. Given reports that endocytosis is required for γ-secretase cleavage of proteins other than Notch, the endosome is an attractive site for Notch proteolytic activation.

DSL-Independent Endocytosis and Trafficking of Notch

In the absence of ligand, Notch is also removed from the cell surface and may be either recycled or targeted for degradation. Since ubiquitin signals endocytosis, sorting and trafficking of cell surface proteins, it is not surprising that a number of E3 ubiquitin ligases are associated with Notch (**Table 1**).

In mammalian cells, the E3 ubiquitin ligases Cbl and Itch/AIP4 have been reported to interact with or modify Notch, but the biological relevance of these findings is unclear. Genetic studies of flies have provided evidence that ubiquitination and endosomal trafficking of Notch are important regulators of Notch activity. The Nedd E3 ubiquitin ligases bind and ubiquitinate Notch, and they may regulate Notch cell surface expression by promoting endocytosis and targeting for lysosomal degradation. Dominant-negative Nedd proteins increase Notch localization to recycling endosomes and at the cell surface, suggesting that losses in Notch ubiquitination promote its return to the cell surface, perhaps for increased exposure to ligands. However, coexpression of dominant-negative Nedd proteins and Notch in *Drosophila* activates signaling independent of ligand. In this case, suppressing Nedd function could protect a positive Notch effector from degradation. In fact, both *Drosophila* and mammalian Nedd family members ubiquitinate Deltex, a positive effector of Notch signaling, marking it for lysosomal degradation. Deltex is a RING-finger E3 ubiquitin ligase that, when overexpressed in flies, results in aberrant accumulation of dNotch in late endosomes/lysosomes and ligand-independent signaling. However, Deltex has also been shown to ubiquitinate dNotch directly for proteosomal degradation that would limit signaling. This negative effect of Deltex on dNotch requires a third protein, Kurtz, the single *Drosophila* ortholog of β-arrestin. Interactions between β-arrestin and dNotch may mediate integration of Notch with other signaling pathways given that in mammalian cells β-arrestins act as scaffolds/adaptors for signaling effectors.

Membrane trafficking defects result in aberrant accumulation of Notch within endosomes and in some cases unliganded Notch is activated (**Table 1**). In the early endosome, Notch is localized by the Hrs protein to the ESCRT machinery that promotes trafficking for lysosomal degradation. Defects in either ESCRT or Hrs proteins promote endosomal accumulation of Notch, but ectopic signaling is only activated with loss of ESCRT components (vps23 (erupted)/tsg101 and vps25) or a novel cytoplasmic protein, Lethal giant discs (Lgd), which is epistatic to Hrs. This ligand-independent Notch activation requires γ-secretase and further indicates that Notch can be activated intracellularly.

The ability of some, but not all, endosomal trafficking defects to activate Notch inappropriately provides insight into the intracellular locale where Notch is activated. Differences in endosomal acidity could limit γ-secretase activity to a specific compartment where Notch accumulates and the pH is low enough to promote conformational changes and/or receptor dissociation for ligand-independent activation. Proteolysis is possible since Notch activating proteases are also transmembrane proteins that could be trapped with Notch in the same compartment when membrane trafficking is blocked. During normal membrane trafficking, Notch and its associated proteases would transit quickly though the endosome, thereby avoiding aberrant activation.

Regulation of Notch Target Gene Expression

Transcriptional Complexes

Structure–function analyses have identified several regions of the intracellular domain necessary for transcriptional activation (**Figure 2(a)**) in addition to two nuclear localization sites that function in transport of NICD to the nucleus. The RAM domain, juxtaposed to the cytoplasmic face of the membrane-spanning region, is responsible for strong interactions with CSL, the major effector of Notch signaling. In addition to the RAM domain, seven ankyrin (ANK) repeats also interact with CSL proteins and are both necessary and sufficient for Notch transcriptional activity. Downstream of the ANK repeats is a region of high homology required for transcriptional activation (TAD), and at the C-terminus PEST sequences regulate turnover of NICD protein in the nucleus. Although all four mammalian Notch proteins are proteolytically processed to generate active NICD-like fragments, the individual TADs vary in potency, which may reflect differences in target gene activation induced by the different Notch receptors.

Genetic studies indicate that the CSL genes Su(H) and LAG-1 are required for normal Notch/LIN-12/GLP-1 function but act downstream of the receptors to positively regulate signaling (**Figure 1**). Typically, the CSL proteins function as transcriptional repressors; however, activation of Notch signaling in cells converts these DNA-binding proteins into transcriptional activators. Complexes containing NICD and CSL physically associate with DNAs containing CSL binding sites, and these interactions correlate with transcriptional activation of Notch target genes such as Enhancer of split (E(spl)) and the homologous vertebrate genes of the Hes and HERP family (**Figure 3**). In fact, induced expression of E(spl) and its homologs has been a convenient marker to monitor and identify cells undergoing Notch signal transduction, although some interpretation is needed because these genes can be regulated independently of Notch.

A crucial component of the NICD/CSL activation complex is mastermind (MAM). There are three mastermind-like proteins in mammals, MAML1–3, that *in vitro* have differing abilities to potentiate the activity of NICD/CSL complexes. Structure studies indicate that MAM binds to a composite site that consists of both the ANK repeats of Notch and a portion of CSL (**Figure 3**). Only the NT 80 amino acids of MAM are necessary for binding to the CSL/NICD interface and on their own function as a dominant-negative pan-Notch inhibitor, presumably by forming inactive complexes with activated NICD. This implies that the bulk of MAM binds and recruits other proteins required for activated transcription. In fact, in response to ligand, NICD, MAM, p300, CBP, and SKIP (Ski-interacting protein) are found associated with the Hes1 promoter. Since CSL binds to its cognate binding sites in the absence of Notch protein, it is constantly present on the Hes1 promoter (**Figure 3**).

In the absence of activated Notch, CSL transcriptional repression has been linked to a number of corepressor proteins and their associated histone deacetylases (HDACs) (**Figure 3**). Mammalian CSL proteins physically interact with two different corepressor complexes, one containing SMRT (silencing mediator of retinoid and thyroid hormone receptors), HDAC-1, and SHARP/MINT (msx-interacting protein), and the other consisting of corepressors CIR (CBF1-interacting corepressor), SAP30, and HDAC-2. Notch can displace the corepressors bound to CSL to activate transcription, whereas SMRT or CIR can compete with NICD for binding to CSL that results in a suppression of NICD-mediated CSL activation. As might be expected for a protein that requires cofactors to actively repress transcription, 'de-repression' resulting in transcriptional activation can also occur either in the absence of corepressors or due to removal of CSL from its DNA-binding sites. KyoT2, a LIM-only protein, may act in this manner because it can block CSL binding to DNAT and compete with activated Notch1 for binding to CSL.

Notch Target Genes Regulated by CSL

The best genetically and biochemically characterized direct targets are the E(spl)/Hes/HERP genes. These genes encode basic helix–loop–helix (bHLH) transcription factors that in response to Notch signaling repress both the expression and the function of genes that encode activating bHLH transcription factors, such as the proneural achaete–scute complex (AS-C) in *Drosophila* and the vertebrate homologs of the AS-C genes, MASH, neuroD, neurogenin, and MATH.

The vertebrate homologs of E(spl), the Hes genes, also serve as Notch effectors downstream of NICD/CSL activation. Three of the Hes genes (Hes1, -5, and -7) and all three Hes-related genes (HERP1–3) have been shown to be direct transcriptional targets of Notch. As in the fly, genetic loss of Hes genes often, but not always, leads to Notch-like phenotypes. Although the Hes/HERP family of bHLH repressors represents a major subset of Notch target genes that lead to repression of downstream targets, it has become evident that CSL binding sites are present in a number of promoters and that NICD can lead to transcriptional

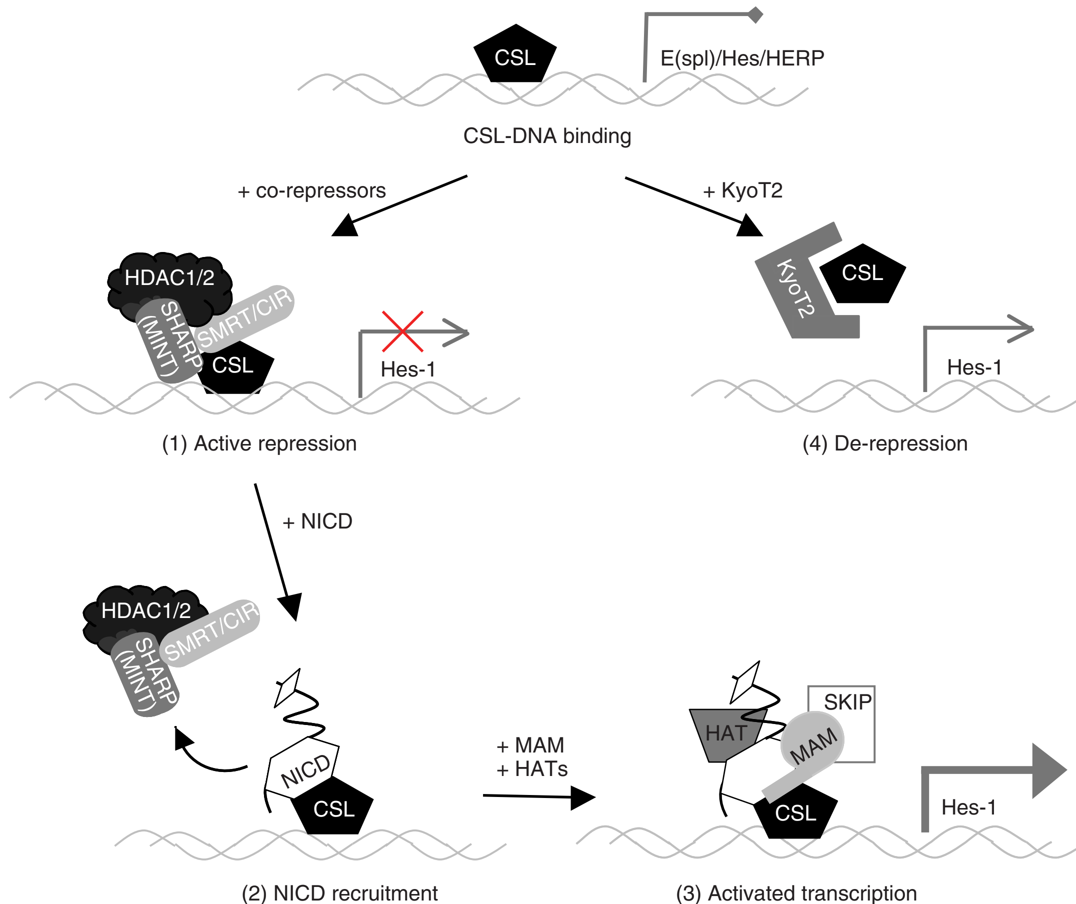

Figure 3 CSL transcriptional regulation. The DNA-binding CSL protein is common to both repression and activation complexes. In the absence of activated Notch, CSL interacts with known corepressor proteins (SMRT and SHARP(MINT)) that recruit histone deacetylases (HDAC1 or -2) to repress transcription (1). Upon Notch activation, NICD enters the nucleus and displaces the corepressor complex (2). NICD is thought to interact with CSL in a manner analogous to SMRT, although the Notch ICD and SMRT contain no identifiable sequence similarities. NICD binding to CSL creates a novel interaction site for the coactivator mastermind (MAM) that further recruits histone acetyltransferases (HAT), kinases, and elongation factors (not shown) to activate transcription (3). Loss of CSL DNA binding activity, either through interaction with KyoT2 or mutation of CSL, leads to 'de-repression' (4). In the absence of repression (and activation) complexes, some transcriptional activity is detected.

upregulation of these genes as well. The growing list includes Erbb2, glial fibrillary acidic protein, brain lipid-binding protein, GATA2, and IκBα.

Elaborations on Notch Signaling, Both Canonical and Noncanonical

The seemingly simple and direct mode of Notch signal transduction is at odds with the myriad of functions attributed to Notch, especially given the relatively small number of receptors and ligands identified for this signaling pathway. However, diversity in the output of canonical Notch signaling is most likely achieved by cross-interactions with other signaling pathways, and further diversity is achieved using noncanonical forms of Notch signaling. For example, in vascular development, the canonical Notch pathway involving NICD/CSL can cooperate with the hypoxia-inducible factors to promote blood vessel formation, perhaps by collaborating to drive expression of the Hey/HERP/Esr genes. The canonical Notch pathway can also interact with the TGF-β superfamily receptor signaling system through direct interactions between their downstream effectors. Smads activated following TGF-β binding to its cognate receptors can directly bind to NICD, leading to enhanced NICD–CSL activation of the target genes Hes/HERP. Finally, interactions have also been reported between canonical Notch signaling and the Wnt signaling pathway, but in this case the outcome is antagonistic. Cross-talk between Notch signaling and many of the major signaling pathways is likely to emerge in the future as more is learned about the global changes that occur in cells when they integrate multiple signals.

Diversity in Notch signal transduction is also achieved using pathways that diverge away from the

core one using CSL. For example, noncanonical ligands that lack DSL domains, such as DNER and F3/contactin/NB-3, have been reported to activate Notch and drive glial-specific genes such as myelin-associated glycoprotein through interactions with the downstream effector Deltex. Binding of these atypical Notch ligands leads to proteolytic activation of Notch and treatment with γ-secretase inhibitors blocks signaling. Although DNER and F3/contactin/NB-3 activation of Notch induces the translocation of NICD to the nucleus, transcriptional activation of glial-specific genes occurs independent of CSL and does not appear to involve Hes1. Diversity in Notch signaling may also be achieved using pathways that are activated independently of the transcriptional effects of NICD. In one report, for instance, DSL ligand-mediated Notch proteolysis has been proposed to induce rapid cytoplasmic responses, which involve a series of phosphorylation events on PI(3)K, Akt, and STAT3 that culminate with the translocation of phosphorylated STAT3 to the nucleus, where it stimulates Hes3 transcription. By an unknown mechanism, Hes3 leads to the production of sonic hedgehog to promote survival of neural stem cells, all independent of CSL transcriptional activation. Finally, diversity in Notch signaling may involve a pathway in which DSL ligand activates a nonheterodimeric form of Notch. Importantly, biochemical studies have documented that uncleaved, full-length Notch is endogenously expressed on the surface of a number of different mammalian cell types, and this form appears to be the major species found in *Drosophila* cells. This type of signaling has been reported to block myogenic differentiation in the absence of CSL. Notably, a full-length, furin-resistant, uncleaved Notch receptor can respond to ligand to suppress myogenesis but not activate a CSL reporter. These results indicate that an alternative form of the Notch receptor can mediate signaling which is both CSL independent and proteolysis independent.

Summary

In the past few years, there has been rapid growth in the understanding of Notch signaling mechanisms and functions. Genetically amenable model organisms that require Notch signaling have led to the identification and characterization of this essential signaling pathway. The growing number of interactions identified for Notch with other signaling pathways, as well as the isolation of additional Notch activators, likely account for the diverse and extensive nature of this highly conserved and ubiquitous signaling system. As additional components of endocytosis and membrane trafficking are linked to Notch signaling, requirement for endocytosis in Notch signaling should become clearer. What is becoming clear is that the more we investigate Notch signaling mechanisms, the more we realize just how pervasive its use is in both developmental and disease settings.

See also: Notch Pathway: Lateral Inhibition; Retinal Development: An Overview.

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Helix–Loop–Helix (bHLH) Proteins: Proneural

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bHLH Transcription Factor Family

The basic helix–loop–helix (bHLH) family of proteins comprises transcriptional regulators of a variety of developmental processes including cellular differentiation and lineage commitment, not only in the nervous system but also in the pancreas, muscle, and heart. Data collected from several model organisms have implicated bHLH transcription factors in the determination of neuronal lineages and the specification of distinct cell fates. *Drosophila* studies have underscored the importance of bHLH factors in the acquisition of neuronal identity in the ectoderm. In this context, bHLH proteins are both necessary and sufficient to confer a neuronal fate to ectodermal cells; consequently, several bHLH factors, including achaete–scute and atonal, have been dubbed 'proneural genes,' while inhibitory bHLH factors such as hairy and enhancer-of-split suppress neurogenesis. Vertebrate homologs of the *Drosophila* proneural genes have been isolated and neural expression and function appears to be conserved.

The bHLH proteins mediate their effects by protein dimerization and DNA recognition. The binding to a core hexa-nucleotide e-box, CANNTG, is mediated by the ~60-amino-acid bHLH motif. The crystal structures of the bHLH proteins E47 (Tcfe2a, MGI) and Myod1 bound to DNA demonstrated that the bHLH region is composed of two α-helices separated by a loop. The basic region contacts DNA in the major groove, while the HLH domain is involved in protein dimerization. The bHLH proteins are classified by three criteria: (1) dimerization capabilities, (2) DNA binding specificities, and (3) tissue distribution. Two broadly defined groups are class A (class I) and class B (classes II–VII). Class A proteins are broadly expressed and are often called E-proteins. These E-proteins, including HEB (Tcf12, MGI), E2-2 (Tcf4, MGI), and the two splice variants of E2a (Tcfe2a, MGI), E12 and E47, in vertebrates, and Daughterless in *Drosophila*, form homodimers, as well as heterodimers with class B proteins. Class B bHLH proteins are further categorized into six subclasses termed classes II–VII based upon protein motifs and activity.

Different members of these subclasses of bHLH factors act to either inhibit or promote transcription of downstream targets. Particularly important for neural differentiation and cell-type specification is the neural expressed subset of class II bHLH factors that include Mash1 (Ascl1, MGI), Math1 (Atoh1, MGI), Math5 (Atoh7, MGI), Ngn1,2,3 (Neurog1,2,3, MGI), and Neurod1. These factors form heterodimers with E-proteins, bind to DNA at e-box elements, and act as positive regulators of neurogenesis and neuronal specification. **Table 1** lists some class II bHLH factors chosen for their expression in progenitor zones in the neural tube. In each case, loss-of-function studies have determined their importance in the formation of discrete neural cell types. In addition, class VI factors that include Hes proteins (homologs to *Drosophila* hairy and enhancer-of-split) also play essential functions in these processes but they act to suppress neurogenesis. The class II and class VI bHLH factors are in a regulatory loop that involves cell–cell signaling via the Notch–Delta signaling pathway and serves to limit the number of cells differentiating from the progenitor domain (**Figure 1**). In one cell, the Class II proneural bHLH increases levels of Notch ligand Delta. Notch signaling is activated in the neighboring cell resulting in an increase in Hes levels. Hes proteins suppress levels of the proneural bHLH factor. The cell with high levels of the proneural bHLH will undergo neuronal differentiation, whereas the cell receiving Notch signaling is more likely to remain as a progenitor, or to later become an astrocyte. This pathway was worked out originally using *Drosophila* genetics, but many components and mechanisms appear to be conserved in vertebrates as well.

Neuronal Differentiation

Several lines of evidence have determined roles for bHLH factors in neural development, particularly those of the achaete–scute and atonal classes. Members of these subclasses of bHLH factors are transiently expressed in restricted progenitor domains throughout the developing nervous system. Loss-of-function studies have demonstrated their absolute requirement for the formation of specific subsets of neurons, while overexpression assays have been particularly important in revealing their function in neural differentiation. In *Drosophila*, the achaete–scute complex genes (AS-C) are required for external sensory organs, and atonal is required for chordotonal organs and photoreceptor cells. In the absence of these bHLH factors, neuroblasts fail to form. Conversely, overexpression of achaete, scute, and atonal led to neuronal hyperplasia.

Table 1 Vertebrate neural bHLH factors and neural tissues where they function

bHLH	*Population/region*	*References*
Math1 (Atoh1)	Cerebellar granule cells	Ben-Arie (1997) *Nature* 390: 169.
	Gut epithelium	Yang (2001) *Science* 294: 2155.
	Hair cells of the inner ear	Bermingham (1999) *Science* 284: 1837.
	Merkel cells of the skin	Ben-Arie (2000) *Development* 127: 1039.
	Pontine nuclei	Ben-Arie (2000) *Development* 127: 1039.
	Spinal cord dI1 interneurons	Gowan (2001) *Neuron* 31: 219.
Math3 (Neurod4)	Retinal horizontal cells	Akagi (2004) *Journal of Biological Chemistry* 279: 28492.
	Retinal amacrine cells	Inoue (2002) *Development* 129: 831.
	Retinal bipolar cells	Hatakeyama (2001) *Development* 128: 1313.
Math5 (Atoh7)	Retinal ganglion cells	Wang (2001) *Genes Development* 15: 24.
		Kanekar (1997) *Neuron* 19: 981.
Mash1 (Ascl1)	Adrenal medulla-chromaffin cells	Huber (2002) *Development* 129: 4729.
	Gabaergic neurons cortex	Casarosa (1999) *Development* 126: 525.
		Fode (2000) *Genes Development* 14: 67.
	Enteric serotonergic neurons	Blaugrund (1996) *Development* 122: 309.
	Hindbrain serotonergic neurons	Pattyn (2004) *Nature Neuroscience* 7: 589.
	Noradrenergic neurons of brainstem	Hirsch (1998) *Development* 125: 599.
		Lo (1998) *Development* 125: 609.
	Noradrenergic neurons of PNS	Guillemot (1993) *Cell* 75: 463.
	Paracardiac ganglia	Guillemot (1993) *Cell* 75: 463.
	Primary olfactory neurons	Guillemot (1993) *Cell* 75: 463.
	Retinal neurons	Tomita (1996) *Genes to Cell* 1: 765.
	Spinal cord dI3 and dI5 interneurons	Helms (2005) *Development* 132: 2709.
	Spinal cord V2 interneurons	Li (2005) *Proceedings of the National Academy of Sciences of the United States of America* 102: 10688.
Ngn1/2 (Neurog1/2)	Distal cranial sensory ganglia (Ngn2)	Fode, *Neuron* 20: 483
	Proximal cranial sensory ganglia (Ngn1) (1998).	Ma (1998) *Neuron* 20: 469.
	Glutamatergic neurons of cortex	Fode (2000) *Genes Development* 14: 67.
	Dorsal root sensory ganglia	Ma (1999) *Genes Development* 13: 1717.
	Motor neurons (with Olig2)	Mizuguchi (2001) *Neuron* 31: 757.
		Scardigli (2001) *Neuron* 31: 203.
	Spinal cord dI2 interneurons	Gowan (2001) *Neuron* 31: 219.
	Spinal cord ventral interneurons (V1–3)	Scardigli (2001) *Neuron* 31: 203.
	Ventral midbrain dopaminergic neurons	Kele (2006) *Development* 133: 495.
Ngn3 (Neurog3)	Enteroendocrine cells of stomach	Lee (2002) *Genes Development* 16: 1488.
	Pancreatic Islet of Langerhans	Gradwohl (2000) *Proceedings of the National Academy of Sciences of the United States of America* 97: 1607.
	Ventral spinal cord glial cell differentiation	Lee (2003) *Developmental Biology* 253: 84.
Olig1/2	Motor neurons	Novitch (2001) *Neuron* 31: 773.
		Mizuguchi (2001) *Neuron* 31: 757.
	Oligodendrocytes of spinal cord and brain	Zhou (2001) *Neuron* 31: 791.
		Zhou (2002) *Cell* 109: 61.
Olig3	Spinal cord dI1–3 interneurons	Müller (2005) *Genes Development* 19: 733.
Ptf1a	GABAergic neurons of the cerebellum	Hoshino (2005) *Neuron* 47: 201.
	Spinal cord GABAergic dI4 and dIL^A interneurons	Glasgow (2005) *Development* 132: 5361.
	Pancreatic exocrine cells	Krapp (1998) *Genes Development* 12: 3752.

A role for vertebrate neural bHLH factors in inducing neuronal differentiation has been repeatedly demonstrated in multiple paradigms across a variety of species. Overexpression of Mash1, Ngn1, Neurod1, Neurod2, and Math1 conferred neuronal properties to the mouse embryonal carcinoma cell line, P19, by inducing cell cycle exit and expression of neuronal specific genes. In *Xenopus*, injection of mRNA for XNgnr1, Xneurod, Xath1, and Xash1/3 increased primary neurogenesis to varying extents at the expense of ectodermal cells. Forced expression of Ngn1 in primary rat cortical cultures induced neurogenesis while suppressing gliogenesis. Additionally, forced expression *in ovo* of Mash1, Math1, Ngn1, and Ngn2 in chick neural tube has clearly demonstrated the activity of these factors in driving neuronal differentiation. In these experiments, the proliferating progenitors in the ventricular zone were forced out of the cell cycle, moved laterally out of the ventricular zone into the marginal zone, and began expressing neuronal specific genes. In some cases, the interpretation of loss-of-function mutations

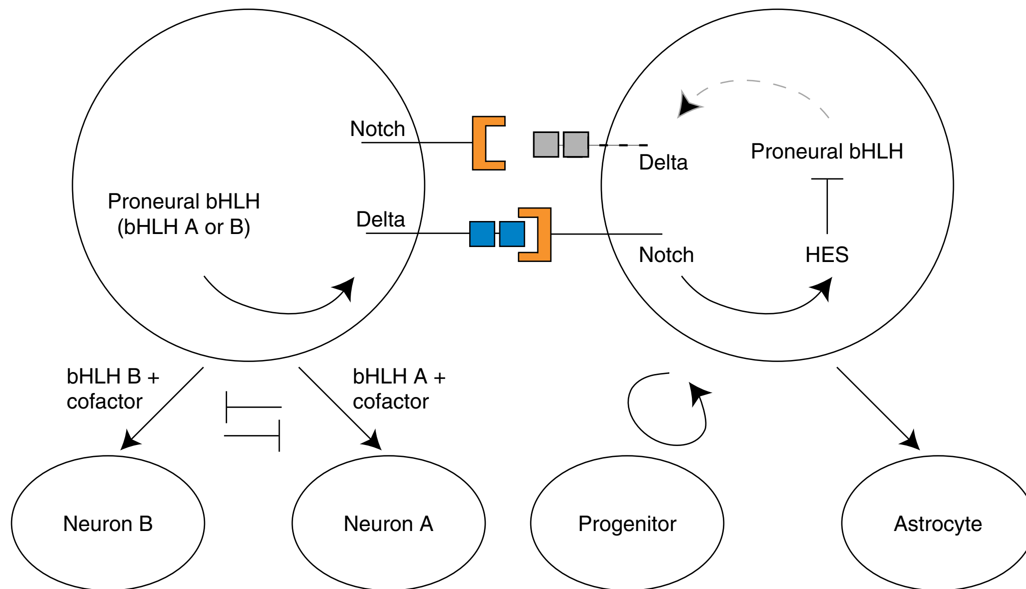

Figure 1 Proneural bHLH transcription factors and the Notch–Delta signaling pathway interact to control neuronal differentiation. In a cell with high levels of a proneural bHLH protein, expression of Delta ligand is enhanced. This in turn signals the neighboring cell, through the Notch receptor, to turn on the Hes bHLH transcription factors that repress the proneural bHLH proteins in that cell. The cell with high levels of Notch signaling and low levels of the proneural bHLH will remain as a progenitor cell or ultimately differentiate to an astrocyte. The cell with low levels of Notch signaling and high levels of the proneural bHLH will undergo neuronal differentiation. The type of neuron that is generated depends on the combination of the proneural subtype plus additional transcription factors.

in mouse are more difficult to interpret in regards to neuronal differentiation, since in the absence of one neural bHLH factor, neighboring or co-expressed bHLH factors can compensate. While compensation is a major issue, there are many examples where loss of one bHLH factor has dramatic consequences for neurons in a specific region (see **Table 1**).

Neurons versus Glia

The bHLH factors are not only involved in the differentiation of various neuronal lineages but they are also intrinsic mediators of the decision between neuronal and glial cell fates. As mentioned above, in cortical cultures, Ngn1 induces neurogenesis while suppressing astrocyte development. *In vivo*, neuronal and glial cell generation are temporally separated, where neurons are generated first followed by overlapping waves of oligodendrocytes and astrocytes. The loss of neural bHLH factors such as Mash1, Math3 (Neurod4, MGI), and Ngn1/2 results in an increase in astrocytes. This is seen in the cortex in the absence of Mash1 and Ngn2; likewise, loss of Mash1 and Math3 in the midbrain and hindbrain results in an increase in astrocytes. Single mutants do not clearly show the increase in astrocytes, probably due to redundant function of these bHLH factors in neurogenesis. Indeed, in mouse models where Ngn2 replaces Mash1 or vice versa, to a first approximation their neurogenesis activity is interchangeable. Studies in retina have also contributed to establishing a role for several bHLH factors in promoting neuronal fate and inhibiting glial fate. Overexpression of Math3 or Mash1 in retinal explant cultures increased neuron production while inhibiting the generation of Müller glial cells. Consistently, the absence of Mash1 results in an increase in the production of Müller glial cells. Together, these results demonstrate the requirement for class II neural bHLH factors to produce neurons; in their absence, glial cells form. As opposed to the function of the class II, class VI bHLH factors, particularly Hes1 and Hes5, suppress neuronal differentiation. In mutants for this class of bHLH proteins, premature neuronal differentiation is detected.

Oligodendrocytes are another major class of glia cells, and they also require bHLH proteins. Olig1 and Olig2 are required for oligodendrogenesis and maturation in brain and spinal cord. In the developing ventral spinal cord, Olig2 and Ngn2 are involved in the specification of oligodendrocytes and motor neurons. A precursor cell expressing Ngn2 and Olig2 will generate motor neurons. However, when Ngn2 levels are reduced, the Olig2 cells generate oligodendrocytes. Motor neurons and oligodendrocytes are not generated from a common Olig1/2-expressing progenitor; instead, these divergent cell types appear to be generated sequentially from neuroepithelial stem cells.

As stated previously, multiple lines of evidence place Mash1 as a neuronal differentiation factor.

However, there are increasing reports that Mash1 functions in oligodendrocyte formation as well. Mash1 is present in progenitors to neurons early and then oligodendrocytes later in the spinal cord. Mash1 is also present in the subventricular zone (SVZ) and rostral migratory stream (RMS) in the telencephalon in progenitors to neurons and oligodendrocytes. When cells from this region were cultured from Mash1 mutants, there was a loss of neurons and oligodendrocytes but not astrocytes relative to that seen with wild-type cultures. Together, these studies demonstrate the importance of some bHLH factors for generation of neurons, such as Ngn1 and Ngn2, and also reveal the importance of bHLH factors in oligodendrocyte development, such as Olig1, Olig2, and Mash1. In the absence of these bHLH factors, excess astrocytes are formed. This balance between neurons and oligodendrocytes on the one hand, and astrocytes on the other, involves the activation of Notch signaling (described above), a pathway known to be activated by the proneural bHLH factors (**Figure 1**). Through Notch signaling, the Hes bHLH factors are induced, which in turn suppress the proneural bHLH factors, and, thus, suppress neurogenesis.

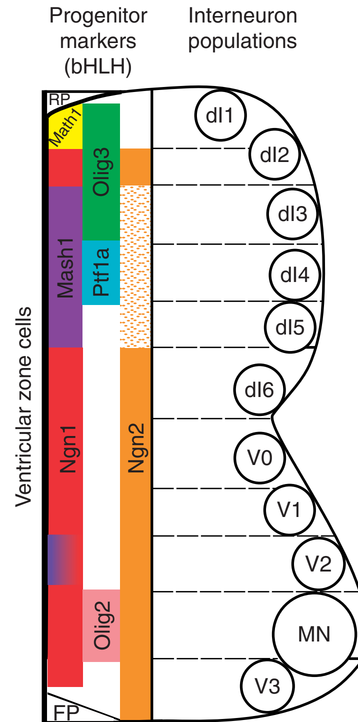

Figure 2 Class II bHLH factors are present in distinct populations of neuronal progenitor cells that are required for neuronal differentiation and cell-type specification. The pattern of expression of a subset of bHLH factors is shown in the ventricular zone of the spinal neural tube. Some progenitor cells express multiple bHLH factors. The dorsal interneurons (dI1–dI6), ventral interneurons (V0–V3), and the motor neurons (MN) require a distinct combination of bHLH factors to develop. RP, roof plate; FP, floor plate.

Neuronal Subtype Specification

Once a cell has committed to the neuronal lineage, it is specified to a certain neuronal cell type. Neural bHLH proteins in some regions of the nervous system have been shown to play roles in this aspect of nervous system development as well (see **Table 1**). In many regions of the developing embryo, the expression domains of the bHLH factors and the neuronal populations they specify are tightly linked. Expression of Mash1, Math1, and Ngn1 throughout the neural tube is nonoverlapping. Loss-of-function mutations in each of these bHLH factors have established their requirement for formation of specific neuronal populations. For example, in the dorsal spinal cord where interneuron populations are defined by the combination of homeodomain transcription factors they express, Math1 is required for dI1 neurons, Ngn1 is required for dI2 neurons, Mash1 is required for dI3 and dI5 neurons, and Ptf1a is required for dI4 neurons (**Figure 2**). Furthermore, ectopic expression of Math1, Ngn1, and Mash1 in ventricular zone cells in the chick neural tube results in an increase of a specific neuronal subtype at the expense of the others. This neuronal specification function of the bHLH factors is thought to work in combination with other factors. For example, when Math1 is ectopically expressed in the dorsal neural tube, it appears to convert dI2 and dI3 neurons to dI1, but when it is expressed ventrally it does not induce dI1 neurons, suggesting that a factor present dorsally but not ventrally is required for dI1 cell specification in combination with Math1. In addition, ectopic expression of Mash1 with another bHLH Olig3 increases dI3 neurons while Mash1 plus the homeodomain factor Lbx1 increases dI5. Thus, the generation of each interneuron population in the dorsal neural tube requires a distinct neural bHLH factor, but to specify the specific neuronal cell type, additional factors are required.

Neurotransmitter phenotype is a major facet of neuronal subtype that has also been shown to be specified, at least in part, by bHLH factors. GABAergic neurons in the dorsal horn of the spinal cord and in the cerebellum have an absolute requirement for the bHLH factor Ptf1a. However, the relationship between the requirement for a specific neural bHLH factor and a specific neurotransmitter phenotype is usually not this clear. For example, Mash1 is required for adrenergic neurons in the sympathetic nervous system and in the hindbrain, serotonergic neurons in the enteric nervous system and hindbrain, and GABAergic neurons in the cortex. Similarly, Ngn1

and Ngn2 are required for glutamatergic neurons in the cortex, sensory ganglia, and cholinergic neurons in the ventral spinal cord. Although the bHLH subtype does not appear linked to the neurotransmitter subtype when assessed in this global manner, regionally, neurons of specific neurotransmitter subtype require distinct bHLH factors. In the cortex, the GABAergic neurons migrate from ventral regions and are Mash1 derived, while glutamatergic neurons are generated dorsally and are Ngn1 and Ngn2 derived.

The requirement for factors in addition to the bHLH proteins for neuronal subtype specification, particularly the homeodomain class of transcription factors, is best illustrated from studies of retina development. In the retina, as in the brain and spinal cord, both gain- and loss-of-function studies have demonstrated the importance of the bHLH factors in both neuronal differentiation and in subtype specification. And although specific retinal cell types are lost or reduced when neural bHLH factors are absent, ectopic expression of these factors is not sufficient to specify the correct cell type. In addition, even with loss of function, often more than one bHLH must be mutated to lose a cell type. Retinal ganglia cells require Math5, amacrine cells require both NeuroD and Math3, and bipolar cells require Mash1 and Math3. The requirement for the concerted activity of bHLH and homeodomain factors for specification of these cell types was shown in overexpression assays. For example, to obtain bipolar cells in retinal explants requires the activity of the homeodomain containing factor Chx10 together with Math3 or Mash1. A similar situation arises in the specification of amacrine cells where the homeodomain factors Pax6 or Six3 are required with Math3 or Neurod1 to get specification to this cell type. Thus, although bHLH factors are important components of the code that determine the neuronal subtype, they appear to work in combination with homeodomain factors. The mechanism by which these two classes of transcription factors interact is largely unknown. Synergistic interaction between LIM homeodomain proteins and two bHLH factors, Ngn2 or NeuroM, that activate transcription of the Hb9 enhancer may play a role in motor neuron specification.

Control of bHLH Protein Expression

Controlling expression of the bHLH factors is critical for normal nervous system formation. The different neural bHLH factors can be classified into those expressed in mitotically active cells of the ventricular zone, to those expressed in newly postmitotic cells, to those that are maintained in mature neural cells in the adult. This article highlights the factors expressed and functioning at the early stages of neural differentiation (**Table 1**). In some cases, the later-expressed bHLH factors are downstream targets of the early factors, as is seen in Ngn1 and Ngn2 regulation of the postmitotic bHLH factor NeuroD. There are many additional mechanisms controlling bHLH expression including autoregulation, repression by Notch signaling (**Figure 1**), response to signaling pathways, and cross-repression between different bHLH factors. Autoregulation includes positive autoregulation seen with Math1 and Math5, and negative autoregulation seen with Mash1. The latter likely involves a regulatory loop with Notch/Delta signaling where Mash1 induces Delta, which in turn activates Notch signaling in the neighboring cell, resulting in Hes repression of Mash1 (see **Figure 1**). As discussed earlier, this may control the timing of expression of a bHLH factor that drives neuronal differentiation. Patterning signals influence the spatial expression pattern of a bHLH factor. For example, bone morphogenetic protein (BMP) signaling is required for expression of Math1 and Ngn1 in the dorsal neural tube, and Math1 in the cerebellum. And finally, cross-repression of expression was demonstrated in the dorsal neural tube where Math1 and Ngn1 suppressed expression of neighboring bHLH factors. This mechanism ensures strict boundaries of expression that may be important in generating the correct composition of neurons.

Concluding Remarks

Overwhelming evidence implicates bHLH transcription factors in the neuronal differentiation and cell-type specification in multiple regions of the developing nervous system. The bHLH family of transcription factors includes factors that are positive regulators of neurogenesis, negative regulators of neurogenesis, and positive regulators of oligodendrogenesis. Even with these different subclasses of bHLH factors, there are complexities that arise from formation of homodimers and heterodimers across these distinct subclasses of bHLH proteins. These complexes may bind DNA and activate or repress transcription, they may form inactive complexes that bind DNA but have no transcription activity, or they may not bind DNA at all. Other intricacies that modulate the function of the neural class II factors include the formation of higher-order complexes. For instance, the bHLH factor Ptf1a that is required for GABAergic neurons in the dorsal horn and cerebellum, is known to form a trimer complex in pancreas with an

E-protein (HEB, E12, or E2-2) and Rbpsuh, an effector of Notch signaling. Another example is the putative complex formed between bHLH heterodimers, and tetrameric or hexameric complexes containing Lim homeodomain factors. Interactions between these classes of transcription factors are suggested by their synergistic activity in the ventral spinal cord in generating motor neurons and ventral interneurons. Together, there is extensive evidence supporting essential roles for neural bHLH transcription factors in the timing and process of neuronal differentiation, and in the specification of neuronal subtype. However, the mechanistic complexities involved in these functions remain a fertile area for future studies.

See also: *Drosophila* Apterous Neurons: from Stem Cell to Unique Neuron; Helix–Loop–Helix (bHLH) Proteins: Hes Family; Notch Pathway: Lateral Inhibition; Notch Signal Transduction: Molecular and Cellular Mechanisms; Oligodendrocyte Specification.

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Helix–Loop–Helix (bHLH) Proteins: Hes Family

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Structure and Transcriptional Activity

Hes genes are mammalian homologs of *Drosophila hairy* and *Enhancer of split* [*E*(*spl*)], which are known to inhibit neurogenesis. There are seven members in the Hes family, and each member has a conserved basic helix–loop–helix (bHLH) domain (**Figures 1**(**a**) and **1**(**b**)), which is required for dimer formation and DNA binding. Hes1 forms homodimers and binds with high affinity to the DNA motif, the N-box (CACNAG) or the class C site (CACGCG), and with low affinity to the E-box (CANNTG). In contrast, many other bHLH factors, such as the proneural bHLH factors Mash1 and E47, form heterodimers and bind only to the E-box with a higher affinity. In the basic region, all Hes factors have a proline residue (**Figures 1**(**a**) and **1**(**b**), asterisk), unlike other bHLH factors, suggesting that this proline residue might be responsible for the Hes-specific DNA binding activity. At the C-terminus, Hes factors have Trp-Arg-Pro-Trp sequence (the WRPW domain), which functions as a repression domain by recruiting the co-repressors TLE/Grg factors, homologs of *Drosophila* Groucho (**Figures 1**(**a**) and **2**(**a**)). Thus, Hes factors act as transcriptional repressors, while proneural bHLH factors function as transcriptional activators. One of the targets of Hes1 is the proneural bHLH gene *Mash1*. There are class C sites in *Mash1* promoter, and Hes1 represses *Mash1* expression by directly binding to these class C sites. Hes1 also forms heterodimers with bHLH activators such as Mash1 and E47, but these heterodimers do not bind to the DNA, indicating that Hes1 inhibits the activities of proneural bHLH activators by forming non-DNA-binding heterodimers (**Figure 2**(**b**)). Thus, Hes factors repress gene expression by two different mechanisms: by recruiting co-repressors (active repression) and by forming non-DNA-binding heterodimers (passive repression) (**Figure 2**).

There are several Hes-related factors such as Hey/Hesr/Herp. They have a Hes-related bHLH domain and WRPW-related sequence and form heterodimers with Hes. Interestingly, Hes/Hey heterodimers bind to the class C site with a higher affinity and repress transcription more efficiently than Hes and Hey homodimers.

Between the bHLH and WRPW domains, Hes factors have the Orange domain (**Figure 1**(**a**)), which consists of two amphipathic helices. Some bHLH factors preferentially form heterodimers with each other while others do not, and this specificity for bHLH factor interaction is regulated by the Orange domain. This domain is also involved in transcriptional repression, although a co-repressor mediating this repression is not known.

Hes3 has two forms, Hes3a and Hes3b, which are generated by alternative promoters and first exons. Hes3a lacks the amino-terminal half of the basic region and thus cannot bind to the DNA, although it forms a non-DNA-binding heterodimer complex with other bHLH factors. Hes3a is specifically expressed by Purkinje neurons in the cerebellum, but the function of Hes3a in Purkinje neurons remains to be determined. In contrast, Hes3b has a complete basic region and represses transcription by binding to the N-box. Hes3b is expressed by neural progenitors (see following sections), unlike Hes3a. In the rest of this article, Hes3b is designated as Hes3.

Unlike other Hes factors, Hes6 does not bind to the N-box or the E-box, although it has a complete bHLH domain. Furthermore, Hes6 has an opposite activity to Hes1: Hes6 antagonizes Hes1 by physical interaction and supports Mash1 activity, thereby promoting neuronal differentiation. The loop region of Hes6 is shorter than that of the other Hes factors (**Figure 1**(**b**)), and it is likely that this short loop region is responsible for such unique activities of Hes6.

Regulation of *Hes* Gene Expression

Hes1 and *Hes5* expression is regulated by Notch signaling in the nervous system (**Figure 3**). The transmembrane protein Notch is activated by its ligands such as Delta. Upon activation, the intracellular domain of Notch (ICD) is cleaved from the transmembrane region and translocated into the nucleus. In the nucleus, the ICD forms a complex with the DNA-binding protein RBP-J, which binds to the *Hes1* and *Hes5* promoters. Before Notch activation, RBP-J interacts with a histone deacetylase co-repressor complex and functions as a transcriptional repressor, but Notch activation disrupts the repressor complex and leads to the formation of the ICD–RBP-J complex, which functions as a transcriptional activator. Thus, Notch activation induces expression of *Hes1* and *Hes5* genes. However, while *Hes5* expression is lost in the absence of Notch signaling, *Hes1* expression is not in some regions, suggesting that *Hes1* expression is additionally controlled

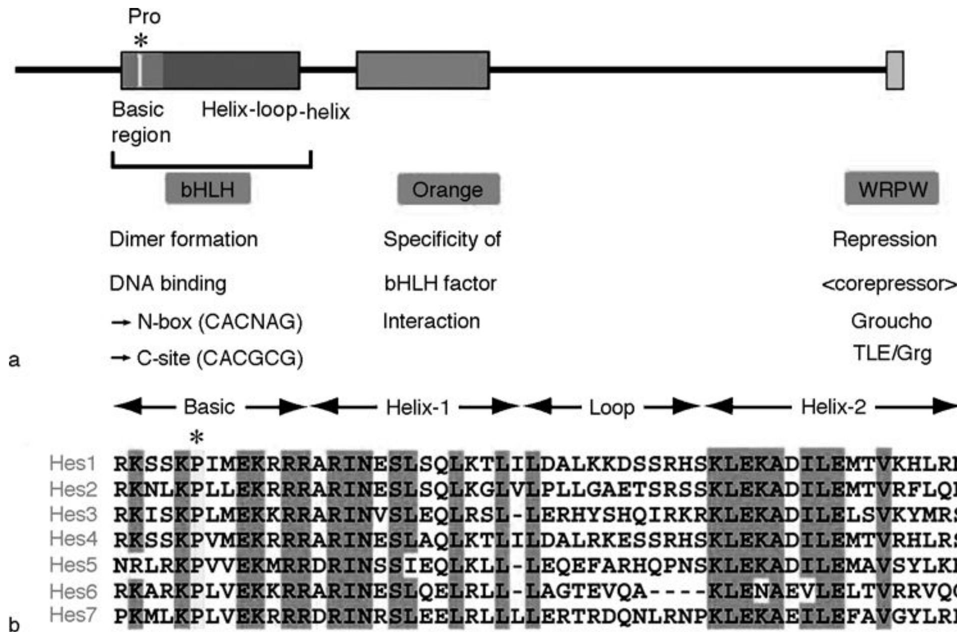

Figure 1 Features of Hes bHLH factors. (a) The bHLH, Orange, and WRPW domains and their functions. (b) Sequence alignment of the bHLH domain of Hes factors. Proline is conserved in the middle of the basic region (asterisk). The loop region of Hes6 is shorter than that of other Hes factors.

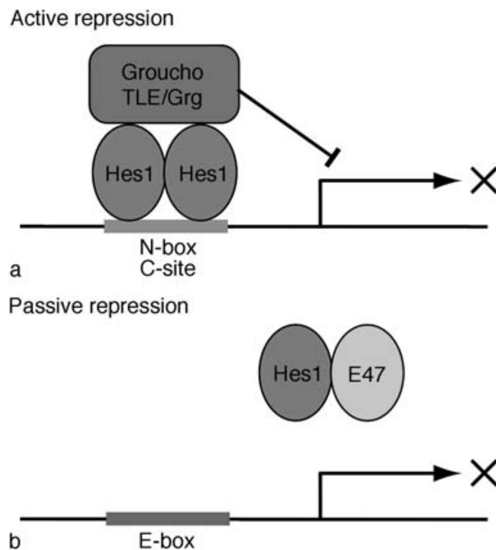

Figure 2 Two different mechanisms of Hes1-mediated transcriptional repression. (a) Active repression. Hes1 homodimer binds to the N-box and the class C site, and the co-repressors Groucho/TLE/Grg interact with the WRPW domain. This complex actively represses transcription by recruiting the histone deacetylase. (b) Passive repression. Hes1 forms a non-DNA-binding heterodimer with bHLH activators such as E47 and thereby represses transcription.

by other factors. In agreement with this notion, Wnt, Shh, and BMP have been reported to upregulate *Hes1* expression. *Hey* gene expression is also induced by Notch signaling, indicating that Hes and Hey function together in Notch signaling.

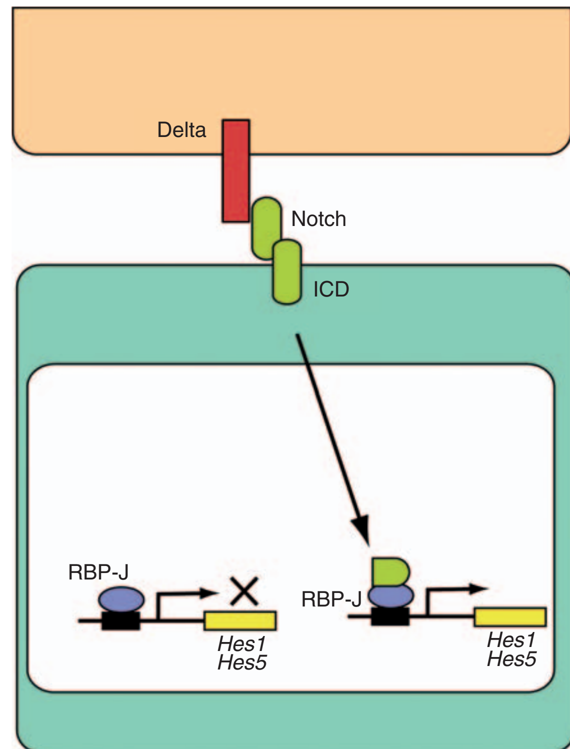

Figure 3 Notch signaling. The transmembrane protein Notch is activated by its ligands such as Delta. Upon activation, the intracellular domain (ICD) of Notch is cleaved and translocated into the nucleus. In the nucleus, the ICD forms a complex with the DNA-binding protein RBP-J, which binds to the *Hes1* and *Hes5* promoters. RBP-J alone is a transcriptional repressor, but the ICD–RBP-J complex is a transcriptional activator. Thus, Notch activation induces *Hes1* and *Hes5* expression.

Another important feature regulating *Hes* expression is that there are multiple N-box sequences in the *Hes1* promoter where Hes1 can bind and autorepress its own expression. Strikingly, when the promoter is activated, *Hes1* autonomously starts oscillatory expression with the periodicity of about 2 h (**Figure 4**). This oscillation depends on the negative feedback, as follows. *Hes1* promoter activation leads to accumulation of Hes1 protein, which represses its own transcription. This repression immediately leads to the disappearance of Hes1 mRNA and protein because they have extremely short half-lives (about 20 min). Disappearance of Hes1 protein then relieves the negative feedback, allowing the next round of transcription. In this way, *Hes1* displays oscillatory expression when the promoter is activated. Similarly, Hes7 expression also oscillates with the periodicity of about 2 h in the presomitic mesoderm. This oscillation is thought to be an integral component of the segmental clock that cycles every 2 h in the mouse, thus allowing another bilateral pair of somites to bud off at the anterior end of the presomitic mesoderm. Indeed, in the absence of Hes7, cycling of the clock is disrupted and somites are severely fused. It is likely that *Hes1* also acts as a 2-h-cycle biological clock in many cell types including neural progenitors. Supporting this idea, immunohistochemical analysis shows that Hes1 protein levels are variable in neural progenitors (see the following section), although further studies are required to determine whether or not *Hes1* expression oscillates in these cells *in vivo*.

Hes Expression in the Developing Nervous System

Differentiated neural cell types are generated from the neuroepithelial cells that line the ventricles of the developing brain and spinal cord. When they first appear in development, neuroepithelial cells initially undergo self-renewal by symmetric cell division (**Figure 5**). As development proceeds in the forebrain and midbrain, the neuroepithelial cells become radial glial cells, with cell bodies located to the ventricular zone and with long processes (radial fibers) reaching the pial surface (**Figure 5**). Radial glia have long been thought of as specialized glia (this is why they are named as such), but it has now been revealed that they are the second form of embryonic neural progenitors. Radial glial cells undergo asymmetric cell division, by which each radial glial cell produces one radial glial cell and one neuron (or neuronal precursor). Some radial glial cells undergo symmetric cell division, giving rise to two radial glial cells or two neurons. Neurons migrate along the radial fibers of radial glial cells toward the outer layers. Radial glial cells also give rise to ependymal cells, the internal

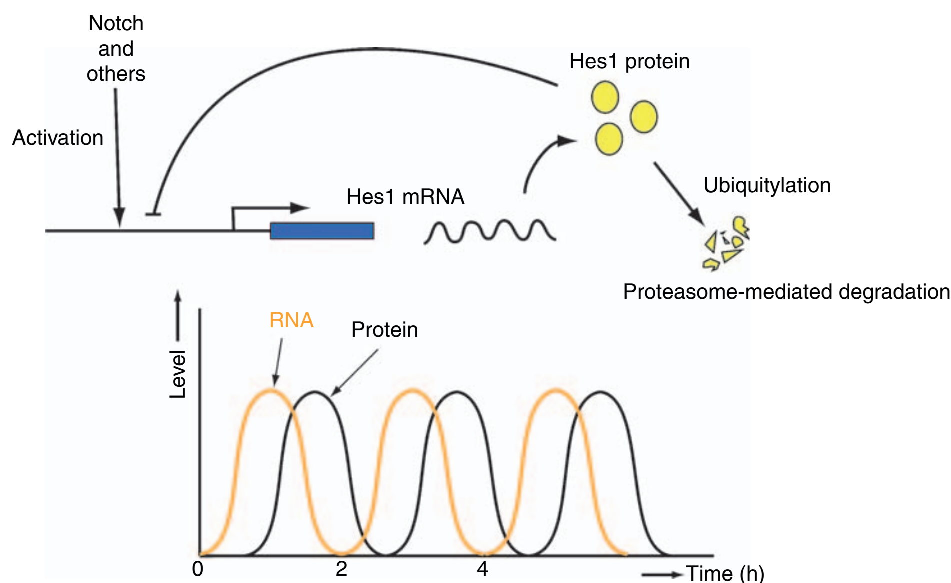

Figure 4 Oscillatory expression of Hes1. *Hes1* promoter activation leads to accumulation of Hes1 protein, which represses its own transcription. This repression immediately leads to disappearance of Hes1 mRNA and protein because they have extremely short half lives (about 20 min). Disappearance of Hes1 protein then relieves the negative feedback, allowing the next round of transcription. In this way, *Hes1* displays oscillatory expression when the promoter is activated.

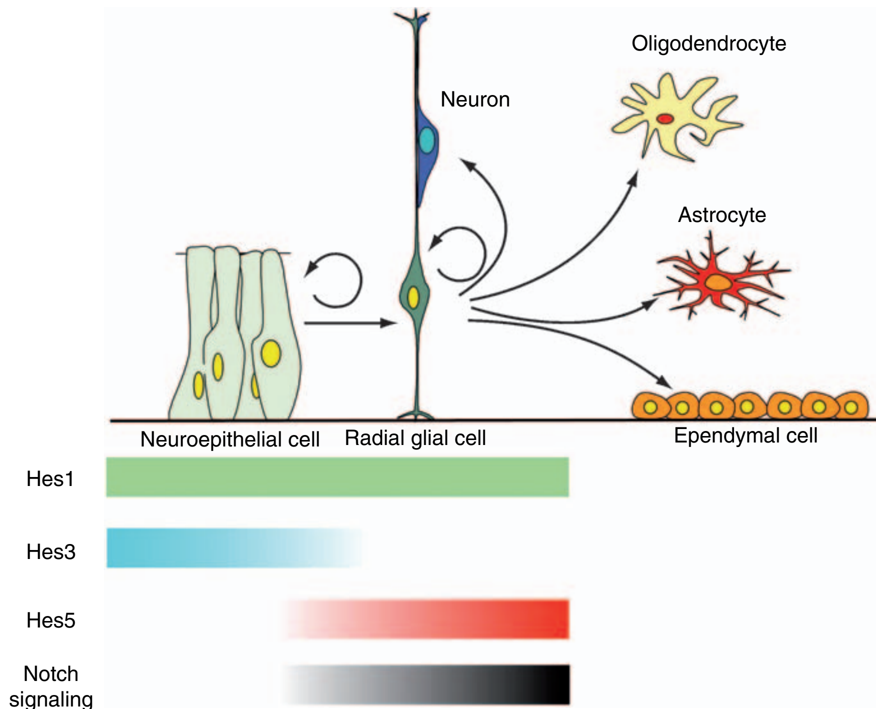

Figure 5 Expression patterns of *Hes* genes. In the developing nervous system, neuroepithelial cells are initially formed. Later, they become radial glial cells, which give rise to neurons, ependymal cells, oligodendrocytes, and astrocytes. *Hes1* and *Hes3* are expressed by neuroepithelial cells. *Hes1* is also expressed by radial glial cells while there is a switch from *Hes3* expression to *Hes5* expression during transition from neuroepithelial cells to radial glial cells. Notch signaling is also activated during this transition.

lining of ventricles (**Figure 5**). After production of neurons, radial glial cells are differentiated into glial cells (oligodendrocytes and astrocytes) (**Figure 5**). After birth, radial glial cells mostly disappear, but neural progenitors remain in a few neurogenic regions including the subventricular zone of the forebrain and the subgranular zone of the hippocampus. Thus, neural progenitors change their morphology and competence over time.

Initially, *Hes1* and *Hes3* are widely expressed by neuroepithelial cells (**Figure 5**). This expression occurs before *Notch1* and its ligands *Deltalike* (*Dll*) *1* and *Dll3* are expressed, indicating that *Hes1* and *Hes3* expression is independent of Notch signaling. During the transition from neuroepithelial cells to radial glial cells, *Hes3* expression is gradually downregulated and finally lost while *Hes5* expression starts together with *Notch1* and *Dll1* expression (**Figure 5**), suggesting that *Hes5* expression is controlled by Notch signaling. In contrast, Hes1 expression is maintained by radial glial cells (**Figure 5**) but becomes mostly complementary to *Hes5* expression (**Figure 6**). *Hes1* expression gradually overlaps with *Notch1* and *Dll1* expression, suggesting that *Hes1* expression is also controlled by Notch signaling at later stages. Interestingly, in the absence of *Hes1*,

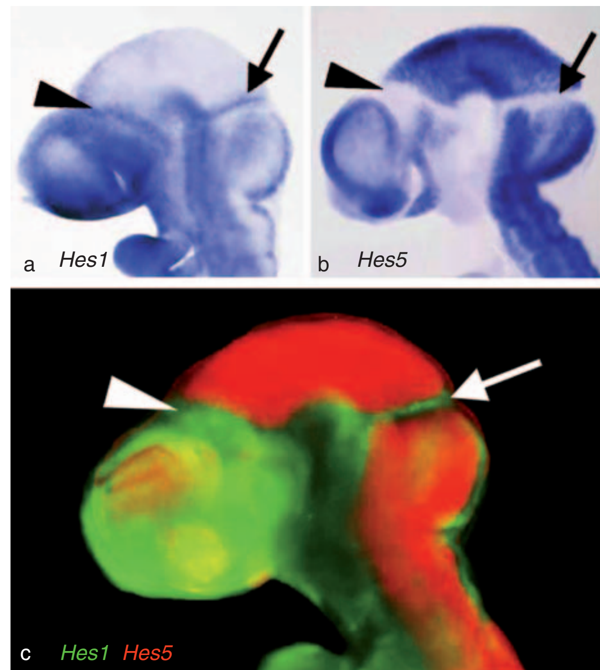

Figure 6 *Hes1* and *Hes5* expression in the developing brain. *Hes1* and *Hes5* expression are mostly complementary to each other at E9.5 in mouse embryos. *Hes1* is highly expressed in the zona limitans intrathalamica (arrowhead) and the isthmus (arrow) while *Hes5* is not.

Hes5 is expressed in the regions that normally express *Hes1* only, and vice versa, suggesting that *Hes1* and *Hes5* compensate each other. As development proceeds, *Hes1* and *Hes5* expression is downregulated but is retained in both the slowly dividing, niche localized neural stem cells, and the rapidly amplifying progenitors in the subventricular zone of the lateral ventricles, and the subgranular zone of the dentate gyrus. Thus, *Hes* genes are continuously expressed by neural progenitors. *Hes* genes are also expressed by subsets of glial cells such as Müller glia in the retina.

Hes Genes Regulate Maintenance of Neural Stem Cells

Many aspects of Hes function in the developing nervous system are explained by their ability to inhibit neurogenesis, thereby preventing neuronal differentiation of progenitors at earlier stages and promoting gliogenesis at later stages. Interestingly, in the absence of proneural bHLH genes such as *Mash1*, *Neurogenin* (*Ngn*), and *Math3*, progenitor cells that should normally become neurons are inhibited from neuronal differentiation and remain undifferentiated for a while but then adopt the glial fate prematurely. Thus, it is likely that proneural bHLH genes not only promote neuronal fate determination but also inhibit premature gliogenesis. In agreement with this notion, the proneural bHLH factor Ngn1 activates neuronal-specific gene expression and inhibits glial-specific gene expression by sequestering a p300/CBP–Smad complex. The phenotypes of proneural bHLH gene inactivation, inhibition of neurogenesis, and promotion of gliogenesis, are very similar to those of misexpression of *Hes* genes, suggesting that *Hes* genes exert their phenotypes by repressing proneural bHLH genes. Proneural bHLH genes are also required for *Hes* expression and maintenance of neural progenitors. These genes induce Delta expression, thereby activating Notch signaling in neighboring cells. In the absence of proneural bHLH genes, Notch is not activated, and *Hes* expression is downregulated, resulting in loss of neural progenitors. Thus, *Hes* and proneural bHLH genes form a network to regulate maintenance of neural progenitors and the formation of neurons and glial cells.

Hes1, Hes3, and Hes5 likely act to inhibit neurogenesis by repressing proneural gene expression directly. For example, the proneural bHLH gene *Mash1* contains class C binding sites in its promoter, and Hes1 represses *Mash1* expression by directly binding to these sites. In agreement with this result, *Mash1* expression is upregulated in the absence of *Hes1* and is even more severely upregulated in the absence of *Hes1*, *Hes3*, and *Hes5*. Furthermore, other proneural genes such as *Ngn* and *Math1* are also highly upregulated in the absence of these *Hes* genes, suggesting that Hes1, Hes3, and Hes5 negatively regulate proneural bHLH gene expression. Hes proteins might also inhibit neurogenesis through other targets, including the cyclin-dependent kinase (CDK) inhibitors p21 and p27, both of which inhibit cell cycle progression by retarding G1 phase. It is likely that Hes1, Hes3, and Hes5 promote cell cycle progression, and thus the maintenance of neural progenitors, by repressing p21 and p27. However, paradoxically, it has been also shown that persistent and high levels of Hes1 expression inhibit cell proliferation (see following section). Because p21 and p27 are also required for assembly of cyclin D1–CDK4 complex and nuclear import of cyclin D, complete repression of these CDK inhibitors by persistent and high levels of Hes1 may be inhibitory on cell cycle progression.

Based on their expression patterns and mutant phenotypes, the Hes1, Hes3, and Hes5 proteins subserve different although overlapping functions within the developing neuroepithelium. In the absence of *Hes1* and *Hes5*, radial glial cells prematurely differentiate into neurons and are depleted without generating later-born cell types. Premature depletion of radial glial cells leads to intermingling of neurons from the left and right walls of the neural tube and disruption of the neural tube structures (**Figures 7(f)–7(h)**, compare with **Figures 7(c)–(e)**), because radial glial cells have the junctional complex and the basal lamina at the apical and basal side, respectively, both of which serve as barriers to prevent neurons from spilling over. Conversely, transient misexpression of *Hes1* or *Hes5* in mouse brain at embryonic day 13.5 inhibits neuronal differentiation and maintains radial glial cells, leading to the increase of later-born neurons and glial cells. In contrast to the peripheral nervous system, where transient Notch activation instructs a glial fate (Schwann cells), transient Notch activation or transient misexpression of *Hes1* or *Hes5* in the central nervous system (CNS) does not seem to instruct gliogenesis. Instead, Hes1 and Hes5 seem to be required to maintain undifferentiated neural progenitors until later stages, when neurogenesis ceases and gliogenesis begins. Thus, *Hes1* and *Hes5* maintain neural progenitors without affecting their temporally changing competency in the CNS. *Hey1* and *Hey2* also have a similar activity: transient misexpression of *Hey1* or *Hey2* does not instruct gliogenesis but promotes maintenance of neural progenitors.

In the absence of *Hes1*, *Hes3*, and *Hes5*, not only radial glia but also neuroepithelial cells are not properly maintained and prematurely differentiated into neurons. However, even in *Hes1*:*Hes3*:*Hes5*

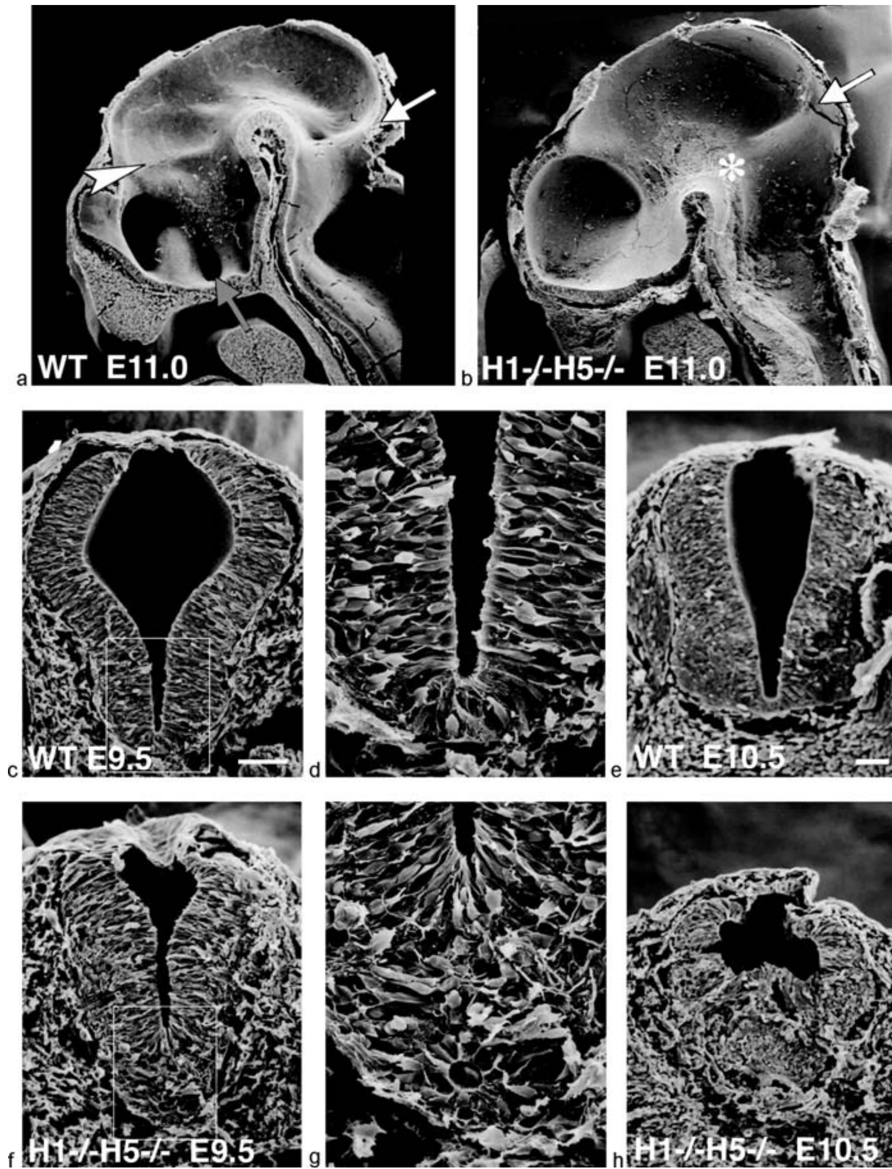

Figure 7 Structural defects of *Hes1*;*Hes5*-double-null mice. Mouse embryos were analyzed by scanning electron microscopy. (a) The optic vesicle (yellow arrow), the zona limitans intrathalamica (zli) (arrowhead), and the isthmus (white arrow) are formed in the wild type. (b) In the absence of *Hes1* and *Hes5*, the optic vesicle and the zli are not formed. The isthmus is only partially formed (asterisk and arrow). (c–e) Cells are aligned radially in the walls of the neural tube in the wild type. (f–h) Cells from the left and right walls are intermingled with each other at E9.5 in the ventral part of *Hes1*;*Hes5*-double-null embryo (f, g). This defect becomes severer at E10.5 (h). Boxed regions in (c) and (f) are enlarged in (d) and (g), respectively. Scale bar = 100 μm.

triple knockout mice, neuroepithelial cells are initially formed, indicating that *Hes* genes are required for maintenance of, but not formation of, neuroepithelial cells. It remains to be determined which factors are responsible for initial formation of neuroepithelial cells. Based on these phenotypes of *Hes*-mutant mice and expression patterns of *Notch* and *Hes* genes, the neuroepithelium develops stepwise in the following way: (1) an initial *Hes*- and *Notch*-independent phase, (2) a subsequent *Hes*-dependent but *Notch*-independent phase, and (3) a *Hes*- and *Notch*-dependent radial glial phase (**Figure 8**). During the second phase, the neuroepithelial cells go through a transitory stage, in which they usually do not give rise to any neurons, but without *Hes* genes, prematurely differentiate into neurons.

Based on the expression patterns, it is likely that *Hes1* and *Hes3* are mainly responsible for transitory neuroepithelial cells, while *Hes1* and *Hes5* are required for radial glial cells. *Hes1* and *Hes5* expression in radial glial cells may be controlled by Notch

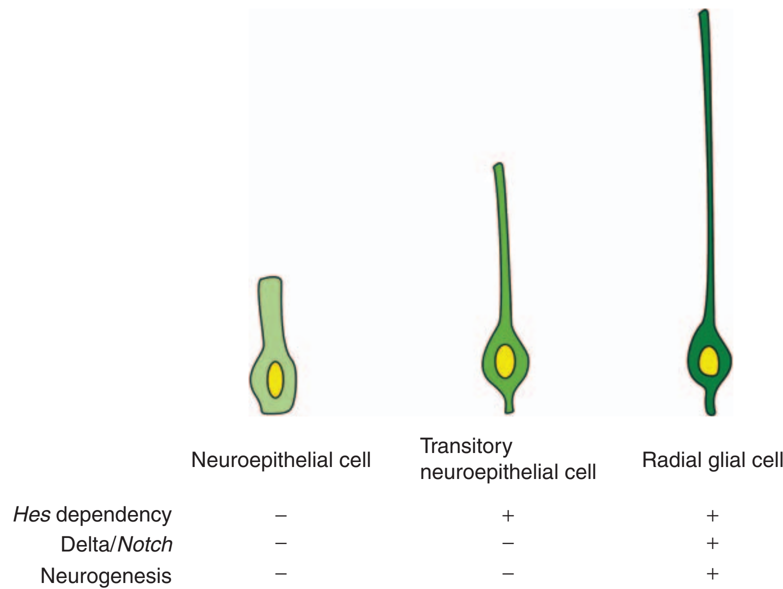

Figure 8 Temporal changes of neural progenitor characteristics. Embryonic neural progenitors change their characteristics as follows: (a) the initial *Hes*- and *Notch*-independent neuroepithelial cells, (b) the subsequent *Hes*-dependent but *Notch*-independent transitory neuroepithelial cells, and (c) *Hes*- and *Notch*-dependent radial glial cells. *Hes*-dependent but *Notch*-independent transitory neuroepithelial cells prematurely differentiate into neurons in the absence of *Hes* genes.

signaling (**Figure 5**), but it remains to be determined which factors regulate *Hes1* and *Hes3* expression in transitory neuroepithelial cells. While transitory neuroepithelial cells usually undergo symmetric cell division and do not produce any neurons, radial glial cells give rise to neurons by asymmetric cell division. Interestingly, during the switch from transitory neuroepithelial cells to radial glial cells, expression of both *Notch* and its ligands begins together, suggesting that neural stem cells undergoing asymmetric cell division express Notch signaling molecules whereas those undergoing symmetric cell division do not. Thus, it is likely that expression of Notch signaling molecules is a key feature of the switch from symmetric to asymmetric cell division.

Eye formation also depends on *Hes1* and *Hes5*. In the absence of *Hes1*, neuronal differentiation is accelerated and retinal neural stem cells are prematurely depleted, resulting in disruption of the neural retina structures. Strikingly, in the absence of *Hes1* and *Hes5*, the optic vesicles are not formed (**Figure 7**(**b**), compare with **Figure 7**(**a**), yellow arrow), although the homeodomain gene *Rax*, which is essential for eye formation, is expressed normally in the prospective eye regions. Normally, no neurogenesis occurs during the optic vesicle formation, but without *Hes1* and *Hes5*, neurogenesis is severely accelerated in the prospective eye regions. Thus, *Hes1* and *Hes5* are required for maintenance of retinal progenitors from the earliest stage of eye development and seem to function together with *Rax*.

Hes Genes Regulate Boundary Formation

The CNS is partitioned into many compartments by boundaries (**Figure 9**), which are formed by specialized neuroepithelial cells or radial glial cells. Boundaries act as organizing centers which function by producing morphogens, thereby patterning nearby compartments and specifying regional aspects of neural differentiation. For example, the isthmus, a midbrain–hindbrain boundary, expresses Fgf8 and regulates neuronal specification in the midbrain and the hindbrain while the zona limitans intrathalamica (zli), a boundary between the dorsal and ventral thalamus, expresses Shh and regulates specification of dorsal and ventral thalamic neurons. Boundary cells restrict cell migration and do not proliferate as efficiently as neuroepithelial and radial glial cells of compartments. In addition, neurogenesis usually does not occur in boundaries. *Hes1* is expressed at high levels in most boundaries, while *Hes3* is expressed in the isthmus. *Hes5* is not expressed in boundaries, but it is ectopically expressed in some boundaries in the absence of *Hes1*.

Hes genes are not only required in neuropithelial cells within the compartments but also for the formation of neuroepithelial cells that comprise the boundaries. In the absence of *Hes1* and *Hes3*, the isthmus is not maintained and differentiated into neurons. As a result, Fgf8 expression is prematurely lost, resulting in defects of midbrain- and hindbrain-specific neuronal differentiation. Similar defects occur in the

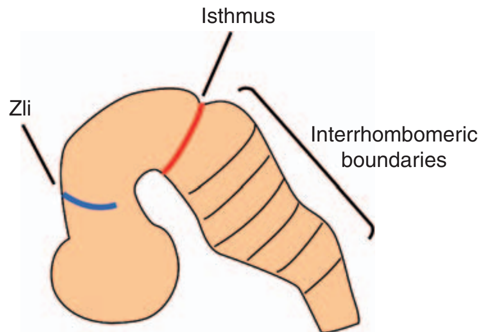

Figure 9 Boundaries in the developing CNS. The developing CNS is partitioned into compartments by boundaries, which function as organizing centers. Hes1 is highly expressed in boundaries and, without *Hes* genes, boundaries are prematurely lost or not formed. zli, zona limitans intrathalamica.

isthmus of *Hes1*;*Hes5*-double-null embryos (**Figure 7(b)**, arrow and asterisk, compare with **Figure 7(a)**, white arrow). In addition, in the absence of *Hes1* and *Hes5*, the zli is not formed (**Figure 7(b)**, compare with **Figure 7(a)**, arrowhead) and Shh expression does not occur. In the absence of *Hes1*, *Hes3*, and *Hes5*, ectopic neurogenesis and loss of the organizer activities occur in all boundaries, resulting in defects of regional-specific neuronal differentiation. *Hes1* is persistently expressed at high levels by many boundary cells (**Figures 6(a)** and **6(c)**, arrow and arrowhead). In contrast, *Hes1* expression is variable in compartments: some cells express Hes1 protein at high levels while others express at lower levels or do not express Hes1 protein. When Hes1 expression is reduced, proneural bHLH factor expression is upregulated, displaying an inverse correlation between Hes1 and proneural bHLH factor expression (**Figure 10(a)**, upper panel). As stated above, Hes1 expression could oscillate in compartmental progenitors (**Figure 10(a)**, lower panel). In boundaries, it is likely that persistent and high levels of Hes1 expression constitutively repress proneural bHLH gene expression and thereby block neurogenesis (**Figure 10(b)**). In agreement with this notion, inactivation of *Hes1*, *Hes3*, and *Hes5* leads to ectopic expression of proneural bHLH genes and premature loss of organizing activities in boundaries. Furthermore, when *Hes1* is misexpressed at persistent and high levels in compartmental neural progenitors, these cells encounter blockade of neurogenesis and reduction of proliferation rates, two important features of boundary cells. Thus, expression mode of Hes1 is different between compartments (variable) and boundaries (persistent) (**Figure 10**), and it is likely that this different expression mode regulates compartment versus boundary cell characteristics. Interestingly, both Hes1 mRNA and protein are highly expressed in boundaries, suggesting that the negative feedback does not work in

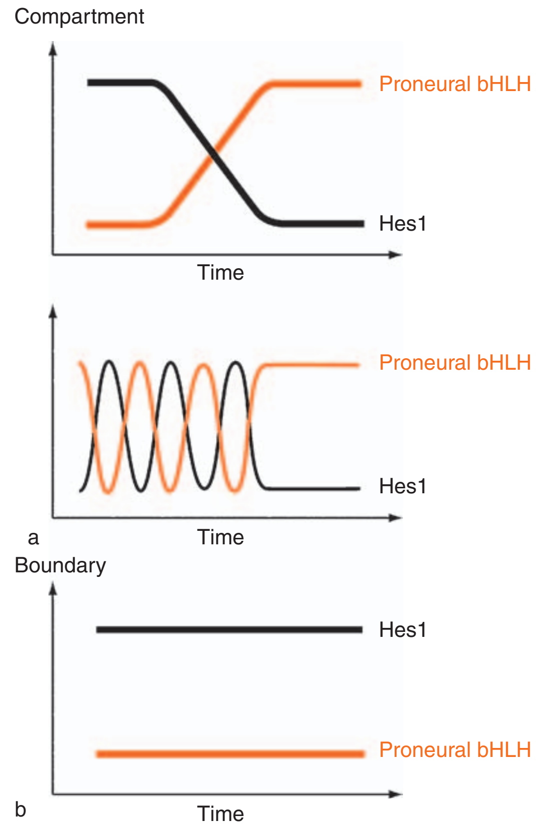

Figure 10 Different Hes1 expression modes in compartments and boundaries. (a) In compartments, Hes1 expression is variable: either gradual downregulation or oscillatory expression. Downregulation of Hes1 expression leads to upregulation of proneural bHLH gene expression. (b) In boundaries, persistent and high levels of Hes1 constitutively repress proneural bHLH gene expression, thereby maintaining neuron-free zones and organizing centers. It is likely that dynamic versus persistent expression of Hes1 regulates compartment versus boundary formation.

boundaries. Further studies are required to demonstrate how this unique expression mode is regulated.

Summary

In sum, *Hes* genes act to inhibit neurogenesis by repressing proneural bHLH genes but do so in different ways along the neuraxes. In the neurogenic compartments, *Hes* gene expression tends to be dynamic, and a loss of *Hes* gene function results in accelerated neuronal differentiation and a premature loss of neural progenitors at the expense of later-born cells. Thus, *Hes* genes are required in neurogenic regions to ensure the generation of differentiated cells in both the correct numbers and full diversity. In regions of the neuroepithelium that form boundaries, *Hes* gene expression persists at high levels and thereby constitutively represses proneural bHLH gene expression. In the absence of *Hes* genes, ectopic neurogenesis occurs in boundaries and their organizing activities are

lost. These different aspects of *Hes* gene function are likely to depend on the different modes by which their expression is regulated (i.e., dynamic versus persistent).

See also: Helix–Loop–Helix (bHLH) Proteins: Proneural; Notch Pathway: Lateral Inhibition; Notch Signal Transduction: Molecular and Cellular Mechanisms; Stem Cells and CNS Repair; Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging.

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Sox Gene Expression

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Introduction

A suite of transcription factor families that are essential for the regulation of gene expression in both the developing central and peripheral nervous systems has been identified. One such group is the SOX family. SOX proteins serve many important functions throughout the development of the nervous system, including the induction of neural competence, the maintenance of progenitor characteristics, and cell fate determination. They were identified based upon their sequence identity to the founding member of the SOX family, the mammalian testis-determining factor SRY (sex-determining region Y). The *SRY* gene contains a single 79-amino-acid DNA-binding motif known as the high mobility group (HMG) domain. SOX family members are classified based upon their sequence similarity to the *SRY* HMG domain, with all members sharing at least 50% homology. SOX proteins are grouped into subfamilies based upon their degree of sequence similarity, with approximately 90% similarity in HMG domains within subfamilies. Currently in vertebrates, there are over 20 individual SOX proteins divided into eight subfamilies (A–H), with some subfamilies further subdivided based upon similarities outside of the HMG domain. A number of these SOX subfamilies are expressed in the developing nervous system (**Table 1**).

SOX factors regulate their downstream targets through multiple mechanisms, including chromatin modification, direct transcriptional activation or repression, posttranslational modification, and protein–protein interactions. All SOX proteins, as well as the related TCF/LEF family of transcription factors, contain a single 'HMG box'; these proteins have been shown to bind the DNA sequence $^{A}/_{T}$ $^{A}/_{T}CAA^{A}/_{T}G$ with high sequence specificity compared to the classical HMG domain proteins HMGB1 and HMGB2, which contain multiple HMG domains but do not bind DNA with any sequence specificity. Generally, SOX proteins bind the minor groove of DNA, which in turn results in an acute bend in the DNA strand, suggesting that SOX proteins are capable of structurally modifying the DNA. However, SOX proteins have also been demonstrated to act as classical transcription factors, with their C-termini capable of acting as either an activator or repressor domain. In addition, posttranslational modification of SOX proteins also plays a role in their regulation of diverse genes. For example, the SUMO-1 (small ubiquitin-like modifier) protein can bind to SOX9 and SOX10 lysine residues in both the activation domain and immediately 5′ of the proposed E1 protein–protein interaction region, potentially modifying the proteins' stability, localization, and protein–protein interactions. These findings have propelled further studies to identify additional mechanisms that can modify SOX signaling.

The diverse functions of SOX proteins, as well as their broad and overlapping temporal and spatial expression patterns, suggest that SOX factors work in conjunction with tissue-specific cofactors to regulate the expression of their target genes. For instance, SOX2, a member of the SOXB1 subfamily, is expressed in multiple cell lineages and, in each tissue type, interacts with unique factors to regulate the expression of downstream targets. In embryonic stem (ES) cells, SOX2 has been shown to directly cooperate with OCT3/4 (octomer-binding factor) to regulate FGF4 (fibroblast growth factor) expression and thus maintain cell pluripotency. Similarly, in neuroepithelial (NEP) cells, SOX2 interacts with another POU factor, BRN2, in the regulation of the *Nestin* gene, a marker for neural stem/progenitor cells. On the other hand, in the developing lens, SOX2 directly interacts with PAX6 to bind and activate the expression of *δ1-Crystallin*. These data demonstrate the importance of identifying and understanding the interactions of SOX members and their binding partners and how they regulate transcription in the context of neural development.

Role of SOX Factors in Defining Neural Competence

Sox genes are expressed from the very onset of embryonic development. One family, the *SoxB*1 group (*Sox1*, *Sox2*, and *Sox3*), is essential for the acquisition of neural competence in primitive ectodermal cells and for their commitment to a neural fate. As mentioned previously, SOX2 is expressed in ES cells and is required to maintain the pluripotency of these cells in conjunction with other transcription factors, such as OCT3/4 and NANOG. During germ layer specification, both SOX2 and SOX3 are expressed in all ectodermal cells, including those that will constitute the prospective neuroectoderm. In the developing mouse embryo, the restriction of SOX2 and SOX3 expression to ectodermal cells committed to a neural fate coincides with the onset of SOX1 expression in these cells. As evidence for an evolutionarily conserved role of *Sox1*, the *Drosophila* orthologs of *Sox2* and *Sox1*, *Dichaete* (or *Fish-hook*) and *Sox-Neuro*, respectively, show similar confined expression

Table 1 SOX factor function in nervous system development

Family		*Member*	*Function*
B	1	1	Maintains neural progenitor characteristics in mouse embryonic stem and P19 cells
			Regulates migration of mouse telencephalic neurons
			Required for differentiation of neurons in mouse ventral striatum
		2	Maintenance of SOX2 expression biases *Xenopus* animal cap cells and mouse embryonic stem cells toward neural fate
			Maintains neural progenitor characteristics in embryonic stem cells, chick. spinal cord, and mouse retinal and rat oligodendrocyte progenitor cells
			Required for neuronal differentiation in anterior thalamus, dorsal striatum, and septum
			Required for maintenance of chick neural crest progenitor identity
		3	Maintains neural progenitor characteristics in chick spinal cord
			Required in a subset of mouse hypothalamic neurons that regulate the hormonal output of the anterior pituitary
	2	14	Promotes neuronal differentiation, cell cycle exit, and delamination of chick neural progenitors
		21	Promotes neuronal differentiation, cell cycle exit, and delamination of chick neural progenitors
C		4	Promotes activation of differentiated neuronal markers in chick embryonic spinal cord
		11	Promotes activation of differentiated neuronal markers in chick embryonic spinal cord
			Regulates neuronal survival and neurite outgrowth of chick neurons
D		5	Represses specification and terminal differentiation and influences mouse oligodendrocyte migration patterns
		6	Represses specification and terminal differentiation and influences mouse oligodendrocyte migration patterns
E		8	Regulates *Xenopus* and chick neural crest cell migration
		9	Required for neural crest induction in *Xenopus*, chick, and mouse embryos
			Promotes chick oligodendrocyte differentiation
		10	Maintenance and survival of neural crest progenitors in *Xenopus* and mouse embryos
			Induction and survival of glial lineages in *Xenopus* and mouse embryos
			Promotes oligodendrocyte differentiation

patterns in the developing fly. Ultimately, all three SOXB1 factors are then expressed in central nervous system (CNS) neural progenitors throughout ontogeny. Functional studies suggest that these proteins are essential for the establishment of the neural lineage, as inhibiting SOXB1 function in *Xenopus* embryos results in a lack of formation and differentiation of neural tissue. Conversely, overexpression of SOXB1 factors in naive ectodermal cells in *Xenopus* initiates neural differentiation in conjunction with FGF. The precise temporal and spatial restriction of the *SoxB1* genes to the neural ectoderm suggests that they are regulated by neural inducing signals. For instance, overexpression of the neural inducer chordin upregulates SOX2, while overexpression of the antagonistic target of chordin, bone morphogenetic protein (BMP), suppresses SOX2 expression in the *Xenopus* embryo. Similarly, Dpp and Sog, the fly counterparts of BMP4 and chordin, regulate the expression of the *Drosophila* ortholog of *SoxB1* genes, *SoxNeuro*. Furthermore, direct evidence for the regulation of SOX2 expression by factors that regulate neural induction stems from a series of elegant studies in the chick embryo showing that FGFs and WNTs can regulate a *Sox2* enhancer element and activate its expression to initiate neural plate development. Despite these findings, the precise roles of SOXB1 proteins in the extensive regulatory network controlling neural competence remain to be further elucidated.

Role of SOX Factors in the Maintenance of Identity and Differentiation of Neural Progenitor Cells of the Central Nervous System

Individual members of the SOX transcription factor family play essential roles not only in the acquisition of neural fate but also in the maintenance and differentiation of neural progenitor cells of the CNS (**Figure 1**). The SOXB1 proteins mark a common transcriptional state shared by diverse populations of neural progenitors throughout the CNS during development and in the adult. The expression of *SoxB1* genes directly correlates first with the commitment of cells to a neural fate. Next, after neural induction, all three genes are co-expressed in proliferating neural progenitor cells along the entire anteroposterior axis of the developing vertebrate CNS and are maintained in neurogenic regions of the postnatal and adult CNS. Furthermore, SOXB1 factors mark proliferating neural progenitors in derivatives of the CNS, including the neural retina, the olfactory epithelium, and the inner ear. The importance of SOXB1 factors in the nervous system has been highlighted both by results from misexpression and dominant interfering studies as well as by genetic analyses. Gain-of-function and dominant interference experiments in *Xenopus* and chick embryos, as well as in

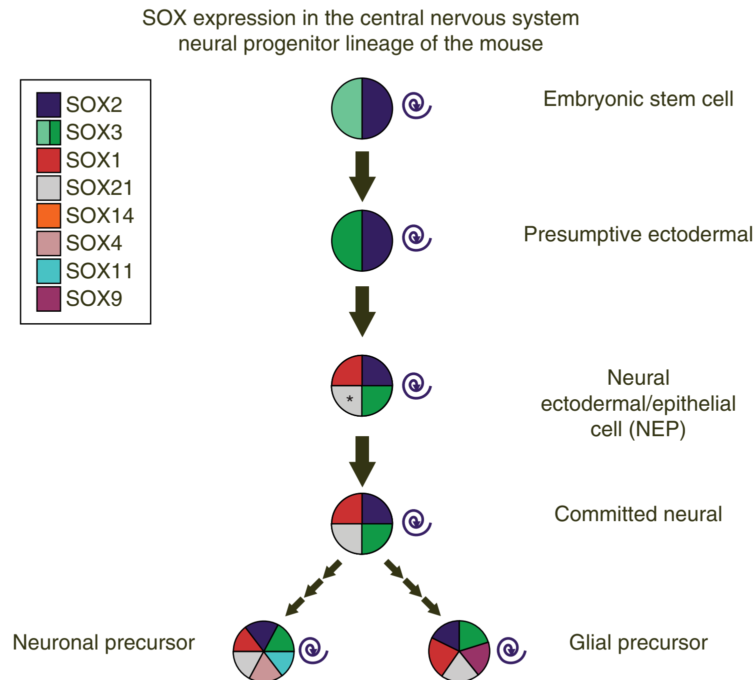

Figure 1 Temporal expression of different SOX proteins in neural progenitor cells of the mouse central nervous system. The asterisk denotes SOX21 expression in the ventricular zone. Light and dark green represent low and high levels of SOX3, respectively; spirals denote proliferative capacity.

mouse cell lines, have shown that SOXB1 signaling plays an essential role in maintenance of neural progenitor identity. These data provide evidence that inhibition of SOXB1 signaling in neural progenitor cells results in their premature delamination from the ventricular zone, their exit from the cell cycle, and the onset of their neuronal differentiation. Conversely, constitutive expression of SOX2 results in the maintenance of progenitor characteristics. These experiments also provide evidence that SOXB1 factors function by antagonizing the actions of proneural genes. Specifically, studies in the chick embryo have shown that the ability of proneural genes to promote neuronal differentiation inversely correlates with the level of SOXB1 expression.

The requirement of SOXB1 factors for the maintenance of neural progenitor identity has been confirmed by genetic studies in a number of species. Analyses of conditional and hypomorphic mutations of *SoxB1* genes in *Drosophila*, zebra fish, mice, and humans have not only verified the absolute requirement of SOXB1 factors but have revealed a dosage-dependent role for them in the maintenance of neural progenitor identity. The identification of hypomorphic mutations and the generation of compound mutations in the *Drosophila Dichaete* and *SoxNeuro* genes, as well as the generation of conditional, hypomorphic, and compound mutations in mouse *SoxB1* genes, permit the assessment of the function of SOXB1 factors in the CNS. In the fly, *SoxNeuro/Dichaete* double-mutant embryos show severe neural hypoplasia throughout the CNS, as well as dramatic loss of Achaete-expressing proneural clusters. These data suggest that members of the *Drosophila SoxB1* subfamily act upstream and in parallel to genes of the *Achaete–Scute* complex. Furthermore, analysis of a *Sox2* hypomorphic allele (Dr11) and directed transgene expression has demonstrated that Dichaete function is necessary for the correct development of midline glia of the CNS. In the mouse, analysis of the tissue-specific conditional ablation of SOX2 in neural progenitor cells, dependent on the Cre–loxP system, has provided the first genetic evidence for the requirement for SOX2 in the maintenance of neural progenitor cell identity. Specifically, retinal progenitor cells with conditionally ablated *Sox2* lose competence to both proliferate and terminally differentiate. In contrast, in *Sox2* hypomorphic/null mice, a reduction of SOX2 expression causes variable microphthalmia as a result of aberrant neural progenitor differentiation. In *Sox2* hypomorphic mutant retinas the decrease in levels of SOX2 expression directly correlates with a decrease in the levels of Notch1 and its direct downstream effector, HES-5, expression. Moreover, and consistent with the observations made in the fly embryo, in mouse neural retinal progenitor cells that express decreased levels of SOX2 due to germ line *Sox2* hypomorphic mutations, the expression of proneural genes such as *Atoh7* (*Math5*) and *Neurod1* is prematurely upregulated. It is important to note that although SOXB1

factors are generally restricted to and are clearly essential for the maintenance of neural progenitor cell identity, their expression has been detected in small subsets of postmitotic neurons in the developing and adult CNS. For example, it has been shown that SOX1 is not only expressed in, but also functions to regulate, the migration of telencephalic neurons of the ventral striatum, and deletion of SOX3 in mice leads to defects in pituitary function and abnormal development of Rathke's pouch.

Unlike the members of the SOXB1 subfamily, for which expression is generally downregulated as neural progenitor cells exit the cell cycle, expression of members of the SOXB2 subfamily, including SOX14 and SOX21, and members of the SOXC subfamily, SOX4 and SOX11, is maintained in postmitotic neurons. Consistent with this, these factors have been shown to play a crucial role to promote neuronal differentiation. Misexpression of either SOX14 or SOX21 in chick neural progenitor cells results in their premature exit from cell cycle, delamination to the differentiated mantle zone, and the onset of expression of markers of mature postmitotic neurons. In contrast, SOX4 and SOX11 function further downstream of proneural basic helix–loop–helix (bHLH) proteins and are essential for the establishment of panneuronal protein expression. Misexpression of SOXC factors results in the aberrant expression of markers of differentiated neurons but does not cause progenitor cells to exit the cell cycle or commit to neuronal differentiation.

SOX factor expression and function is maintained in restricted progenitor cells of both the neuronal and glial lineages. For example, it has been shown that members of the SOXD (SOX5 and SOX6) and SOXE (SOX8, SOX9, and SOX10) families are expressed in the oligodendrocyte lineage. Interestingly, and analogous to the antagonistic function of SOXB1 and SOXB2 factors during neuronal differentiation, both genetic and molecular data suggest that SOX5 and SOX6 directly interfere with the functions of group E SOX proteins. In contrast to SOX9 and SOX10, which promote oligodendrocyte specification and terminal differentiation, SOX5 and SOX6 repress the specification and terminal differentiation of oligodendrocytes and influence their migration.

Role of SOX Factors in the Maintenance and Differentiation of Neural Progenitor Cells of the Peripheral Nervous System

The peripheral nervous system (PNS) is derived from neural crest cells, a subset of dorsal neuroepithelial cells. Similar to the initial specification of neural fate during neural induction, neural crest cell fate is also defined by the functions of SOX family members. Human neuropathies, as well as spontaneous mutations found in the mouse, have provided evidence that one SOX family, SOXE, plays a pivotal role in the PNS. In addition, group C members SOX4 and SOX11, group D member SOX5, and the group B1 member SOX2 are also found expressed transiently during neural crest development (**Figure 2**).

All three SOXE members (SOX8, SOX9, and SOX10) are initially expressed in the developing neural crest in *Xenopus*, chick, and mouse, although the relative timing of expression onset and the expression level of each SOXE protein is species specific. Generally, as development proceeds, SOX9 is maintained in migrating cranial neural crest progenitors (which later form cranial neurons and glia), while SOX10 is maintained in trunk neural crest progenitors (which give rise to the PNS). SOX8 is maintained in both cell populations.

As mentioned previously, SOX10 is expressed in trunk neural crest progenitor cells that give rise to the PNS. Upon differentiation, SOX10 is maintained in all cells fated for the glial lineage but lost in all neuronal derivatives. Initially, SOX10 does not appear to be necessary for neural crest specification or migration, as SOX10 knockout mice are capable of generating some migrating neural crest cells. However, SOX10 does appear essential for the survival of the neural crest cells, as postmigratory neural crest cells undergo apoptosis in these mice. This, in addition to knockin experiments in which SOX10 was replaced with SOX8, demonstrates the inability of SOX8 to compensate for SOX10 loss. Interestingly, SOX10 morpholino knockdown in *Xenopus* blocks the expression of early neural crest makers SNAIL2 (SLUG), SOX9, and FOXD3. However, given that SOX10 expression succeeds that of these markers, it is suggested that SOX10 maintains the expression of these genes in progenitors.

In addition to regulating the survival of neural crest cells, SOX10 has also been demonstrated to maintain the pluripotency of these cells and to prevent premature neuronal differentiation. Interestingly, SOX10 is also necessary for the establishment of the neuronal lineage, as demonstrated by its functional requirement for the induction of the neurogenic transcription factors MASH1 and PHOX2B in the mouse, as well as Ngn1 in *Xenopus*. Therefore, how can the loss of SOX10 be required for neuronal differentiation, yet its expression is necessary for neurogenesis? This discrepancy appears to be dependent upon the level of SOX10 expression. In mice with a single functional *Sox10* allele, the maintenance of neurogenic potential is unaltered, as evidenced by the maintenance of MASH1 and PHOX2B expression. However, in these same

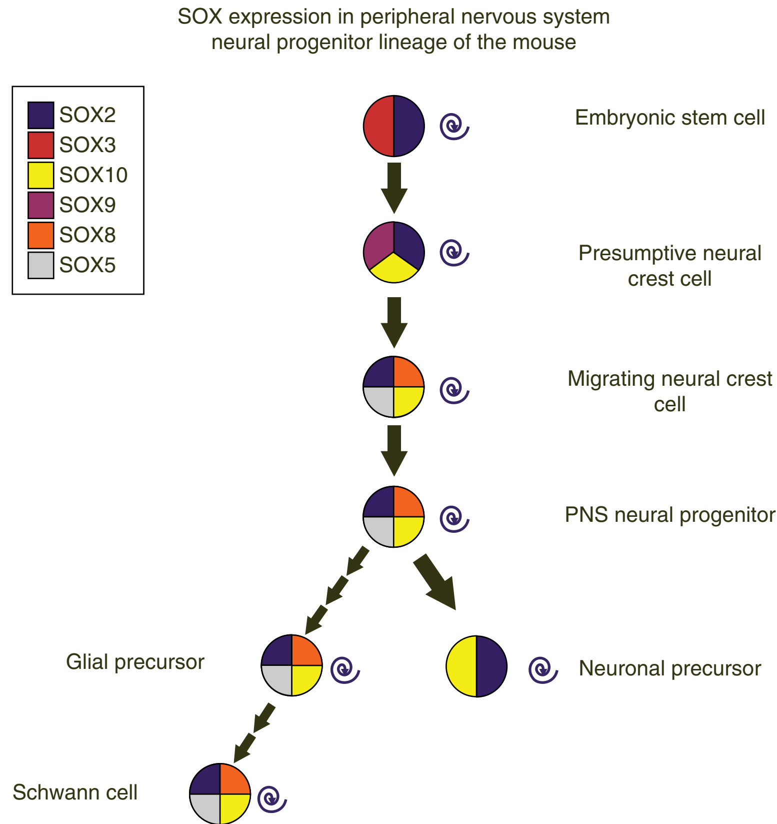

Figure 2 Temporal expression of different SOX proteins in neural progenitor cells of the mouse peripheral nervous system. Expression of SOX proteins in nonneural lineages is not shown; spirals denote proliferative capacity.

animals, the ability to inhibit neuronal differentiation is decreased, as observed by a reduction in the expression of PHOX2A, a marker for autonomic neuronal differentiation. This suggests that SOX10 is downregulated in cells that begin to differentiate but is maintained at sufficient levels to drive them toward a neuronal fate.

The maintenance of SOX10 in all glial cells of the PNS is important for glial fate, and its loss results in complete ablation of all peripheral glia. Molecularly, it has been shown that SOX10 can stimulate gliogenesis by regulating ERBB3 expression and neuregulin signaling. Furthermore, similar to its function in CNS oligodendrocytes, SOX10 also regulates myelination in PNS Schwann cells by controlling myelin protein-zero expression through interactions with the EGR/KROX20 transcription factor. Connexin-32 and ciliary neurotrophic factor (CNTF) are also regulated by SOX10, the latter being important for neuronal maintenance.

Consistent with other vertebrate species, SOX10 is required in human neural crest, and mutations in SOX10 are found in some human neuropathies. For instance, SOX10 mutations result in Waardenburg–Shah syndrome, which is characterized by skin hypopigmentation, deafness, and Hirschprung's disease (aganglionic megacolon), in addition to severe dysmyelination. Mice with little or no SOX10 expression present with similar phenotypes. Additionally, Charcot–Marie–Tooth syndrome may also involve SOX10 mutations.

SOX9, on the other hand, has been shown to be important for neural crest specification, though again species-specific differences are observed. For instance, early knockdown of SOX9 in *Xenopus* and zebra fish results in a lack of induction of neural crest markers such as FOXD3, whereas SOX9 knockout mice maintain normal FOXD3 expression. In addition, ectopic expression of SOX9 in the chick neural tube can activate expression of neural crest markers in the neuroepithelium, though these cells are unable to delaminate and migrate. These results suggest that SOX9 is involved in neural crest specification, but its contributions to this process may vary by species. This is supported by recent studies that have shown that SOX9 is able to bind and activate SNAIL2, a transcription factor necessary for the epithelial–mesenchymal transition and migration of neural crest cells, as well as by the identification of a neural crest specific enhancer for SOX9 that is dependent upon Wnt–β-catenin signaling. Currently, the

role of SOX9 in the survival of neural crest cells is still unclear. Conditional ablation of SOX9 specific to neural crest cells using Wnt1/Cre-expressing mice results in cranial neural crest defects but no observable abnormalities in trunk neural crest. This suggests that either SOX9 or SOX10 (which is expressed in trunk neural crest) is necessary to maintain neural crest survival.

In the mouse, SOX8 appears to serve a minimal role in neural crest development; SOX8 knockout mice do not exhibit any neural crest phenotype, potentially due to compensation by SOX9/SOX10 expression. However, in *Xenopus*, morpholino-mediated knockdown of SOX8 results in defects in proper neural crest migration. Therefore, the relative importance of SOX8 in the developing PNS remains to be determined.

SOXB1 proteins also appear to be involved in the establishment of the PNS, though to what extent remains unclear. The most studied of these proteins is SOX2. In the chick, SOX2 is downregulated, but not extinguished, in cells as they become biased toward the neural crest fate. This low level of expression is maintained in migrating neural crest cells, but upon reaching their targets SOX2 is upregulated in neural precursors in the developing dorsal root ganglia and is ultimately maintained in some glial lineages. Furthermore, neural populations of neural crest cells contain SOX2-specific enhancer activity, and SOX2 was demonstrated to inhibit neural crest formation and to regulate proliferation and differentiation in the PNS neural progenitors. Thus, SOX2 appears to play a similar neurogenic role in both the PNS and the CNS.

The functions of other SOX factors in PNS development are still unclear. Ectopic expression of SOX5 in the chick neural tube has been demonstrated to induce the expression of many neural crest transcription factors. Also, SOX11 has been shown to function in the regulation of neuronal survival and neurite outgrowth in both neural cell lines and primary dorsal root ganglia neuron cultures. Clearly, however, our understanding of the multiple functions of SOX proteins in PNS development is far from exhaustive. The upstream and downstream targets of individual SOX members are only beginning to be elucidated, as are the numerous cofactors with which SOX proteins interact. Only by identifying these mechanisms will the role of SOX proteins in the regulatory network controlling PNS development be understood.

Conclusion

The SOX family of transcription factors is essential for the establishment, development, and maintenance of the nervous system. Though highly conserved, each member appears to serve a distinct function, depending upon the tissue it is expressed in, the level of its expression, and the cofactors that are co-expressed in the cell. The mechanisms by which SOX proteins act are equally diverse, as are the downstream targets regulated by SOX function. These highlight the importance of SOX proteins in neural development and, in conjunction with *Sox* mutations associated with human neuropathies, demonstrate the necessity for further understanding of their functions.

See also: Neural Crest; Neural Crest Cell Diversification and Specification: ErbB Role; Neural Crest Cell Diversification and Specification: Melanocytes; Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation.

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Transcriptional Networks and the Spinal Cord

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Introduction

One of the challenges facing the developing embryonic nervous system is how to ensure the production of multiple neuronal subtypes at the correct time, place, and number during development. One of the areas of the central nervous system (CNS) where the mechanisms for regulating cell fate have been extensively studied is the spinal cord. The spinal cord is embryologically derived from dividing multipotential neuroepithelial cells comprising the neural tube at early stages of development. Over the past two decades, there has been tremendous progress in our understanding of the signaling pathways and transcription factors that instruct neuroepithelial cells to differentiate into functionally specialized subpopulations of postmitotic neurons and glia. Spinal neuronal differentiation signals are distributed along the dorsoventral (D/V) and rostrocaudal (R/C) axes, leading to the generation of specific cell types at stereotypical positions along these axes.

D/V Patterning of the Spinal Cord

Interneurons that process and relay sensory signals are primarily restricted to the dorsal region of the spinal cord, whereas motor neurons and the interneurons that regulate their activity are concentrated ventrally. A key signaling molecule working to pattern the ventral spinal cord is sonic hedgehog (shh), a factor that is used at multiple sites within the embryo to control cell specification and growth. Shh is a glycoprotein secreted from notochord cells located underneath the neural tube. Later in development, shh is also produced by the floor plate, a glial wedge that forms at the ventral midline of the neural tube. A critical feature of shh's action on neural tube progenitor cells is its ability to function as a morphogen, namely to signal the differentiation of different cell types in a concentration-dependent manner. Within the neural tube, ventrally located progenitor cells closest to the floor plate and notochord encounter high levels of shh whereas cells located more dorsally are exposed to lower levels of the factor (**Figures 1(a)** and **1(b)**). Thus, the position of a cell within the ventral neural tube dictates the level of shh it will encounter. Cells exposed to different concentrations of shh activate distinct differentiation programs, express unique genes, and ultimately acquire different cell fates (**Figure 1(a)**).

Shh signaling controls cell specification in the ventral spinal cord by establishing molecularly distinct classes of precursor cell populations, also referred to as progenitor cell domains. Each of the separate precursor populations established by shh signaling expresses a unique combination of homeodomain-class transcription factors that control gene expression (**Figure 1(b)**). This group of so-called progenitor factors can be divided into two classes based on how they are regulated by shh; class I factors are expressed by default and become repressed by shh, whereas expression of class II factors is activated by shh. The ventral spinal cord is divided into five separate progenitor cell domains referred to as p0, p1, p2, pMN, and p3 that serve as the precursor cell populations for V0–V2 interneurons, motor neurons, and V3 cells, respectively (**Figure 1(a)**). Each of the postmitotic neuronal progeny that form from the progenitor domains exhibit different phenotypic characteristics, including unique cell migration patterns, axon targeting, and in the cases examined distinct physiological properties.

Insight from studies on ventral cell patterning has helped us to understand some of the general principles that operate to control dorsal interneuron specification in the neural tube. The precursors of dorsal sensory classes of interneurons (dI) are similarly divided into progenitor cell domains (dp1–6) that represent the precursors for six early forming (dI1–6) and two late developing (dIL^A and dIL^B) neuronal subtypes (**Figure 1(a)**). These interneuronal cell types can be broadly divided into two groups based on the signals required for their specification. Class A cells, consisting of dorsal interneurons dI1–3, are dependent on secreted factors from the roof plate. In contrast, class B neurons (dI4–6 and dIL^A and dIL^B) are able to differentiate even in the absence of roof plate-derived signals. The roof plate secretes at least two different types of signaling molecules that contribute to class A cell specification. One group of related proteins are members of the transforming growth factor-β (TGF-β) family and include activin, bone morphogenic protein 4 (BMP4), BMP5, BMP7, dorsalin1, and growth differentiation factor 7 (GDF7). Another family of signaling proteins expressed by roof plate cells are the wingless related factors Wnt1 and Wnt3A. Numerous genetic experiments have clearly implicated TGF-βs in dorsal interneuron specification. For example, overexpression of BMPs in the spinal cord increases the

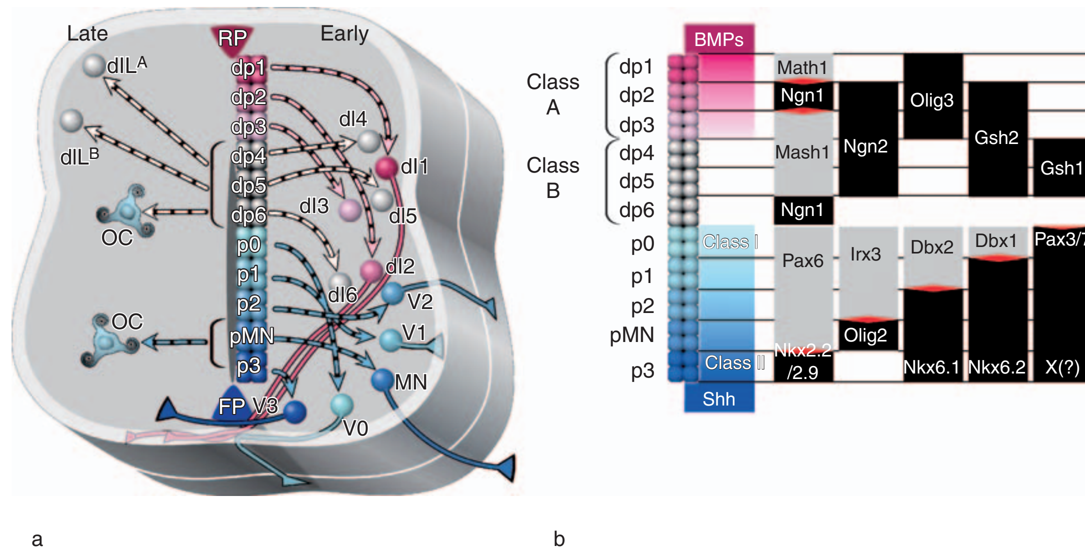

Figure 1 Progenitor domains give rise to distinct cell types. (a) The ventricular zone is divided into progenitor domains based on the differential expression of specific genes. The ventral spinal cord is divided into five progenitor domains (p0–p3 and pMN) that give rise to distinct classes of mature neurons (V0–V3 and MN) that migrate to stereotypical locations and extend axons along specific pathways. Later in development, pOC domains form that gives rise to oligodendrocytes (OC) that disperse throughout the spinal cord. In the dorsal spinal cord, six progenitor domains (dp1–6) give rise to six early-born (dI1–6) and two late-born (dIL^A and dIL^B) classes of interneurons which migrate laterally to stereotypic positions. Where known, the axonal projections are indicated. RP, roof plate; FP, floor plate. (b) In the ventral spinal cord, gene expression patterns are established by a low dorsal to high ventral concentration of shh. Progenitor transcription factors fall into two classes: class II factors (black bars) that are induced by shh and class I factors (gray bars) that are repressed by shh. The shh gradient thus leads to cells expressing a unique combination of progenitor factors. In addition to the graded responses to shh, progenitor factors exist in cross-repressive pairs that are incompatible with co-expression in the same cell, causing discrete boundaries between progenitor domains to form. In the dorsal spinal cord, progenitor domains are classified into two subtypes, the roof plate-dependent class A (dI1–3) and roof plate-independent class B (dI4–6 and dIL$^{A/B}$) neurons. bHLH factors have prominent roles for patterning the progenitor domains in the dorsal spinal cord, whereas homeodomain factors are more common ventrally. Similar to the ventral spinal cord, cross-repression (between Mash1, Ngn1, and Math1) is an important mechanism delineating progenitor boundaries. Other progenitor factors are also shown.

number of dI1 progenitors and interneurons at the expense of other interneurons, whereas inhibition of BMP signaling by expression of the BMP antagonist noggin or inactivation of both BMPr1a and BMPr1b receptors results in reduced numbers of dI1 and dI2 interneurons. Interestingly, dI3 interneurons are still generated in these BMP receptor, mutants, consistent with recent findings suggesting that activated activin receptors promote dI3 interneuron fate independently of BMP signaling. The wnts appear to have complementary functions to those of the TGF-β family, as *Wnt1*/*Wnt3A* double-mutant mice also exhibit defects in dI1–3 interneuron specification.

Negative Gene Regulation: Cross-Repression and Derepression

One of the key features establishing D/V identity in the neural tube is the proper interpretation of the graded shh signal into decisive differentiation programs for cell fate specification, even under conditions when cells reside at the signaling threshold for two different fates. The strategy used by progenitor cells appears to be based on the genetic interactions that take place between the numerous downstream transcription factors that become expressed in progenitor cells. Transcription factors expressed by adjacent progenitor cell populations have been found to repress one another's expression, a process referred to as cross-repression. Class I factors repressed by shh oppose the expression of class II factors induced by shh in adjacent cells, and similarly class II factors repress adjacent class I factors (**Figure 1(b)**). Thus, mutations in one factor of a cross-repressive pair leads to an expansion of the opposing factor into an inappropriate cell domain. This alteration results in misspecification of neuronal cell fate from the altered precursor cells in the progenitor domain. The cross-repressive interactions between progenitor cell transcription factors likely help to ensure that cells do not acquire mixed or hybrid characteristics even at the progenitor boundaries.

In the dorsal spinal cord, cross-repressive interactions between basic helix–loop–helix (bHLH) class transcription factors such as Math1, Ngn1, and Mash

1 delineate progenitor domains in a manner that is analogous to the homeodomain factors in the ventral spinal cord (**Figure 1(b)**). Similarly, studies of mouse mutants and gain-of-function experiments in chick embryos have established key roles for Ngn1, Math1, and Mash1 in the specification of dorsal neuron fate. Additional bHLH and homeodomain transcription factors such as Olig3, Gsh1, and Gsh2 are also expressed by dorsal progenitor cells, but do not appear to participate in cross-repression. Nevertheless, genetic experiments have established a role for Olig3 in dI1–3 cell specification and Gsh2 in dI3 interneuron development (**Figure 1(b)**).

It is likely that the chromatin modifications directed by the progenitor factors have a central role controlling cell fate specification in the developing spinal cord. The groucho/TLE cofactor physically interacts with many of the progenitor cell transcription factors and is essential for cross-repression. Eight of the 11 known transcription factors expressed by ventrally located progenitor cells have an Engrailed homology (eh1) domain that binds to groucho/TLE corepressors. The recruitment of groucho/TLE proteins to DNA inhibits gene expression by recruiting the histone deacetylase Rpd3/HDAC1. This enzyme catalyzes the removal of acetyl groups on histone tails, creating a chromatin structure that is less favorable for transcription. Thus, it is predicted that progenitor factors cross-repress the expression of neighboring progenitor factors by creating a multiprotein enzymatic complex that limits the accessibility of RNA polymerase and other factors needed for transcription.

The finding that progenitor factors are transcriptional repressors helps to explain how they prevent genes from adjacent cell domains from becoming expressed, but it raises further questions about how cells acquire unique identities using gene silencing as a mechanism. Although there is precedence for bifunctional transcription factors that can activate or repress gene expression based on their context, the cell fate specifying activity of the progenitor factors appears to be mediated entirely through transcriptional repression. Fusion of just the DNA-binding homeodomain of the progenitor factors to the eh1 groucho/TLE interaction domain of Engrailed generates constitutive repressors that are found to mimic the activity of the native progenitor factors. Because progenitor cell fate is regulated by gene silencing, it is thought that 'derepression' is important for proper expression of genes. The derepression model predicts that progenitor factors function by preventing the expression of inappropriate genes for alternative cell fates rather than activating the expression of genes for the development of a specific cell type. For this derepression model to work, however, it is thought that general (noncell type-specific) transcriptional activators are poised to activate genes for spinal neuron differentiation unless prevented from doing so by the recruitment of histone deactylases by progenitor factors. Expression of effector genes for a specific cell identity would thus be determined by the absence of binding sites for a particular progenitor factor.

Cell Specification Factors Downstream of Progenitor Factors

Although molecular differences are apparent in the profile of transcription factors expressed by progenitor cells within the ventricular zone of the spinal cord, the process of cell fate specification is best described as a sequential cascade in which progenitor factors control the expression of a second layer of genes that play active roles in regulating the phenotypic characteristics of the differentiating neuronal subtypes. Thus, many of the progenitor cell transcription factors are only expressed by cells within the ventricular zone and become downregulated as neuronal differentiation proceeds. Many of the downstream factors that become expressed in differentiating neurons represent additional homeodomain transcription factors. For example, V0 cells express Evx1, V1 cells express EN1, V2 cells express Chx10, MN cells express Hb9 and Isl1/2, and V3 cells express Sim1.

Gain- and loss-of-function experiments in mouse and chick embryos have helped to define the role of many of the downstream homeodomain transcription factors involved in specifying the phenotype of cells within the spinal cord. Some factors are essential for establishing the complete identity of particular cells, whereas other factors appear to regulate the expression of a subset of the phenotypic characteristics. In *Evx1* mutant mice, for example, V0 commissural interneurons extend axons ipsilaterally as if they have acquired the identity of V1 cells. Mutations in *En1* cause V1 neuron axon pathfinding defects and a slowing in the activity of the spinal locomotor circuitry involved in rhythmic stepping. The loss of En1 function, however, does not appear to be associated with a wholesale conversion in the identity of V1 cells to that of another cell type in the ventral spinal cord. Mutation of the *Isl1* gene expressed by motor neurons results in a block in motor neuron differentiation. As a consequence, the progeny of pMN cells abruptly apoptose in *Isl1* mutants rather than develop with an alternative cell identity. The loss of Hb9 function from motor neurons, in contrast, has a more subtle phenotype in which motor neurons still develop but they inappropriately express V2 interneuron genes. The hybrid motor neuron/V2 interneuron cells that

form in *Hb9* mutants are unable to extend axons to proper muscle targets in the periphery and as a result the animals exhibit severe locomotor defects.

Neurogenesis

Although progenitor cells exposed to roof plate- and floor plate-derived signals become predisposed to produce specific cell progeny, there is also an ancient evolutionarily conserved regulatory pathway that is superimposed on the process of cell specification which controls whether precursor cells continue to divide or begin their terminal differentiation. Gene expression for this regulatory check point termed neurogenesis is controlled by bHLH transcription factors. This family of proteins is defined by the bHLH signature motif consisting of 60 amino acids. The N-terminal 15 amino acids encompass the basic domain that binds to DNA. The remaining C-terminal domain forms a helix–loop–helix that functions as a dimerization domain that mediates the formation of homo- and heteromeric complexes. bHLH dimers bind to a DNA sequence called the E box (CANNTG) found in many neuronal genes. As noted above, the dorsal spinal cord expresses several bHLH transcription factors within the dI progenitor cells, and these factors exhibit cross-repressive interactions, cell fate-specifying attributes, and drive neurogenesis. Thus, a remaining question is to understand how each of these processes is regulated in a context-dependent manner.

The function of proneural bHLH factors is controlled at both the transcriptional and posttranscriptional level. Id-family proteins bind directly to proneural bHLH factors such as Mash1, Ngns, and NeuroD and generate non-DNA binding heterodimers. This inactivation occurs because Id proteins contain helix–loop–helix domains for dimerization but lack the basic region required for DNA binding. In addition, the transcription of proneural bHLH factors is negatively regulated by the Hes1 transcriptional repressor. Thus, in order for neurogenesis to proceed, Hes and Id must be downregulated and/or inactivated in progenitor cells to allow assembly of functional proneural bHLH complexes.

Once neurogenesis is initiated, the differentiation of neurons appears to involve the sequential activity of several bHLH factors that encompass the period when progenitor cells cease dividing, emerge from the ventricular zone as a postmitotic neuron, and mature into a specialized neuronal subtype. Developmental studies suggest that the proneural bHLH factors expressed transiently just prior to neuronal differentiation, including the Ngn family, Mash1, and Math1, initiate neurogenesis programs, while members of the NeuroD/Nex family are involved in the terminal differentiation and maturation of the cells. For example, Ngn2 overexpression decreases the proportion of proliferating progenitors and induces the expression of cell division kinase (CDK) inhibitors which stop the cell cycle, whereas NeuroD expression is more prominent in postmitotic neurons and coincides with their expression of pan-neuronal genes such as neurofilament.

Precursor cells are found to produce neurons at early stages and later give rise to glial cell derivatives. Thus, the check points that control neurogenesis have an important influence on the differentiation of cells within the spinal cord. Oligodendrocytes are produced from the pMN and dp4–5 progenitor domains, whereas astrocytes are generated from the other progenitor cell populations. The differentiation of oligodendrocytes from the pMN domain is initiated when Ngn2 expression is lost from the Olig2-expressing cells. The mechanistic basis for Ngn2's suppression of glial fate may be based on a process of transcription cofactor 'squelching.' This is predicted from the finding that the related factor, Ngn1, inhibits astrocyte formation by sequestering CREB binding protein (CBP), which is an obligate cofactor whose availability is needed in order to efficiently transcribe astrocyte genes.

LIM Networks in Postmitotic Neurons

Many different types of neurons within the spinal cord express LIM homeodomain (LIM-HD) transcription factors as they emerge from the ventricular zone and begin to differentiate. There are 12 LIM-HD genes in higher vertebrates, most of which are matched with homologs in invertebrates that have been shown to control cell fate specification. The regulation of LIM-HD expression in developing neurons remains poorly understood, but these factors are clearly downstream of the progenitor cell factors. Thus, the prevailing model of the transcriptional networks that operate in the neural tube posits that extrinsic factors such as shh and TGFβs regulate progenitor cell factors which in turn regulate the expression of LIM-HD genes, resulting in a sequential cascade of regulatory factors being expressed as development progresses.

A hallmark of the LIM-HD factors is the zinc-binding LIM domain found in their N-terminus. The LIM motif was first identified as a conserved structure in two *Caenorhabditis elegans* genes, *lin-11* and *mec-3*, and in the insulin enhancer binding protein *Isl1*. The LIM domain mediates protein–protein interactions with the cofactor nuclear LIM interactor (NLI). NLI is present in many cell types and possesses its own self-dimerization domain which allows NLI to drive the formation of complexes containing multiple LIM-HD proteins.

A critical finding regarding the LIM-HD factors was that they function in a combinatorial fashion to control cell fate. Although the expression of a single LIM-HD factor does not segregate with neuronal subtypes, the combinatorial expression of LIM-HD factors has been used to define distinct subtypes of neurons. Thus, individual members of the LIM-HD gene family are typically expressed by several neuronal subtypes within the spinal cord, but each neuronal subtype is often associated with a unique mix of LIM-HD proteins, referred to as the LIM code. During neuronal differentiation, LIM-HD factors appear to control several aspects of cell fate in a sequential manner. For example, as motor neurons develop from pMN progenitors, they express LIM-HD factors Isl1 and Lhx3 which specify a generic motor neuron phenotype. This early motor neuron LIM code is transient, however, and is soon followed by new combinations of LIM-HD factors that drive the further diversification of motor neuron identity leading to the formation of motor columns – a subdivision of motor neurons that corresponds to cells that settle together within the spinal cord and extend axons along common pathways in the periphery (**Figures 2(a)** and **2(c)**).

The advantage of a LIM code is that diversity can be generated by mixing and matching a small number of elements; however, a by-product of a combinatorial code is that the elements are reused repeatedly. For example, Lhx3 alone contributes to V2 interneuron development, but Lhx3 combined with Isl1 triggers motor neuron formation. How do LIM-HD factors function in a combinatorial manner to control gene expression? The solution appears to be based on cell-specific protein–protein interactions mediated by the LIM domains. Within V2 interneurons, an Lhx3:NLI complex forms that regulates distinct target genes from the Lhx3:NLI:Isl1 complex that triggers motor neuron development. This example illustrates that the LIM domain is a critical determinant for controlling how LIM-HD factors regulate different cell fates in a combinatorial manner.

R/C patterning, Hox Genes, and Motor Pools

There are distinct classes of neurons that align along the R/C axis of the spinal cord in approximate coordination with the peripheral targets they innervate, ensuring for example that limb-innervating motor neurons are generated in register with the limbs. Motor columns form one of the basic units of motor neuron organization in the spinal cord, with distinct columnar subtypes innervating specific muscle targets. Lateral motor columns (LMCs), for example, are generated at fore- and hindlimb levels and innervate limb musculature, whereas autonomic motor neurons in the preganglionic motor column (PMC) form at thoracic levels (**Figures 2(a)** and **2(c)**). Within motor columns,

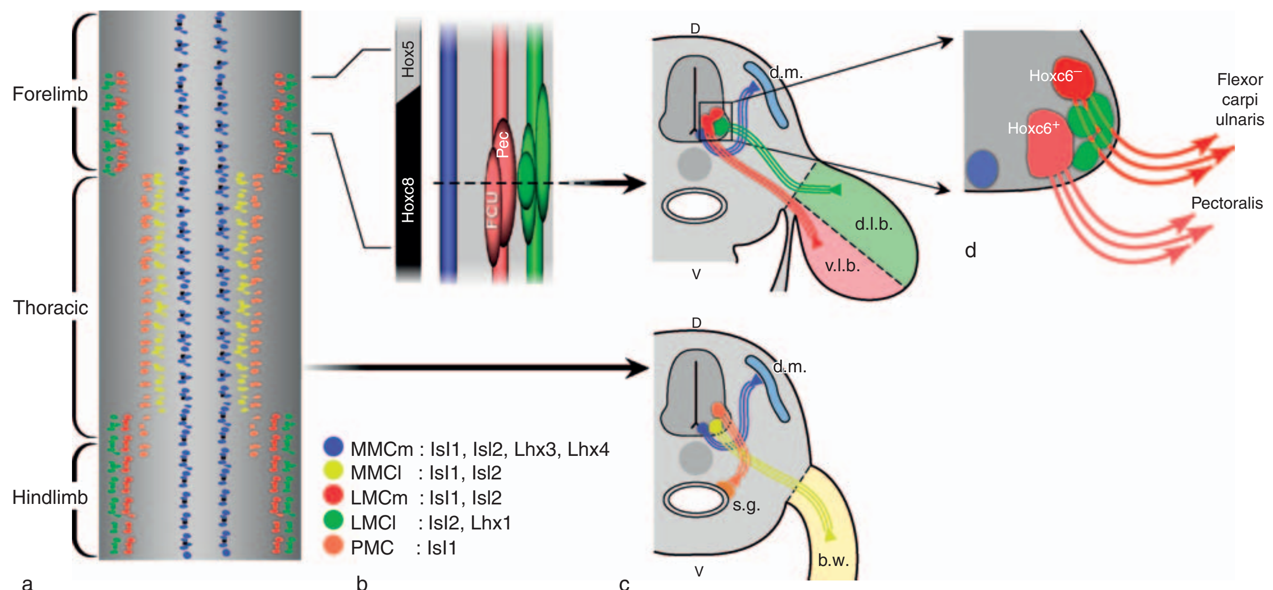

Figure 2 The LIM and Hox codes define columnar and pool identity of motor neurons. (a) Schematic representation of the spinal cord showing the approximate location of the motor columns marked by unique combinations of LIM-HD factors (color coded). (b) An expanded view of the brachial spinal cord showing the relative position of several motor pools. The position of motor pools innervating the pectoralis (Pec) and flexor carpi ulnaris (FCU) muscles are determined by Hox5 (Hoxa5 + Hoxc5) and Hoxc8 expression. (c) Transverse sections of the brachial and thoracic spinal cord are diagramed showing axonal projections to muscle targets. d.m., dermamyotome; d.l.b., dorsal limb bud; v.l.b., ventral limb bud; s.g., sympathetic ganglia; b.w., body wall. (d) Within a motor column (in this case the LMC, red), motor pools emerge that connect to specific muscle targets. The intrasegmental distinction between FCU and Pec cells depends on the status of Hoxc6 in combination with other transcription factors.

motor neurons can be further subdivided into motor pools representing cells that form selective connections with individual muscles (**Figures 2(b)** and **2(d)**). Although the LIM code plays an important role in assigning columnar identity, it is insufficient to account for the entire range of motor neuron subtypes. Hox-class homeodomain transcription factors play a major role in the further subdivision of motor columns into motor pools.

The Hox gene family is organized into four clusters in which the expression of the 3′-most genes is initiated at rostral levels of the CNS while the 5′-positioned genes are progressively expressed at more caudal regions. Exposure to retinoic acid (RA) is required for expression of more 3′ Hox genes, whereas fibroblast growth factors (FGFs) activate the expression of more 5′ genes. Recent studies have demonstrated that Hox protein expression segregates with motor neuron columnar and pool identity and that sequential phases of Hox protein expression are required to properly specify motor neuron subtype identity. The basis for this highly precise segregation in Hox expression is analogous to progenitor factors and is due in part to cross-repressive regulatory interactions among the Hox genes. The restriction of Hox proteins to specific motor pools is critical for establishing pool identity and proper muscle connectivity. Hoxc6, for example, is found in motor pools of the LMC that innervate the pectoralis muscle but not the flexor carpi ulnaris (**Figures 2(b)** and **2(d)**). Consequently, misexpression of Hoxc6 causes a switch in motor pool identity leading to muscle targeting errors.

Large muscles are innervated by large motor pools and small muscles by small pools. An important feature of the Hox network is that the inherent strength of the cross-repression mediated by each Hox factor could potentially determine the size of the motor pool. Small motor pools might therefore be defined by weak repressors, whereas large pools could be generated using strong represses. In this way, the number of cells comprising the motor pool could be more accurately matched with the size of the peripheral muscle target.

Conclusions

One of the largest remaining questions in developmental neurobiology is how neuronal migration, axon guidance, and other crucial aspects of a neuron's phenotype are determined by the early gene regulatory events that occur as the neuron's identity is established. It is clear that there exists a sequential transcriptional cascade involving bHLH, homeodomain, LIM-HD, and Hox factors operating at multiple steps. In the first stage, as the neural plate folds to form the neural tube, progenitor domains within the ventral spinal cord are defined by the expression of homeodomain progenitor factors that are either induced (class II) or repressed (class I) by shh. In the second stage, the profile of the class I and II homeodomain transcription factors leads to the expression of determinants of neuronal subtype identity. In the third phase of the transcriptional hierarchy, neuronal subtype transcription factors including LIM-HD and Hox factors act in conjunction with neurogenic bHLH factors to coordinate pan-neuronal with subtype-specific programs of differentiation. Defining the full repertoire of target genes of these transcriptional networks may provide the link between neuronal cell identity and neuronal phenotype.

See also: Hox Gene Expression; Sonic Hedgehog and Neural Patterning; Transcriptional Silencing; Wnt Pathway and Neural Patterning.

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Transcriptional Silencing

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Introduction

Transcriptional silencing is the process by which regions of the eukaryotic genome, ranging from single genes to entire chromosomes, are made transcriptionally inactive through the reorganization of chromatin structure. Chromatin is the material in the nucleus of a eukaryotic cell comprising the nuclear DNA and all of the proteins associated with it. The bulk of this protein content is histones, basic proteins which package the DNA into nucleosomes, each nucleosome consisting of 146 bp of DNA wrapped around a histone octamer containing two copies each of the four core histones H2A, H2B, H3, and H4 (**Figure 1**). Linker DNA separates adjacent nucleosomes and is, in many species, associated with histone H1. There are also many other proteins in chromatin, including transcriptional activators and repressors, enzymes that modify DNA and histones, enzymes that cleave and repair DNA, components of the replication and recombination machinery, and proteins involved in higher order packaging. Under the microscope, this packaging is revealed by the presence of two different forms of chromatin – densely staining heterochromatin, which is for the most part transcriptionally inactive, and pale euchromatin, which is generally transcriptionally active. Transcriptional silencing reflects the fact that heterochromatic DNA is largely inaccessible to RNA polymerase and other components of the transcriptional machinery. Silencing differs from general transcriptional repression in that the former can extend over large regions of DNA, up to and including entire chromosomes, whereas the latter is specific to individual genes (or in some cases gene clusters). Silencing is also an epigenetic phenomenon which, once established, can be maintained passively through successive cell divisions, whereas gene-specific repression depends on the active maintenance of DNA–protein interactions within the promoter and other regulatory elements of the target gene. Transcriptional silencing is a natural phenomenon which is used to regulate gene expression during the development of the nervous system and as part of it normal function in adulthood. However, it is also used as a defense mechanism to protect the genome against invasive nucleic acids, such as transposable elements, viruses, and endogenous repeat sequences.

Mechanisms of Transcriptional Silencing

Histone Modification

Transcriptional silencing is generally achieved by the structural reorganization of chromatin, a process known as chromatin remodeling. In the brain, chromatin remodeling brought about by histone modification is fairly well characterized, and although much remains to be learned about the precise mechanisms involved, certain key events are now understood in detail. Histones are subject to various forms of reversible posttranslational modification, including the addition and removal of small chemical groups (acetylation, methylation, phosphorylation), larger chemical adducts (ADP-ribosylation), and small proteins (ubiquitylation, SUMOylation). Histones have a characteristic N-terminal tail which contains lysine and arginine residues and in some cases serine/threonine and glutamate. The lysine and arginine residues may be subject to acetylation and methylation, lysine residues may also be ubiquitylated or SUMOylated (SUMOs are small ubiquitin-like modifier proteins), the serine/threonine residues may undergo phosphorylation, and glutamate residues may undergo ADP-ribosylation. The roles of many of these modifications are unclear and may be context specific, but hyperacetylation usually corresponds to active chromatin whereas hypoacetylation correlates with inactive, silenced chromatin. It is thought that histone modification alters the affinity between histones and DNA, and/or between the histones within the nucleosome core, such that the DNA becomes either more or less accessible to the transcriptional machinery. Histone modification may also result in the specific recruitment of certain transcriptional activators and repressors, or proteins that sequester DNA into higher-order structures. There may well be a histone code, in which specific combinations of modifications contribute to defining a state of activity or inactivity which can persist through multiple rounds of DNA replication.

Histone modification is mediated by specific enzymes, and those controlling acetylation and methylation are the best understood. Histone acetyltransferases (HATs) catalyze the acetylation of lysine and/or arginine residues, and a number of known transcriptional activators have been subsequently identified as HATs. Histone deacetylases (HDACs) catalyze the removal of acetyl groups and associate with transcriptional repressors. The actual state of chromatin in any region of the genome depends to a large extent on the balance of these two opposing enzyme activities. In a similar way, the methylation of lysine or arginine residues is

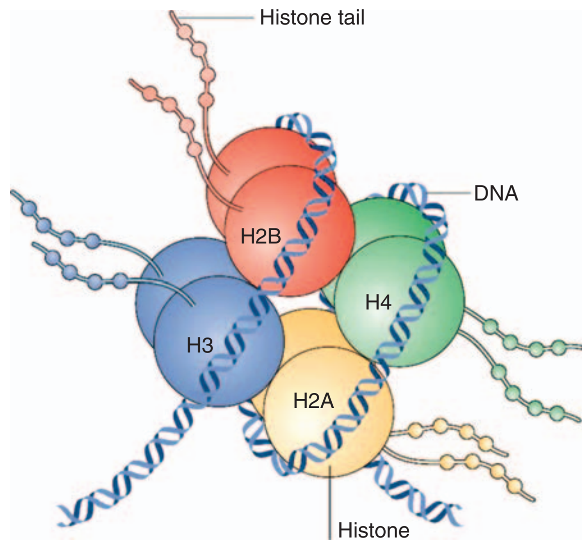

Figure 1 Nucleosome structure showing 148 bp of DNA wrapped around a histone octamer consisting of two copies each of the four core histones H2A, H2B, H3, and H4. Reproduced from Tsankova N, Renthal W, Kumar A, et al. (2007) Epigenetic regulation in psychiatric disorders. *Nature Reviews Neuroscience* 8: 355–367, with permission from Nature Publishing Group.

promoted by histone methyltransferases (HMTs) and reversed by histone demethylases (HDMs), although the role of methylation in gene silencing is not always so clear-cut, as methylation may be associated with either active or inactive chromatin in different contexts.

DNA Methylation

The structure of eukaryotic DNA is remarkably invariant, but epigenetic regulation by DNA methylation constitutes an important exception. In mammals, DNA is methylated at the 5′ position of cytidine residues in the context of the dinucleotide sequence CpG. The methylation of DNA is catalyzed by DNA methyltransferases (DNMTs), which transfer the methyl group from an *S*-adenosylmethionine (SAM) donor. Generally, CpG dinucleotides are constitutively methylated and tend to mutate to TpG by deamination over evolutionary timescales, resulting in significant underrepresentation in today's mammalian genomes. However, those in promoters and other regulatory regions are relatively undermethylated, and therefore escape the evolutionary pressure to mutate, resulting in clusters of dinucleotides known as CpG islands, which have been used to pinpoint the location of genes in genomic DNA clones. Within such islands, the level of DNA methylation corresponds to the degree of transcriptional silencing.

As might be expected, patterns of DNA methylation correspond to patterns of histone modification that establish inactive chromatin. This is achieved through the presence of methyl-CpG-binding proteins

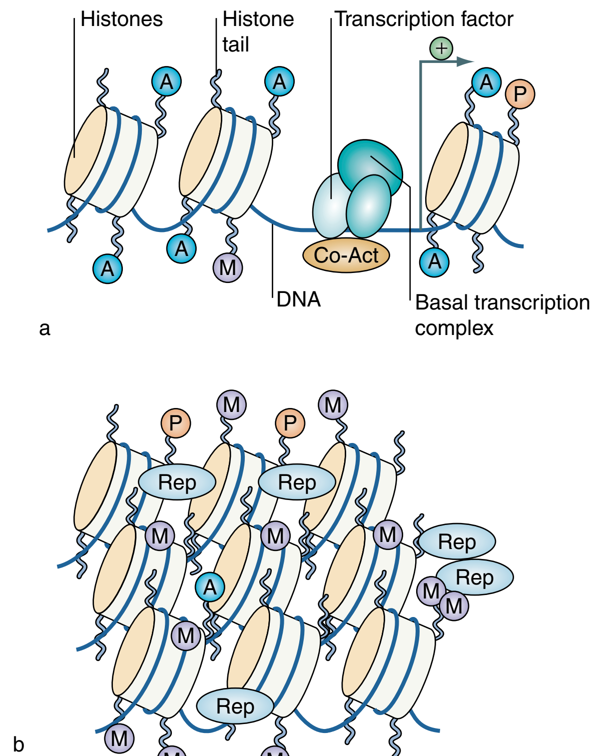

Figure 2 (a) Active chromatin (euchromatin) consists of loosely packed nucleosomes in which the histones are heavily acetylated (A) but are methylated (M) and phosphorylated (P) at low levels. This enables the basal transcription complex and coactivators (Co-Act) to assemble on the promoter, and transcription to commence. (b) Repressed chromatin (heterochromatin) consists of tightly packed nucleosomes in which histones are heavily methylated but are acetylated at low levels. Further repressor proteins (Rep) are also present. Reproduced from Tsankova N, Renthal W, Kumar A, et al. (2007) Epigenetic regulation in psychiatric disorders. *Nature Reviews Neuroscience* 8: 355–367, with permission from Nature Publishing Group.

(MeCPs) that associate with protein complexes containing HDACs and HMTs. DNA methylation, histone methylation, and histone deacetylation are therefore intrinsically linked in order to facilitate transcriptional silencing, establish epigenetic markers, and allow the silenced state to persist through DNA replication and cell division (**Figure 2**).

Other Chromatin Remodeling Mechanisms

In addition to histone modification, chromatin remodeling can be brought about by a number of other, less well-characterized mechanisms. One example is the phenomenon of histone replacement. Here, instead of modifying histones *in situ* to alter their affinity for DNA and components of the transcriptional machinery, one set of histones is simply swapped for another with properties that are more suitable. Another example is nucleosome sliding, an ATP-dependent process in which the histone octamer is transiently displaced,

allowing the transcriptional machinery to assemble at the promoter. Such repositioning of nucleosomes has been observed directly *in vitro* and is thought to be mediated by proteins of the SWI/SNF family.

Higher-Order Packaging and Nuclear Organization

A key difference between euchromatic and heterochromatic DNA is that the latter is folded into higher-order chromatin structures that maintain the DNA in an inaccessible state. This higher-order packaging is mediated by proteins that recognize the types of histone modification associated with inactive chromatin. For example, heterochromatin protein 1 (HP1) binds specifically to nucleosomes containing histone H3 methylated on lysine-9. A second mechanism for regulating access to the DNA then becomes evident and this involves the spatial positioning of genes within the nucleus. Interphase DNA in the nucleus does not diffuse about randomly, but is instead organized into discrete areas by attachment to the nuclear matrix. Labeling experiments have shown that chromosomes, and even small chromosome regions, tend to have a specific address, known as a chromosome territory, within the nucleus. Not much is known about the precise way in which the territories are established and occupied.

The nucleus is also organized into zones which are enriched with proteins that favor either transcriptional activity (active zones) or transcriptional silencing (inactive zones). Nuclear zones and territories are not aligned, and it appears that the zones are actually superimposed on the territorial organization so that they can incorporate whole chromosomes (e.g., the inactivated X chromosome in female mammals), contiguous chromosome segments, or even regions of similar transcriptional activity on different chromosomes which come together in the same locality. Thus, regulation of gene expression can occur at many levels involving the local chromatin environment, chromosomal territories, and the nuclear zones (**Figure 3**).

Recruitment to the different zones is mediated by protein–protein interactions, as illustrated by HP1, which not only interacts with modified histones but also with components of the nuclear envelope, thereby positioning heterochromatin within the inactive zone at the nuclear periphery. Specialized DNA elements known as insulators or boundary elements are thought to separate adjacent chromatin regions and allow them to occupy different zones, perhaps by binding to the nuclear matrix and preventing the propagation of heterochromatin structures from one domain to the next (see later, discussion of position effects).

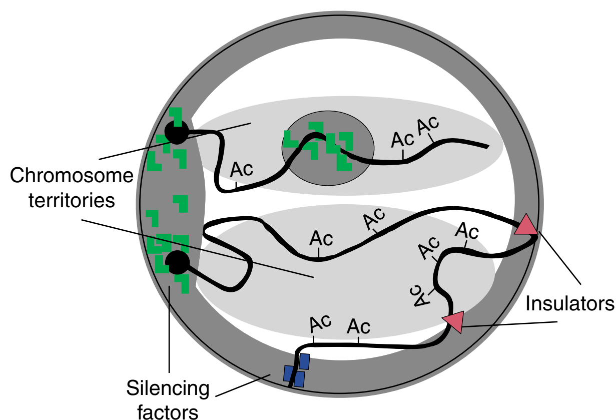

Figure 3 Nuclear organization: the nucleus contains territories (light gray) and silencing zones (dark gray periphery). Histone acetylation (Ac) is shown over active chromatin, while hypoacetylation is associated with inactive chromatin, such as that found at centromeres (black circle) and telomeres. Small domains within a chromosome can acquire properties similar to those of heterochromatin and form a silencing zone (shaded circle). Silencing factors (green and blue shapes) are involved in chromatin compaction, gene silencing, and tethering to the nuclear periphery. Insulator elements (red triangles) partition chromosomes into transcriptionally related domains and assist in zonal organization. Reproduced from Tsankova N, Renthal W, Kumar A, et al. (2007) Epigenetic regulation in psychiatric disorders. *Nature Reviews Neuroscience* 8: 355–367, with permission from Nature Publishing Group.

Events That Trigger Transcriptional Silencing

Endogenous Signaling Pathways

Transcriptional silencing is widely used during the development of the nervous system to maintain states of determination and differentiation as permanent developmental decisions. It is also becoming apparent that transcriptional silencing is used in mature neurons as a mechanism to implement long-term changes in response to external signals. Most brain disorders are characterized by dysregulation of gene expression, resulting in long-lasting effects on behavior. However, such observations clash with what is known about conventional gene regulation, as well as with evidence from animal models showing that the levels of transcription factors and other regulators return to normal within hours or days of perturbation, yet abnormal behavior persists. The possibility that epigenetic mechanisms such as the imposition or lifting of transcriptional silencing could mediate these long-term changes is therefore an attractive one, and a number of neuronal signaling pathways have been shown to fit with this idea. For example, the transcription factor cyclic AMP response element-binding protein (CREB) is phosphorylated on a serine residue by extracellular signal-regulated kinase (ERK) in response to signals that elicit either cAMP or Ca^{2+} as second messengers. Phosphorylated CREB can then recruit CREB-binding

protein (CBP) into a complex, and the intrinsic HAT activity of CBP acetylates histones, making the target gene transcriptionally active. In hippocampal neurons, increases in intracellular Ca^{2+} lead to the activation of Ca^{2+}/calmodulin-activated protein kinases (CaMKs), which phosphorylate class II HDACs. This phosphorylation triggers the shuttling of the enzyme out of the nucleus and results in increased histone acetylation. In both cases, the epigenetic mark for transcriptional activity would persist until erased by HDAC activity.

It is also interesting to note that certain neuroactive drugs are known to induce rapid changes in histone modification. For example, cocaine induces the acetylation of histone H4 and the phosphoacetylation of histone H3 in the promoter of the immediate-early gene c-*fos*. This occurs specifically in the striatum, which mediates the behavioral effects of cocaine.

DNA Rearrangement and Integration

In addition to being a significant feature of normal gene regulation, transcriptional silencing can also occur due to abnormal or unanticipated events. In chromosome rearrangements such as inversions and translocations, DNA at the breakpoint is often subject to what is known as a position effect, where active genes can undergo transcriptional silencing due to the spreading of inactive chromatin. This occurs because the breakpoint brings active and inactive chromatin into juxtaposition without the normal insulator or boundary element to separate them into distinct nuclear zones. What then occurs is down to chance, reflecting the extent of histone modification activity and the abundance of heterochromatin proteins available in the nuclei of different cells. A well-characterized model is the developing eye of the fruit fly *Drosophila melanogaster.* Under normal circumstances, the *white* gene (which encodes an enzyme involved in pigment synthesis and therefore confers the normal red eye color when active) is located far from any heterochromatin. A rare chromosomal inversion brings the *white* gene much nearer, and removes an insulator which normally keeps the heterochromatin restricted to its own zone. The heterochromatin then spreads along the chromosome depending on the availability of the necessary components and enzymes. In some cells of the developing eye, the heterochromatin fails to reach the relocated *white* gene, which remains active. In others, it spreads through the *white* gene and inactivates it. Since the epigenetic mark is heritable, each cell gives rise to a clone of cells in the adult eye, which becomes either a white or red patch. The overall eye is therefore mottled, or variegated, a phenomenon known as 'position effect variegation.'

Positional silencing can also occur when DNA integrates into a heterochromatic region, although it is necessary to establish whether the absence of gene expression is genuinely because an intact transgene has integrated into inactive chromatin, or whether the integration event has resulted in transgene rearrangement or mutation.

Repetitive DNA

Repetitive DNA sequences (both endogenous sequences and transgenes) are often subject to transcriptional silencing because they act as nucleation centers for heterochromatin formation. This occurs both at tandem repeats, such as those found in the centromeric region of chromosomes, and at dispersed repeats, such as transposable elements and integrated transgenes. In some cases, repeated sequences may trigger heterochromatin formation because they attract sequence-specific DNA-binding proteins which are in turn recognized by HDACs or DNMTs, or proteins such as HP1. In yeast, for example, repetitive DNA sequences found at the telomeres are recognized by the DNA-binding protein Rap1, which interacts with components of the heterochromatin machinery and ensures localization at the nuclear periphery. Since mammalian transposable elements and transgenes are diverse in their sequences, it is unlikely that a sequence-specific mechanism is involved and suggests that the cell possesses some kind of mechanism for sensing repeated DNA sequences and targeting them. Models have been proposed based on the formation of DNA–DNA pairs that act as nucleation sites, as well as models involving the production of aberrant RNA species, invoking the RNA-mediated interference (RNAi) pathway (see later).

Invasive DNA

Transgene silencing can occur even following the integration of a single-copy gene at a euchromatic site, suggesting that cells can under some circumstances recognize integrating DNA sequences as invasive and deal with them by sequestering them into heterochromatin following *de novo* DNA methylation. It is possible that cells possess some form of genome scanning apparatus that looks for sequences with unusual base composition compared to the surrounding genomic context. The same mechanism is probably responsible for the *de novo* methylation of integrating human immunodeficiency virus (HIV) genomes, and vectors based thereon, as well as retroviral vectors used to introduce transgenes into mammalian embryos. Prokaryotic DNA appears to be a strong trigger for *de novo* methylation, since the co-integration of vector backbone sequences (which are

generally of bacterial origin) often leads to silencing, whereas clean DNA sequences (just the promoter, coding region, and polyadenylation site) are more successful. It is also possible that invasive DNA somehow induces the RNAi pathway (e.g., through the expression of aberrant RNA species from integrated transgenes, or through the generation of double-stranded RNA intermediates during viral replication).

RNA Silencing

Small RNA molecules can also silence gene expression, but generally do so posttranscriptionally, either by cleaving homologous mRNA molecules (RNA interference) using short interfering RNAs (siRNAs) or by regulating translation (micro-RNAs). However, the RNA silencing apparatus also has significant interactions with chromatin proteins, including histone-modifying enzymes and DNA methyltransferases. Recent evidence has accumulated that the RNAi machinery can set epigenetic marks in *Drosophila* and mammalian cells. Although the mechanisms involved are complex and yet to be elucidated fully, it appears that repetitive regions of the genome such as tandem repeats and transposons (and perhaps integrated transgenes) can generate long double-stranded RNA molecules, which are processed into siRNA-like structures known as repeat-associated siRNAs (rasiRNAs). These are cleaved by the enzyme Dicer to form complexes known as RNA-induced initiation of transcriptional silencing (RITS); these complexes mediate histone modification and DNA methylation.

Nervous System Transcriptional Silencing

Transcriptional Silencing in Neural Development

Transcriptional silencing is used to establish and maintain cell fates in the developing nervous system and, most importantly, to suppress neuronal fates in nonneuronal cells. Embryonic cells and their derivatives have the potential to differentiate into neural cells if grown in the correct conditions (e.g., mouse P19 embryonal carcinoma cells will differentiate into neurons in the presence of retinoic acid), so it is apparent that this developmental lineage must be suppressed in normal development. There is evidence that this is achieved through chromatin remodeling, since HDAC inhibitors can induce neural differentiation in embryonic cortical cells and suppress the differentiation of oligodendrocytes. A more specific example is the neuron-restrictive silencing factor (NRSF), a transcriptional repressor expressed in glia and other nonneuronal cells. The function of NRSF is to repress neuronal differentiation; NRSF associates with a number of other proteins, including transcriptional repressors and the histone deacetylase HDAC2. In this way, the promoters of neuronal genes are maintained in an inactive state in nonneuronal cells. NRSF is also expressed in some neurons later in development, when its function is to regulate the activity of neuronal genes.

Transcriptional Silencing in Mature Neurons

Transcriptional silencing is also used to control gene expression in mature neurons, and a particularly striking example is the regulation of the *BDNF* gene (encoding brain-derived neurotrophic factor). The organization of this gene is very unusual in that there are at least nine distinct promoters, each paired with a unique noncoding exon. When the gene is expressed, one of these noncoding exons is spliced onto a common coding exon to generate one of at least nine possible mature transcripts. The promoters are independently regulated and have different levels of activity and different expression patterns, allowing BDNF to be expressed in different patterns depending on which promoter is activated by a given external signal. This activation is mediated by chromatin remodeling. For example, acute membrane depolarization in mouse cells releases intracellular Ca^{2+}, which results in the activation of Ca^{2+}/calmodulin-activated protein kinase II, causing the phosphorylation of MeCP2 and favoring transcription from promoters P1 and P4. In rats, electroconvulsive seizures or *in vivo* administration of pilocarpine increase histone H4 acetylation at promoter P2, strongly elevating transcription from this promoter.

See also: Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation; Neuromuscular Junction: Neuronal Regulation of Gene Transcription at the Vertebrate; Transcriptional Networks and the Spinal Cord.

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Motor Neuron Specification in Vertebrates

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Motor Neuron Progenitors: Extrinsic Signals

During early stages of neural tube development, multiple signaling activities intersect with one another and are responsible for specifying the identity of neuronal subtypes within the spinal cord. In the ventral neural tube where motor neurons and multiple classes of interneurons differentiate, cell fate specification occurs in a highly stereotypical manner based on the position of cells along the rostrocaudal (RC) and dorsoventral (DV) axes. Embryonic cell position and fate are linked because the location of undifferentiated progenitors is correlated with the types and concentrations of extrinsic factors they encounter – and it is the extrinsic factors that activate signaling pathways involved in establishing cell identity.

Motor neurons are located within the caudal regions of the nervous system, including the hindbrain and spinal cord. In the absence of extrinsic factors, progenitor cells default to an anterior identity, and as a consequence their progeny have characteristics of forebrain neuronal classes. A variety of extrinsic factors produced by Hensen's node and the mesoderm flanking the neural tube have been implicated in 'caudalizing' progenitor cells, including wnts, retinoic acid (RA), fibroblast growth factors (FGFs), and growth/differentiation factor 11 (gdf11). Although the interplay between these factors is still poorly understood, their graded activity appears to shift cells toward a progressively more posterior fate. Caudalizing signals play a critical role in establishing precursor cells that can give rise to motor neurons, whereas progenitors located at anterior levels do not encounter these transforming signals and therefore are not competent to generate motor neuron progeny. Based on studies of RC signaling in embryos, *in vitro* differentiation protocols using mouse and human embryonic stem cells to generate motor neurons typically incorporate RA and FGF to shift the rostrocaudal identity of the neural progenitors to a caudal identity appropriate for motor neuron specification.

RC patterning is not only involved in establishing progenitor cells competent to produce motor neurons but also contributes to the further diversification of motor neurons into individual subclasses. In large part, motor neurons develop in RC register with their peripheral targets. For example, limb-innervating motor neurons are located at limb levels (lower cervical and lumbar), whereas craniofacial-innervating motor neurons are located within the hindbrain. Grafting studies in chick embryos have shown that the early RC position of cells within the neural tube has a major influence on the eventual formation of motor neuron subclasses. Studies that alter RA, FGF, and/or Gdf11 signaling have shown that the identity of developing motor neuron subclasses is shifted along the RC axis of the neural tube. The influence of these signals also contributes to the precise assignment of motor neuron 'pools,' the unit representing a group of motor neurons that target specific muscles. Thus, caudalizing signals not only play a role in establishing precursor cells that can give rise to motor neurons but also operate at an extremely high resolution in defining what subtypes of motor neurons will develop along the RC axis of the embryo. These RC signals likely begin to influence progenitor cell identity prior to, and independently of, the DV signals that trigger motor neuron development.

Progenitor cells within the ventral portion of the caudal neural tube generate many cell types, including astrocytes, oligodendrocytes, floor plate cells, a diverse array of interneuronal classes (e.g., V0–V3), and motor neurons. Classical cell grafting studies have shown that graded signals along the DV axis of the neural tube influence cell fate specification. Sonic hedgehog (Shh) is produced as a secreted glycoprotein from the notochord and floor plate, establishing a high ventral-to-low dorsal gradient. With remarkable accuracy, neuroepithelial cells are able to readout different concentrations of Shh and respond by initiating specific differentiation programs. *In vitro* studies suggest that Shh activates signaling pathways for altering gene expression in the 0.5–10 nM range. Concentration differences as small as twofold lead to the activation of entirely different differentiation programs. At spinal cord levels, a narrow range of Shh triggers motor neuron development, whereas higher or lower levels of Shh trigger the differentiation of other cell types. Although Shh appears to only trigger the differentiation of a single generic population of ventral-exiting motor neurons (v-MNs) at spinal cord levels, the situation is more complex at hindbrain levels. Here, graded Shh appears to contribute to the specification of two major motor neuron classes. In the hindbrain, high concentrations of Shh have been linked to the development of dorsal-projecting visceral motor neurons (d-MNs), whereas

progenitor cells located more dorsally in the neural tube encounter lower levels of Shh and respond by differentiating as ventral-projecting somatic motor neurons (v-MNs). Thus, the intersection of RC and DV signals is integrated to specify motor neurons of appropriate subtypes at specific locations within the developing nervous system.

Motor Neuron Progenitors: Intrinsic Factors

RC and DV extrinsic signals are converted into intrinsic regulatory programs by activating the expression of multiple families of transcription factors, many of which contain a homeodomain for DNA binding. Along the DV axis, graded levels of Shh lead to the expression of unique combinations of transcription factors that define five separate progenitor cell domains within the ventral neural tube. An interesting cell-intrinsic mechanism is used to ensure that cells which fall close to the threshold level of Shh for two different progenitor cell domains are decisively resolved into a genetic program for a single cell type. This is achieved by having transcription factors for one progenitor cell type negatively regulate the expression of the factors expressed in adjacent cell domains. If cells initially start to express transcription factors for two different cell types, the reciprocal inhibition of the factors ensures co-expression is not tolerated, which rapidly leads to one set winning the cross-inhibitory 'battle.' Thus, a gradient of Shh is converted into all-or-nothing responses at the level of gene regulation controlled by progenitor domain-specific transcription factors.

The progenitor cells for motor neurons within the spinal cord reside within a 'domain' called the pMN region of the ventricular zone. pMN cells express a unique constellation of homeodomain transcription factors that distinguishes them from the progenitor cells for other neuronal types in the spinal cord. Furthermore, progenitor cells for d-MNs (restricted to hindbrain and upper cervical levels), such as trigeminal and spinal accessory neurons, arise from a unique pMN domain marked by Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2, and phox2b, whereas pMN cells that generate v-MNs express Nkx6.1, Nkx6.2, Pax6, and Lhx3. v-MN progenitors also express basic helix–loop–helix (bHLH) transcription factors that control the process of neurogenesis, a genetic subroutine involved in converting neural stem cells into postmitotic neurons. Two bHLH factors have been identified in pMN cells that have antagonistic functions toward one another: Olig2, which seems to hold pMN cells in a dividing progenitor cell state, and Ngn2, which favors neuronal differentiation. The counterrelationship between Olig2 and Ngn2 might serve as a mechanism for first expanding the pMN precursor population before allowing progeny cells to differentiate since precocious motor neuron differentiation would likely deplete the number of progenitor cells for motor neurons. The coordination of pMN growth and differentiation appears to be based on the timing of Olig2 and Ngn2 expression in pMN cells: Olig2 is expressed initially, but then it activates the expression of Ngn2, which begins to accumulate in the cells as development proceeds. The high Olig2 level in early pMN cells favors their growth, whereas the accumulation of Ngn2 beyond a threshold level over time appears to drive neuronal differentiation (neurogenesis) at later stages.

The high caudal-to-low rostral gradient of FGF in the embryo influences the expression of nested sets of Hox class transcription factors along the RC axis of the neural tube. Motor neurons that innervate the same muscle cluster their cell bodies into pools within the spinal cord, which typically span two to four spinal cord segments, vary in cell number in relationship to the size of the muscle they innervate, and extensively overlap with other motor pools spread along the RC axis. Gain- and loss-of-function studies have shown that Hox transcription factors are involved in establishing motor pool subtype identity. Like the progenitor cell factors involved in DV patterning, Hox genes involved in the development of different motor pools have cross-inhibitory regulatory interactions that help to convert graded extrinsic signals of RC position into unambiguous patterns of gene regulation for cell identity. By tuning the relative strength of the cross-inhibitory interactions for Hox factors, this could represent a mechanism for allowing some motor pools to expand in size at the expense of others so that motor neuron numbers within individual motor pools are roughly matched to the size (i.e., muscle fiber number) of the peripheral target.

Stepwise Progression in Motor Neuron Diversification

As progenitor cells generate postmitotic motor neurons, many changes in gene expression occur with the final cell division. The postmitotic birth of d-MN cells is tightly linked to the initiation of expression of the LIM-homeodomain (LIM-HD) factor Isl1 and the homeodomain factor Phox2a. On the other hand, v-MN cells express Isl1 and homeodomain factor Hb9 when they are born and maintain their expression of the LIM-HD 'progenitor' factor Lhx3 (**Figure 1**). Nevertheless, many of the other progenitor transcription factors found in pMN cells such as Olig2 and Pax6 are downregulated in newly formed postmitotic motor neurons. Little is known about the molecular basis for the further diversification of d-MN cell

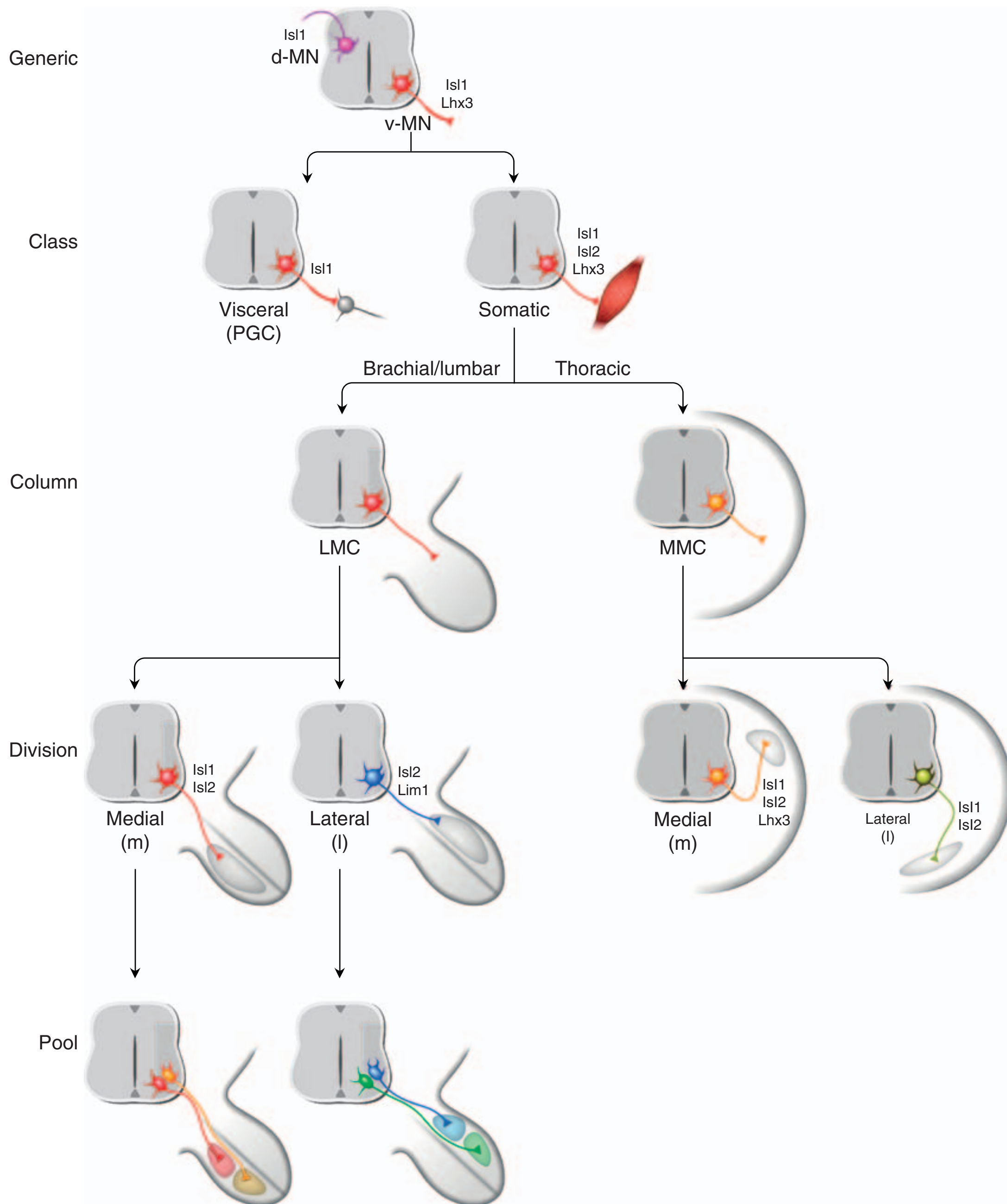

Figure 1 Motor neuron subtypes and their stepwise specification by LIM-HD factors. Motor neurons are classified based on their cell body position, axonal trajectory, and targets. Generic: Dorsal-projecting motor neurons (d-MNs) located at the hindbrain/upper cervical levels occupy dorsolateral positions and extend axons dorsally from the neural tube. There are many subclasses of d-MNs (not shown), and each has a slightly different cell body position and axonal trajectory. This diagram is a schematic representation of d-MNs that is not intended to indicate their precise position and axonal trajectory. Ventral-projecting motor neurons (v-MNs) extend axons ventrally from the neural tube forming ventral roots. Class: v-MNs at spinal levels are divided into two subclasses – somatic motor neurons innervate skeletal muscles and visceral motor neurons (forming a preganglionic motor column (PGC) in the intermediolateral spinal cord) innervate sympathetic neurons. Column: Somatic motor neurons are composed of multiple motor columns named based on their mediolateral cell body position – median motor column (MMC) neurons and lateral motor column (LMC) neurons. Division: Individual motor columns are divided into medial and lateral subdivisions – medial MMC cells extend axons dorsally and innervate axial muscles, lateral MMC cells extend axons ventrally and innervate body wall (intercostal) musculature, medial LMC (LMCm) cells extend axons into the ventral limb, and lateral LMC (LMCl) cells extend axons into the dorsal limb. Pool: Subsets of motor neurons clustered together within a single motor column such as the LMCl and LMCm that innervate the same muscle. The expression profile of LIM-HD factors is listed. Lhx3 has a paralogous gene Lhx4 (Gsh4) that has the same expression as Lhx3 and therefore is not listed for simplicity.

types, but numerous additional steps in the subspecialization of v-MN cells have been defined (**Figure 1**). Newly formed v-MN cells all extend axons out through the ventral root of the neural tube, but soon after this step additional changes occur in their profile of transcription factor expression reflecting the emergence of different v-MN subtypes. These dynamic and highly selective patterns of gene expression correlate with the migration of subclasses of v-MN cell bodies to specific locations within the neural tube and the growth of their axons along specific pathways in the periphery (**Figure 1**).

At thoracic spinal cord levels, v-MN cells can be divided into two major subclasses: somatic motor neurons innervating skeletal muscles and visceral motor neurons innervating sympathetic targets. Somatic motor neurons upregulate the LIM-HD factor Isl2, whereas visceral motor neurons downregulate Lhx3 and Hb9 but continue to express Isl1 as their cell bodies migrate dorsomedially to form a preganglionic motor column (PGC). Somatic motor neurons contribute to the formation of many different motor columns that are classified as either median (MMC) or lateral (LMC) based on their mediolateral cell body position in the ventral horn. The median and lateral motor columns are further subdivided into medial and lateral divisions to create four motor columns containing cells that innervate distinct peripheral targets: MMCm cells innervate axial muscles, MMCl cells innervate body wall musculature, LMCm cells innervate ventral limb targets, and LMCl cells innervate dorsal limb targets (**Figure 1**). Each of these motor columns is marked by a specific combinatorial pattern of LIM-HD genes. MMCm cells express Isl1, Isl2, Lhx3 (also called Lim3), and Lhx4 (also called Gsh4); MMCl cells found only at thoracic levels and LMCm cells found only at limb levels express Isl1 and Isl2; and LMCl cells express Isl2 and Lim1 (also called Lhx1). A variety of genetic studies have shown that the LIM-HD factors function in a combinatorial manner to control the expression of genes within motor columns that mediate axon pathfinding.

Motor columns can be further subdivided into motor pools which correspond to cells that are clustered together within a column and are related to one another by virtue of innervating the same muscle, expressing the same pattern of cadherins, and being gap junctionally interconnected. Individual motor pools express specific patterns of Hox and Ets class transcription factors. Ets factors such as Pea3 and Er81 are upregulated in motor pools by retrograde neurotrophin signaling activated by neurotrophin 3 (NT-3) and glial-derived neurotrophic factor (GDNF) produced by cells encountered by motor axons in the periphery. The Ets factors mediate a number of later functions in motor pools, whereas gain- and loss-of-function studies with *Hox* genes such as *Hoxc8*, *Hox5*, and *Hoxc6* have linked these factors to the actual specification of motor pool identity within the brachial (lower cervical) spinal cord. Taken together, the stepwise process whereby the subclass identity of 'generic' v-MN spinal motor neurons becomes progressively more refined is reflected in the sequential changes in the transcription factor 'coding' that take place during postmitotic stages of motor neuron development (**Figure 1**).

The LIM Code and Motor Neuron Diversification

LIM-HD genes evolved in early metazoans, and many studies have linked their function to the regulation of cell fate during the development of numerous tissues. The *Caenorhabditis elegans* LIM-HD factors Mec-3 and Lin11 are involved in specification of mechanosensory neurons and vulval cells, respectively. In *Drosophila*, the LIM-HD factor Apterous specifies dorsal cell identity during wing development. Likewise, LIM-HD factors in vertebrates are found in diverse organs, such as the developing pituitary, retina, limbs, pancreas, spinal cord, and brain, and are expressed with highly restricted expression patterns in specific cell types. As noted previously, motor neurons express unique combinations of LIM-HD genes during several phases of their development (**Figure 1**). v-MN cells express Isl1, Lhx3, and the Lhx3 paralog Lhx4 when they extend axons out through the ventral root, whereas d-MN cells express only Isl1. The first demonstration of the combinatorial function of LIM-HD genes was based on the finding that ectopic expression of Lhx3 in d-MN cells converted them to v-MNs, and conversely double knockouts of Lhx3 and Lhx4 converted v-MNs into d-MNs. Thus, the presence or absence of Lhx3/4 activity in newly formed motor neurons is thought to control the v-MN versus d-MN fate decision.

Interestingly, LIM-HD factors also contribute to the further diversification of v-MN subclasses at later stages of motor neuron development. Following the generation of v-MN cells, the emergence of motor columns is associated with the appearance of unique combinatorial arrays of five different LIM-HD factors (**Figure 1**). The refinement of motor neuron subtype identity is associated with both up- and downregulation of specific LIM-HD genes at postmitotic stages of neuronal development. Genetic studies in mice have shown that at later stages Lhx3 is involved in MMCm motor column development, Lim1 (Lhx1) is involved in LMCl development, and Isl2 is involved in PGC development. Taken together, functional studies on

the LIM-HD transcription factors indicate that they contribute to the development and subclass refinement of motor neurons by acting in a combinatorial manner at several stages in the stepwise development of these cells. Thus, they contribute to motor neuron subtype diversification at two levels: by functioning at multiple time points and by functioning in a combinatorial manner. These features of the LIM-HD factors enhance the ability of this relatively small gene family to contribute to the generation of an extraordinary level of cellular diversity within the spinal cord. Since all 12 members of the LIM-HD gene family are expressed by developing neurons in the peripheral and central nervous system, it is not surprising that studies have defined functions for these transcription factors in cortical, retinal, pituitary, and sensory neuron development as well.

The Molecular Underpinnings of the LIM Factors

The LIM motif is a cysteine–histidine-rich sequence which creates a tandem zinc finger structure that functions as a protein–protein interaction domain. Although numerous studies support the view that the LIM domain mediates interactions for higher-order ternary complexes, only a limited set of binding partners are well defined. Structural analyses and biochemical studies have demonstrated that the LIM domains derived from the nuclear LIM proteins encoded by 4 LIM-only genes and 12 LIM-HD genes can interact with the nuclear LIM interactor (NLI) protein (also called Clim and Ldb) (**Figure 2**). NLI is a widely expressed (probably ubiquitous) nuclear factor containing a self-dimerization domain and a LIM interaction domain. The protein-interaction characteristics of NLI suggest that it serves as a scaffold for assembling complexes with multiple LIM-HD proteins (**Figure 2**). A simple example of these LIM-HD complexes is found in V2a interneuron development – a population of Lhx3-expressing cells that form dorsal to motor neurons in the spinal cord. Biochemical and structure–function studies indicate that stoichiometric complexes of 2-Lhx3:2-NLI molecules regulate gene expression and specify V2a interneuron cell identity (**Figure 2**).

v-MN motor neurons expressing Isl1, Lhx3, and NLI represent a more complicated situation that illustrates a general issue with combinatorial codes based on reusing elements in a context-dependent manner. The co-expression of multiple LIM-HD factors, such as Isl1 and Lhx3 in the case of v-MN cells, raises the possibility that a mix of LIM complexes could form (i.e., 2-Lhx3:2-NLI, 2-Isl1:2-NLI, and Lhx3:2-NLI:Isl1). However, if this occurred, it is expected that the 2-Lhx3:2-NLI complex would inappropriately activate V2a interneuron genes, leading to a hybrid phenotype in v-MN cells. In this case, biochemical studies found that the LIM domain of Lhx3 is also able to bind with high affinity to the C-terminal portion of Isl1, leading to the formation of a hexameric complex composed of 2-Lhx3:2-Isl1:2-NLI (**Figure 2**). The binding of Lhx3 to Isl1 results in a competitive

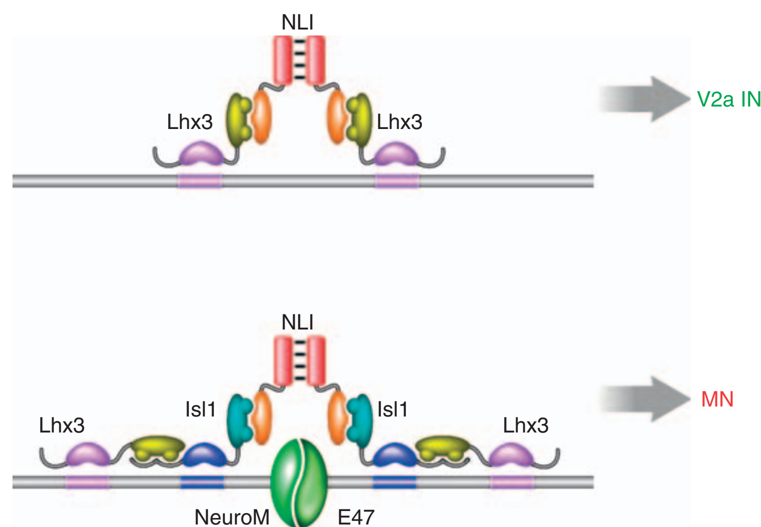

Figure 2 Ternary LIM-HD complexes regulate gene expression. The LIM domain of Lhx3 can bind to both NLI and Isl1. In V2a interneurons in which Lhx3 is expressed without Isl1, Lhx3 binds to NLI, which dimerizes to form a tetrameric 2-Lhx3:2-NLI complex for DNA binding. In v-MN motor neurons, Isl1 displaces Lhx3 from NLI, thereby preventing the V2a interneuron tetrameric complex from forming. Instead, Lhx3 binds to Isl1's C-terminus leading to the formation of a hexameric complex composed of 2-Lhx3:2-Isl1:2-NLI. This complex synergizes with bHLH factors such as Ngn2, NeuroM, and NeuroD (green) to activate high transcription levels. This synergy couples the activity of LIM-HD factors to proneural bHLH factors ensuring that neuronal differentiation and subtype specification are linked.

situation whereby Lhx3–NLI interactions needed for V2a interneuron gene regulation are depleted at the expense of forming hexameric complexes for v-MN specification. Thus, neuron-specific protein–protein interactions represent one way in which LIM-HD factors can be used to regulate gene expression in different cellular contexts.

Although Isl1 and Lhx3 specify v-MN cell identity, until recently it was unclear how the activity of LIM-HD factors was tied to bHLH class transcription factors in developing motor neurons. Ngn2, NeuroM, and NeuroD are expressed by motor neuron progenitor cells and differentiating motor neurons and have proneural activity, meaning that they trigger cell cycle exit and activate generic neuronal genes such as β-tubulin. Transcription studies have found that the hexameric LIM complex containing Isl1 and Lhx3 (**Figure 2**) has a low inherent transactivating capacity. However, the binding sites of Isl1 and Lhx3 within the motor neuron-specific gene Hb9 promoter were found to flank E-box elements for bHLH factors. In the presence of Ngn2, NeuroM, or NeuroD, the activity of the LIM-HD and bHLH factors synergizes to greatly enhance v-MN gene activation. This synergistic link requires the dimerization of NLI (**Figure 2**), suggesting a conformational change occurs that mediates efficient transcription in the presence of LIM-HD and bHLH transcription factors. This cooperative link serves as a coincident detector that provides a means for integrating and coordinating the activity of factors for neuronal differentiation with those that control subtype properties.

Motor Neuron Soma Migration

Once generated, newborn motor neurons undergo stereotypical patterns of cell migration. Individual subclasses of motor neurons often settle in different positions from one another. For example, somatic motor neurons within the spinal cord typically settle in the ventral horn in specific medial and lateral columnar and pool positions (**Figure 3**). In contrast, thoracic visceral motor neurons migrate dorsomedially away from the ventral horn and settle at intermediate positions in response to reelin. Multiple transcription factors have been implicated in the control of motor neuron cell body positioning. For instance, misexpression of Lhx3 (normally in medial MMC cells) among non-MMCm neurons in *Lhx3* knockin mice shifted the settling position of motor neurons to a medial (MMCm-like) position. Likewise, *Isl2* null mice display defects in the mediolateral distribution of MMC neurons, and LMCl neurons require Lim1 induction for their normal migration. Ets class transcription factors such as Pea3 have been implicated in the positioning of motor pools within motor columns. The clustering of like-motor neurons into motor pools has been linked to the cadherin gene family, encoding

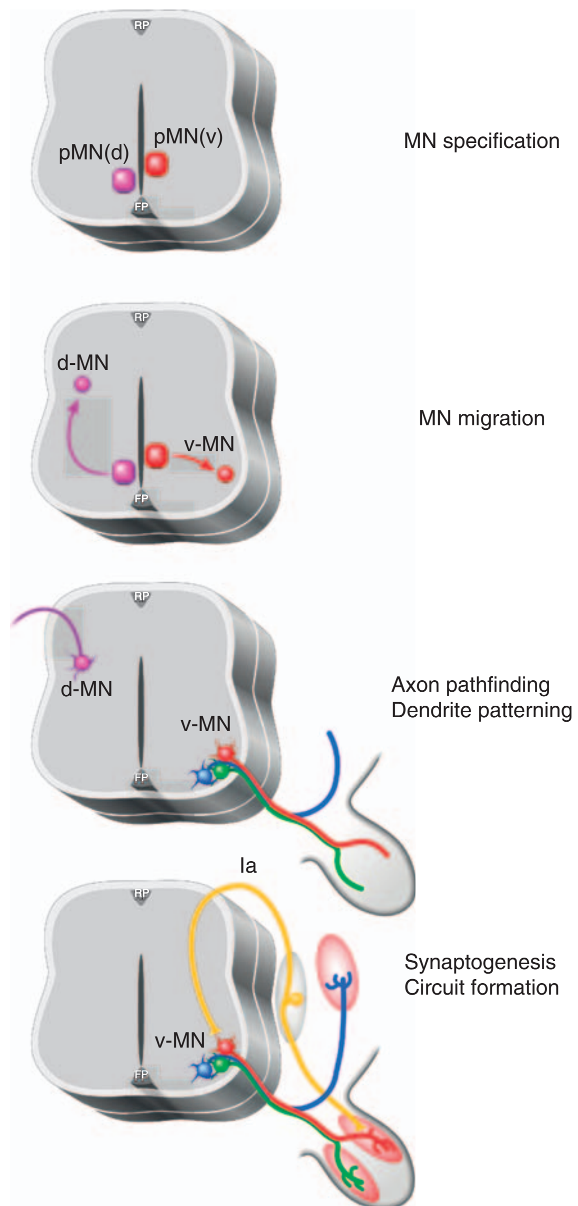

Figure 3 Stages of motor neuron development and maturation. v-MNs and d-MNs arise from different progenitor cell populations (pMN(d), purple; pMN(v), red) generated in response to different levels of Shh. The cell bodies of d-MNs move dorsolaterally and their axons exit the neural tube dorsally. The cell bodies of somatic v-MNs migrate laterally and form longitudinally aligned columns: medial MMC (MMCm, blue), medial LMC cells (LMCm, green), and lateral LMC cells (LMCl, red). All v-MN axons exit the spinal cord ventrally but select specific pathways in the periphery. LMC neurons grow to the base of the limb. LMCm cells innervate the ventral limb, whereas LMCl cells innervate the dorsal limb. MMCm neurons turn dorsally to innervate axial muscles. As motor neuron subtypes mature, they form peripheral branches and develop characteristic dendritic patterns which may contribute to the formation of specific connections for controlling their activity.

adhesion molecules that are selectively expressed by different motor neuron subtypes.

At hindbrain levels, the cell bodies of d-MNs (also called branchiomotor and visceromotor cranial motor neurons) migrate extensively. These classes of motor neurons often migrate dorsally along dorsolateral pathways, radially to the pial surface, and, in the case of facial cranial motor neurons, caudally along 'tangential' pathways. Although individual classes of d-MN cells follow different migratory pathways, some of the regulatory factors that control diverse migratory behaviors are shared among d-MN cells, including Tbx20 and Nkx6.1. In contrast, factors such as Ebf1, the planar cell polarity signaling pathway, and VEGF164 signaling appear to control a single process – the tangential migration of facial motor neurons.

Axon Pathfinding

Motor neurons share the unique property of extending axons into the periphery. All v-MN cells initially follow a common ventral pathway out of the spinal cord, but soon after exiting they are confronted with a choice point that branches toward three targets: MMCm cells select a dorsal pathway toward axial muscles; LMC and MMCl motor neurons select a ventral pathway toward the limbs and body wall muscles, respectively; and PGC axons grow ventrally toward sympathetic ganglia (**Figure 1**). Once LMC neurons reach the base of the limb, LMCl cells choose to grow into the dorsal region, whereas LMCm neurons enter the ventral limb compartment (**Figure 1**). Although arrays of LIM-HD transcription factors such as Isl1 and Lhx3 initially play a pivotal role in assigning motor neuronal fates, the expression of LIM-HD factors becomes progressively restricted to the selective motor neuron subtypes in postmitotic motor neurons (**Figure 1**). The specific expression of these factors precedes the selective axonal pathway and target innervation of neuronal subtypes, indicating that the combinatorial expression of LIM-HD factors (the LIM code) may guide the subtype-specific axon projection toward the target muscles. For instance, whereas most motor neurons rapidly extinguish Lhx3 and Lhx4 expression, MMCm neurons continue to express Lhx3 and Lhx4. Mouse genetic studies demonstrated that in the absence of Lhx3 and Lhx4, MMCm axons abnormally choose their exit points dorsally from the neural tube. Furthermore, when Lhx3 is stably expressed in all motor neurons as in *Lhx3* knockin mice, non-MMCm neurons extend axons toward axial muscles. This phenotype has been linked to Lhx3's regulation of FgfR1, which mediates chemoattraction toward the Fgf4/8-expressing axial muscles. LMCm neurons which innervate ventral limb muscles express Isl1, whereas LMCl neurons which innervate dorsal limb muscles express Lim1 (Lhx1). LMCl axons grow normally to the base of the limb in *Lim1* mutant mice, but they randomly select both the dorsal and the ventral limb compartment. Lim1 has been linked to regulation of EphA4, which mediates repulsion from the ephrin-A5-expressing region of the ventral limb. Individual motor columns contain motor pools which innervate the same muscle. Misexpression and knockdown studies with Hox genes such as *Hox5* and *Hoxc8* have shown that these factors control motor pool subtype identity and their altered expression influences axonal targeting.

Locomotor Circuitry: Dendrite Patterning and Synaptogenesis

Motor neuron cell bodies occupy specific positions and develop elaborate dendritic arbors for receiving input from presynaptic connections (**Figure 3**). Many classes of interneurons, corticospinal neurons, and sensory neurons synapse with motor neurons to control their activity; however, the mechanisms that control the precise pattern of input are just beginning to emerge. Individual motor pools display unique dendritic morphologies with regard to their branching pattern, size, and orientation. Genetic studies of *Pea3* mutant mice have found that the pattern of dendrite organization within specific motor pools requires the Ets transcription factor Pea3. After motor axons reach their target muscles, axons branch across the surface of the muscle and form neuromuscular synapses. Axons of specific motor pools display muscle-specific branching patterns, suggesting the presence of intrinsic branching programs. This is supported by the finding that *Pea3* mutants exhibit defects in the terminal arborization of motor neurons innervating the cutaneous maximus and latissimus dorsi muscles.

Motor neurons receive proprioceptive feedback input from Ia sensory neurons (**Figure 3**). This connectivity develops with incredible precision so that homologous motor and sensory neurons form monosynaptic connections. Interestingly, Ets factors Pea3 and Er81, which are selectively expressed in specific motor pools, are also present in subsets of sensory afferent neurons. Labeling studies have shown that there are preferential connections between sensory neurons expressing a particular Ets factor and the motor cells expressing the same factor. This finding suggests that Ets factors control multiple aspects of motor neuron maturation, including cell body position, dendritic pattern, and possibly Ia sensory input.

Conclusion

During the past decade, rapid progress has been made in defining the genetic pathways that control motor neuron subtype diversification. Characterization of the inductive signals and downstream transcription factors in motor neurons has provided great insight into the process of neuronal diversification. It seems likely that other neuronal populations will use similar DV and RC inductive cues and cross-repressive interactions to become diversified. Although our understanding is still fragmentary, the characterization of the factors involved in motor neuron subtype regulation is beginning to provide insight into the processes that control the development of locomotor circuitry.

See also: Anterior-Posterior Spinal Cord Patterning of the Motor Pool; Differentiation: the Cell Cycle Instead; *Drosophila* Apterous Neurons: from Stem Cell to Unique Neuron; Helix–Loop–Helix (bHLH) Proteins: Hes Family; Helix–Loop–Helix (bHLH) Proteins: Proneural; Terminal Differentiation: REST.

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Lissencephaly

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Introduction

The pattern of development of the human cerebral cortex is complex and leads to the unique diversity of cognitive functions that makes us human. Many advances have been made by studying the variations from the basic pattern of forebrain organization in different vertebrates. The mammalian cerebral cortex, a patterned structure, is divided into anatomically distinct and functionally specialized areas leading to a species-specific map. Mature cortical areas differ by their location within the cortex, molecular properties, histological organization, patterns of connectivity, and function. Within the neocortex, rostral regions regulate motor and executive functions whereas caudal regions process somatosensory, auditory, and visual inputs. These different cortical areas have a precise connectivity, particularly with nuclei within the dorsal thalamus, which provides some of the principal inputs to the cerebral cortex. Although many of these areas can be related to each other during vertebrate evolution and share common features of cortical development, many other aspects remain poorly understood. Proliferation, migration, differentiation, and survival of neuronal precursors have been modified during brain evolution to allow for the development of the highly organized primate neocortex. Whenever one of these mechanisms, and in particular neuronal migration, is disrupted or its delicate balance is perturbed, neocortical disorders may arise and lead to different forms of mental retardation or cognitive disabilities and severe epilepsy.

Lissencephaly (LIS) is one of the best characterized and studied human genetic malformations of the cerebral cortex (MCCs). The primary defect underlying the smoothening of the brain of lissencephalic patients (**Figure 1**) is defective neuronal migration. The inability of postmitotic neurons to reach their final destination and correctly populate the cortical plate of the cerebral cortex consequently leads to abnormal cortical thickness and reduced or absent gyri and sulci of its surface. Neuronal migration is driven by complex mechanisms that are orchestrated by many proteins that interact mainly, but not exclusively, to promote the recruitment, organization, stability, movement and function of microtubules. Scientific understanding of the molecular mechanism and the genetic interactions that form the basis of neuronal migration has been aided by the analysis of spontaneous and engineered mouse models, and the gene function of some of the key regulatory proteins has been elucidated. The ability to study the migration features of such mouse models has been greatly facilitated by the development of *in vivo* time-lapse microscopy methods for the imaging of migrating cells. Although our knowledge of neural migration and neocortical development has been partially clarified, the mechanisms and pathways controlling these processes are far from being fully elucidated, and many questions remain unanswered.

Human Genetic Malformations

The development of the cerebral cortex is characterized by three main steps: (1) proliferation and differentiation of the neuronal stem cells into neuroblasts and glia cells, (2) migration of neuronal precursors toward the cortical plate by either radial or tangential movements, and (3) cortical organization into six layers associated with synaptogenesis and apoptosis. These processes are dynamic and occur simultaneously during the different stages of development, and perturbation of these steps leads to MCCs and malfunction. Defects occurring during the early phases of proliferation are usually associated with altered differentiation of both neuroblasts and glia cell precursors, while defects affecting neuronal migration are characterized by malpositioning of neurons along the six layers of the developing cortex. The genetic study of cerebral cortical malformations in humans has identified several genes playing crucial roles during neocortical development. Malformations due to defective neuronal migration include periventricular nodular heterotopia, classical LIS, subcortical band heterotopia (SBH), and Zellweger syndrome. Malformations due to abnormal cortical organization, also called proliferation and patterning disorders, include polymicrogyria and schizencephaly. More-detailed information regarding MCCs may be found in recent reviews. Migration is not the only process responsible for MCCs; proliferation, maturation, and survival of neurons may be critical as well for a proper cortical development. Abnormalities of the brain associated with an impaired proliferation capacity of multipotent stem cells and neuronal precursors may be responsible for microcephaly, a condition characterized by a smaller size of the brain. Compound brain phenotypes may also occur, such as the case of microlissencephaly or the cerebral

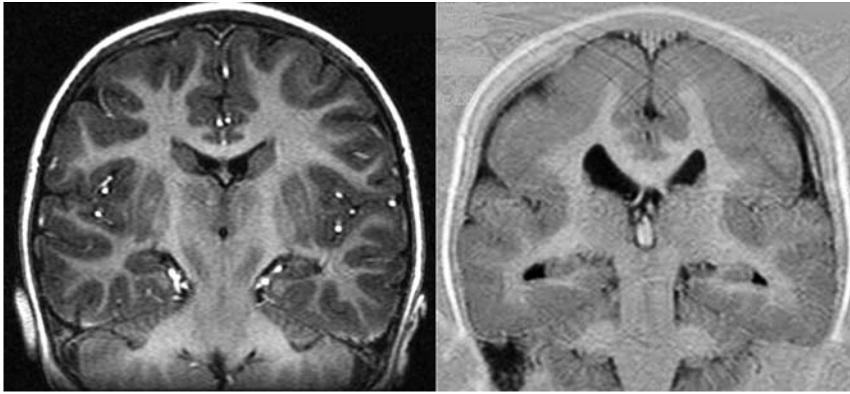

Figure 1 Brain MRI scan, coronal section passing through the hippocampus. The lissencephalic brain (right) shows diffuse cortical thickening (about 1 cm vs. 4 mm of the normal cortex, left) with simplified gyral pattern, especially in the brain convexity. The cortical sulci are shallow, and the number of convolutions is reduced. Courtesy of Dr. Renzo Guerrini, Department of Pediatric Neurosciences, Pediatric Hospital A Meyer and University of Florence, Firenze, Italy.

abnormalities associated with Neu–Laxova syndrome, in which cell survival seem to play and important role. MCCs may be associated with epilepsy that typically tends to be severe, although its incidence and type vary in different malformations; it has been estimated that $\leq$40% of children with drug-resistant epilepsy have a cortical malformation. Some of the causative genes for MCCs have been identified, but most remain unknown; only a dozen or so genes have been found causing epilepsy, and about 400 unbalanced chromosome rearrangements are associated with epilepsy syndromes, thus suggesting that a large number of dosage sensitive genes remain to be discovered.

Neuronal Migration

The mammalian neocortex is organized into six distinct cortical layers, each with distinct neuronal morphology and functions. The distribution of neuronal cells during cortical development occurs mainly by radial and tangential movements. In the neocortex, most neurons and glia arise from radial glia progenitor cells. Radial migration represents the predominant direction and type of movement for the positioning of most neurons in the neocortex departing from the ventricular zone (VZ), where postmitotic neuroblasts arise from radial glia progenitor cells and migrate toward the pial surface. This type of migration is characteristic of the dorsal telencephalon giving rise to the excitatory glutamatergic projection neurons of the cerebral cortex, which sequentially reach the different layers of the cortex. Tangential migration, on the other hand, occurs mainly from progenitors arising in the ventral telencephalon, generating γ-aminobutyric acid (GABA)ergic inhibitory neurons, including basal ganglia neurons, as well as interneurons that migrate tangentially to contribute to the formation of the cortex and to reach the olfactory bulb by following the rostral migratory stream. Cortical layering occurs in the mouse between days 11 and 18 during embryogenesis and in the human between 6 and 20–24 weeks. The generation of the six layers occurs in an inside-out fashion: early migrating neurons will populate the deeper level of the cortex, and later migrating neurons the more superficial ones. The first postmitotic neurons produced in the VZ migrate to form a subpial preplate or primitive plexiform zone. Subsequently, later migrating neurons will form the cortical plate by migrating into the preplate to split it into the superficial molecular layer (or layer I or marginal zone containing Cajal–Retzius neurons) and the deep subplate. The different waves of migrating neurons will pass the subplate and end their migration below layer I, forming cortical layers VI, V, IV, III, and II in an inside-out fashion. Radial migration occurs either by locomotion, in which neurons migrate along the processes of radial glia cells (progenitors guiding migration), or by nuclear translocation, in which migrating neurons extend their leading process toward outer levels of the cortex, to be followed by centrosome–nuclear movement. The two types of radial migration are dynamically different and occur at different times during neocortical development; moreover, recent observations suggest that neuronal migration displays different and complex patterns characterized by spatially and temporally distinct changes in the direction of movement and speed of migration for both pyramidal cells and interneurons.

Time lapse imaging studies using cortical slice cultures have demonstrated that neurons generated in the VZ at later stages of development pass through

a series of distinct stages of migration characterized by changes in cell shape, direction of movement, and speed of migration as they move to the cortical plate. According to these observations, radial migration does not occur directly to the cortical plate but is divided into four different phases. During phase 1, newly generated neurons at the ventricular surface move radially from the VZ to the subventricular zone (SVZ). At this point, phase 2, they pause at the border of the intermediate zone (IZ) with the SVZ and acquire a multipolar stage. This morphological change provides cells with a dynamic capability to change polarity, extending and retracting processes to move within the SVZ. Consequently, they do not appear to depend on radial glia fibers for their movement, as demonstrated by their ability to move tangentially. Most of the neurons go through a third phase characterized by a bipolar state, in which they extend processes toward the VZ, followed by retrograde translocation of the cell body. In the fourth phase, neurons reverse polarity and extend a pial-directed leading process, take on the bipolar morphology of migrating neurons, and begin radial migration to the cortical plate. Some neurons, however, after pausing in the SVZ, do not proceed through phase 3 with a retrograde motion toward the ventricle but instead progress directly from phase 2 to phase 4.

It is interesting that these four distinct phases of neuronal migration may be relevant to partially explain the generation of human migration disorders. For instance, in SBH a band of cortical neurons fails to migrate fully into the cortex and collects in the subcortical white matter. It is possible that these neurons fail to migrate because they are not able to make the transition from phase 2 to phase 3 of migration and therefore remain as immature multipolar neurons in the subcortical white matter. Similarly, periventricular nodular heterotopia may reflect a failure of neurons to transit from phase 3 to phase 4 of migration or inappropriate migration backward toward the ventricular surface. In this regard, *in utero* electroporation experiments indicate a cellular role of filamin A in exit from the multipolar stage; indeed, excess expression of the rodent filamin A or RNAi of Filip1 (which encodes a filamin A-binding protein that targets filamin for degradation) reduces the number of multipolar cells in the SVZ and IZ. By contrast, overexpression of Filip1 and the resulting decrease in Flna expression causes accumulation of cells in the multipolar stage. Filamin A is thus required for migration within the multipolar stage and for the transition into bipolar modes of migration. LIS1 knockdown (see the section titled 'LIS: clinical aspects and molecular genetics') causes accumulation in the VZ and SVZ of multipolar cells that have reduced capacity for migration. Doublecortin (DCX) knockdown in rats (see below) causes cells to accumulate in the multipolar stage in the IZ. Overexpression of DCX, by contrast, causes cells to adopt a highly bipolar morphology. Therefore, similar to FLNA, DCX loss of function and gain of function cause increases and decreases, respectively, in the number of cells in the multipolar stage. Overall, these observations suggest that the multipolar stage is a point of vulnerability to disruption that will require further investigation.

Molecular Pathways of Neuronal Migration

During the past decade, several molecules have been identified that participate in the control of neuronal migration, and several of the mechanisms driving neuronal movement have been partially clarified. The cloning of human disease genes and the study of genetically modified mouse models have helped in the understanding of the function of these gene products. Four main categories can be distinguished in triggering initiation, progression, modulation, and termination of neuronal migration. Filamin-A (an actin-binding protein) and Arfgef2 (or adenosine diphosphate–rybosylation factor GEF2 involved in vesicle trafficking) seem to play an important role in the initiation of migration, whereas DCX, a microtubule-associated protein (MAP), and LIS1, a MAP and dynein regulator, are involved in the regulation of its progression, and mutation of these genes results in slow or delayed migration. Other molecules (MAP1B, MAP2, and Tau) have been described and associated with migration defects, although not yet with human disorders. A second category includes proteins that play a role in lamination, such as reelin (a glycoprotein of the extracellular matrix), Dab1 (an adaptor protein), two reelin receptors (apolipoprotein E receptor 2 (ApoER2), and very low-density lipoprotein receptor (VLDLR)), p35 (an activator of cyclin-dependent kinase 5 (Cdk5), Cdk5 (a serine-serine-threonine kinase), and Brn1/Brn2 (transcriptional activators of Cdk5 and Dab1). Disruption of each of these proteins results in cortical layer inversion. A third category of molecules includes modulators of glycosylation that likely provide stop signals for migrating neurons. These molecules include protein O-mannosyltransferase, protein O-mannose b-1, 2-*N*-acetylglucosaminyltransferase, fukutin (a putative glycosytransferase), and focal adhesion kinase. The last group of molecules acts as modulators of neuronal migration and may be trophic factors such as brain-derived neurotrophic factor and thyroid hormones or neurotransmitters like glutamate and γ-aminobutyric acid.

One molecular pathway important for the progression of neuronal migration ultimately leads to the regulation of dynein motor function via LIS1. A plausible simplified model for this pathway has been proposed regulating nucleokinesis during neuronal migration through the regulation of dynein motor function (**Figure 2**). RELN binds to the VLDLR–ApoER2 complex to activate mDab1. Activated mDab1 associates with cAbl and activates cables. Cables has been identified as a potential link between the RELN pathway and Cdk5. Cables activates Cdk5 through interaction with cAbl and mDab1, bridging the RELN and Cdk5 pathways. Activated Cdk5 then phosphorylates NDEL1 (and perhaps NDE1), and P-NUDEL binds to 14-3-3ε to protect it from phosphatase attack. A LIS1–NudE homolog complex then positively regulates dynein motor function through direct association with CDHC and dynein LC. It appears that Cdk5, LIS1, NDEL1, NDE1, and 14-3-3ε regulate dynein motor function in a positive fashion, since decreasing the activity, decreasing the amount, or eliminating these proteins completely results in decreased dynein motor function and mislocalization of dynein components and centrosomal proteins. Thus, the apparent function of the LIS1 pathway is to activate dynein motors and place them in the proper cellular location for function. This model is undoubtedly an oversimplification of what is likely to be a process that is regulated in a highly ordered and complicated fashion. There may be many branch points to and from this basic backbone, and much of the cross-talk between pathways remains to be identified.

LIS: Clinical Aspects and Molecular Genetics

Classical LIS, or 'smooth brain,' represents a pathological condition of the brain characterized by a simplified pattern or the absence of the normal pattern of

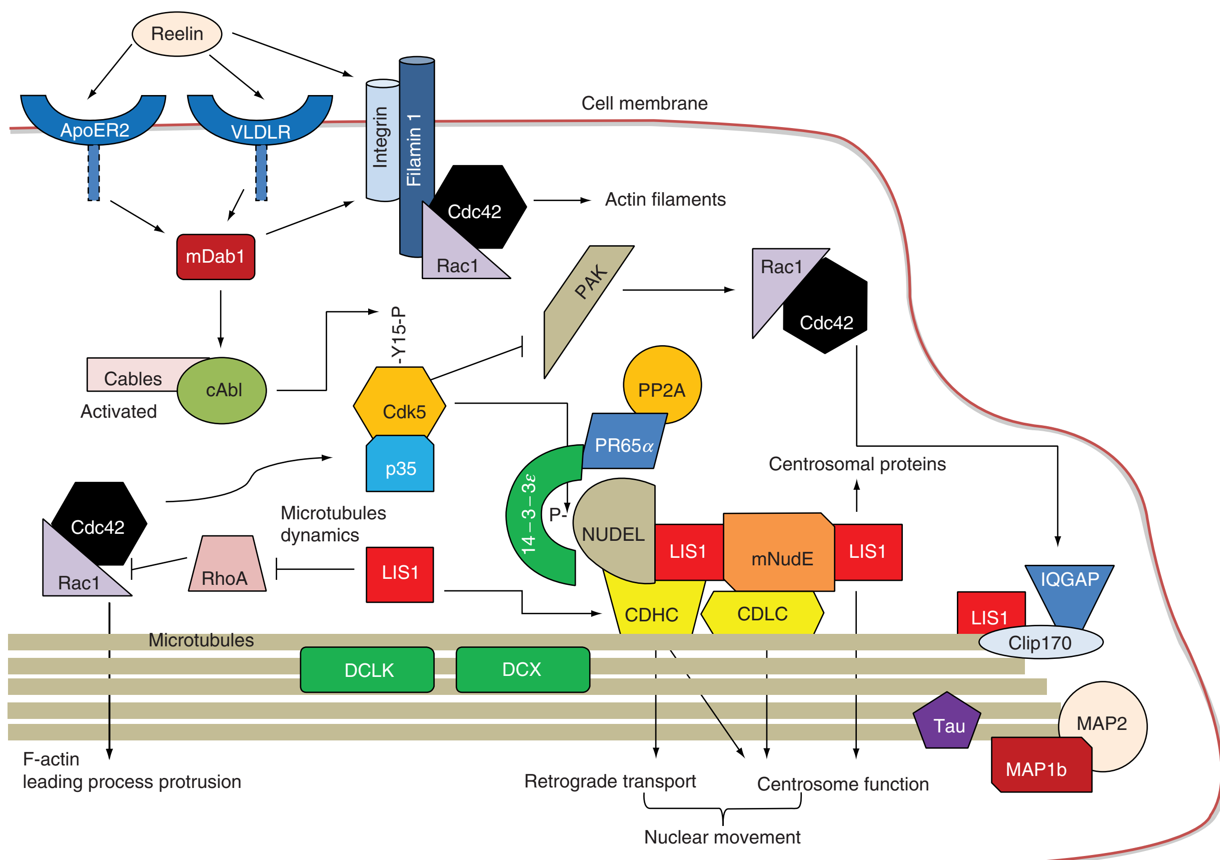

Figure 2 A simplified molecular pathway of the regulation of neuronal migration. The disruption of both actin filaments and microtubule dynamics can inhibit neuronal migration. The molecular cascade is initiated by the binding of reelin to its receptors. The stabilization of microtubules via mitogen-activated proteins (MAPs) and the activation of the dynein motor complex play a critical role in the nucleus–centrosome coupling and nucleokinesis. See text for details. ApoER2, apolipoprotein E receptor 2; Cables, a potential link between the RELN pathway and Cdk5; Cdk5, cyclin-dependent kinase 5; Dab1, an adaptor protein; DCX, doublecortin; CDHC, cytoplasmic dynein heavy chain; CDLC, cytoplasmic dynein light chain; Cdc42, cell division cycle 42; Rho, Rho family GTPase; PP2A, protein phosphatase 2A; VLDLR, very low-density lipoprotein receptor.

gyri and sulci, resulting in a reduction of the cortical surface with abnormal thickness and layer organization (**Figure 1**). This disorder affects about one in 40 000 individuals and is inherited as either an autosomal or an X-linked dominant disorder. Typically, LIS, diagnosed through magnetic resonance imaging, is associated with severe mental retardation and intractable epilepsy that consequently lead to a shortened life span. Syndromes related to LIS include Miller–Dieker syndrome (MDS; a severe form of LIS); double cortex syndrome, or SBH (a less severe condition); and LIS with cerebellar hypoplasia, polymicrogyria, and cobblestone with associated congenital muscular dystrophy and eye disease. In LIS, neurons migrate only partially toward their proper cortical destination so that in the mature cortex, gyri and sulci fail to form. SBH is a disorder in which bilateral bands of grey matter are interposed in the white matter between the cortex and the lateral ventricles. Two major forms of LIS have been differentiated, classical LIS (type I) and cobblestone (type II), although careful delineation of syndromes has revealed multiple forms of LIS.

Two genes have been associated with classical LIS and SBH. The first gene, LIS1 (Online Mendelian Inheritance in Man (OMIM) database number 601545), on chromosome 17p13.3, is responsible for the autosomal form of LIS, while the second gene, DCX (or XLIS, OMIM 300067), is X-linked. Although mutations in either gene can result in either LIS or SBH, the majority of cases of classical LIS are due to deletions or mutations of the LIS1 gene, whereas the majority of cases of SBH are due to mutations of the DCX gene. The most important characteristics of LIS in patients with LIS1 mutations are the very thick (10–20 mm) cortex, gyral malformations that are more severe in posterior than anterior brain regions, and a prominent cell-sparse zone in the cortex, whereas DCX mutations result in LIS more severe in anterior brain regions.

Mouse knockouts for *Lis1* with graded reduction of the protein level displayed a severe developmental brain disorder as the result of an abnormal neuronal migration. Mice with 50% of LIS1 protein displayed cortical, hippocampal, and olfactory bulb disorganization resulting from delayed neuronal migration. Mice with further reduction (35% of LIS1 protein) displayed a more severe brain disorganization, as well as cerebellar defects. These results suggested that *Lis1* has an essential and dosage-sensitive role in neuronal migration during brain development. Reduction of *Lis1* levels has been shown to have a pleiotrophic effect involving interkinetic nuclear migration, neuroblast proliferation, and survival.

Patients with LIS and deletions of 17p13.3 (MDS) have more-severe LIS than do patients with isolated LIS sequence (ILS) in addition to facial abnormalities, thus suggesting that at least a second gene in the deleted region may contribute to the migration defects during cortical development. MDS is always associated with LIS grade 1 (essentially complete agyria), while ILS has been associated with a more variable LIS phenotype ranging from grade 2 to grade 4, in which pachygyria is most frequently observed. The definition of the deleted region in MDS patients (about 400 kb) suggested that 14-3-3ε was the best candidate gene contributing to the severity of phenotype. Indeed, mouse knockouts for *14-3-3*ε displayed abnormal cortical development due to a reduced capacity of neurons to migrate and populate the upper level of the cortex. In addition, double heterozygote mouse mutants for *Lis1* and *14-3-3*ε displayed increased severity of cortical migration defects and hippocampal disorganization compared with the single heterozygotes. The finding of slower migration in the cortex and hippocampus of double heterozygotes, compared with single heterozygotes and wild-type mice, strongly suggested a genetic interaction between *Lis1* and *14-3-3*ε during brain development and neuronal migration, thus partially clarifying the phenotype differences between MDS and ILS patients.

LIS1 encodes a protein that is similar to the β subunit of heterotrimeric G-proteins and has two functions: a regulatory subunit of platelet-activating factor acetylhydrolase (PAF-AH), an enzyme that degrades the bioactive lipid PAF, and a dynein regulator. The importance of LIS1 as part of PAF-AH in neuronal migration is unclear. LIS1 protein has been shown to co-localize with microtubules and to promote their stabilization, and an ortholog of LIS1 in *Aspergillus nidulans*, nudF, mediates nuclear translocation by interacting with microtubules and dynein. Thus, LIS1 exerts its effects on migration through regulating dynein function and microtubule stability. The gene is expressed in neural progenitors within the VZ as well as in differentiated neurons, suggesting a function during multiple stages of neural development. The interaction between LIS1 and dynein is critical for the binding of microtubules to the cell cortex and generation of cellular machinery for neuronal division. LIS1 binds cytoplasmic dynein and dynactin and localizes to the cell cortex and to mitotic kinetochores. It has been demonstrated that the perturbation of LIS1 in cultured cells interferes with mitotic progression and leads to spindle disorientation. The use of *in utero* electroporation of *Lis1* small interfering RNA and dominant-negative forms of LIS1 or dynactin allowed the first real-time imaging of cortical cell progenitors lacking LIS1 or dynactin function. These experiments resulted in the perturbation of the progression of neural progenitors through

the cell cycle in the VZ, causing a reduction of proliferation and the block of postmitotic precursors through the SVZ, leading to the accumulation of multipolar progenitor cells within the SVZ. Additional insights into the role of LIS1 in cortical development are coming from the analysis of human neural precursors isolated from a 33-week postmortem fetus with MDS. This study provides evidence of disruption in cell proliferation, in addition to migration, and suggests that this process might have an important role in the development of LIS.

DCX is a MAP that stabilizes actively polymerized microtubules and is expressed in newly postmitotic migrating neurons in both the central and the peripheral nervous systems. *Dcx* heterozygote and hemizygote knockout mice showed remarkably normal overall brain morphology, with six layers of the cerebral cortex. In contrast to the normal cerebral cortex, however, the hippocampal formation was malformed, affecting the CA fields in both genotypes. The anatomical abnormalities of the hippocampus were shown to be associated with learning and memory deficits. The central role of DCX in neuronal migration, through the organization and stability of microtubules, and the previous finding of LIS1 effects on microtubules, suggest that microtubule regulation is a key component of neuronal migration in the cerebral cortex. In fact, manipulation studies of LIS1, DCX, dynein, and microtubules have demonstrated that LIS1 and DCX mediate nucleus-centrosome coupling in migrating neurons through dynein, and overexpression of either one enhances migration and rescues the reduced nucleus-centrosome distances. Recent data have shown that DCX is also expressed in tangentially migrating neuroblasts in the rostral migratory stream and that it is an important regulator of both radial and tangential migration. Moreover, DCX hemizygosity was responsible for a branched phenotype in neurons that failed to migrate, thus suggesting an additional role of DCX in maintaining the bipolar morphology of migrating neurons, together with its involvement in nuclear translocation.

Conclusion

In the past 10 years, major progress has been made in the diagnostic recognition of MCCs, especially through the use of magnetic resonance imaging. Variations in distribution and depth of cortical sulci, cortical thickness, boundaries between grey and white matter, and signal intensity allow recognition of different malformation patterns. Sophisticated brain imaging techniques in the emerging field of behavioral neurogenetics allow the precise identification of these malformative patterns, greatly contributing to the selection of patients with similar or identical phenotypes. In turn, the improved selection of patients facilitates the analysis and the discovery of the genetic causes for these conditions. The study of patients with LIS has provided an entry point to the molecular pathways governing neocortical development and neuronal migration in particular. Although the basic mechanisms driving the movement of neurons are becoming clearer, many questions remain to be answered, and most likely other pathways remain to be uncovered. Migration is obviously the driving major force for cortical organization, but the importance of proliferation of neuronal precursors and the survival of postmitotic neurons are events that need to be further investigated. One possibility for improving the body of knowledge is to further investigate the molecules or pathways and binding partners that have already been discovered by means of either transgenic mice or sophisticated *in vivo* technologies. An alternative approach would be to identify new candidate genes with positional cloning methods by screening patients with cortical abnormalities and testing these genes with functional tools. Techniques such as RNA interference and the *in vivo* imaging of neurons using slice cultures will be useful for testing new genes and potentially detecting novel players on the migration pathways.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Neural Patterning: Arealization of the Cortex; Neurulation.

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Relevant Website

http://www.ncbi.nlm.nih.gov/ – OMIM – Online Mendelian Inheritance in Man.

Molecular Anatomy of the Mammalian Brain

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Introduction

Given the heterogeneity of the brain, the large numbers of genes encoded in the genomes of higher mammals, and the complexity of processes such as behavior, learning, and memory, how is it possible to identify the genes that regulate these phenotypes? How can we use genetic and genomic information to discover targets for therapeutic intervention for brain diseases? As genomic technologies such as DNA microarrays and high-throughput *in situ* hybridizations have become more commonplace, they have led to significant advances in the study of the molecular anatomy of the mouse, human, and primate brain. Researchers have utilized these data to identify genes associated with specific brain functions, behaviors, and disease-related phenotypes. Technologies, experimental design, and data analysis methods have improved so that it is now possible to reproducibly measure gene expression levels in different regions, nuclei, and even single cells of the brain, and to reliably detect very subtle expression differences under specific conditions.

An important goal in neuroscience is the development of a combined high-resolution map of the molecular, genetic, and physical anatomy of the brain, and this goal, although challenging, is now technically feasible. The purpose of obtaining such a map and of developing the tools to navigate is to provide a more complete context for neurobiological studies in much the same way that the genome sequence provides a genetic context for the study of biology. But like sequence information, such a map is more than just a reference text and a trail guide; it is also a discovery tool which will help neuroscientists to formulate and test hypotheses about the brain, speeding the identification of genes important for brain function and helping to understand the molecular bases of brain processes such as learning and memory and the sources of central nervous system (CNS) diseases.

Integrating Genomic Technologies with Neuroanatomy

The most widely used techniques for studying gene expression levels are *in situ* hybridization, DNA microarrays, quantitative real-time polymerase chain reaction (PCR), and Northern blots. In this article we discuss the use of *in situ* hybridization and microarrays, as these have been the methods of choice in the construction of molecular brain atlases. Both of these methods have their advantages and disadvantages. *In situ* hybridization provides an anatomical context for the gene expression, as it is based on hybridization of a probe complementary to the mRNA of interest on a section of the brain. The disadvantages of *in situ* hybridization are that the method is not very sensitive to small differences in expression levels when two samples are compared with each other, and that the anatomical signal is usually restricted to the nucleus of the cell. This latter problem has been solved in the case of bacterial artificial chromosome (BAC) transgenic mice (see later). Another problem with *in situ* hybridization is that usually only one gene is studied at a time and therefore analysis of large numbers of genes is time consuming, although the method can be automated to a certain extent, as in the case of the Allen Brain Atlas (ABA). The major advantage of microarrays is that expression levels of virtually the whole transcriptome can be studied at the same time. However, the anatomical resolution is dependent on the size of the tissue from which the studied RNA is extracted. Even though microarrays provide better sensitivity than *in situ* hybridization does, very small differences (<20%) in expression levels of the two samples are difficult to distinguish, which might be a problem in the brain, where even subtle changes may have dramatic effects on the phenotype.

Microarrays

Despite a great deal of initial skepticism, it is now clear that genomic approaches, such as parallel expression profiling with DNA microarrays, can be used to help elucidate the workings of the brain at the molecular level. Some of the early skepticism concerning the application of genomic techniques to neuroscience was not wholly unwarranted, however, as the study of the brain brings a variety of somewhat unique and formidable technical challenges. Studies of the nervous system are complicated by the fact that the brain is very heterogeneous, the cells of greatest interest might comprise only a small fraction of the entire tissue, high-quality material can be hard to obtain, the appropriate anatomical divisions between regions are not always clear, dissections may be inconsistent, some anatomical and functional regions are extremely small, cell bodies and processes (axons and dendrites) are often located in different parts of the brain, biologically important expression changes

may be very subtle, and the expression profiles are dynamic and subject to change in response to even minimal perturbations. Nevertheless, there have been great strides made in performing genome-wide gene expression studies in the brain, as detailed in the following sections.

In situ Hybridization

An important step in the process of developing a molecular map of the mammalian brain is also the acquisition of high-quality *in situ* hybridization patterns of genes expressed in the brain. *In situ* hybridization provides an in-depth picture of the neuroanatomical gene expression patterns for brain specific genes. These key brain-specific genes can be used to anchor large-scale microarray gene expression studies in an anatomical context. *In situ* hybridization provides information about the distribution of cells expressing a given gene in a given region (scattered vs. coherent; small population expressing at high levels vs. large population expressing at lower levels), as well as the relationship between gene expression boundaries and neuroanatomical boundaries that cannot be extracted easily from microarray data. By integrating microarray data with *in situ* hybridization data, a fuller picture of the molecular anatomy of the brain is developed. Cross-correlation of *in situ* hybridization patterns with microarray data for a relatively small number of genes makes it possible to identify combinations of microarray parameters that usefully predict whether a given gene is likely to be (1) detectable by *in situ* hybridization and (2) specifically expressed in one or more brain regions.

Brain Tissue Collection Methods

Dissection

Key to the molecular mapping effort in the brain are high-quality dissections that preserve mRNA intact. There are distinct advantages to working with mice as model organisms for the molecular mapping of the mammalian brain, as opposed to humans or other mammals. For example, the handling of the animals and the tissue dissections can be performed systematically and consistently. This is extremely important in order to minimize sample-to-sample variations. In our laboratory, all mice are singly housed for 7 days prior to sacrifice, and the dissections are carried out at specified hours of the day. Dissections are performed on the surface of petri dishes filled with wet ice. Samples are dissected as fast as possible and frozen on dry ice. For microarray studies, pooling of tissue from several animals is to be avoided whenever possible because dissection artifacts from one sample in a pool will invalidate the results of the entire pool. If dissecting small brain regions, such as the amygdala or cortical subregions, we either pool samples from two to five animals and collect them in RNAlater buffer (Ambion) or we use methods for small-sample amplification that do not require pooling.

For microarray studies, we perform many brain dissections by hand using a sophisticated dissection microscope and very fine dissection tools. It is important to dissect the exact anatomical structure of interest. Inclusion of too much additional material might 'dilute' a weak expression difference seen between two regions, and failure of including all small subnuclei might lead to missing important differences. Laser-capture microdissection (LCM) is a method for producing reasonably pure populations of targeted cells from small regions of tissue sections that cannot be obtained otherwise. LCM uses a specially designed microscope and an integrated laser to select and collect cells onto a special transfer film. It has been shown that the detailed morphology of the captured cells is maintained and that DNA and RNA of high quality can be extracted from laser-captured cells and used for a range of different analyses, including gene expression monitoring on DNA microarrays. It is important to use stringent quality control of extracted total RNA. This can be achieved by checking RNA quality on an agarose gel and by an absorption measurement or by using a bioanalyzer. It is clear that gene expression profiling of brain tissue from humans or model organisms is feasible if the special nature of the brain tissue is taken into account and rigorous quality control is performed.

Voxelation

With the advent of sophisticated imaging techniques, such as functional magnetic resonance imaging (fMRI), it is now possible to start to integrate brain imaging technologies with molecular and genetic data. To complement the aforementioned dissection strategies, a voxelation approach has been implemented which allows for microarray analysis of spatially registered voxels, or cubes, taken from the brain. Voxelation creates volumetric maps of gene expression similar to imaging techniques such as fMRI, computed tomography (CT), or positron emission tomography (PET). Voxelation has been performed in both human and mouse studies on Alzheimer's and Parkinson's disease brains. The voxelation approach can lead to volumetric resolution in the human brain from 1 cm^3 to as low as 87 μl and in the mouse brain from 7.5 μl to as low as 1 μl. As the mammalian brain is composed of greater than 700 anatomically distinct major regions and an even larger number of named subregions, hand dissection and LCM currently

remains an impractical high-throughput approach for mapping of the entire mammalian brain. The voxelation approach allows for high-throughput analysis of specific brain areas without dissection biases through the use of mathematical analysis techniques such as singular value decomposition. However, voxelation suffers information loss from what is known as 'voxel inhomogeneity,' which is the dilution or averaging of gene expression signals in any one voxel from various brain regions and cell types. Combining gene expression data from brain regions collected by voxelation, hand dissection, and LCM with *in situ* hybridization data will advance the coverage and resolution of the mammalian molecular brain maps.

Current Large-Scale Projects

Gene Expression Nervous System Atlas

The Gene Expression Nervous System Atlas (GENSAT) BAC transgenic project is a National Institute of Neurological Disorders and Stroke (NINDS)-supported two-stage approach that combines high-throughput *in situ* hybridization screening and the use of BAC transgenic reporter gene analysis. The principal investigators of the study are Nathaniel Heintz and Mary E Hatten, professors at the Rockefeller University. The project is designed to use enhanced green fluorescent protein (EGFP) reporter genes both to map gene expression in the developing and adult brain and to provide experimental access to CNS cell populations. The aim is to identify and map the expression of ~5000 of the most important CNS-expressed genes throughout development. First, a prescreen is done by *in situ* hybridization to identify the most interesting genes, and second, the chosen genes are analyzed at high resolution using BAC transgenic mice. This two-stage approach takes advantage of the sensitivity, dynamic range, and efficiency of *in situ* hybridization methodology to allow parallel analysis of large numbers of CNS-expressed genes, while exploiting BAC reporter gene technology to allow systematic analysis and high-resolution visualization of each cell type expressing a gene of interest. The data generated by the project are available in the GENSAT database, which contains a gene expression atlas of the CNS of the mouse based on BACs (see **Figure 1**). The technology is based on gene replacement, whereby endogenous protein-coding sequences of the studied genes have been replaced by sequences encoding the EGFP reporter gene. Thus, all the cells that would normally express the gene of interest now express the EGFP reporter gene, which can be easily visualized directly or indirectly by immunohistochemistry. This allows the visualization of gene expression patterns in the CNS (and other tissues). The GENSAT database contains histological data from given BAC transgenic mouse lines at three developmental stages: embryonic day 15.5, postnatal day 7, and adult. EGFP is visualized by staining with an anti-EGFP antibody using the diaminobenzidine (DAB) method, or by confocal microscopy of unstained tissue sections. Discoveries in several categories of developmental neurobiology can be made with the dataset, including (1) identification

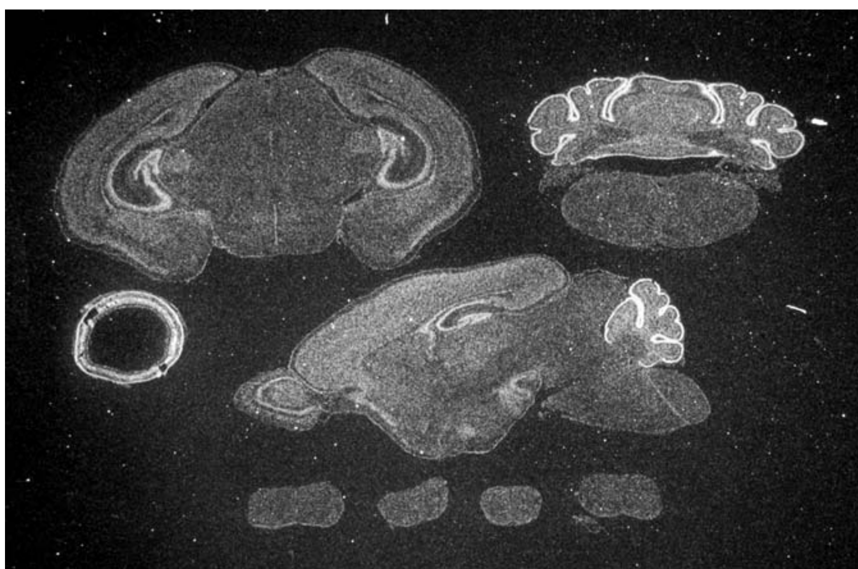

Figure 1 *In situ* hybridization darkfield images of a sagittal section close to the midline and coronal sections of a C57BL/6J postnatal day 7 mouse brain, showing mRNA distribution of the ataxia telangiectasia mutated homolog (*ATM*) gene from GENSAT.

of genes that mark specific regions of the brain, (2) identification of genes that mark specific cell types, and (3) identification of genes that reveal patterns of axonal connections in the emerging neural circuitry of the immature CNS.

Brain Gene Expression Map

The Brain Gene Expression Map is focused on the mouse brain and is housed at St. Jude Children's Research Hospital in Memphis, Tennessee and is associated with GENSAT. This database contains gene expression patterns from mouse nervous system tissues at four time points throughout brain development, including embryonic day 11.5, embryonic day 15.5, postnatal day 7, and adult day 42. Using high-throughput *in situ* hybridization, there are gene expression patterns from approximately 2850 genes, available in a searchable database. The database includes darkfield images from radioactive probes, reference cresyl violet-stained sections, the complete nucleotide sequence of the probes used to generate the data, and all the information required to allow users to repeat and extend the analyses. The database is linked to PubMed, LocusLink, Unigene, and the Gene Ontology Consortium housed at the National Center for Biotechnology Information (NCBI) in the National Library of Medicine in Bethesda, Maryland.

Allen Brain Atlas

The ABA is an interactive, genome-wide image database of gene expression in the mouse brain. It is based on automated high-throughput *in situ* hybridization of adult 8-week-old male C57BL/6J mouse brains. The platform used for the ABA utilizes a nonisotopic approach, with digoxigenin-labeled nucleotides incorporated into a riboprobe produced by *in vitro* transcription. Colorimetric detection of bound probe is generated by the alkaline phosphatase substrates nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP), which produce a blue/purple particulate reaction product that allows cellular localization of the signal. In addition to the *in situ* hybridization data, the project is generating reference atlases of the mouse brain that include both coronal and sagittal planes. This Nissl-stained reference facilitates annotation of the *in situ* hybridization data in the anatomical context. In order to view the *in situ* hybridization data, the project has created a web-based tool that allows searching by gene name and visualizing the corresponding *in situ* hybridization data.

Brain Gene Expression Database

The Brain Gene Expression Database contains gene expression data for various physiological and pathological processes in the mouse brain. All of the data were obtained by adaptor-tagged competitive PCR, an advanced version of quantitative PCR. Assayed genes were selected from the Brain Expressed Sequence Tag (EST) Database containing 3656 genes. RNA samples were taken under unique biological conditions using the C57BL/6J inbred strain. The datasets include postnatal cerebellar and dentate gyrus development at postnatal days 2, 4, 8, 12, and 21, and week 6. The following hippocampal regions are also available at 6 weeks: CA1, CA2, and CA3. The database also analyzes several physiological and pathological processes, such as the effects of ischemia on gene expression in the hippocampus, aging in the whole brain, and antipsychotic drugs (haloperidol and chlozapine) in the frontal lobe.

Microarray-Based Brain Gene Expression Databases

TeraGenomics This database contains microarray gene expression patterns for 24 neural tissues covering the mouse CNS measured in three inbred mouse strains (C57BL/6J, 129S6/SvEvTac, and DBA/2J) using the Affymetrix U74Av2 array. The complete collection of extensively annotated gene expression data along with data mining, visualization, and filtering tools has been made available on a publicly accessible website. A large-scale database, called TeraGenomics, was built to house and provide access to all of the quantitative, brain region-specific gene expression data, along with quality control measures and metadata in accordance with the Minimal Information About a Microarray Experiment (MIAME) standard. The body of metadata contains 75 different sample annotation fields, which include information on sample origin, dissection protocols, sample preparation, and array hybridization parameters. In addition, the anatomical hierarchy of the Neuronames taxonomy has been included as a user-friendly query tool within the database. A reference document for each dissection was created, consisting of photographs, atlas figures, and bregma coordinates of each collected brain region along with step-by-step instructions to illustrate the exact methods used in tissue collection.

SymAtlas The Genomics Institute of the Novartis Research Foundation (GNF) has a publicly available microarray database composed of expression profiles for 79 human tissues (20 brain regions), 61 mouse tissues (10 brain regions), and approximately 15 rat brain structures in as many as four different rat strains. These human, mouse, and rat gene expression atlases are available online and can be accessed through the SymAtlas web application. The web application provides searching and visualization by

Table 1 Publicly available brain atlases

Group	*Online address*
Gene Expression Nervous System Atlas (GENSAT)	http://www.ncbi.nlm.nih.gov/projects/gensat/
Allen Brain Atlas (ABA)	http://www.brain-map.org
Brain Gene Expression Map	http://www.stjudebgem.org
Brain Gene Expression Database	http://genome.mc.pref.osaka.jp/BGED/
TeraGenomics	http://www.barlow-lockhartbrainmapnimhgrant.org/
SymAtlas	http://symatlas.gnf.org/SymAtlas/

keyword, accession number, gene symbol, genome interval, sequence, and expression pattern. Queries can be partitioned by expression location and fold change above or below the median to assist in identifying genes with similar expression patterns. All of the Affymetrix datasets are also available for download. **Table 1** displays online addresses of these large-scale gene-expression-based brain atlases.

Molecular Relationships within the Mammalian Brain

Using the aforementioned resources and tools, we and other neuroscientists have set out to understand how regional gene expression patterns in the brain are related to anatomical brain architecture and organization, and to identify relationships between brain regions based on both shared and restricted gene expression patterns. The existing framework to organize the mammalian nervous system is based on studies of anatomy, embryology, and evolution. It is now possible to begin to understand the mammalian brain from a molecular viewpoint.

In developmental biology, gene expression patterns for small groups of genes have been used to identify particular brain regions during embryogenesis. Dynamic changes in expression patterns have also been used to understand functional relationships between brain regions during development. Distinct neural cell precursors form at different positions along the anteroposterior (AP) and dorsoventral (DV) axes of the developing neural epithelium, which is critical for the establishment of a structured pattern of differentiated neurons. The concept of an established genetic pattern during embryogenesis has been strengthened by the expression of genes that mark morphogenetic fields during brain development, such as homeobox transcription factor genes. Spatially restricted expression of transcription factors specifies regional identity in the developing nervous system. The expression of these transcription factors underlies a crucial stage in neuronal patterning: the partitioning of the neural epithelium into domains with distinct identities that later form different groups of neural cell types.

The role of transcription factors in the developing mouse CNS has been studied using *in situ* hybridization. As mentioned previously, transcription factors are known to play a critical role in brain development by directing the formation of neurons and glia from progenitor cells. The aim of a recent study was to identify transcription factor gene expression patterns that are spatially and temporally restricted within the brain. Gene expression of 1174 transcription factors was visualized at embryonic days 10.5 and 13.5 and postnatal day 0, as well as on sections through the postnatal cerebellum at days 7, 15, and 21. The *in situ* hybridization data showed that gross anatomical diversity based on seven general areas within the CNS could be described by the expression of specific transcription factors in specific brain areas. These seven areas included the cortex, striatum, thalamus, hypothalamus, midbrain, hindbrain, and spinal cord. This study focused on embryogenesis and found that transcription factors known to play a role in developmental patterning, such as homeobox and forkhead genes, had the most restricted expression and were the most informative about anatomical diversity within the brain.

Whole-genome microarray expression data now suggest that the imprinted genetic program established during embryogenesis is still evident in the mature brain. Using gene expression patterns measured from 24 spatially registered (based on bregma coordinates) neural tissues covering the mouse CNS, it was shown that the adult brain demonstrates a transcriptional pattern consistent with where those tissues were derived embryologically. In addition to this imprinted genetic program, significant numbers of embryonic patterning and homeobox genes with region-specific expression in the adult nervous system were observed (see **Figure 2**). Several studies of the developing brain have demonstrated that similar sets of embryonic patterning and homeobox genes are used to establish a particular anatomical region and to maintain the cell–cell relationships of the differentiated region. The global gene expression pattern relationships between different anatomical regions and the nature of the observed region-specific genes suggest that the adult brain retains a degree of overall gene expression

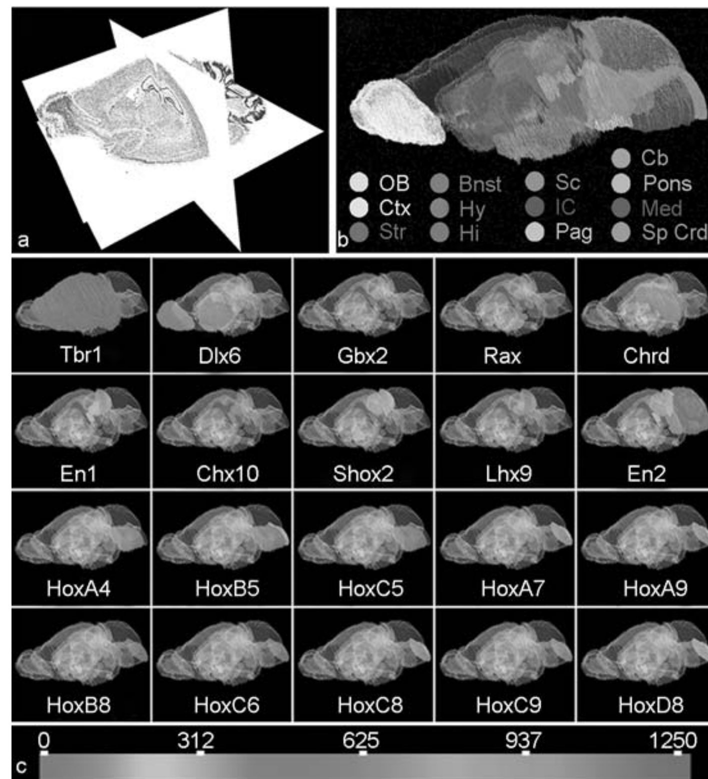

Figure 2 Genes with region-specific expression patterns function in development, pattern specification, and morphogenesis. (a) Reference brain atlas displayed in the three orthogonal planes. This atlas of Nissl-stained C57BL/6N mouse brain comprises 462 coronal sections at 30 μm thickness, digitized at a resolution of 1.3 μm per pixel. The sagittal and horizontal planes are 'virtual' sections dynamically constructed from the coronal sections. (b) Three-dimensional atlas of brain regions. Specific brain regions along the rostrocaudal neuraxis are color coded. OB, olfactory bulb; Ctx, cerebral cortex; Str, striatum; Bnst, bed nucleus of stria terminalis; Hy hypothalamus; Hi, hippocampus; SC, superior colliculus; IC, inferior colliculus; Pag, periaqueductal gray matter; Cb, cerebellum; Med, medulla; Sp Crd, spinal cord. (c) The expression levels of some of the homeobox and other embryonic patterning genes expressed in the adult mouse brain are digitally represented for each region. Adapted from Zapala MA, Hovatta I, Ellison JA, et al. (2005) Adult mouse brain gene expression patterns bear an embryologic imprint. *Proceedings of the National Academy of Sciences of the United States of America* 19: 10357–10362, with permission.

patterning, established during embryogenesis, that is important for regional specificity and for the functional relationships between brain regions in the adult.

There have also been smaller scale studies to investigate the molecular architecture of specific brain nuclei in the mouse. In a previous regional analysis of the amygdala, *in situ* hybridization had revealed that the majority of the genes specifically expressed in the amygdala displayed intra-amygdaloid expression boundaries corresponding to neuroanatomically defined subnuclei. Microarray data further confirmed these findings in that the gene expression patterns of specific amygdaloid nuclei were found to respect the ontogenetic origins of the subnuclei which derive embryologically from both pallial and subpallial structures. Similarly, the study of specific hippocampal subregions (CA1, CA2, and dentate gyrus) found that the relative enrichment and absence of genes in the hippocampal subregions support the conclusion that there is a molecular basis for the previously defined subregions, and these genes could define unique functions of these hippocampal subregions.

It has also become possible to examine anatomically distinct regional brain gene expression patterns in humans. For example, gene expression differences in the cerebellum and in the cortical areas in humans have been studied. Functional classification using gene ontology tools identified functional gene families enriched in the cerebellum and cerebral cortex. Genes with an enriched cortical gene expression pattern belong to categories such as 'calmodulin binding,' 'brain development,' 'receptor protein kinase,' and 'peptide hormones.' Several genes identified as being specifically enriched in the cortical areas have been previously implicated in psychiatric disorders, such as regulator of G-protein signaling 4 in schizophrenia; neuropeptide Y in bipolar disorder; cholecystokinin in depression; somatostatin in mania, schizophrenia,

and Alzheimer's disease; and 5-hydroxytryptamine receptor 2A in major depression and suicide.

Conclusions

There are several ongoing projects which have generated high-quality, spatially specific brain gene expression datasets. These molecular mapping efforts have provided invaluable information about specific gene expression patterns in anatomically distinct brain nuclei. They form a basis for other studies that aim to discover previously unknown functional elements in the brain, such as circuits involved in clinically important phenotypes. It is now possible to begin integrating and leveraging these unique data repositories to gain new insights into the molecular underpinnings of complex brain functions. Thus, it appears that not only are we closer to understanding the molecular anatomy of the mammalian brain, but we are also poised to begin to comprehend how it regulates complex behaviors or how disturbances in brain function manifest directly as specific neuropathologies.

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Terminal Differentiation: REST

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Introduction

In 1990, Gail Mandel and David Anderson independently reported the first data suggesting that expression of neuron-specific genes might be under the control of *cis*-acting negative regulator elements within these genes. These insights quickly led to the discovery in 1992 of the sequence of such an element, which was called RE1 by Mandel and neuron-restrictive silencer element (NRSE) by Anderson. Since NRSE/RE1-like sequences were found in at least three genes specifically expressed by neuronal cell types, it was immediately postulated that NRSE/RE1-dependent silencing might be a general mechanism to silence neuronal genes in nonneuronal cell types, mediated by a *trans*-acting silencing factor expressed in nonneuronal cells but not in neuronal cells. Indeed, separate cloning efforts by both groups, published in 1995, led to the identification of this silencer factor as the RE1-silencing transcription factor (REST) or neuron-restrictive silencer factor (NRSF). As had been hypothesized, REST/NRSF expression was observed to be generally high in nonneuronal cells types, with concomitantly low levels of transcripts of genes with an NRSE/RE1 element, and vice versa.

Although REST/NRSF's initially proposed function as a silencer of a large set of neuronally enriched genes has acquired widespread acceptance, evidence has accumulated in the past few years indicating that REST/NRSF plays additional, much more dynamic roles in normal development and adult brain function, as well as in disease. This view is supported by the discovery of a large network of REST/NRSF targets that includes many genes not exclusively expressed in neurons.

In this article, we review the architecture of the REST/NRSF gene and protein, including regulation of REST/NRSF expression, and present the current knowledge of its gene targets. In our discussion of its role in the developing and adult organism, we also briefly touch upon REST/NRSF regulation in neurological disease and cancer. Lastly, because the control of target gene expression by REST/NRSF is critically dependent on the action of its protein interaction partners, we also provide insight into REST/NRSF-recruited cofactors and how these are thought to modulate the function of this transcription factor.

REST/NRSF: Gene Organization, Alternative Splicing, and Protein Structure

The REST/NRSF gene and its exon structure are evolutionarily conserved from human to fugu (pufferfish). The gene is not found in flies or nematodes, suggesting that REST/NRSF is specific to the vertebrate lineage. It consists of three alternative first exons (exons I–III), located in the 5′ untranslated region (5′ UTR); three constitutively spliced exons (IV–VI); and a short (28 base pairs) alternatively spliced internal exon (exon N). The full-length REST/NRSF protein comprises three known functional domains: a DNA-binding domain and two repressor domains, which are located at the N- and C-termini of the protein. The two repressor domains function independently of each other and serve to recruit distinct transcriptional regulation complexes (**Figure 1(b)**). The protein also has lysine- and proline-rich regions; however, their significance is unknown. REST/NRSF's DNA binding domain encompasses an array of eight highly conserved zinc fingers, small independently folded nucleic acid binding motifs found in many other sequence-specific nucleic acid binding proteins. Thus, with its repressor and DNA binding domains, REST/NRSF shows an architecture typical of most transcription factors.

Although little is known about the function of alternative splicing events that affect the 5′ UTR of the REST/NRSF gene, the splice variant produced by inclusion of exon N, located within the region of the gene that encodes the DNA binding domain, has received a great deal of attention because it is primarily found in neurons. Its protein product, termed REST4, is a truncated version of REST/NRSF that terminates after the fifth zinc finger and therefore lacks the C-terminal repressor domain (**Figure 1(a)**). Thus, REST4 is likely to have a function distinct from full-length REST/NRSF, which will be discussed later.

NRSE/RE1: The DNA Binding Element of REST/NRSF

REST/NRSF regulates target genes by direct binding to its cognate DNA silencer element, the NRSE/RE1 (**Figure 1(c)**), which was originally identified in the promoters of the superior cervical ganglia 10 and type II sodium channel. With its long span of approximately 21 base pairs (bp) and its rather low degree of sequence degeneracy (**Figure 1(c)**), the NRSE/RE1 is a highly unusual transcription factor binding site.

This considerable level of sequence conservation has enabled stringent bioinformatic searches for candidate

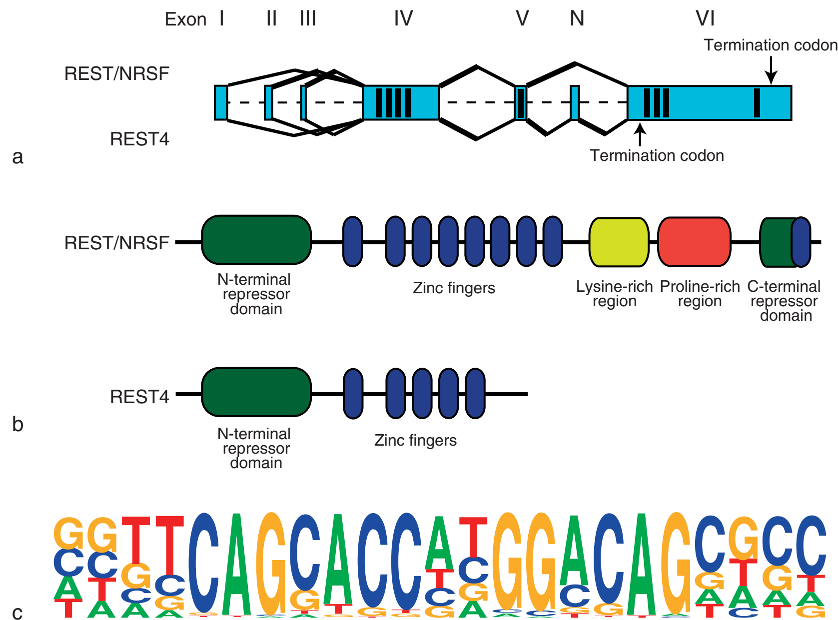

Figure 1 (a) Structure of the REST/NRSF gene and alternative isoform REST4. Exons are indicated as boxes and introns as dashed lines. Exon numbers in roman characters from I to VI are shown above the respective exons; the neuronal-specific exon located between exons V and VI is indicated as exon N. Exons I–III are alternative 5′ UTRs that are spliced to exon IV, represented by lines connecting exons at the intron–exon boundaries. Lines above the primary transcript represent the REST/NRSF primary isoform, and lines below the primary transcript represent the REST4 alternative isoform. Arrows indicate termination codons for REST/NRSF and REST4. (b) REST/NRSF protein includes eight zinc fingers near the N-terminal repressor domain and one zinc finger in the C-terminal domain. REST/NRSF contains a lysine-rich and a proline-rich region upstream of the C-terminal repressor domain. REST4 includes exon N, leading to a truncated protein encompassing the N-terminal repressor domain and five zinc fingers. (c) The conserved REST/NRSF DNA binding site, termed NRSE/RE1, is approximately 21 bp of long and contains of two highly conserved half-site motifs that are separated by two non conserved nucleotides and are flanked by several poorly conserved nucleotides. In this depiction of conserved mammalian NRSE/RE1 sites, generated with pictogram (http://genes.mit.edu/pictogram), the degree of conservation at each position is represented by the size of the nucleotide letter.

NRSE/RE1 sites in the genomes of multiple organisms, and several hundred conserved potential REST/NRSF binding sites in mammalian genomes are now known. Although most of these NRSE/RE1 sequences remain to be experimentally confirmed as functional (i.e., occupied by REST/NRSF in cells), they are enriched near the transcriptional start sites of genes typically expressed in neurons, consistent with the canonical role of REST/NRSF to specifically restrict the expression of genes typical of the neuronal phenotype. These neuronal genes encode ion channels, neurotransmitters, growth factors, hormones, and factors involved in axonal guidance and vesicle trafficking, as well as molecules involved in maintenance of the cytoskeleton and extracellular matrix.

NRSE/RE1 sites have also been found proximal to genes of neuronally enriched microRNAs, a class of small noncoding RNAs known to negatively regulate gene expression at the posttranscriptional level. Since these REST/NRSF-regulated microRNAs extend REST/NRSF's influence on gene regulation beyond NRSE/RE1-containing genes, their action is likely to contribute critically to the establishment of proper gene expression patterns during development and in the adult.

REST/NRSF in Development

The prevalence of NRSE/RE1 sites in the promoters of neuronal genes and the observation that REST/NRSF is most highly expressed in nonneuronal cells initially inspired the simple model that in cells in which REST/NRSF is expressed, it acts as a suppressor of the neuronal cell fate and/or silences the expression of neuronal-specific genes. However, REST/NRSF loss-of-function experiments conducted in mouse, chick, and frog revealed that REST/NRSF's role in early development is much more complex.

From these studies, it has emerged that impairment of REST/NRSF function does not always result in widespread neuronal gene expression in nonneuronal cells within the developing organism, nor does it lead to massive precocious expression of these genes in neural progenitors. Thus, the popular 'master neuronal

regulator' hypothesis of REST/NRSF does not appear to apply in early development. This is likely due to the fact that REST/NRSF-independent mechanisms act redundantly with REST/NRSF to control the timing and extent of neuronal gene expression.

However, even in cases in which REST/NRSF might be the sole repressor of a gene, de-repression of that locus by perturbation of REST/NRSF function will lead to robust transcription of that gene only when activators are available and recruited subsequent to departure of REST/NRSF. The failure to observe catastrophic gene deregulation upon REST/NRSF inactivation may therefore be due to the lack of such activator molecules. Indeed, REST/NRSF, when converted into a transcriptional activator by fusing its DNA binding domain to a powerful activator domain from a viral protein, thereby overcoming the requirement for endogenous activators, can guide the differentiation of myoblasts to a physiologically active neuronal phenotype *in vitro*. This experiment impressively demonstrates the vast network of neurogenic and neuronal genes that REST/NRSF controls. It appears that *in vivo*, however, REST/NRSF is not involved in specifying the fate of nonneuronal cells and tissues, and that de-repression of REST/NRSF-regulated genes alone is not sufficient to direct cells toward the neuronal lineage. Support for this view comes from gain-of-function experiments in the developing chick embryo. Here, forced expression of REST/NRSF does not interfere with neurogenesis, although neurons constitutively expressing REST/NRSF show axon pathfinding errors.

The master neuronal regulator hypothesis of REST/NRSF was placed under more scrutiny when NRSE/RE1 sites were discovered in several nonneuronal genes. Indeed, it is now known that aside from functioning in central nervous system development, REST/NRSF is important in the function of the fetal and adult heart, smooth muscle cells, and pancreatic islet cells. These functions are mediated, at least in part, by REST/NRSF-dependent regulation of these NRSE/RE1-containing nonneuronal genes. It is likely that roles for REST/NRSF in the development of other organs will emerge. These insights further substantiate the notion that REST/NRSF function in neurogenesis is just one aspect of this transcription factor's multifaceted role as a master regulator of a diverse set of gene expression programs.

REST/NRSF in the Adult

Several lines of evidence strongly implicate REST/NRSF in regulating the expression of NRSE/RE1-containing genes in mature neurons. Transcripts of several REST/NRSF isoforms, including full-length REST/NRSF, are detectable, albeit at low levels, in mature neurons within several regions of the adult brain, most prominently in the hippocampus. Experiments in animal disease models strongly implicate this limited amount of REST/NRSF protein in regulating gene expression responses to pathological states of the brain. Similarly, *in vitro* studies have hinted at a role for REST/NRSF in activity-dependent modulation of gene expression.

Induction of status epilepticus (seizure) by systemic administration of the glutamate analog kainate triggers an immediate transient elevation of REST/NRSF transcript and protein in the rodent hippocampus. Because the kainate-induced upregulation of REST/NRSF is preceded by upregulation of known targets of REST/NRSF, including brain-derived neurotrophic factor (BDNF) and the AMPA-responsive Ca^{2+} receptor subunit, encoded by the GluR2 gene, it has been suggested that REST/NRSF serves to dampen spiking expression levels of these genes. Indeed, deletion of the NRSE/RE1 in the BDNF promoter results in increased induction of BDNF upon kainate treatment.

Likewise, global ischemia, a brain insult associated with cardiac arrest, leads to transiently increased levels of REST/NRSF in neurons of the CA1 hippocampal subfield. These CA1 pyramidal neurons are known to eventually die following the ischemic insult, and it is thought that cell death is dependent on changes in transcription. Since several genes that have been implicated in mediating this ischemia-induced cell death, such as the μ opioid receptor 1 and GluR2, are directly regulated by REST/NRSF, it is likely that REST/NRSF is involved in controlling the timing and/or extent of cell death. These observations point to a central role of REST/NRSF in neuroprotective cellular responses. They also highlight the rather dynamic nature of REST/NRSF-dependent modulation of gene expression in the adult brain, which contrasts REST/NRSF's role as a stable silencer in most terminally differentiated nonneuronal cells.

Regulation of REST/NRSF

Despite the often dramatically different levels of REST/NRSF transcripts and protein observed in tissues and cultured cells, our knowledge regarding the transcriptional, translational, and posttranslational regulation of REST/NRSF remains limited. Experiments in the developing chick spinal cord indicate that the canonical Wnt pathway, which is critically involved in orchestrating growth and patterning of neural tissue, may directly control the expression of REST/NRSF. The Wnt-activated β-catenin/TCF complex upregulates REST/NRSF by recognizing a sequence element located within the first exon of REST/NRSF. Since this

element is conserved in human and mouse, it is conceivable that this pathway may regulate REST/NRSF expression in mammals as well. In agreement with a conserved role for Wnt in controlling REST/NRSF transcription, human embryonic carcinoma cells, when treated with constitutively active Wnt protein, demonstrate upregulation of REST/NRSF mRNA levels.

Cell culture experiments have shown a mechanistic switch in the downregulation of REST/NRSF during neuronal differentiation. When mouse-derived pluripotent embryonic stem cells are induced to differentiate into neural stem/progenitor cells, the rate of REST/NRSF transcription remains unchanged, yet REST/NRSF protein decreases substantially because it is specifically targeted for degradation by the proteasome. Upon cell cycle exit and further differentiation into mature neurons, however, REST/NRSF is repressed at the transcriptional level through the action of the unliganded retinoic acid receptor repressor complex acting on the REST/NRSF gene. These mechanisms may also regulate REST/NRSF protein levels at corresponding differentiation stages *in vivo*.

The control of subcellular localization of REST/NRSF is emerging as an additional layer of regulation of REST/NRSF activity. Experiments in a rat model of self-sustained status epilepticus show that REST/NRSF accumulates in the nuclei of CA1 hippocampal pyramidal neurons. The mechanism controlling seizure-induced REST/NRSF compartmentalization is unknown. However, a nuclear envelope protein termed RILP has been shown to specifically bind REST/NRSF and control nucleocytoplasmic shuttling of REST/NRSF, making RILP a good candidate for controlling the distribution of REST/NRSF within the cell. Lastly, the huntingtin protein, which is mutated in Huntington's disease, interacts with REST/NRSF and sequesters a portion of the cellular REST/NRSF pool within the cytoplasm. Mutant huntingtin loses its interaction with REST/NRSF, and the resultant increase in nuclear levels of REST/NRSF causes repression of NRSE/RE1-containing genes. Thus, dysregulation of REST/NRSF subcellular localization may contribute to disease phenotypes in some cases, whereas in other cases intracellular REST/NRSF redistribution may be an adaptive cellular response to counter aberrant gene expression patterns induced by pathological insults.

The Splice Variant REST4

As mentioned previously, REST4 is a splice variant of REST/NRSF that lacks the C-terminal repressor domain found in full-length REST/NRSF, and it also has a truncated DNA binding domain. Initially identified in neurons, REST4 is now known also to be expressed in many cancer cells lines. Although the precise mechanisms that regulate alternative splicing of the REST/NRSF gene are unclear, evidence suggests that protein kinase A regulates as yet unidentified splicing factors responsible for promoting the alternative splicing of the REST/NRSF transcript to produce REST4.

Despite extensive studies, the biological function of REST4 remains controversial. Although *in vitro*, REST4 has much reduced affinity to DNA compared to REST/NRSF, this residual binding activity might nevertheless be sufficient to allow REST4 to occupy DNA sites *in vivo*, especially in cells in which REST4 is highly expressed. REST4 and REST/NRSF can interact with each other directly *in vitro*, so REST4–REST/NRSF hetero-oligomerization may have a function *in vivo*, for example, by sequestering REST/NRSF in an complex that is incapable of binding to DNA. Alternatively, the REST4–REST/NRSF complex might bind NRSE/RE1 sites in a conformation that impairs its ability to recruit corepressors.

Supporting evidence for an antagonistic effect of REST4 on REST/NRSF function comes from experiments in which introduction of REST4 into cells can block REST/NRSF-dependent repression of NRSE/RE1-containing genes. Likewise, expression levels of some NRSE/RE1-containing genes in neuroblastoma- and small cell lung cancer-derived cell lines that also express REST/NRSF is inversely correlated with their levels of REST4 expression, lending further credence to the notion that REST4 might de-repress REST/NRSF-regulated genes.

REST/NRSF and Cancer

In addition to increased expression of REST4 and similar truncated forms of REST/NRSF in cell lines derived from neuroblastomas, aberrantly high levels of REST/NRSF have also been found in these cancers of the sympathetic nervous system. In keeping with a REST/NRSF-antagonistic role for REST4, the expression of the neuroendocrine character typical of these malignancies despite high levels of REST/NRSF has been suggested to be due to the de-repressive action of REST4.

REST/NRSF dysregulation is a hallmark of several other cancers. Medulloblastoma, the most common brain tumor, is thought to originate from neuroectodermal stem/progenitor cells in the cerebellum. Primary tumors and cell lines derived from them express extremely high levels of REST/NRSF, and it has been suggested that the failure to downregulate REST/NRSF in undifferentiated normal brain cells contributes to their malignant transformation. However, since REST/NRSF overexpression by itself has been

shown to be insufficient to induce tumorigenesis in several animal and cell culture models, it appears that the additional deregulated expression of oncogenes, such as c-myc, is required.

Other cancers, however, show markedly reduced and sometimes undetectable levels of REST/NRSF, including breast, colon, and small cell lung cancers. Moreover, a significant number of tumors and cell lines have deletions of the chromosomal locus encompassing the REST/NRSF gene. Consistent with this observation, REST/NRSF was found to be a candidate in a stringent unbiased screen for tumor suppressors in a mammary epithelial cells.

REST/NRSF-Mediated Transcriptional Repression and Silencing

These apparently contradictory observations make formulating a unifying hypothesis for REST/NRSF function a daunting task. Downregulation of REST/NRSF is seen both in terminally differentiated postmitotic neurons and in rapidly dividing cancer cells. Likewise, REST/NRSF has properties of a tumor suppressor but is observed to be dramatically upregulated in some cancers and is high in proliferating stem cells. The answer to these conundrums is likely to be found in the fact that different cellular environments contain different relative levels of available cofactors that REST/NRSF relies on to exert its function. It is therefore imperative that we examine more closely the proteins that REST/NRSF recruits and the chromatin changes that are induced by them (**Figure 2**).

In embryonic stem cells, REST/NRSF occupies NRSE/RE1 sites in promoters and introns of genes and is associated with the corepressors mSin3a and CoREST, recruited by its N-terminal and C-terminal repressor domains, respectively (**Figure 1**(**b**)). These corepressors exist in the nucleus as separate complexes, both of which harbor the class I histone deacetylases (HDACs) 1 and 2. By catalyzing the deacetylation of specific lysine residues within the N-terminal tails

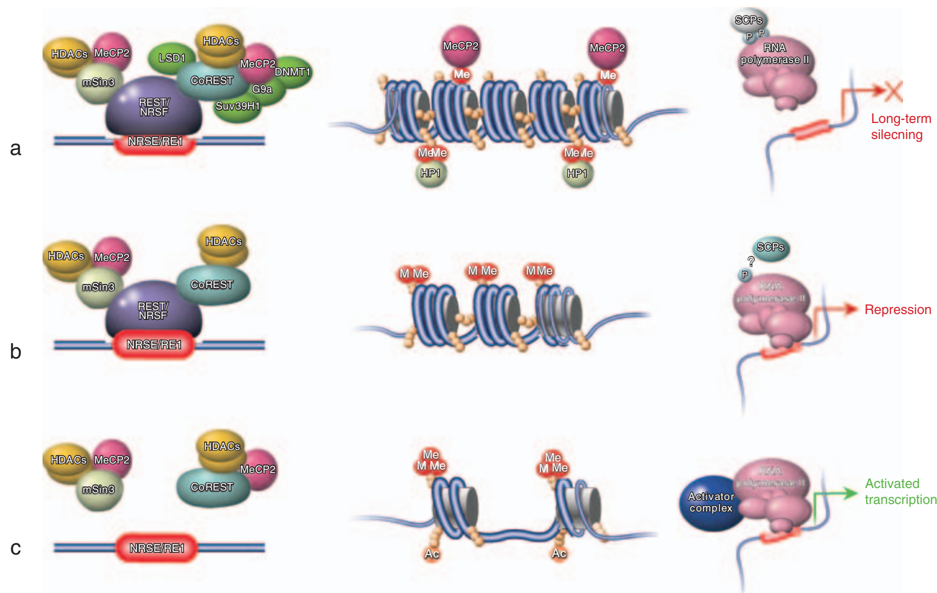

Figure 2 REST/NRSF recruits celltype-dependent complexes to gene regulatory regions, thereby altering chromatin status and transcriptional activity. (a) In terminally differentiated non-neuronal cell types and tissues, genes containing an NRSE/RE1 element are in a REST/NRSF-dependent stably silenced state. In addition to mSin3a/b-mediated recruitment of histone deacetylases (HDACs) and the methyl-DNA-binding protein MeCP2, the REST/NRSF–CoREST complex recruits chromatin modification enzymes associated with long-term silencers (green ovals) (left). These include the histone H3 lysine 9 (H3K9) methyltransferases G9a and SUV39H1, the H3K4 demethylase LSD1, and probably the DNA methyltransferase DNMT1. Methylation (red spheres) of H3K9 and DNA recruits heterochromatic protein 1 (HP1) and MeCP2, respectively. These modifications lead to heterochromatic compaction of chromatin and long-term silencing (middle). RNA polymerase II (pink) is absent from promoters and/or transcriptionally inhibited through phosphorylation (blue spheres) of its C-terminal domain (CTD) by small CTD phosphatases (SCPs) (right). (b) In multipotent embryonic stem cells and neuronal progenitors, NRSE/RE1-regulated genes are in an inactive but permissive state, characterized by the absence or low occupancy levels of long-term silencers but the presence of HDACs, which are generally considered as mediators of dynamic repression (left). Chromatin shows markers of actively transcribed genes, such as hypermethylated H3K4, but is kept in a repressed state by histone deacetylation (middle). RNA polymerase II is found at promoters, albeit at low levels, leading to limited but detectable transcript levels. The role of SCPs at this stage is unknown (right). (c) In most terminally differentiated neurons, REST/NRSF has been downregulated, leading to its departure from most NRSE/RE1-controlled genes (left). Chromatin is highly acetylated and found in a transcriptionally permissive, relaxed state (middle). Transcriptional activators recruit the RNA polymerase II complex to promoters. Downregulated nuclear SCPs levels lead to increased CTD phosphorylation levels of pol II, thereby relieving inhibition of transcription (right).

of histones, HDACs create a chromatin state that is transcriptionally repressive. Because deacetylation can be readily reversed by the action of histone acetyltransferases, this modification is generally considered transient and associated with a dynamically regulated chromatin environment.

In contrast to the situation in terminally differentiated nonneuronal cells, REST/NRSF occupancy at NRSE/RE1 sites is not accompanied by complete silencing of genes in embryonic stem cells (**Figure 1(b)**). Instead, this transcriptional 'twilight state' near NRSE/RE1 sites is characterized, on the one hand, by rather low levels of other epigenetic markers of repressed chromatin, such as methylated DNA, methylated lysine 9 of histone 3 (H3-meK9), and the histone methyl transferase G9a, which creates this modification. On the other hand, the histones in these regions of the genome are associated with active chromatin marks, such as H3meK4. Polymerase activity at these loci is small but detectable, and the net result of these opposing forces is a low level of transcriptional output that may be further reduced by the action of REST-recruited small CTD phosphatases, which remove regulatory phosphate marks on the C-terminal domain of RNA polymerase II and thereby interfere with transcriptional elongation. Thus, in pluripotent cells REST/NRSF maintains a chromatin signature and transcriptional state that is sufficiently repressive to prevent manifestation of the neuronal phenotype but is dynamic enough to allow de-repression upon neuronal differentiation cues.

As embryonic stem cells transition toward a neuronal lineage via a neuronal progenitor stage, REST/NRSF occupancy at NRSE/RE1 sites is gradually relieved. This process is initiated by REST protein degradation and subsequently by repression of the REST gene transcription. In most terminally differentiated neurons, REST can no longer be detected at promoters, the chromatin has changed to a permissive state, and transcription is activated (**Figure 1(c)**). Some gene promoters retain subsets of REST-recruited corepressors, despite REST departure from NRSE/RE1 sites, thus maintaining a responsive chromatin state that can be modulated in response to extracellular stimuli. This subset of genes is likely to be involved in the REST/NRSF-dependent regulation of neuronal plasticity described previously.

In terminally differentiated nonneuronal cell types, REST/NRSF maintains a stably repressed chromatin state by recruiting, via CoREST, not only the aforementioned HDACs but also additional complexes that confer a long-term silenced state on the surrounding chromatin (**Figure 1(a)**). CoREST binds to at least three histone modification enzymes: G9a and SUV39H1, which are both H3-K9 methyl transferases, and LSD1, which is an H3-K4 demethylase. The chromatin modifications created by these enzymes, in turn, serve as binding sites for repressive chromatin binding proteins, such as heterochromatic protein 1 (HP1), which binds to methylated H3-K9. CoREST also recruits the methyl-CpG binding protein 2 (MeCP2), a chromatin regulator protein whose dysfunction is implicated in several neurodevelopmental disorders, most prominently in Rett syndrome. These chromatin modifiers act in concert to maintain a stably repressed transcriptional state at NRSE/RE1-containing neuronal genes.

REST/NRSF as an Activator

The observation in embryonic stem cells that gene transcription can occur despite promoter occupancy by REST/NRSF is surprising given its canonical role as a repressor or silencer of transcription. However, several lines of evidence show that the presence of REST/NRSF at promoters is not incompatible with active transcription. In fact, REST/NRSF might even act as the activator of transcription in some contexts. In transgenic mouse experiments using a reporter gene construct, mutation of the NRSE/RE1 element of the gene that encodes the L1 cell adhesion molecule led to loss of L1 reporter expression in several adult brain structures, including cortex, striatum, and hippocampus. Likewise, expression of a β_2-nicotinic acetylcholine receptor reporter was lost in several structures of the peripheral nervous system upon introduction of a point mutation into its NRSE/RE1. Similar observations have been made in cell culture experiments in which NRSE/RE1 elements in the several other genes were disrupted.

In differentiating neural stem/progenitors cells from the adult brain, a small noncoding double-stranded RNA containing an NRSE/RE1 sequence initiates a switch in the cofactor composition of the REST/NRSF-recruited complex. This process is presumably dependent on the association of the RNA with DNA-bound REST/NRSF and is characterized by a reduction of HDAC association, concomitant with recruitment of proteins and acquisition of histone modifications characteristic of actively transcribed loci. Consequently, these genes are transcribed.

Consistent with these observations, *in vitro* experiments show that REST/NRSF can associate with factors of the basal transcription machinery as well as with the SWI/SNF chromatin remodeling complex, which is capable of remodeling chromatin into both active and repressed states. Since activator properties of REST/NRSF are most often observed in cells and brain structures in which REST/NRSF levels are relatively low, it is likely that REST/NRSF mode of function – activator, repressor, or silencer – is

dependent on its abundance in the nucleus. Clearly, additional parameters, particularly the balance of cofactors recruited by REST/NRSF, are critical in determining the net effect of REST/NRSF promoter association on transcription.

Conclusions

Since its discovery as a silencer of terminal neuronal genes more than a decade ago, REST/NRSF has come to be appreciated as a regulator of a much more complex set of gene expression programs. Advances in the knowledge of the genes targeted by REST/NRSF and the diversity of REST/NRSF-recruited cofactors have contributed to this development. It has also become clear that a quantitative approach toward studying REST/NRSF is invaluable because its function is likely to be critically dependent on both the concentration of available REST/NRSF and that of its cofactors. Large-scale screens aimed at elucidating changes in REST/NRSF promoter occupancy at target genes as well as spaciotemporally targeted REST/NRSF gain- and loss-of-function experiments during development and in the adult will undoubtedly help to build a parsimonious model of REST/NRSF function.

See also: Differentiation: the Cell Cycle Instead; Neurogenesis in the Intact Adult Brain; Transcriptional Silencing.

Further Reading

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Drosophila Apterous Neurons: From Stem Cell to Unique Neuron

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Introduction

Animals contain large numbers of different cell types, and this cellular diversity is profound in the nervous system, where estimates in mammalians are as high as 10 000 unique cell types. Understanding how this remarkable cellular diversity is generated, and with such precision, remains one of the challenges of neurobiology. Although tremendous progress has been made during the past two decades with respect to our understanding of the molecular genetic mechanisms controlling nervous system development, due to the complexity of this system, in many cases research has not progressed beyond the descriptive level. For instance, in more complex tissues such as the mammalian, or even *Drosophila*, adult brains, we are far from even completing a survey of the cellular complexity, let alone understanding the genetic mechanisms involved in generating that diversity. Moreover, although one may argue that it is not necessary to understand the regulatory details underlying the specification and differentiation of each and every *Caenorhabditis elegans* and *Drosophila* neuron, this is obviously not true for the human nervous system. Here, the ultimate goal is to characterize such mechanisms for every one of the 10 000 different neuronal subtypes since they are all likely involved in some intriguing aspect of human biology and are likely linked to various human diseases. As exemplified here, by the regulatory complexity underlying specification of a few cell fates in the powerful *Drosophila* model system, decoding the molecular genetic mechanisms controlling the generation of the complete developing human nervous system remains a major challenge.

This article reviews studies on neuronal cell-type specification of a unique neuronal subset, the apterous (Ap) neurons in the *Drosophila* ventral nerve cord. The focus is on a subset of Ap neurons – the Ap cluster of four cells located in each thoracic hemisegment – and studies that address the origin, generation, specification, differentiation, and pupal remodeling of these neurons are reviewed.

The Apterous Neurons of the *Drosophila* Ventral Nerve Cord

The *Drosophila* central nervous system can be subdivided into the brain and the ventral nerve cord (VNC). These tissues are functionally equivalent to the mammalian brain and spinal cord, respectively. The VNC is segmentally organized into three thoracic and eight abdominal segments, and it contains approximately 10 000 cells, the majority of which are neurons. In the VNC, only 90 cells express the LIM-homeodomain gene *apterous* (*ap*), but these neurons, the Ap neurons, are remarkably diverse. They differ in axon pathfinding; most Ap neurons extend their axons in a common fascicle, whereas the Tv neuron innervates a peripheral secretory gland, the dorsal neurohemal organ (DNH), which is a specialized glial-derived structure present in the three thoracic VNC segments (**Figure 1**).

Ap neurons also differ in neurotransmitter expression and approximately half are peptidergic. In this peptidergic subclass, the Tv cell selectively expresses the neuropeptide gene *FMRFamide*, whereas dAp and Tvb cells selectively express the neuropeptide gene *Nplp1* as well as a dopamine type I receptor gene, *Dop-R*. Importantly, for these terminal differentiation genes, their expression within the VNC is confined to these subsets of Ap neurons; *FMRFa* is only expressed in 6 and *Nplp1/Dop-R* in 28 of the 10 000 cells present in the VNC, respectively. How is this remarkably specific gene expression controlled?

Genetic Mechanisms of Apterous Neuron Specification

Studies have identified a number of regulatory genes that act postmitotically in the specification and differentiation of the Ap neurons. These include the transcription factors *ap* (mammalian *Lhx2a-b*), the Col/Olf-1/EBF family member *collier/knot* (*col*) (mammalian *ebf1–3*), the zinc finger gene *squeeze* (*sqz*) (mammalian *CIZ*), and the basic helix–loop–helix gene *dimmed* (*dimm*) (mammalian *Mist1*) as well as the transcriptional cofactors *eyes absent* (*eya*) (mammalian *Eya1–4*) and *dachshund* (*dac*) (mammalian *Dach1–2*). In addition, FMRFa expression in the Tv neuron is critically dependent on a target-derived transforming growth factor-*β* (TGF-*β*)/bone morphogenetic protein (BMP) signal – the first identified case of a retrograde instructive signal. These regulatory genes act in a combinatorial manner such that a combinatorial code of *ap/sqz/dac/eya/dimm* and target-derived TGF-*β*/BMP signaling dictates Tv neuron identity and activates *FMRFa* expression, and *ap/eya/dimm/col* dictates Tvb/dAp identity and activates *Nplp1/Dop-R* expression (**Figure 2**).

As outlined previously, using the neuropeptides FMRFa and Nplp1 as terminal cell identity markers, as well as using independent axonal markers for these

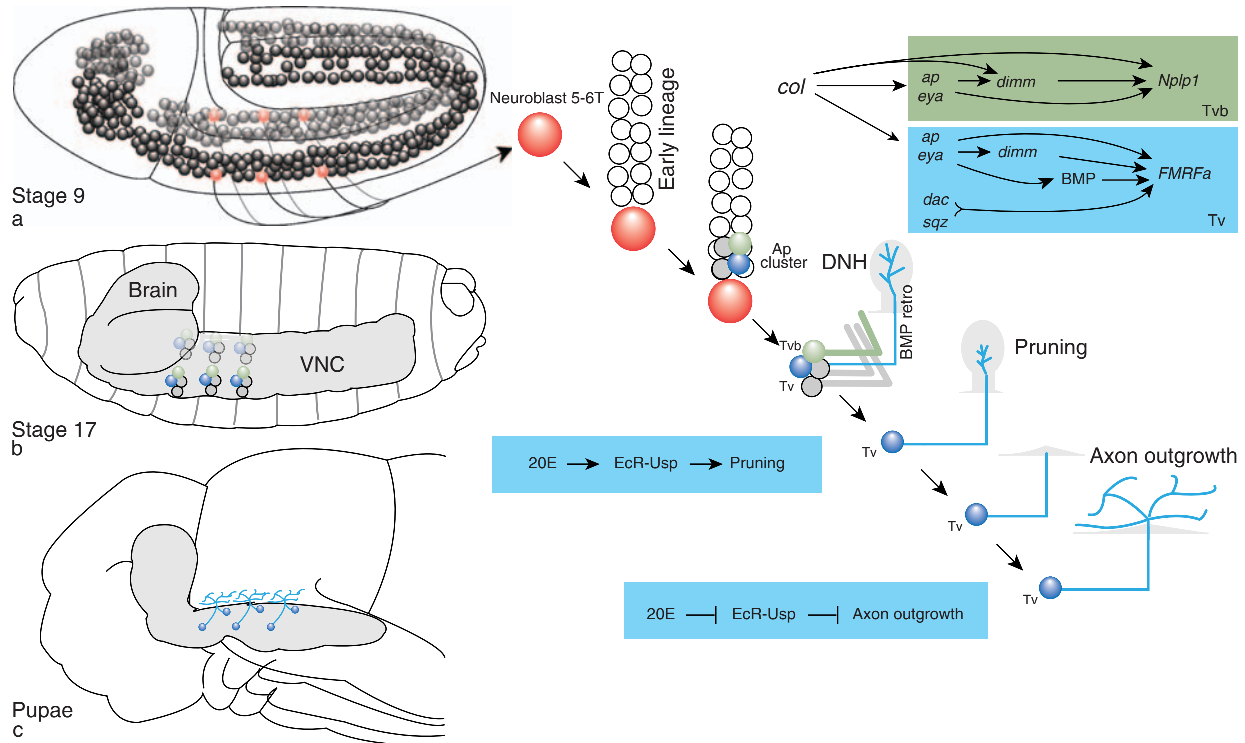

Figure 1 (a) The *Drosophila* central nervous system is generated from a defined set of neuroblasts (gray circles) that delaminate from the ectoderm during embryonic stages 9–11. The six NB 5-6T neuroblasts are located laterally in the three thoracic hemisegments (red circles). NB 5-6T undergoes a series of divisions to generate an early lineage of neurons and glia, after which the four Ap cluster neurons are generated. A complex regulatory cascade, unique to the Tvb (Nplp1 neuropeptide expressing; green circles) and Tv (FMRFa expressing; blue circles) neurons, ensures that these two neurons acquire proper identity. (b) After completion of embryonic neurogenesis, the *Drosophila* VNC is segmentally organized into the three thoracic and eight abdominal segments. The VNC contains an estimated 10 000 cells, of which only 90 express the *ap* gene, with the prominent Ap cluster of four cells locates in each of the six thoracic hemisegments. All Ap cluster neurons have now extended their axons, and the Tv neurons (blue) have received the TGF-β/BMP ligand Glass bottom boat that finally triggers expression of the *FMRFa* neuropeptide gene, whereas the Tvb neurons (green) now express the *Nplp1* neuropeptide gene. (c) During metamorphosis, the Tv neurons undergo elaborate changes in their axonal projections, involving pruning, axon outgrowth, and branching. These events are under the control of the steroid hormone 20E that acts via the EcR-Usp nuclear receptor signaling system. The function of the 20E-EcR-Usp system during these events is rather complex, and a simplified version if depicted here.

neurons, a number of genes have been identified that are important for specification of Tv/FMRFa and/or Tvb/Nplp1 cell fate. However, how do these regulators act – in parallel or in a genetic cascade? Using a multitude of markers and experimental approaches, such as loss of function, rescue (gene A of mutant A), cross-rescue (gene A of mutant B), misexpression, co-misexpression, gene expression analysis, and temporal analysis of gene function using transgenic RNAi (*UAS-RNAi*), it has been determined that the identified regulators act in rather complex genetic cascades (**Figure 1**). It is noteworthy that not all regulators participate in all events during Ap cluster specification and differentiation. For instance, *ap* and *eya*, but not *sqz*, *dimm*, or *dac*, are involved in axon pathfinding of Ap neurons. Similarly, *sqz* plays a unique role in determining the proper composition of Ap cluster neurons, and *sqz* mutants display both ectopic Ap cluster neurons and ectopic Tvb neurons. Thus, there exists a clear division of labor, with most regulators participating in more than one, but not all, events during Ap cluster specification and differentiation.

The Apterous Cluster Is Generated by the Stem Cell Neuroblast 5-6T

Studies have led to the identification of the stem cell that generates the lateral four-cell clusters of Ap neurons located in the three thoracic segments that include the Tv and Tvb neurons (**Figure 1(a)**). A large body of work during the past 20 years has been dedicated to understanding the development of the *Drosophila* VNC, revealing how a specific region of the ectoderm acquires neurogenic potential; how this neuroectodermal sheet is patterned; and how precursor cells, central nervous system stem cells

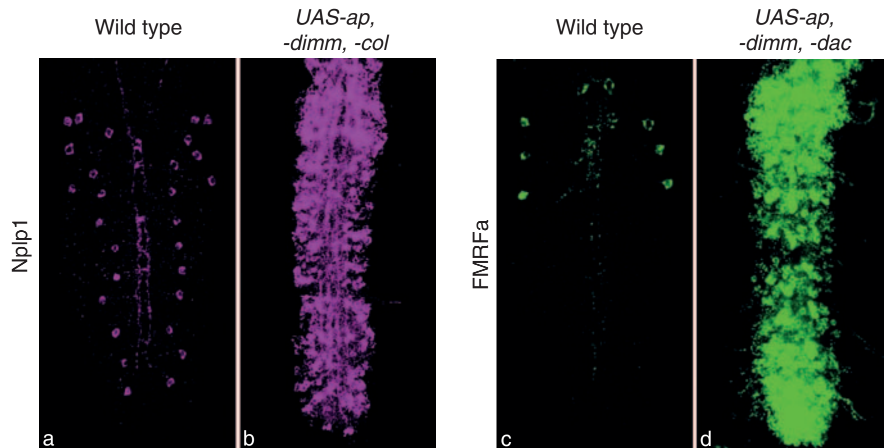

Figure 2 (a, c) Expression of the neuropeptides Nplp1 and FMRFa is confined to 28 and 8 of 10 000 cells in the wild-type VNC, respectively. (b, d) Misexpression of specific combinatorial codes of regulators in all neurons, by crossing a transgenic *elav-Gal4* driver to transgenic lines carrying *UAS-cDNA* constructs, triggers widespread ectopic neuropeptide expression. Adapted from Baumgardt M, Miguel-Aliaga I, Karlsson D, Ekman H, and Thor S (2007) Specification of neuronal identities by feedforward combinatorial coding. *PLoS Biology* 5: 295–308, *Public Library of Science*.

(denoted neuroblasts in *Drosophila*), are determined within this sheet. These early patterning events result in the generation of a set of 30 neuroblasts in each developing hemisegment, each expressing a unique combinatorial code of regulatory genes – a code that will dictate a stereotyped number of asymmetric divisions that generate a unique set of neurons and glia. By taking advantage of this previously generated information, it has been determined that the four Ap cluster cells are generated from the thoracic neuroblast 5-6 (NB 5-6T) (**Figures 1**(**a**) and **1**(**b**)). Having identified NB 5-6 as the stem cell generating the Ap cluster, now the goal is to decipher the mechanisms by which global regulatory mechanisms, acting along the anterior–posterior, dorsal–ventral, and segmental axes, integrate with temporal transitions in stem cells to generate the four Ap cluster neurons.

Remodeling of Apterous Tv Neurons during the Pupal-to-Adult Transition

After 3 or 4 days of life, *Drosophila* larvae leave the food, attach themselves to a rigid surface, and undergo a fascinating transformation – the metamorphosis of holometabolous insects. During this stage, many larval tissues undergo histolysis (tissue degradation) and many new, adult-specific structures are formed. At this stage, studies using live imaging time-lapse videos to follow the pruning and extension events in great detail have revealed that one of the Ap neurons, the Tv neuron, undergoes a dramatic remodeling of its axonal arbors, involving an initial retraction (pruning) followed by axon outgrowth and extensive branching onto the surface of the VNC (**Figure 1**(**c**)). Steroid hormones play a central role during insect metamorphosis and have been found to play an important role also in the Tv neuron. One of the key players in this regulatory system is the hormone ecdysone and its metabolite, 20-hydroxyecdysone (20E). 20E binds to a nuclear hormone receptor consisting of a heterodimer of two nuclear receptors, EcR and the RXR-ortholog Ultraspiracle (USP), and this heterodimer binds to target sites in the DNA to regulate gene expression. By analyzing EcR mutants, misexpressing dominant negative EcR forms, and by blocking EcR by transgenic RNA interference (*UAS-RNAi*), it has been demonstrated that EcR plays an important role, particularly during the pruning phase. For example, by driving expression of a dominant negative EcR construct from the FMRFa enhancer (*FMRFa-Gal4/UAS-EcRDN*), it was found that the pruning was severely impaired and axon branch extension delayed and reduced. Since 20E-EcR-Usp signaling likely affects a multitude of neurons in different ways, it would be interesting to learn how this system integrates with neuronal subtype-specific regulatory mechanisms. Moreover, whether other Ap neurons also undergo remodeling during metamorphosis has not been addressed.

Important Themes during Neuronal Subtype Specification

Studies of Ap neurons have revealed several interesting mechanisms of neuronal subtype specification, and the more pertinent ones are highlighted in this section.

Combinatorial Coding versus Master Regulators

In-depth genetic studies, particularly of the Tv and Tvb neurons, have revealed an intriguing division of labor between the identified regulators. Notably, whereas some features of both Tv/FMRFa and Tvb/Nplp1 neuron identity are under strict combinatorial control – expression of FMRFa requires the combined action of *ap/sqz/dac/eya/dimm* and target-derived TGF-*β*/BMP signaling and expression of *Nplp1* depends on *ap/eya/dimm/col* (**Figure 2**) – other features, such as neuropeptide processing capacity, are under discreet control of one regulator, namely *dimm*, which is necessary and sufficient to activate gene expression of the neuroamidase gene *PHM* in both Tv and Tvb. In fact, *dimm* can activate *PHM* expression in most, if not all, neurons and can thus be viewed as a 'master' regulatory gene, perhaps better termed a 'selector' gene, for one specific neuronal subtype property – neuropeptidergic identity.

Feed-Forward Loops

Genetic network studies in single cell systems such as *Escherichia coli* and yeast have identified a common regulatory loop denoted the 'feed-forward loop.' In this loop, gene A will activate gene B, and A/B then act together to activate C. Studies of the Ap neurons have revealed the existence of such feed-forward loops during both Tv and Tvb specification. This is clearly exemplified by the action of *col* in the early postmitotic Tvb neuron, in which it initially activates *ap* and *eya*, subsequently acts with *ap/eya* to activate *dimm*, and, finally, acts with all three regulators to activate *Nplp1* expression. In adjacent Ap cluster neurons, *col* plays only an early postmitotic role to activate *ap* and *eya*. Thus, the nature of *col* expression has an informative value: Transient *col* expression activates *ap/eya* and generates a 'generic' Ap cluster fate, whereas persistent *col* expression leads to subsequent activation also of *dimm* and, finally, *Nplp1*, and thus it has a Tvb-specific value.

Target-Derived Instructive Signals

The results of many studies of mammals during the past few decades have led to the development of the well-known neurotrophic model, in which mammalian neurons are strongly dependent on target-derived signals for their survival. These signals primarily revolve around two different receptor-tyrosine kinase signal transduction pathways – the neurotrophin and glial cell line-derived neurotrophic factor systems. Genetic studies in *Drosophila*, however, have revealed that the TGF-*β*/BMP signal transduction system is also used in a target-derived manner, specifically to modulate the arbor size of developing motor neurons. However, studies of the Tv neurons have revealed that the TGF-*β*/BMP system is also used in an instructive manner to dictate terminal identity of unique neurons, and they have found that expression of FMRFa is strictly dependent on a TGF-*β*/BMP signal from the DNH target gland. It is tempting to speculate that such instructive target-derived signals may indeed be quite common also in the mammalian nervous system, and that their identification may have been hindered by the prevailing target-derived survival signals – that is, malfunction of axon transport or axon misrouting may often lead to cell death, thus occluding any effects on neuronal cell fate determination.

Remodeling

When is a neuron terminally differentiated? The Tv neurons illustrate this issue: Their axons innervate the DNH target gland in the mid- to late embryo and begin expressing the FMRFa neuropeptide, only to go through a complete remodeling after 3 or 4 days of larval life. It is tempting to speculate that this morphological change may be accompanied by other changes in its function and neurotransmitter profile, and it will be interesting to relate the genetic mechanisms involved in generating the Tv neuron in the embryo to the mechanisms acting to remodel this cell in the pupae.

Conclusion

As revealed by the Ap neurons, there exists an amazing complexity of gene regulatory networks that act to specify unique neuronal cell identity. Similar highly complex networks have been identified that act to specify other well-studied neuronal subtypes, such as the ASER/L neurons of *C. elegans* and the spinal motor neurons of the chick and mouse spinal cord. The following are some of the more pressing questions that may now be addressed in this and other models: (1) How are the correct numbers of cells specified (i.e., only 6 Tv and 28 Tvb neurons per VNC)? (2) Are early specification genes used again during later remodeling and 'homeostasis' of mature neurons? (3) How is context specificity achieved – that is, most of the regulators identified that act to specify Ap neuron identity also act in other developmental events – how is their distinct role in each event determined? It is hoped that answering these questions will help us address neuronal subtype specification in humans.

See also: Differentiation: the Cell Cycle Instead; Motor Neuron Specification in Vertebrates; Neural Crest Cell Diversification and Specification: Melanocytes; Oligodendrocyte Specification; Terminal Differentiation: REST.

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Macroglial Lineages

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Introduction

Macroglia together with neurons make up the two classes of neural cells in the central nervous system (CNS); both macroglia and neurons have a common neuroectodermal embryonic origin. The three major subtypes of macroglia are astrocytes, oligodendrocytes, and the ependymal cells. The fourth major glial cell type in the CNS is the microglia, which are nonneuronal cells that have a mesodermal origin; microglia originate from macrophages that invade the brain during early development. There are also many glia in the peripheral nervous system (PNS), mainly Schwann cells, together with satellite cells of sensory and sympathetic ganglia and glial cells of the enteric nervous system of the gastrointestinal tract. CNS and PNS neural cells have clearly distinct lineages from an early stage of embryonic development.

Astroglia and oligodendroglia are the most numerous and functionally important macroglial cell types; ependymal cells are simple cuboidal cells that line the ventricles in the brain and the central canal in the spinal cord. Astrocytes are morphologically and functionally diverse and have a widespread distribution throughout the brain and spinal cord; astrocytes have the archetypal feature of expressing glial fibrillary acidic protein (GFAP). The two major classes of astrocytes are protoplasmic astrocytes and fibrous astrocytes, which are found in the gray and white matter, respectively. In addition, there are specialized astroglial cells throughout the CNS; for example, radial glia are bipolar astroglial cells that predominate in the developing brain, and Müller glia and Bergmann glia are specialized astroglia restricted to the retina and cerebellum, respectively. Unlike astroglia, oligodendroglia are highly specialized cells and consequently have a more restricted distribution; oligodendrocytes form the myelin sheaths that insulate axons in the CNS and are most numerous in white matter. Until recently, the accepted model was that macroglia and neurons were derived from separate glial and neural progenitors. However, glial and neuronal lineages are much more closely related than was previously thought. Astrocytes and oligodendrocytes develop from multipotent neural stem cells that also generate neurons. Indeed, astroglial stem cells and oligodendrocyte progenitor cells (OPCs) persist in the mature brain and are potential sources of new neurons in the adult.

Neuroepithelium

Neurons and macroglia derive from the neuroepithelium, which at the earliest stages of development forms the neural plate. As the neural plate thickens, the neural groove forms and the neural plate closes dorsally to form the neural tube, the lumen of which becomes the ventricular system. The cells lining the ventricles of the neural tube form a primary proliferative zone termed the ventricular zone (VZ), and a secondary proliferative zone called the subventricular zone (SVZ) emerges later from the VZ. The VZ ultimately disappears, but the SFV persists into adulthood within the forebrain. As the neural tube expands, the rostral area delineates into the forebrain, midbrain, and hindbrain, and the caudal neural tube forms the spinal cord. During this regionalization, neural crest cells differentiate from the neural tube and migrate to specific regions to form the neurons and glia of the PNS. Thus, CNS and PNS neural cells have clearly distinct lineages from an early stage.

Radial Glia Are Neural Stem Cells

The neuroepithelial cells of the VZ can be considered multipotent neural stem cells in the sense that their progeny gives rise to all of the neurons and macroglia in the CNS. The first cells that emerge from the neuroepithelium are the radial glia. These are distinguished from neuroepithelial cells by the expression of a number of antigens, including the astroglial markers GFAP, vimentin, and the calcium-binding protein S-100β, as well as the glutamate transporter GLAST, nestin, and tenascin-C. The somatas of radial glial cells are located in the VZ and their processes extend to the opposite wall of the neural tube or the pial surface. Radial glia are the main neural stem cells during development and are responsible for most of the subsequent neurogenesis, giving rise first to neurons and later to astrocytes and some oligodendrocytes by a process of 'transdifferentiation.' The majority of oligodendrocytes, however, originate at an early stage of development from glial precursors that are generated in specific sites in the brain and spinal cord. Astrocytes are generated later in development, both from radial glia and from glial precursors that also give rise to oligodendrocytes; the proportion of the final population of astrocytes derived from radial glia and glial precursors depends on the region of the CNS. Radial glia not only produce neurons and glia but also form a scaffold along which newborn progenitors migrate from the SVZ. Moreover, the radial glial cells and astrocytes that differentiate from them retain the function of stem cells in the adult brain.

Glial Restricted Precursors

Multipotent neural stem cells (neuroepithelium) do not differentiate into astrocytes and oligodendrocytes directly but, rather, through intermediate lineage-restricted stages. The first of these to develop are the glial-restricted precursors, which are distinguished from neuroepithelial cells by expression of A2B5 and nestin and are localized to the ventral neural tube. Several stages in the development of astrocytes and oligodendrocytes have been distinguished, with the main ones being oligodendrocyte and type-2 astrocyte precursors (O2A cells), oligodendrocyte precursors, astrocyte precursors, and NG2-expressing glia. The first stage in the differentiation of oligodendrocytes and probably astrocytes is the bipotential O2A glial precursor, which *in vitro* can generate either type of glial cell; O2A cells are distinguished by expression of platelet-derived growth factor-α receptors (PDGF-αRs) and NG2, in addition to A2B5. It is uncertain to what extent O2A cells are bipotential *in vivo*, where they may serve predominantly as OPCs which generate only oligodendrocytes. OPCs express the early oligodendrocyte lineage markers PDGF-αR and NG2 and later the homeobox transcription factor Nkx2.2 and the oligodendrocyte lineage gene Olig2 (basic helix–loop–helix factor). Astrocyte precursors have been isolated from numerous regions of the embryonic CNS; they express A2B5 but not GFAP until a relatively late stage of differentiation, and they are therefore difficult to distinguish from glial precursors and O2A cells. Expression of the extracellular matrix transmembrane protein CD44 may serve as an astrocyte precursor marker. Early glial precursors are small cells, with one or more processes, and are highly mobile, migrating from multiple sites to colonize the entire CNS white and gray matter. They do not express early glial antigens such as PDGF-αR and NG2 until they exit the SVZ, and as they migrate, they begin to acquire markers of OPCs and astrocytes. The relative contributions of O2A cells, OPCs, and astrocyte precursors to gliogenesis *in vivo* are not clear; they display many similarities and may represent different stages in glial precursor differentiation or distinct lineages that arise separately from neural precursors. Indeed, at least *in vitro*, glial precursors are not truly lineage restricted in that they can generate neurons, astrocytes, and oligodendrocytes under appropriate culture conditions. In addition, after generating oligodendrocytes during development, a significant population of NG2 glia persists throughout the white and gray matter of the adult brain; the functions of adult NG2 glia are unresolved, but they serve in part as a pool of adult OPCs and they may even be multipotent adult neural stem cells. There are also GFAP-positive astrocyte-like neural stem cells in specific regions of the adult CNS, and these are multipotent and self-renewing.

Regional Variations

In the forebrain, glial precursors in the SVZ migrate along radial glia to their final sites in the white matter and cortex to generate oligodendrocytes and some astrocytes, in addition to adult NG2 glia. Astrocytes are derived from migratory glial precursors and from radial glia. In early forebrain development, oligodendrocyte precursors are generated in localized sites ventrally and migrate dorsally. Later, glial precursors that originate from a large dorsolateral SVZ near the lateral ventricle give rise to oligodendrocytes and some astrocytes. Oligodendrocytes and the diverse astrocyte cell types in the cerebellum are derived predominantly from migratory glial precursors from an area dorsal to the fourth ventricle; Bergmann glia arise from embryonic radial glia and share a common lineage with Purkinje neurons. In the embryonic retina, Muller glia arise from a common precursor with neurons, and astrocytes develop at a later stage from glial precursors that migrate into the retina via the optic nerve; oligodendrocytes are absent from the retina of most species. Most astrocytes in the optic nerve are derived from intrinsic radial-like astroglia, whereas oligodendrocyte precursors migrate into the nerve during the perinatal period. In the spinal cord, oligodendrocytes originate from neural stem cells in the ventral neuroepithelium, which first generate neurons and then oligodendrocyte precursors. Later, oligodendrocyte precursors also originate from dorsal areas. Spinal cord astrocytes appear to be derived from separate astrocyte precursors after neurons and oligodendrocytes; as in other areas of the CNS, astrocytes most likely also arise from radial glia.

Gliogenesis in the Adult Central Nervous System

Gliogenesis occurs at a slow rate throughout the adult CNS. New glial cells are born locally, predominantly from parenchymal multipotent glial precursor cells and also, in the case of astrocytes, from division of mature cells. The type of cell produced is determined by locality of the precursors; they mostly produce oligodendrocytes in white matter, whereas astrocytes and oligodendrocytes are produced in the same quantities elsewhere. These glial precursors do not express GFAP, but they do express nestin and NG2. It is not clear that all NG2-expressing glia in the adult CNS are glial precursors. NG2 glia are slowly proliferating

cells that can generate oligodendrocytes *in vivo* and can also generate astrocytes and neurons *in vitro*. Nonetheless, the substantial majority of NG2 glia in the mature CNS appear to be nonmitotic, but like astrocytes, they may retain the function of stem cells in the brain throughout maturation and adulthood. In addition, self-renewing and multipotent neural stem cells are widespread in the ventricle walls of the adult CNS. These neural stem cells have morphological, physiological, and biochemical/immunological characteristics of astrocytes; those located in the hippocampus and olfactory subendyma generate neurons throughout life. The relative contributions of parenchymal NG2 glia and 'stem' astrocytes to gliogenesis in the adult remain to be resolved.

See also: Neural Stem Cells: Adult Neurogenesis.

Further Reading

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Dopaminergic Differentiation

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Introduction

Dopaminergic (DA) neurons in the mammalian midbrain region are critically affected in several neuropsychiatric disorders, such as schizophrenia, and they are specifically lost in Parkinson's disease (PD). This article reviews how the DA neurons are generated through the interactions of multiple extrinsic and intrinsic factors during embryogenesis. The molecular identities and the mechanisms of action of an increasing number of such factors have been elucidated. Many of the same factors have been successfully used to induce efficient differentiation of DA neurons *in vitro* from embryonic or neural stem cells. These advances promise to have significant impacts on science and medicine in the future.

Overview: The Medical Importance of DA Neuron Development

Dopamine is a major catecholamine neurotransmitter of the mammalian central nervous system (CNS). It is also present in noradrenergic and adrenergic neurons as a biosynthetic precursor to noradrenalin and adrenaline. Several distinct populations of DA neurons can be identified in the CNS. They include a subset of interneurons in the olfactory bulb, a subset of amacrine interneurons of the retina, distinct neuronal groups in the hypothalamus, and the ventral mesencephalon, which includes substantia nigra (SN, the so-called nigrostriatal system), and ventral tegmental area (VTA, the so-called mesolimbic system). Medically, the most important group of DA neurons, which is also the largest population by number, of the mammalian CNS is in the ventral mesencephalon. The nigrostriatal DA neurons are selectively lost in PD, whereas dysfunction of the mesolimbic DA neurons is implicated in obsessive–compulsive disorder, schizophrenia, and substance abuse.

Since PD is marked by progressive loss of the nigrostriatal DA neurons, 'dopamine supplementation' therapy such as L-dopa and dopamine receptor agonists has been the mainstay of the symptomatic management of this most common movement disorder. However, the standard dopamine supplementation gradually becomes ineffective after the first 2–4 years of treatment, presumably due to the inexorable loss of nigrostriatal DA neurons and eventually their synaptic targets. Even when effective, the current DA agonist medications are frequently accompanied by fluctuations, dyskinesias, and psychotic side effects, which can be attributed at least in part to improperly regulated and ectopic dopamine transmission. Consequently, the concept of 'DA cell replacement' gradually emerged as a potential alternative to dopamine supplementation. Transplantation of DA neurons or their precursors in the striatum or in the substantia nigra may restore physiologically regulated and anatomically precise dopamine release. However, there are severe technical and ethical constraints on the use of human tissue for transplantation. As a result, attention has been directed increasingly toward deriving DA neurons from embryonic stem (ES) cells as well as from adult-derived neural stem cells. Understanding the mechanism by which normal mesencephalic DA neurons develop, therefore, has become important, not only as a model of neuronal subtype differentiation in neural development, but also for its medical implications. This article focuses on the developmental mechanism of DA neurons in the ventral midbrain.

Extrinsic Factors in Mesencephalic DA Neuron Development

Mesencephalic DA neurons are generated in the mid–hindbrain boundary (MHB) under the influence of two major signaling centers, the floor plate and the isthmus, of developing embryos. Sonic hedgehog (Shh) and fibroblast growth factor-8 (FGF-8) are the principal secreted signals underlying the activity of each of these signaling centers, respectively. Shh determines the location of DA neurons along the dorsal–ventral axis, whereas FGF-8 positions the mesencephalic DA neurons along the anterior–posterior axis of the neural tube. The Gli proteins are a conserved family of zinc finger transcription factors that function as both activators and repressors in transducing the Shh signal inside the cells. Forced Gli-1 expression in transgenic mice induced ectopic mesencephalic DA neurons while Gli-2-deficient mouse embryos failed to develop mesencephalic DA neurons. These results further underscore the importance of the Shh signaling pathway in the development of mesencephalic DA neurons.

How do these early patterning signals originate during embryogenesis? Shh expression in the floor plate was shown to be controlled by the forkhead transcription factor FoxA1/HNF3β in mouse and zebra fish, the basic helix-loop-helix (bHLH) and PAS domain containing transcription factors Sim-1 and -2 in the mouse, and directly by TGFβ family factor Cyclop in

the zebra fish. Likewise, FGF-8 expression in the MHB is controlled by a complex network of regulatory genes including a key homeodomain transcription factor Pax2. These early developmental events that initiate, refine, and maintain the pattern and signaling centers along the anterior–posterior and dorsal–ventral axes in the MHB have been reviewed extensively elsewhere.

In addition to the patterning signals intersecting at the ventral MHB of the early embryo, several other secreted factors can induce the differentiation or support the survival of DA neurons *in vitro* and *in vivo*. Among them, glia-derived neurotrophic factor (GDNF) is the most potent trophic factor for mesencephalic DA neurons. The GDNF−/− mice initially develop a full complement of midbrain DA neurons, but these mice die soon after birth, precluding the analysis of later survival and function of DA neurons in the absence of GDNF. When transplanted into the striatum of a 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine-lesioned wild-type mouse, the GDNF mutant (−/−) donor tissues exhibit a greatly reduced survival of DA neurons compared with the wild-type (+/+) donor tissues. Moreover, such a defect can be rescued by immersion of the GDNF−/− donor tissues with a high concentration of GDNF prior to transplantation. Thus GDNF is needed for continued survival of mesencephalic DA neurons in the adult. This is particularly significant in view of early promising clinical studies in which continuous and localized delivery of recombinant GDNF into the SN by a pump led to a rapid and significant rescue of the DA neurons of PD patients. Significant technical hurdles, such as efficient drug diffusion in the striatum, remain to be resolved in order to achieve optimal outcome in such applications of a potentially neuroprotective or regenerative agent.

Intrinsic Factors in Mesencephalic DA Neuron Development

Once the DA neuronal fate is induced by the extrinsic signals in the ventral MHB region, several cell-autonomous transcription factors are required for initiation and maintenance of the expression of DA cell-specific genes as well as for survival of DA neurons. They include several homeodomain transcription factors (En1, En2, Msx1, Lmx1a, Lmx1b), a bHLH transcription factor (Ngn-2), and a nuclear receptor family transcription factor (Nurr1) as described below in more detail.

Two homeodomain transcription factors, En-1 and En-2, are highly expressed in the developing and mature mesencephalic DA neurons as well as other cell types in the MHB. Although the midbrain DA neurons are initially specified in single- or double-En knockout mice, their survival requires the En1 and -2 genes in a dosage-dependent manner. In the En1/En2 double knockout mice, a very small population of TH-positive mesencephalic DA neurons remains, but the expression of α-synuclein, a gene mutated in a familial form of PD, is completely lost in the ventral midbrain, suggesting that En genes may directly regulate α-synuclein expression in the DA neurons. The loss of α-synuclein expression, however, is unlikely to be responsible for the loss of DA neurons in the En1/En2 double knockout mice since the α-synuclein knockout mice maintain a full complement of midbrain TH neurons.

A LIM homeodomain transcription factor, Lmx-1a, and another homeodomain protein, Msx-1, are specifically turned on by Shh in the ventral MHB. Lmx-1a is sufficient and required to induce DA neuron differentiation *in vivo*. One function of Lmx-1a is to induce the expression of Msx-1, which further turns on the proneural gene Neurogenin-2. The absence of Neurogenin-2 in mice resulted in progressive loss of DA neurons in both the SN and the VTA. It is important to note that forced expression of Lmx-1a can drive robust DA cell differentiation in ES cell culture. It is intriguing to note that Lmx-1a and Msx-1 are both expressed in dorsal neural tube and required for the development of the roof plate. These two dorsal factors are somehow co-opted to serve in the development of a ventral cell type, that is, the DA neurons, specifically in the MHB region.

Lmx-1b, another LIM homeodomain transcription factor, is initially highly expressed in the MHB and the dorsal neural tube and later expressed in developing and adult mesencephalic DA neurons. The early expression of Lmx-1b is induced and/or maintained by FGF-8, and retroviral-mediated Lmx-1b expression is able to induce ectopic Wnt-1 expression, another secreted signal of the MHB. In the absence of Lmx-1b, the ventral mesencephalic DA neurons retain expression of Nurr1 and TH, but most of them lose the expression of a homeodomain transcription factor Pitx3. These results suggest that Lmx-1b and Pitx3 form a regulatory cascade independent of the expression of Nurr1 or TH in DA neurons.

Another gene that attracted significant attention is Nurr1, an orphan nuclear receptor that is widely expressed in the CNS including in the mesencephalic DA neurons. In the absence of Nurr1, mice initially still develop a group of Pitx-3-, Lmx-1b-, and HNF3β-expressing cells in the ventral MHB region but fail to initiate TH expression. These ‘prospective’ DA cells eventually degenerate and disappear, resulting in a complete loss of mature DA neurons. Conversely, forced expression of Nurr1 in the adult hippocampus-derived progenitors can induce TH expression without affecting other DA-specific genes,

suggesting a direct regulation of TH expression by Nurr1. Furthermore, overexpression of Nurr1 can induce the differentiation of a mature DA neuronal phenotype by a cerebellum-derived immortalized cell line in the presence of the type 1 astrocytes. Therefore, Nurr1 appears to be a transcriptional regulator of TH expression and is essential for DA neuronal differentiation and survival.

Development of Distinct Subtypes of Mesencephalic DA Neurons

The mesencephalic DA neurons are mainly distributed in two distinct anatomical structures, SN DA neurons, innervating the striatum, and VTA DA neurons, innervating the nucleus accumbens and the prefrontal cortex. Little is known about how these two groups of DA neurons develop since they largely express a common set of genes. Raldh-1 (class 1 aldehyde dehydrogenase) is one of the first genes characterized to exhibit a subgroup-specific expression in mesencephalic DA neurons. The Raldh-1-expressing axonal terminals form a gradient in the striatum and nucleus accumbens such that its density is highest in the rostral and dorsal region of the basal forebrain. Such a graded distribution suggests that Raldh-1 is preferentially expressed in the DA neurons of SN instead of those of the VTA. Given the importance of retinoic acid in early embryogenesis, hindbrain patterning, and spinal motor neuron development, such a striking expression pattern of a retinoic acid-synthesizing enzyme suggested that retinoic acid and/or its derivatives may play a role in the development of subsets of DA neurons.

Some other genes, while not necessarily exclusively expressed in any specific subset of DA neurons, are nevertheless required for the development or maintenance of only a subpopulation of mesencephalic DA neurons. Genetic deletion of mouse transforming growth factor-α (TGF-α), for instance, leads to a selective reduction of DA neurons in SN, but not in VTA. This appears to reflect an early, possibly a developmental, requirement of TGF-α since such a defect was already present in the newborns. Neither the site of expression nor the mechanism of TGF-α action in a subset of the SN DA neurons is known.

The brain-derived neurotrophic factor (BDNF) and its receptor trkB are expressed in the mesencephalic DA neurons. BDNF can promote DA neuronal survival and differentiation in experimental culture conditions. When researchers conditionally deleted the BDNF gene by crossing the floxed allele of BDNF and a Wnt–Cre line, BDNF expression was nearly completely abolished in the MHB region. Such mouse mutants exhibit a $\sim$23% reduction in tyrosine hydroxylase (TH)+ DA neurons in the substantia nigra compacta but not in the VTA, indicating a preferential requirement of BDNF in DA neuron development of the SN.

One transcription factor that regulates the differentiation or maintenance of a subset of DA neurons is Pitx-3/Ptx-3, a paired-type homeodomain transcription factor that is expressed almost exclusively in all the mesencephalic DA neurons. It is interesting that the number of DA neurons fell dramatically in the SN, but not the VTA, in the Aphakia mutant mice that are deficient in the Pitx-3 gene. This is accompanied by $\sim$90% loss of dopamine in the dorsal striatum and significant deficits in sensorimotor coordination behaviors. These deficits can be reversed by peripheral administration of L-dopa. Finally, the Aphakia mutant mice also displayed denervation hypersensitivity to L-dopa in the dorsal striatum, making Aphakia one of the best mouse models with all the major symptomatic and pharmacologic characteristics of the early clinical stages of PD.

The Control of DA Axonal Development

DA neurons project their axons in the rostral direction to form the medial forebrain bundle (MFB) before reaching the final targets in the forebrain, which include the corpus striatum, nucleus accumbens, and the prefrontal cortex. MHB and DA neuron co-culture experiments have shown that the MHB tissue alone is not repulsive to the DA axons, suggesting that no localized repulsive signal from the MHB and rostral hindbrain region prohibits the DA axons from entering the hindbrain. In contrast, the DA axons do turn abruptly when forced to grow into a stratum of the reverse polarity, suggesting that locally distributed directional cues guide the rostral projection of DA axons to their precise positions in their physiological targets. The absence of repulsive signals in the hindbrain suggests that the DA axons may turn rostrally in response to attractive guidance cue(s) distributed in the vicinity of ventral midbrain.

Unlike many ascending axons originating from the spinal cord and elsewhere, the mesencephalic DA axons never cross the midline. Once turned rostrally, the DA axons join additional ascending axons to form the MFB, a symmetrical pair of axonal bundles running in parallel to the ventral midline of the forebrain. Studies in the *Drosophila* embryo have shown that longitudinal axonal pathways are kept from crossing the midline and maintained at a constant distance from the midline by the repulsive signal Slit. Similarly, mammalian Slit-1 and Slit-2 are both expressed in the ventral midline of the CNS. The TH-positive axons, as well as other axons in the MFB, are displaced ventrally in Slit2−/− mutant mice

and frequently enter the hypothalamus in Slit1−/−; Slit2−/− double mutants. Therefore, the repulsive Slit signals contribute to the maintenance of DA axonal trajectory in the ventral forebrain.

Finally, DA axons invade selected targets in the forebrain, that is, the striatum and the prefrontal cortex, in a topographic manner. The SN DA axons preferentially innervate the dorsal striatum, while the VTA DA axons the ventral striatum (mainly nucleus accumbens) and the prefrontal cortex. The Eph receptor–ligand system has been implicated in the formation of the DA topographical map. EphB1 is preferentially expressed in the SN DA neurons, while its ligand ephrin-B2 shows a graded expression in the DA axonal targets, with the highest level in the ventral striatum. Furthermore, ephrin-B2 specifically inhibits neurite outgrowth of SN but not VTA, suggesting that repulsive interaction between EphB1 and ephrin-B2 may underlie at least in part the formation of the DA topographic map. This proposal awaits confirmation by gain and loss of function experiments *in vivo*.

DA Neuron Differentiation *In Vitro*

Current understanding of the mechanisms by which the mesencephalic DA neurons are generated during normal development has been successfully extended to useful methods of promoting DA neuronal differentiation from the totipotent ES cells. Addition of Shh and FGF-8 to the ES cell culture increases the efficiency of DA cell differentiation from ∼5% to 20–30%. Similar results were also obtained with clonal ES cell lines derived from nuclear transplantation of adult donor cells (ntES cells). It is interesting that different ntES cell lines produced DA neurons at highly variable frequencies, with one line generating 50% DA neurons, suggesting that the inherent potential of DA neuron differentiation is ES cell line-dependent.

Such intrinsic factors with the ability to influence DA neuron differentiation have been identified, including Lmx-1a and Nurr1. As mentioned earlier, Lmx-1a expression in mouse ES cells can drive up to 60% TH+ neuronal differentiation in the presence of basic fibroblast growth factor, FGF-8, and Shh. Essentially all these TH+ neurons also express the correct mesencephalic DA neuronal phenotype, including dopamine transporter, Lmx1a, En, Nurr1, and Pitx3. A separate study found that the efficiency of DA differentiation can be increased up to 80% by adding Shh and FGF-8 to an ES cell line forced to express Nurr-1. The DA cells derived from these experimental protocols express not only dopamine-synthesizing enzymes and dopamine receptor and transporter but also molecular characteristics that are specific to the differentiating and mature mesencepahlic DA neurons (e.g., cRet, Pitx-3, En-1). When transplanted into the striatum, these cells can correct the amphetamine-induced ipsilateral rotation of rats whose mesencephalic DA neurons were ablated by 6-hydroxydopamine in one side. Moreover, animals grafted with Nurr-1-expressing ES cells not only exhibited contralateral turning on amphetamine stimulation but also manifested spontaneous contralateral rotations. This phenomenon, which results from overproduction of dopamine in one side of the brain, demonstrates a robust differentiation of Nurr-1 ES cells into functional dopamine-producing DA neurons *in vivo*.

Given the success in inducing ES cells to the DA lineage, promoting the differentiation of adult or fetal mesencephalic neural progenitor cells would seem conceptually straightforward. This approach, however, has generally achieved a lower percentage of DA neuron production compared with ES cells. One possible explanation for this discrepancy relates to the fact that the tissue oxygen pressure in the developing and adult brain ranges from 1–5% whereas the standard techniques expose the neural progenitor cells to the ambient 20% of oxygen pressure. Lowering the tissue culture condition to 3% oxygen significantly increases the proliferation of mesencepahlic progenitor cells, decreases their death, and most important, enhanced efficiency of their differentiation into DA neurons by up to 56% (compared with 18% at the regular 20% oxygen condition). Nurr-1 and Shh expression levels were not altered by low oxygen, but FGF-8, vascular endothelial growth factor (VEGF), and erythropoietin (EPO) messenger RNA levels are significantly increased by low oxygen tension. Neither adding nor blocking VEGF affected DA differentiation. However, FGF-8 exposure enhanced mesencephalic progenitor proliferation and delayed the differentiation of TH+ cells. It is interesting that recombinant EPO protein increased the yield of DA neurons from mesencephalic progenitors at 20% oxygen environment in a dose-dependent manner, and an EPO-neutralizing antibody partially abolished the efficiency of DA differentiation at the 3% oxygen condition. It will be interesting to learn whether and how EPO acts as an inducer of DA neurons *in vivo* as well as in the ES cell culture system.

Conclusion

In the past decade, considerable advances have been made at an ever accelerating pace in understanding the cellular and molecular mechanisms controlling the development of mesencephalic DA neurons. Indeed the extrinsic and intrinsic signals underlying native DA neuron development have turned out to be

highly relevant and useful in efforts at directing DA differentiation from ES cells *in vitro*. Conversely, new observations from *in vitro* culture systems have revealed unexpected molecular mechanisms influencing DA neuron differentiation, which will inspire further studies of their roles *in vivo*. While the recent progress was fueled by the expectation that *in vitro* production of DA neurons would be useful as a form of cell replacement therapy for PD, it is quite possible that 'neuroprotective' or 'regenerative' medicines for PD can also derive from the knowledge of DA neuron development.

An update on latest advances is as follows:

1. DA neurons were found to be greatly reduced in mice in which Dicer, a critical enzyme essential for micro-RNA formation, has been conditionally deleted in DA neurons. Furthermore, a specific micro-RNA, miR133b, is enriched in the midbrain DA neurons and can negatively regulate Pitx3 expression and the maturation of dopaminergic neurons.
2. As a definitive confirmation of the importance of GDNF in DA neuronal development and maintenance, conditional deletion of GDNF in adult mice resulted in severe hypokinesia and loss of DA neurons.

See also: Drosophila Apterous Neurons: from Stem Cell to Unique Neuron; Motor Neuron Specification in Vertebrates; Neural Crest Cell Diversification and Specification: ErbB Role; Neural Crest Cell Diversification and Specification: Melanocytes; Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation.

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Olfactory Neuron Patterning and Specification

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Overview of Olfactory Epithelium and Vomeronasal Organ Structure and Cell Types

In terrestrial vertebrates, the primary relay neurons of the olfactory system are contained within two specialized sensory epithelia in the head – the main olfactory epithelium (OE), which lines the nasal cavity and is involved in detection of odors, and the vomeronasal sensory epithelium, which lines the vomeronasal organ (VNO; a bean-shaped structure located in the anterior–ventral region of the nasal septum) and mediates detection of nonvolatile pheromones (**Figure 1(a)**). Cell bodies of olfactory receptor neurons (ORNs) lie within the epithelia of the OE and VNO. ORNs are bipolar primary sensory neurons: they have apical dendrites, with cilia- or microvilli-covered surfaces that extend into the nasal or VNO cavities, and they extend axons that synapse upon neurons within the central nervous system. In the case of the OE, the axons of ORNs form the first cranial nerve and synapse upon cells of the main olfactory bulb (MOB) of the forebrain. The sensory neurons of the VNO extend axons to form the vomeronasal nerve, which synapses upon the accessory olfactory bulb (AOB), a prominent structure in rodents that lies immediately adjacent to the MOB.

Because rodents have a highly developed sense of smell and have been used for many years as model organisms in the study of olfaction, information provided in this article concentrates primarily on studies of OE and VNO in rodents, particularly rats and mice. Studies of primary olfactory pathway development in mouse mutants have shown that the process of neurogenesis in both the OE and the VNO is regulated to a large extent by the same genes, which are expressed by cells at distinct stages in the neuronal lineages of the two epithelia (**Figure 1(b)**). The OE and VNO are also similar in that, once their structures are established during the final third of gestation, the neural cell types within them are arranged in roughly comparable laminar organizations (**Figure 1(c)**). Thus, in both epithelia, neural stem cells and committed neuronal progenitors are located in the basal compartment (near the basement membrane, which forms the boundary between sensory epithelium and underlying connective tissue stroma – i.e., the lamina propria). Committed neuronal progenitors give rise to terminally differentiated sensory neurons (ORNs), which comprise the majority of cells within both epithelia. Another major cell type within the two sensory epithelia is the supporting, or sustentacular, cell, best characterized in the main OE. The cell bodies of supporting cells lie in a distinct layer in the apical compartment of the OE and their processes extend to the basal lamina; they appear to be analogous to glial cells of the brain, and studies of mouse mutants suggest that ORNs and supporting cells are both derived from a common neural stem cell in the OE.

Another cell type found associated with the OE is the olfactory ensheathing cell (OEC). OECs, which encircle bundles of ORN axons in the stroma underlying OE, possess characteristics of both Schwann cells and astrocytes. Interestingly, transplanted OECs appear to promote recovery in a variety of nerve lesion models, and it has been proposed that their presence is responsible for the ability of lesioned ORN axons to regenerate and reinnervate the central nervous system. Some experimental embryology studies suggest that OECs may originate from the olfactory placode, and tissue culture studies have also reported that cells resembling OECs arise in cultures made from OE proper. However, no definitive lineage-tracing study has been performed to demonstrate unequivocally that OECs arise from the olfactory placode or OE proper, and at present their origin remains uncertain. Thus, although the provocative possibility exists that OECs are, like ORNs, products of a multipotential OE stem cell, this hypothesis remains to be proved, and OECs are not discussed further in this article.

Neuronal Cell Types of the OE and VNO

Newly generated neurons in the OE and VNO are products of differentiation pathways known to contain at least three distinct proliferating cell types. These cell types have been most extensively characterized in the main OE (**Figure 1(b)**): (1) neural stem cells divide to give rise to a population of (2) committed neuronal progenitor cells that express the proneural gene *mammalian achaete scute homolog 1* (*Mash1*; also known as *Ascl1*), and *Mash1*-expressing progenitor cells are committed to a neuronal fate and undergo amplifying divisions to rise to (3) immediate neuronal precursors (INPs), which express a different proneural gene, *neurogenin1* (*Ngn1*; also known as *Nurog1*). Studies using retroviral lineage tracing and

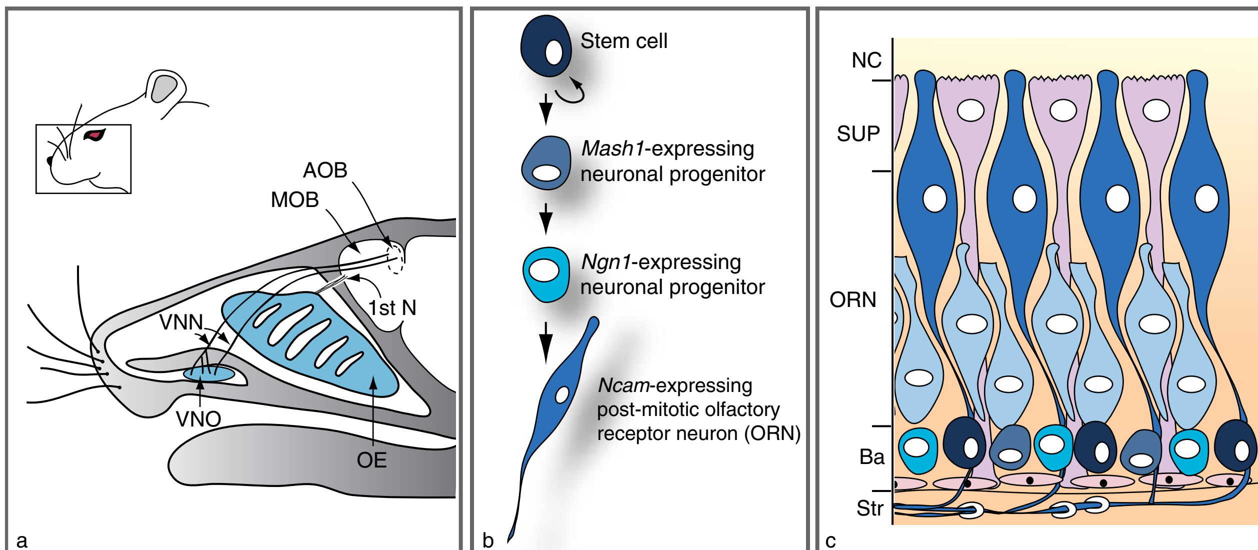


Figure 1 Location of main olfactory epithelium (OE) and vomeronasal organ (VNO) in mouse head, and lineage and distribution of different cell types within the OE. (a) Sagittal section through an adult head reveals the position of the VNO relative to the OE. While the vomeronasal nerves (VNN) target the accessory olfactory bulb (AOB), axons of olfactory receptor neurons (first cranial nerve; 1st N) target the main olfactory bulb (MOB). (b) Scheme of the neuronal differentiation pathway in the OE. (c) Histological arrangement of cells in mature OE. Neuronal stem cells (black) located at the basal layer (Ba) adjacent to the stroma (Str) give rise to transit-amplifying progenitors expressing the *Mash1* gene (round gray-blue cells) followed by *Ngn1*-expressing precursors (turquoise cells). The immature ORNs (light blue elongated cells) arise from the *Ngn1*-positive cells and mature into *Ncam*-expressing neurons (dark blue elongated cells). Supporting cells (SUP) lie in a single layer on the apical surface of the OE, at the nasal cavity (NC), while olfactory ensheathing cells wrap around nerve bundles in the underlying stroma. The VNO (not shown) has a similar histological arrangement, with basal cells at the boundary between epithelium and mesenchyme and neurons populating the bulk of the epithelium. Adapted from Kawauchi S, Beites CL, Crocker CE, et al. (2004) Molecular signals regulating proliferation of stem and progenitor cells in mouse olfactory epithelium. *Developmental Neuroscience* 26: 166–180, with permission. Copyright 2004 by S. Karger AG, Basel, Switzerland.

molecular markers for different cell types indicate that, in the established OE (see later), INPs, *Mash1*-expressing neuronal progenitors, and neural stem cells are all found in the basal compartment of the epithelium. INPs are, like *Mash1*-expressing progenitors, transit-amplifying cells, but INPs are committed ultimately to generating daughter cells that undergo terminal differentiation into ORNs. ORNs express many genes generally characteristic of terminally differentiated neurons, as well as genes specific to their sensory function (odorant receptors, cell-type-specific channels, etc.). Two well-known markers for ORNs are the neuronal cell adhesion molecule (NCAM1) and the olfactory marker protein (OMP). Interestingly, in the main OE, the differentiation and maturation of ORNs from stem and progenitor cells observed during embryonic development appear to be maintained during regenerative neurogenesis, which occurs when ORNs in the main OE have been lost due to disease or environmental insult.

ORNs of the main OE and VNO express different classes of odorant receptors (the catalytic receptors that transduce odor stimuli into cytoplasmic signals), reflecting their different functions: detection of 'common' odors in the main OE, and pheromone detection in the VNO. Odorant receptors in both sensory epithelia are seven-transmembrane, G-protein-coupled receptors. In the main OE, each ORN appears to express only one odorant receptor gene (of over 1000 estimated receptor genes), and those ORNs expressing the same odorant receptor converge their axons homotypically onto selected glomeruli of the MOB. Thus, a specific set of ORNs, with receptors that have been activated by a specific odorant, will stimulate postsynaptic neurons (mitral and tufted cells) within a specific set of MOB glomeruli, which then transduce this information to higher-order olfactory structures in the brain. In the VNO, two families of receptors, the V1Rs and V2Rs, serve as pheromone receptors, and expression of different receptors from these two families appears to divide VNO sensory neurons into two distinct populations: neurons within the apical zone of the VNO neuroepithelium express V1Rs, and their axons project to the anterior portion of the AOB, while neurons in the basal zone of the VNO neuroepithelium express V2Rs and project to the posterior portion of the AOB.

Two Phases of Olfactory Neurogenesis

Both anatomical studies and, more recently, studies of mouse mutants indicate that olfactory neurogenesis can be divided into two phases. The first is an early, morphogenetic phase, in which the neuronal lineage is established and the basic structures of the primary olfactory pathway (OE and VNO) are set up (primary neurogenesis); the second phase is established neurogenesis, during which the characteristic morphology of the nasal cavity and mature pattern of distribution of different cell types within the epithelia emerge, and the signaling systems that control expansion and regenerative neurogenesis come to predominate in the regulation of neuronal cell number. These phases of neurogenesis are depicted in **Figure 2**.

During primary neurogenesis, the OE forms from the olfactory placodes, thickenings of embryonic ectoderm that first appear as two oval epithelial patches in the anterolateral region of the head around day 9 of gestation (e9) in the mouse. As development proceeds, the olfactory placodes invaginate to form the nasal pits, which by e10.5 are already lined by neuroepithelium that contains cells at every stage of the neurogenic pathway. By e11.5, the nasal pits continue to deepen and fold, and it is during this period that the VNO becomes recognizable as a thickening in the epithelium of the medial wall of the nasal pit. At e12.5, the end of primary neurogenesis, developing nasal turbinates – the elaborate foldings of which will permit a greatly expanded OE surface area – begin to be recognizable in the main olfactory cavity. It is during this period that VNO morphogenesis takes place, as the ventral medial fold of developing neuroepithelium invaginates and pinches off from the main nasal cavity, forming a tube that is closed posteriorly, and anteriorly communicates via its own duct with the developing oral cavity, nasal cavity, or sometimes both.

In the OE that lines the developing nasal pit as early as e10, the three mitotic neuronal cell types (neural stem cells, *Mash1*-expressing progenitors and *Ngn1*-expressing INPs), as well as *Ncam*-expressing ORNs, are all present. These cell types are initially localized in concentric patterns, with the least differentiated cells lying closest to the rim and the most differentiated cells (ORNs) at the center (**Figure 3(a)**). Expression of *Sox2*, which encodes a marker for OE neural stem cells during established neurogenesis, defines the entire neuroepithelial domain of the developing nasal pit during primary neurogenesis. However, a small group of *Sox2*-expressing cells, which lie closest to the rim of the pit, also express *fibroblast growth factor 8* (*Fgf8*). *Fgf8* encodes a signaling molecule known to be required for OE neurogenesis, nasal cavity formation, and development of the VNO (see later); the cells in the rim of the olfactory pit that co-express *Sox2* and *Fgf8* are thought to be primordial neural stem cells responsible for initiating primary olfactory neurogenesis (by analogy to the primordial germ cells that ultimately give rise to gametes) (**Figure 3(b)**).

Around e14.5 in the mouse, the overall number of mitotic figures in the OE starts to decrease in the

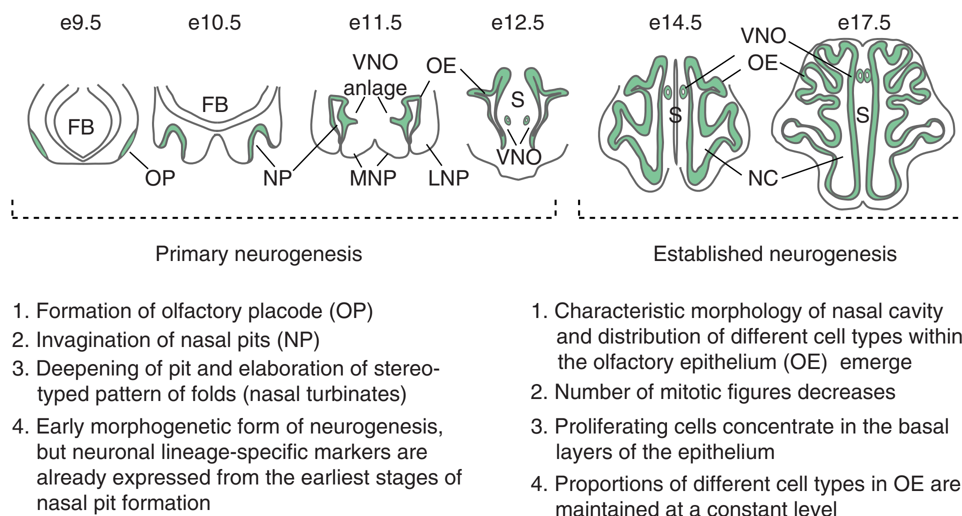


Figure 2 Schematic model and description of primary and established neurogenesis during mouse olfactory epithelium development. Coronal sections through heads at days 9.5–12.5 of gestation (e9.5–e12.5) are depicted for the phase of primary neurogenesis, while horizontal sections at e14.5 and e17.5 (immediately before birth) are portrayed for the phase of established neurogenesis. FB, forebrain; MNP, medial nasal process; VNO, vomeronasal organ; LNP, lateral nasal process; S, nasal septum; NC, nasal cavity. Adapted from Kawauchi S, Shou J, Santos R, et al. (2005) Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse. *Development* 132(23): 5211–5223.

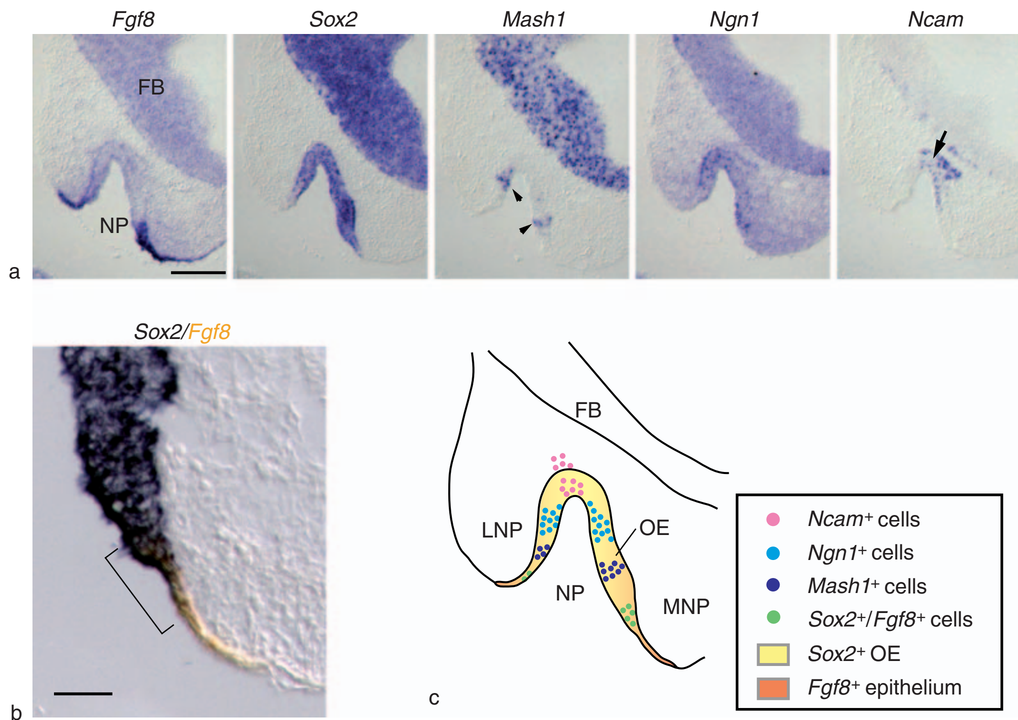


Figure 3 Expression of *Fgf8* and neuronal cell markers in developing olfactory epithelium (OE). (a) Five successive images show *in situ* hybridization for *Fgf8* and OE neuronal lineage markers in invaginating nasal pit (NP) at day 10.5 of gestation (FB, forebrain). While *Sox2* is expressed throughout the neuroepithelium, *Fgf8* is localized to the borders of the invaginating pit. *Mash1* (arrowheads) expression is located next to the *Fgf8*-expressing cells at the inner rim of the nasal pit, while *Ncam*-expressing neurons (arrow) are located at the center of the pit. (b) Double-label *in situ* hybridization for *Fgf8* (orange) and *Sox2* (blue) demonstrates overlap of the two markers in a small rim of surface ectoderm and adjacent invaginating neuroepithelium (bracket). (c) Model of peripheral-to-central process of neuronal differentiation in developing OE and origin of *Sox2*-expressing neural stem cells from *Fgf8*-expressing ectoderm (LNP, lateral nasal process; MNP, medial nasal process). Scale bar = 200 μm (a), 50 μm (b). Adapted from Kawauchi S, Shou J, Santos R, et al. (2005) Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse. *Development* 132(23): 5211–5223.

epithelium, and different cell types of the OE begin to take on the laminar positions that they will ultimately maintain throughout life. This is the onset of the established phase of neurogenesis, when the mature structure of the nasal cavity also emerges: turbinates and definitive septum form, the sensory epithelium becomes localized to the posterior and dorsal nasal cavity, and the more anterior nasal cavity comes to be lined with respiratory epithelium. Within the OE proper, supporting (sustentacular) cells emerge as a distinct cell population at this time, and their cell bodies begin to form the apical cell layer of the neuroepithelium. *Ncam*-expressing ORNs now lie in the middle compartment of the epithelium, and *Mash1*- and *Ngn1*-expressing neuronal progenitors predominate in the basal compartment of both OE and VNO (**Figure 4**). With the emergence of this organized pattern begins a new phase of neurogenesis Scale bar = 200 μm (a), 50 μm (b) that is dedicated to maintaining and regulating ORN number. From this time on, the proportions of different cell types are maintained at a fairly constant level within the OE, and the locations of populations expressing different cell-type-specific genes within the epithelium remain essentially the same throughout life.

During the established phase of neurogenesis, an asymmetry develops in the VNO epithelial lining, such that its lateral wall is lined with nonsensory epithelium, whereas the medial wall is lined with a thicker sensory neuroepithelium. This is analogous to the asymmetry that develops in the main nasal cavity, wherein nonsensory, respiratory epithelium predominates in the lining of the anterior and ventral regions of the cavity, whereas the thicker sensory neuroepithelium containing ORNs tends to be localized to more posterior and dorsal regions, covering the turbinates and the posterior two-thirds of the nasal septum.

Intrinsic Factors: Transcription Factors Regulating Mitotic Cell Populations

Neurogenesis and nerve cell renewal take place throughout life in both the OE and the VNO. This

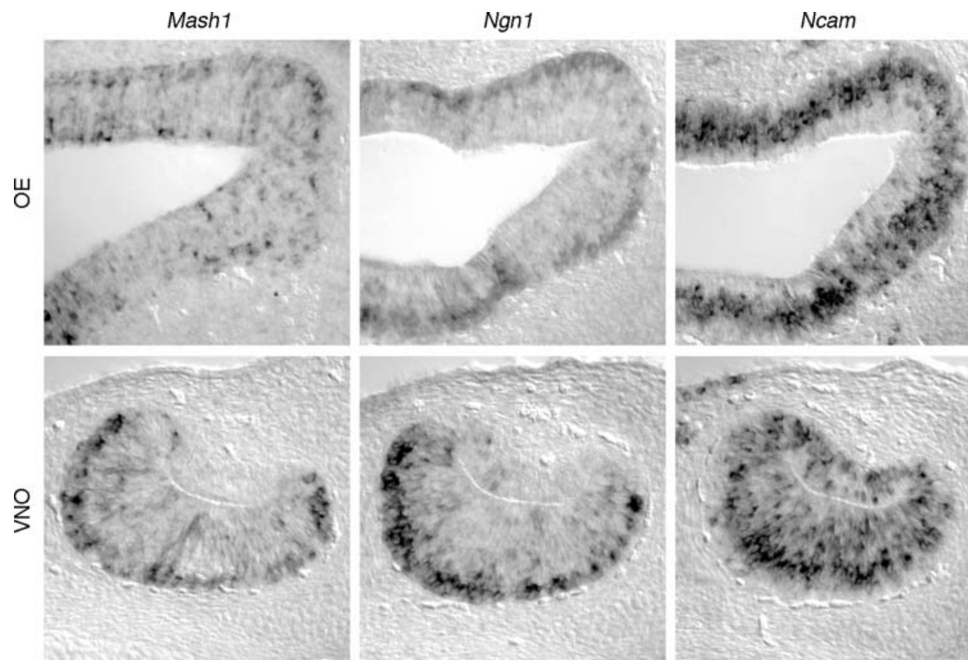


Figure 4 Cell-type-specific markers for committed neuronal progenitors and differentiated olfactory receptor neurons in the established phase of neurogenesis in main olfactory epithelium (OE) and vomeronasal organ (VNO). *In situ* hybridization is shown for mRNAs encoding *Mash1*, *Ngn1*, and *Ncam* in the OE and VNO of mice at day 14.5 of gestation, when the pattern of neurogenesis becomes established in the two sensory epithelia. Even at this stage in prenatal development, the characteristic laminar patterns of cells are apparent, and the relative proportions of cells at different stages of differentiation are similar to what is seen in the postnatal epithelia. (Top panel) *Mash1* and *Ngn1* messages are all expressed in basal areas of the OE, while some apical cells express *Mash1* but not *Ngn1*. *Ncam*, an olfactory receptor neuron marker, is expressed throughout the OE but is absent from the supporting cell layer that lines the nasal cavity. (Bottom panel) *Mash1* and *Ngn1* are similarly located in the VNO, at the boundary between basal epithelium and underlying connective tissue. *Ncam*-positive cells populate the majority of the sensory epithelium, from the concave side of the lumen to the basal layer. Adapted from Murray RC, Navi D, Fesenko J, et al. (2003) Widespread defects in the primary olfactory pathway caused by loss of Mash1 function. *Journal of Neuroscience* 23(5): 1769–1780, with permission. Copyright 2003 by the Society for Neuroscience.

capacity for ongoing neurogenesis is coupled with the ability to regenerate the sensory neuron compartment quickly, at least in the main OE. When ORN number is reduced dramatically by surgical or chemical ablation, progenitor cells in the basal compartment of the OE rapidly upregulate proliferation and produce new differentiated ORNs to replenish the damaged epithelium. These observations imply that stem and neuronal progenitor cells, as well as the microenvironment in which they reside, produce signals that stimulate proliferation and differentiation. Moreover, it must also be the case that OE neuronal stem and progenitor cells express intrinsic factors that endow them with the capacity to respond to these signals.

Work from a number of groups has demonstrated that as cells progress through the OE neuronal lineage, they successively express transcription factors that are characteristic of, and required for, differentiation of stem cells into committed neuronal progenitors and, ultimately, ORNs. Thus, expression of these cell-intrinsic factors both provides unique molecular 'signatures' for neuronal progenitor cells at specific developmental stages and determines the ultimate fates of these cells. This feature makes the OE an ideal system for studies of the regulatory roles that such transcription factors play in neurogenesis, and also provides the molecular markers needed to decode effects of extrinsic factors, such as signaling molecules, on the regulation of neurogenesis. Indeed, studies of mice with mutations in genes encoding regulatory transcription factors have proved to be extremely informative in understanding the roles that such factors play in regulating ORN and VNO development.

Although no definitive stem cell marker has been found for OE and VNO stem cells, a likely candidate is the transcription factor, Sox2. Sox2, a transcription factor of the SoxB1-type SRY transcription factor family, is thought to be a general neuronal stem cell marker: It is expressed throughout the neural primordium in rodents, is an important regulator of embryonic development, and has been shown to direct neural progenitor identity. Moreover, in a number of

neural tissues, *Sox2*-expressing cells have been shown to be capable of both self-renewal and differentiation, suggesting that *Sox2* gene expression is a trait shared by stem cells in many neural systems. During primary neurogenesis in the OE, expression of *Sox2* mRNA defines the neuroepithelial domain of the invaginating olfactory pit, and co-expression of *Sox2* and *Fgf8* has recently been reported to define a population of primordial neural stem cells that will give rise to all subsequent neural stem and progenitor cell types of the OE (**Figure 3**). Indeed, in mice in which the *Fgf8* gene is inactivated in anterior neural structures, this *Sox2–Fgf8* co-expressing cell population undergoes apoptosis, leading to a failure in subsequent OE neurogenesis, nasal cavity formation, and morphogenesis of the VNO (see later). Thus, although no stem cell marker has been identified for the VNO, the observation that the VNO fails to develop at all in *Fgf8* conditional mutant mice strongly suggests that the *Sox2–Fgf8* co-expressing primordial neural stem cells, observed to play a critical role in the early stages of primary neurogenesis in the OE, give rise to the neurogenic population of the VNO as well.

During primary neurogenesis at e10–e11, cells expressing the proneural gene, *Mash1*, are found in close apposition to *Sox2–Fgf8* co-expressing primordial neural stem cells near the rim of the invaginating olfactory pit (**Figure 3**). A day or so later in development (e12.5), *Mash1* mRNA can be detected in cells found in the apical, middle, and basal compartments of the OE, coincident with the location of mitotic figures at this age. As the OE matures and enters the phase of established neurogenesis, *Mash1*-expressing cells come to be located primarily in the basal compartment of the OE, suggesting that the action of *Mash1* is required early in the ORN lineage. *Mash1* has been shown in genetic studies to be required for ORN development, and in studies *in vitro*, in OE cultures, and *in vivo*, in surgical models of induced neurogenesis, *Mash1* has been shown to be expressed by early-stage transit-amplifying progenitors of the ORN lineage (**Figure 1(b)**). Indeed, in mice with targeted inactivation of the *Mash1* gene, *Ngn1*-expressing INPs, as well as ORNs, fail to develop, indicating that *Mash1* acts upstream of *Ngn1* to direct neuronal differentiation in the OE.

Recent studies indicate that, in the absence of *Mash1* function, the OE reverts to a state in which it maintains high levels of both proliferation and apoptosis. Proliferating cells express *Sox2*, the *Mash1* 3′-untranslated region (3′-UTR; which is still present in the targeted mutant), and *Steel*, a marker of supporting cells. This has led to the hypothesis that these proliferating cells in the OE of *Mash1*$^{-/-}$ mice are 'frozen' at an early stage of differentiation, and the fact that these cells co-express markers of both supporting cells (*Steel*) and early neuronal progenitors (*Mash1* 3′-UTR) suggests that they would be capable, if they did not undergo apoptosis (the precipitating cause of neurogenic failure in the *Mash1* mutant), of giving rise to both ORNs and supporting cells. These observations provide indirect evidence that the neural stem cell of the OE is a bipotential stem cell, capable of giving rise to both glial and neuronal cell types. Interestingly, neurogenesis also fails in the VNO of *Mash1*$^{-/-}$ mice, and in the same manner as in main OE: *Ngn1* expression fails to occur and neurons fail to form, while the *Mash1* 3′-UTR is expressed in abundant proliferating cells that undergo high levels of apoptosis. Thus, the developmental hierarchy of gene expression in both the main OE and the VNO appear to be fundamentally similar.

As they progress through the ORN lineage, *Mash1*-expressing progenitors lose expression of *Mash1* and upregulate expression of a different proneural gene, *Ngn1*. *Ngn1* expression defines the immediate neuronal precursor, which has been shown by tissue culture and genetic studies to be committed to ORN differentiation after one to two rounds of division. Evidence for the function of *Ngn1* as a neural determination gene first came from studies in *Xenopus*. In *Xenopus*, misexpression of an *Ngn1* homolog can convert nonneurogenic ectodermal cells to neurons. In *Ngn1* mutant OE, most ORNs fail to develop and differentiate (at least by the end of primary neurogenesis at e12.5), suggesting that mammalian *Ngn1* plays a role similar to that of its *Xenopus* counterpart. Moreover, although *Ngn1* expression is severely reduced in the OE of *Mash1* mutant mice, *Mash1* expression is not significantly affected in the OE of *Ngn1*-null mice, indicating that *Mash1* and *Ngn1* expression are essential at different stages of differentiation and that *Mash1* acts upstream of *Ngn1* in the ORN lineage.

A number of other transcription factor genes play roles in regulating neuronal differentiation in the OE, including *RunX1* and *NeuroD*. *Runx1* encodes a member of the Runt/Runx family of transcription factors, whereas *NeuroD*, like *Mash1* and *Ngn1*, encodes a basic helix–loop–helix transcription factor. In the OE, expression of *Runx1* and *NeuroD* is restricted primarily to cells in the basal half of the epithelium. Evidence from developmental genetic studies suggests that *NeuroD* is expressed at the stage when late, *Ngn1*-expressing neuronal progenitors are just differentiating into ORNs. Gene expression studies indicate that only a few cells co-express *Runx1* and *Mash1*, whereas virtually all cells expressing *NeuroD* also express *Runx1*. Thus, expression data suggest that both *Runx1* and *NeuroD* act at the time when

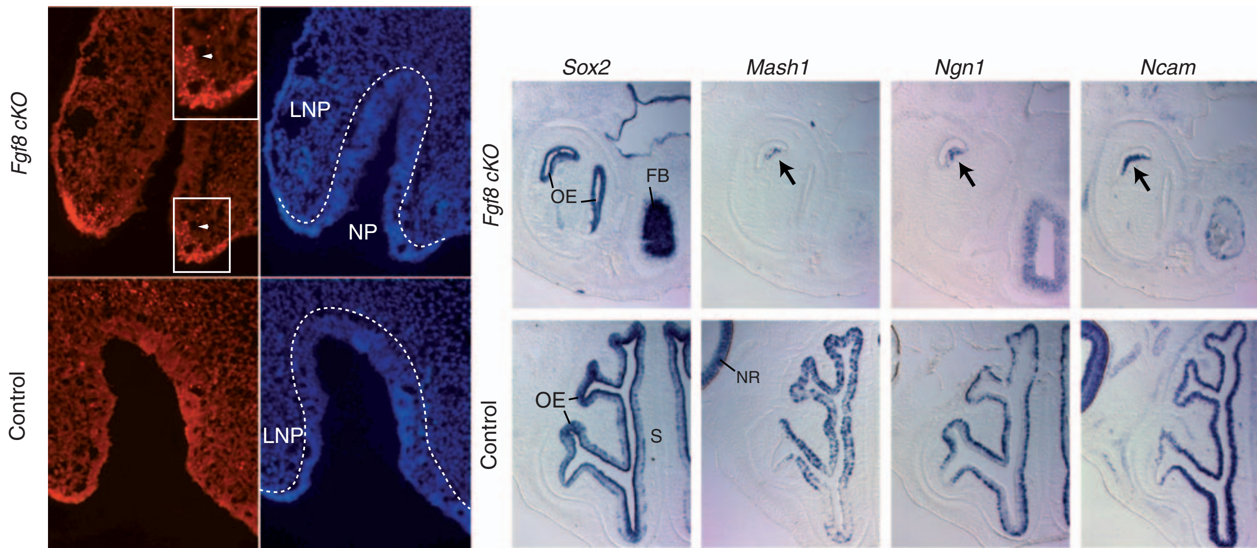
late-stage neuronal progenitors (INPs) are undergoing terminal differentiation into ORNs. OE development in mice with targeted inactivation of the *Runx1* gene has only been examined up to the end of the primary phase of neurogenesis, since homozygous nulls die at e12.5. Interestingly, in the OE of e12.5 *Runx1*$^{-/-}$ embryos, the total number of cells, and the number of *Mash1*-expressing cells, appear to be unchanged; however, there is a decrease in the number of *NeuroD*-expressing cells and an increase in cells expressing the early neuronal marker β-III tubulin. Since Runx1 is also known to repress expression of cyclin-dependent kinase inhibitors (which act as 'brakes' on mitotic cells in the G1/S transition; see later), these observations have been interpreted as showing a role for Runx1 in regulating *NeuroD* expression and terminal differentiation of OE neuronal progenitors into postmitotic ORNs. Since *NeuroD* is expressed in the VNO in a pattern analogous to its expression in OE, by extension it seems possible that Runx1 may function in this tissue as well to regulate sensory neuron differentiation, although this has not yet been investigated.

Control of Neurogenesis by Extrinsic Factors

Developmental transitions in expression of transcriptional regulators, cell proliferation, cell differentiation, and intraepithelial cell location in the OE are directed by the actions of extrinsic signaling molecules. Different signaling molecules appear to predominate during the primary and established phases of neurogenesis, and these factors are produced both within the OE itself and by its underlying mesenchymal stroma (also known as the lamina propria of the mature epithelium). The actions of these secreted signaling molecules in regulating programs of neuronal cell proliferation and differentiation in the OE are classified into two categories: (1) proneurogenic effects, which are positive effects on OE neurogenesis and include stimulation of progenitor cell proliferation and cell survival, and (2) antineurogenic effects, which include suppression of cell proliferation and, directly or indirectly, increases in cell death (apoptosis). Both categories of action are important for OE and VNO morphogenesis and acquisition and maintenance of proper sensory neuron number, and therefore for OE function during development and postnatal life. Over the past several years, studies have revealed that OE neurogenesis is critically dependent on signaling molecules from two different polypeptide growth factor superfamilies, fibroblast growth factors (FGFs) and transforming growth factor-βs (TGF-βs). Each of these superfamilies of signaling molecules includes many members, all of which have specific patterns of expression and differing functions during development and tissue homeostasis. During OE neurogenesis it has been found that different factors from these two superfamilies interact in at least two ways: first, opposing signals converge on neuronal stem and progenitor cells at specific developmental stages in the ORN lineage, regulating proliferation and the stepwise progression of neuronal differentiation. Second, TGF-βs and their secreted antagonists play key roles in feedback loops that regulate the size of progenitor cell pools and the number of ORNs that differentiate from these cell pools. Since these developmental pathways have been worked out almost exclusively in the main OE of rodents, particularly the mouse, the following discussion deals primarily with mouse main OE.

Fibroblast Growth Factors

During OE development, several different FGFs are known to be expressed, and since expression of these FGFs is developmentally regulated, it is thought that the functions of different FGFs predominate at different developmental stages. It has recently been established that FGF8 plays a crucial role in OE development during the early stages of primary neurogenesis. Tissue-specific inactivation of the *Fgf8* gene has shown that *Fgf8* expression, which is highest in the ectoderm and neuroepithelium that outlines the rim of the invaginating pit at e10–e11 in the mouse, defines a morphogenetic center of function that is crucial for OE neurogenesis, nasal cavity morphogenesis, and development of the VNO. As mentioned previously, a subpopulation of *Sox2*-expressing neural stem cells, located in the outermost rim of the invaginating OE neuroepithelium, also express *Fgf8* (**Figure 3**); these cells have been termed 'primordial' neural stem cells of the OE. In mice with tissue-specific inactivation of *Fgf8*, these *Sox2*–*Fgf8* co-expressing cells undergo apoptosis (**Figure 5**(**a**)). As a result, OE neurogenesis fails, as does nasal cavity formation; indeed, by e14.5, only a small vestige of remaining OE, with just a few neural cells, can be observed – and this is seen only in the least severely affected mutants (**Figures 5**(**b**) and **5**(**c**)). These animals also lack any VNO, indicating that FGF8 is a crucial signal for morphogenesis and neurogenesis in the VNO as well as in the main OE. The effects of the neurogenic deficits resulting from absence of FGF8 are long lasting: although animals survive until birth, no OE or VNO ever forms, and only a few, scattered neural cells are observed in the malformed oral–nasal cavity that does develop. These observations indicate



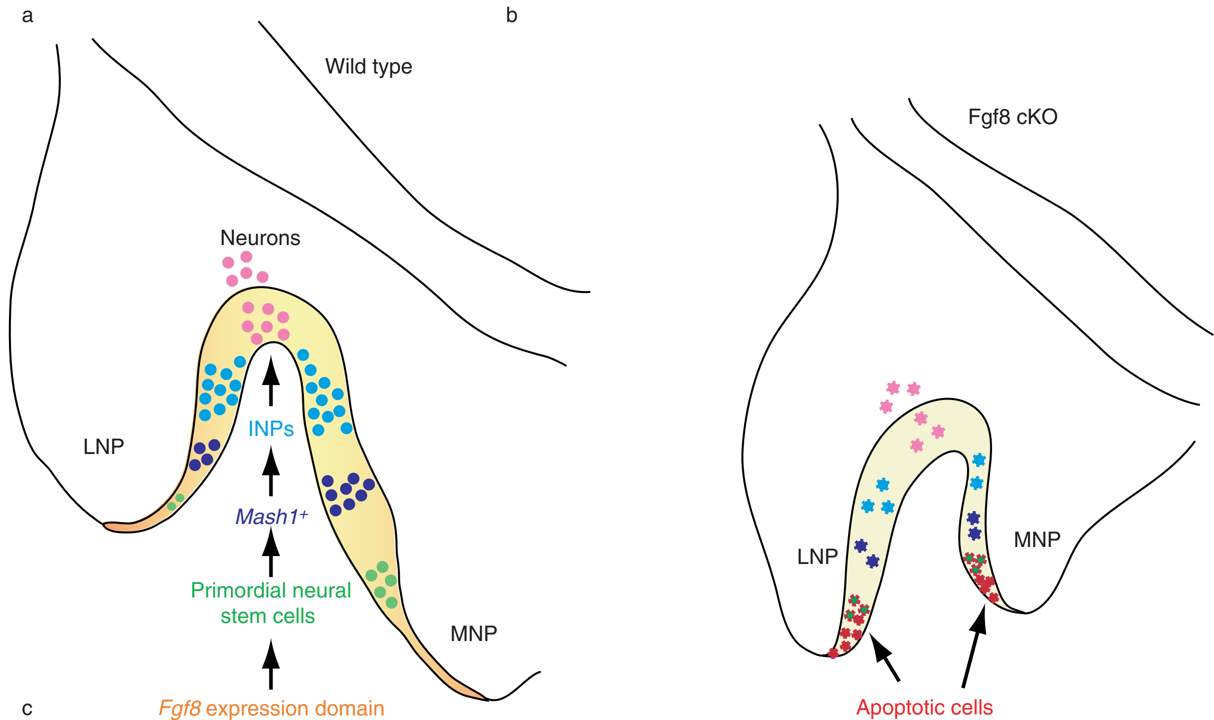


Figure 5 *Fgf8* is required for cell survival in the neurogenic domain. (a) Terminal dUTP nick end labeling (TUNEL) of olfactory epithelium of mutant (cKO; conditional knockout) and control littermates at day 10.5 of gestation shows a high number of apoptotic cells in mutants in ectoderm and olfactory epithelium (white arrowhead; magnified in inset) of invaginating nasal pit (NP). Hoescht panel (blue, right) shows extent of invaginating NP. Broken white line indicates the boundary of the neuroepithelium lining the NP and lateral nasal process (LNP). (b) *In situ* hybridization on horizontal sections of mutant and control littermates (day 14.5 of gestation) shows the near absence of OE in the mutants and the lack of neuronal markers in the remaining OE (arrow) (FB, forebrain; S, nasal septum; NR, neural retina). (c) Role of *Fgf8* in olfactory neurogenesis. Schematic of primary neurogenesis at day 10.5 of gestation in wild-type OE and *Fgf8* mutant OE, illustrating the relative positions (MNP, medial nasal process) of the *Fgf8* expression domain and different neuronal cell types: *Fgf8* expression domain, orange; *Sox2*-expressing neuroepithelium, yellow; *Sox2*- and *Fgf8*-expressing primordial stem cells, green; *Mash1*-expressing progenitors, blue; immediate neuronal precursors (INPs),turquoise; *Ncam*-expressing olfactory receptor neurons, pink. In the mutant invaginating pit, cells undergoing apoptosis due to *Fgf8* inactivation are shown in red and apoptotic primordial neural stem cells are green with red jagged borders. Diminished populations of other neuronal cell types are shown in their corresponding colors, but with jagged edges. Adapted from Kawauchi S, Shou J, Santos R, et al. (2005) Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse. *Development* 132(23): 5211–5223.

that FGF8 is required not only for the survival and expansion of the neural stem cell pool during primary neurogenesis (**Figure 5(c)**), but also that it is required for maintenance of primary neurogenesis and, as a result, the initiation of the established phase of neurogenesis. Interestingly, *Fgf8* expression declines by the end of primary neurogenesis (e12.5), when another closely related FGF, encoded by *Fgf18*, begins to be expressed within the neuroepithelium of the OE. It has been hypothesized that FGF18 assumes FGF8's role in maintenance of the stem cell population during the embryonic stages of the established phase of neurogenesis, although this idea has not been tested directly in *Fgf18* mutant mice.

Unlike *Fgf8*, *Fgf2* does not appear to be expressed at significant levels in prenatal mouse OE, but in adult mice, it is expressed throughout the neuronal cell layers of the main OE. By 3 weeks of age, FGF2 expression can also be detected in axon bundles that converge below the basal lamina, en route to the olfactory bulb. Like FGF8, FGF2 can also function as a proneurogenic factor: In cultures of OE, addition of FGF2 increases the number of proliferating INPs, by promoting multiple rounds of INP divisions before INP daughter cells undergo terminal differentiation into ORNs; FGF2 has also been shown to be capable of promoting survival and proliferation of putative stem cells in OE cultures. Taken together with the pattern of *Fgf2* expression, these observations suggest that FGF2 is likely to be involved in maintaining neuronal stem and progenitor cells, as well as in serving as a mitogen for these cells, in order to regulate ORN number in postnatal OE.

TGF-β Superfamily

Bone morphogenetic proteins Many studies indicate that the bone morphogenetic proteins (BMPs), a subfamily of TGF-βs structurally related to the *Drosophila* signaling molecules Dpp and 60A, have important roles in regulating neuronal fate determination and neurogenesis during vertebrate development. For example, endogenous BMP4 promotes acquisition of an epidermal fate, at the expense of neural tissue, in developing ectoderm, and BMP2 and BMP4 have been shown to inhibit proliferation and/or induce apoptosis of neural progenitor cells in several systems. Studies of the OE have shown that BMPs can exert both pro- and antineurogenic effects, depending on the concentration and identity of the BMP in question and the identity of the target cell that is acted upon.

Bmp2, *Bmp4*, and *Bmp7* are all expressed in and/or near OE proper during embryonic development in the mouse, and all three of the secreted proteins (BMP4, BMP2, and BMP7) can inhibit OE neurogenesis *in vitro* by acting on *Mash1*-expressing neuronal progenitors. Exposure to any of the three BMPs causes these progenitors to target preexisting *Mash1*-encoded protein for proteasome-mediated degradation, resulting in apoptosis and termination of the ORN developmental pathway at the *Mash1*-expressing stage. Interestingly, however, low concentrations of BMP4, but not BMP7, stimulate OE neurogenesis: In this case, the action of BMP4 is to promote survival of newly generated ORNs. Since BMP4 and BMP7 selectively activate different subsets of serine–threonine kinase type I BMP receptors, which are known to be differentially expressed within the OE, it is likely that differential responsiveness of specific OE neuronal stem/progenitor cells to different concentrations of specific BMPs is dictated by the identity of the cell surface receptors that are activated in the various target cell types. The OE is thus proving to be a useful model system for dissecting out the cellular basis of concentration-dependent responses to BMPs, an area of broad interest to both developmental biologists and cancer biologists.

Growth and differentiation factor 11 and feedback inhibition of neurogenesis Studies *in vitro* and *in vivo* have shown that generation of new ORNs by their progenitors is inhibited by a signal produced by OE neuronal cells themselves; this process has been termed feedback inhibition of neurogenesis. Recently, growth and differentiation factor 11 (GDF11), a member of a small subgroup of activin-like TGF-βs that includes the muscle regulatory factor GDF8 (also called myostatin), has been shown to mediate feedback inhibition of INP proliferation during the established phase of OE neurogenesis. *Gdf11* is expressed by both committed neuronal progenitors and newly differentiated ORNs in the OE, as are its serine–threonine kinase transmembrane receptors (ALK5 and ActRIIb). Both *Gdf11* and the gene that encodes its secreted antagonist, follistatin (FST), are first expressed at significant levels at the onset of the established phase of neurogenesis, and their expression continues through adult life (*Gdf11* is expressed only within OE neuroepithelium proper; *Fst* is expressed in OE stroma and, at lower levels, in OE proper). Recombinant GDF11 inhibits OE neurogenesis *in vitro*, by reversibly arresting INP divisions; this action is accompanied by induction of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ in these neuronal progenitors. The antiproliferative effect of GDF11 on INPs is abrogated by addition of FST to OE cultures, but, interestingly, GDF11 actions

predominate over the proliferative effect of FGFs on INPs. *Gdf11*-null mice show increased cell proliferation in the OE, and the number of *Ngn1*-expressing INPs is increased, as is overall OE thickness, relative to that of wild-type control littermates. These supernumerary INPs go on to give rise to ORNs, as demonstrated by an increase in *Ncam*-expressing cells in *Gdf11*-null OE (**Figure 6**(**a**)). FST, a secreted protein that prevents effective binding and signaling by GDF11, is able to completely antagonize the antineurogenic effect of GDF11 on INPs in OE cultures. Importantly, the number of INPs and ORNs is dramatically decreased in $Fst^{-/-}$ OE, consistent with what would be expected with removal of a crucial brake on the antineurogenic actions of endogenous GDF11 in the OE neuroepithelium (**Figure 6**(**b**)). These observations have led to the proposal of a model in which GDF11 and FST interact to maintain neuron number in the OE by tightly regulating INP divisions and ORN production. Since GDF11 arrests INP proliferation but does not kill INPs, its role in feedback inhibition of OE neurogenesis is likely to be one of maintaining INPs in stasis, such that they are capable of responding rapidly to a decrease in ambient GDF11 levels (as when OE neuronal cells are lost through injury or disease) by rapidly reentering the cell cycle and giving rise to ORNs (**Figure 6**(**c**)).

Summary and Conclusions

In addition to revealing major steps in morphogenesis and neurogenesis of the OE and VNO, studies of the development of these two sensory neuroepithelia have revealed important aspects of the molecular regulation of neurogenesis. Studies of main OE development in particular have led to the concept that neurogenesis in olfactory sensory epithelia proceeds in a linear manner, with expression of specific transcription factors marking the progressive restriction in fate observed as neuronal stem and committed progenitor cells differentiate through the ORN developmental pathway. Both these intrinsic signals, which operate in a cell-autonomous manner, and extrinsic (secreted) signals present in the local microenvironment of developing cells (the stem/progenitor cell niche) play important roles in regulating this process. The factors that predominate during primary olfactory neurogenesis play an expansionary, morphogenetic role. For example, FGF8, which is crucial for increasing the size of the stem cell pool, is of vital importance at this stage in order to establish the ORN lineage in the main OE and to permit formation of the VNO (**Figure 7**). Once the OE neurogenesis enters its established phase, both FGFs and TGF-βs participate in regulating neuronal cell number. Studies of the

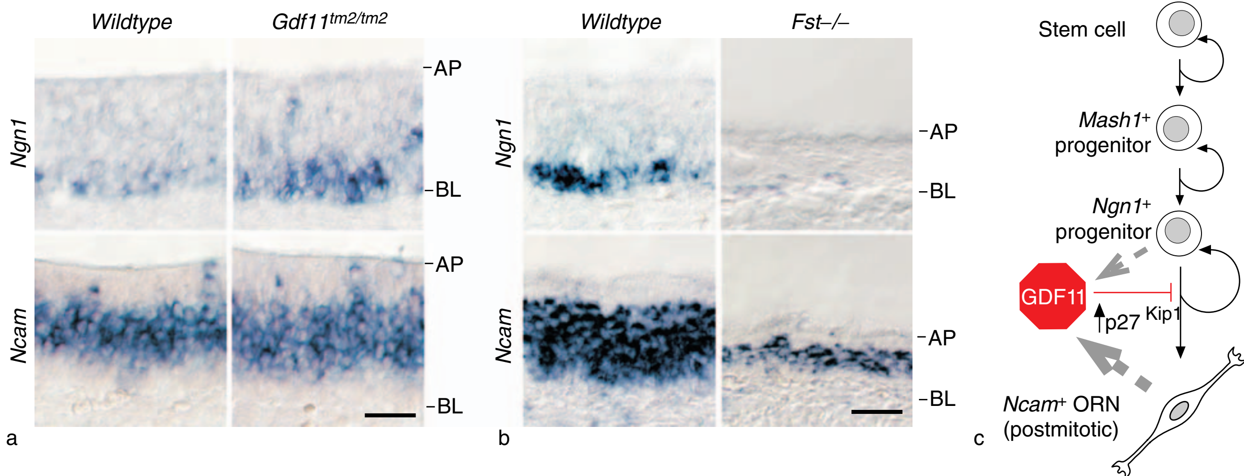


Figure 6 Disruption of neurogenesis in mice with loss or gain of function of *Gdf11*. (a) *In situ* hybridization on horizontal sections (AP, apical layer; BL, basal layer) of olfactory epithelium of $Gdf11^{tm2/tm2}$ mice reveals an increase in *Ngn1*-expressing cells and a corresponding increase in *Ncam*-expressing cells: the *Ncam*-expressing cell layer is thicker by 20% (9 μm), about the diameter of one olfactory receptor neuron (ORN), in $Gdf11^{tm2/tm2}$ olfactory epithelium, compared to in wild type. (b) Mice lacking a functional *follistatin* (*Fst*) gene show decreased olfactory epithelium neurogenesis. *In situ* hybridization for *Ngn1* and *Ncam* shows large decreases in expression of both markers as well as aberrantly thin olfactory epithelium in the *Fst* mutant. (c) Schematic model of growth and differentiation factor 11 (GDF11) action in ORN neurogenesis. GDF11 is produced by both *Ngn1*+ progenitors and *Ncam*+ ORNs (gray broken-line arrows). GDF11 reversibly arrests *Ngn1*+ progenitors through induction of the cycle-dependent kinase inhibitor $p27^{Kip1}$, thus preventing ORN generation. Reproduced from Wu HH, Ivkovic S, Murray RC, et al. (2003) Autoregulation of neurogenesis by GDF11. *Neuron* 37: 197–207, with permission from Elsevier.

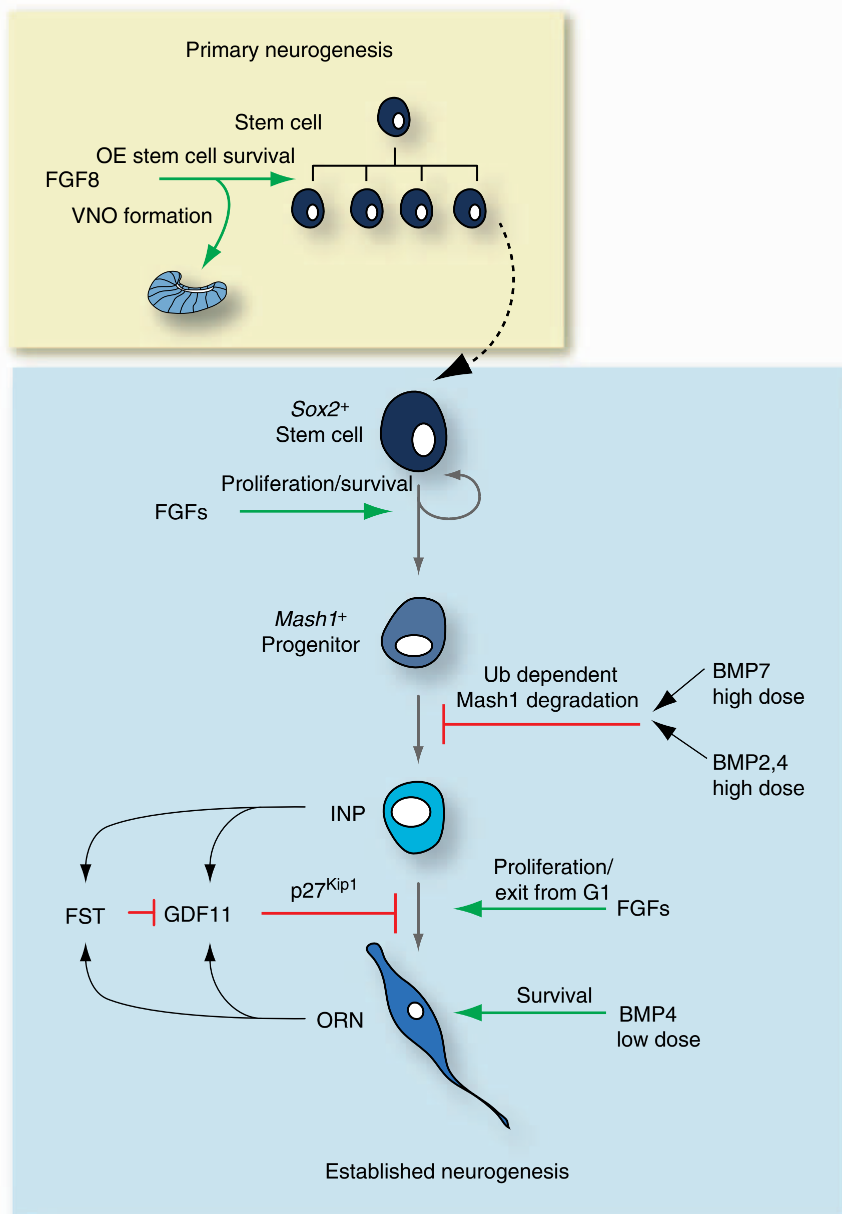


Figure 7 Summary of molecular signals involved in both primary and established olfactory epithelium (OE) neurogenesis. During primary neurogenesis (yellow box), fibroblast growth factor 8 (FGF8) and other factors are involved in producing the stem cell pool and initially establishing the different progenitors in the olfactory receptor neuron lineage. FGF8 is also necessary for initial vomeronasal organ (VNO) formation. After day 12.5 of gestation, once the lineage is established (blue box), FGFs and transforming growth factor-βs (BMP, bone morphogenetic protein; FST, follistatin; GDF, growth and differentiation factor) converge on different cell types in the OE lineage to achieve and maintain proper neuron number. Green arrows indicate stimulatory interactions; red bars indicate inhibitory interactions. Adapted from Kawauchi S, Beites CL, Crocker CE, et al. (2004) Molecular signals regulating proliferation of stem and progenitor cells in mouse olfactory epithelium. *Developmental Neuroscience* 26: 166–180, with permission. Copyright 2004 by S. Karger AG, Basel, Switzerland.

actions of TGF-βs and their secreted antagonists in regulating OE neurogenesis have led to the concept of feedback inhibition of neurogenesis, in which feedback circuits consisting of endogenous secreted antineurogenic factors regulate the size of the stem/progenitor cell pool and total neuron number. Ultimately, determining whether similarities in these processes exist between the OE and other regions of the nervous system should provide important knowledge for understanding how neuronal stem/progenitor cell pools are controlled in all neural tissues, and for overcoming roadblocks to recovery in regions of the

adult nervous system where – unlike the OE – neuronal regeneration is limited or absent.

See also: Drosophila Apterous Neurons: from Stem Cell to Unique Neuron.

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Oligodendrocyte Specification

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Specification of Oligodendrocyte Precursor Cells in the Embryonic Neural Tube

All the neurons and glial cells of the central nervous system (CNS) are derived from the neuroepithelial progenitor cells that form the walls of the embryonic neural tube. The neuroepithelial cell layer (ventricular zone (VZ)) of the spinal cord and brain stem can be subdivided along the dorsal–ventral axis into 11 distinct microdomains, defined by expression of different combinations of transcription factors (**Figure 1**). These progenitor domains generate different types of neurons followed by glial cells (oligodendrocytes or astrocytes). Fully differentiated glia are not formed directly from the neuroepithelium but via intermediate glial precursors. During or after neuronogenesis, the neuroepithelial progenitors give rise to so-called radial glial cells, which express characteristic gene products such as the glutamate transporter GLAST and the radial cell antigen RC2. Radial glia subsequently generate dedicated astrocyte or oligodendrocyte precursors. In a given domain of the VZ, radial glia produce either mainly astrocytes or mainly oligodendrocytes. For example, the pMN domain of the ventral spinal cord (**Figure 1**) generates motor neurons followed by oligodendrocyte precursors (OPCs) but few astrocytes. Overall, approximately 80% of spinal cord oligodendrocytes are derived from ventrally derived OPCs, mainly pMN, and the remaining 20% from OPCs that originate in dorsal domains dP3–5 (**Figure 1**).

Visualizing Oligodendrocytes and Their Precursors *In Situ*

From the earliest stages of their development, migratory OPCs can be visualized by immunohistochemistry or *in situ* hybridization against a characteristic set of markers, including the platelet-derived growth factor receptor (α subunit) (PDGFRA), the proteoglycan NG2, the transcription factor SOX10, and, in chicken only, surface antigens recognized by monoclonal antibody O4. In rodents, O4 antigen does not appear until a later, premyelinating stage (called pro-oligodendrocytes) when the precursors have stopped migrating and are beginning to differentiate into oligodendrocytes. In both birds and rodents, actively myelinating oligodendrocytes can be easily recognized by the expression of proteins (or corresponding mRNAs) that are abundant structural components of myelin, such as myelin basic protein and myelin proteolipid protein.

Using these and other molecular markers, it has been shown that OPCs are first generated in the pMN progenitor domain of the ventral spinal cord at 12.5 days postfertilization (E12.5) in mice, E14 in rats, and E6 or E7 in chick. In human spinal cord, OPCs appear on or before 45 days postfertilization. The following discussion, unless otherwise stated, refers to mouse. After the OPCs first appear at the ventricular surface, they proliferate and migrate throughout the cord, becoming evenly distributed through the gray matter and developing white matter before birth. Starting at approximately E18 (birth is approximately E19 in mice), oligodendrocyte differentiation starts in the ventral white matter and continues for approximately 6 weeks, peaking at approximately postnatal days 10–14. In human spinal cord, myelination of corticospinal tracts continues into the teenage years.

Spatial Control of OPC Generation by Signals from the Ventral and Dorsal Midline

In the ventral spinal cord, specification of both motor neurons and OPCs depends on signaling molecules that are secreted from the notochord and floor plate at the ventral midline, notably the secreted protein Sonic hedgehog (SHH). If chicken embryos are treated *in ovo* with cyclopamine, an inhibitor of SHH signaling, then the appearance of OPCs in the ventral spinal cord is blocked. Conversely, supplying an ectopic source of SHH (e.g., by implanting SHH-expressing cells or fragments of notochord adjacent to the spinal cord) induces extra OPCs in misplaced positions. Members of the bone morphogenetic protein (BMP) family of signaling molecules are also involved in limiting the spatial extent of OPC generation in the ventral cord. BMPs (notably BMP2 and BMP4) are expressed in the dorsal spinal cord and exert long-range influences along the dorsal–ventral axis. This has been demonstrated by surgically removing the BMP-expressing domain *in ovo* or by transplanting cells that express the competitive BMP inhibitor Noggin next to the chick spinal cord. Both these manipulations caused the OPC-producing pMN domain to expand dorsally. The extra OPCs were generated at the expense of astrocytes, which are normally produced in the p2 progenitor domain, immediately dorsal to pMN. Therefore, it seems that the

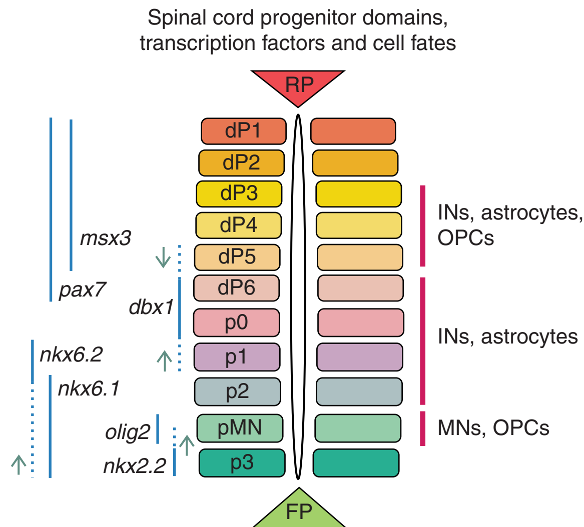

Figure 1 Diagram of progenitor domains in the VZ of the embryonic (~E11) mouse spinal cord. Domains are known as p3, pMN, etc. in the ventral half of the cord and dP6, dP5, etc. in the dorsal half. To the left of the diagram are shown the expression limits of transcription factors, some of which are mentioned in the text. Dotted lines with small arrows indicate that the expression domain expands or shrinks after it is first established. On the right are shown the cell fates of progenitors in the indicated domains (the origins of astrocytes are still tentative). FP, floor plate; INs, interneurons; MNs, motor neurons; OPCs, oligodendrocyte precursor cells; RP, roof plate.

spatial extent of OPC and astrocyte production in the ventral cord is limited by mutually antagonistic actions of SHH and BMPs.

The majority (approximately 80%) of OPCs in the spinal cord are generated in the ventral VZ. The remainder are generated in other progenitor domains, including dorsal domains dP3–5. The dorsally derived OPCs appear later in development than the pMN-derived OPCs (approximately E15 vs. E12.5) and they migrate less widely than their ventrally derived counterparts. It seems unlikely that these dorsal progenitor domains are under the influence of SHH from the floor plate, suggesting that there might be a SHH-independent route to OPC specification. Indeed, OPCs can arise in cultures derived from SHH null spinal cord or in the presence of cyclopamine, if fibroblast growth factor-2 (FGF-2) is also present in the culture medium. OPC generation in the dorsal spinal cord might therefore depend on FGF signaling, possibly combined with a decline in BMP expression in the dorsal cord during late embryogenesis. There might even be a biochemical overlap between SHH and FGF signaling because it has been shown that both pathways depend on MAP kinase activity.

It is not known whether or not ventrally and dorsally derived OPCs in the spinal cord are functionally specialized. However, there is evidence that ventrally and dorsally derived OPCs in the forebrain can substitute for one another, implying that they are functionally equivalent.

OPC Generation in the Forebrain

The adult forebrain, including the cerebral cortex, develops from an embryonic structure called the telencephalon. Like the spinal cord, this starts as a simple neuroepithelial tube, although it becomes progressively more convoluted during development. There is no notochord underlying the telencephalon and no floor plate; however, the ventral neuroepithelial cells express SHH and its receptors, Patched (PTC) and Smoothened (SMO). At approximately E13.5, some cells in the ventral neuroepithelium (medial ganglionic eminence (MGE)) start to express OPC markers such as PDGFRA and SOX10 and migrate away from their origin through the ventral telencephalon. Generation of these early OPCs depends on SHH because they do not appear in embryos that lack SHH expression in the ventral telencephalon (*Nkx2.1* null mice). Later, other parts of the VZ generate OPCs in a temporal wave of production from ventral to dorsal. OPCs from the MGE and lateral ganglionic eminence (LGE) initially populate the developing forebrain including the cerebral cortex before birth, to be joined after birth by OPCs that originate within the cortex. The latter OPCs remain in the cortex and do not migrate ventrally.

The question of whether all these OPCs and the oligodendrocytes that they give rise to are functionally specialized, or equivalent, was addressed by killing specific subpopulations of OPCs at source. This was achieved by targeting diphtheria toxin A-chain expression to subpopulations of OPCs that originate in different domains of the telencephalic neuroepithelium (MGE, LGE, and cortex) by combinatorial use of an oligodendrocyte lineage-specific gene promoter (*Sox10*) and one of several promoters that are active in different parts of the VZ (*Nkx2.1, Gsh2*, or *Emx1*). These experiments showed that when OPCs originating within the cortical VZ (normally approximately 50% of all OPCs in the cortex) were ablated, the remaining ventrally derived populations expanded to make up the loss and the animals survived and lived a normal life span. Even when all telencephalic OPCs from MGE, LGE, and cortex were ablated simultaneously, they were replaced by OPCs that migrated forward from the diencephalon. A normal OPC cell density was restored – with a significant but ultimately harmless delay – indicating that OPCs from different parts of the embryonic neuroepithelium are not intrinsically different from one another despite the very different signaling environments in which they arise.

Of course, there might be subtle differences that would not be detected without detailed behavioral analysis of the ablated mice, but this remains to be investigated.

Role of Transcription Factors in OPC Development: The OLIG Genes

A major step forward in understanding the molecular control of oligodendrocyte lineage development resulted from the discovery of transcription factors that orchestrate OPC specification and differentiation. Prime among these are the oligodendrocyte lineage (OLIG) transcription factors, OLIG1 and OLIG2. These are members of the large family of basic helix–loop–helix factors that also includes the pro-neural proteins NGN1/2 and MASH1 and the cell lineage regulators MYOD and NEUROD. In the developing spinal cord, SHH induces expression of OLIG2 in pMN, prior to and during motor neuron (MN) production. OLIG2 is downregulated rapidly in postmitotic MNs but remains on in OPCs as they proliferate and migrate away through the parenchyma. OLIG2 is required for both MN and OPC specification because both cell types are lost in *Olig2* null spinal cords. Pockets of OPCs persist in the brains of *Olig2* null mice, but no OPCs are found anywhere in the CNS of *Olig1/2* compound nulls. Therefore, OLIG1 might partly compensate for loss of OLIG2 in the brain. There is no effect on OPC generation in either the brain or the spinal cord of *Olig1* null mice, although there can be severe myelination defects later. OLIG1 therefore seems to be mainly involved in the later stages of oligodendrocyte differentiation and myelination.

Note that different laboratories have reported different experiences with *Olig1* knockout mice, depending on whether or not the *PGK-Neo* cassette used to inactivate the locus was removed prior to establishing mouse lines. When the *PGK-Neo* cassette was left in place, there was no obvious dysmyelinating phenotype during development, possibly because of *cis*-acting effects of the *PGK-Neo* transcription unit on the nearby *Olig2* gene. However, even in the latter mice there was a striking effect on remyelination of adult mice that were subjected to gliotoxin-induced experimental demyelination; such demyelinated lesions are rapidly repaired in wild-type mice but remyelination was blocked in the *Olig1* knockouts.

The underlying mechanism by which OLIG2 governs the sequential production of MNs and OPCs ('neuron–glial switch') is not known. Presumably, OLIG2 switches binding partners (transcriptional cofactors) during the transition from MN to OPC production. During the period of MN production, OLIG2 and the homeodomain transcription factor NKX2.2 are mutually repressive so that the OLIG2 and NKX2.2 expression domains (pMN and p3, respectively) are sharply demarcated (**Figure 2**). Later, after MN production has ceased, the cross-repression seems to relax because NKX2.2 expression creeps dorsally into pMN and an overlap region develops. Initially, it was thought that OPCs might be generated specifically within this overlap region under the cooperative activities of OLIG2 and NKX2.2. In chick, it does appear that PDGFRA^{+} OPCs are formed exclusively within the overlap region, but in mice OPCs arise in all parts of the OLIG2-expressing pMN domain, not just the region of overlap with NKX2.2 (**Figure 2**). Moreover, OPC specification is not affected in *Nkx2.2* null mice,

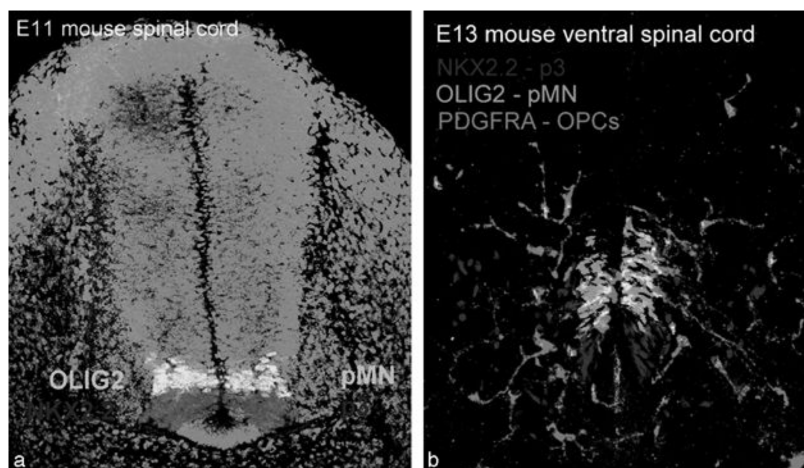

Figure 2 (a) Immunolabeling of ventral progenitor domains p3 and pMN in the mouse spinal cord with antibodies against transcription factors NKX2.2 and OLIG2, respectively. Motor neurons are still being generated at E11. (b) Three-color immunolabeling of NKX2.2 (p3, green), OLIG2 (pMN, blue), and PDGFRA (newly forming OPCs, red) in the mouse ventral spinal cord at E13. The first OPCs are formed mainly within the OLIG2-expressing pMN domain in mouse, outside the NKX2.2-expressing p3 domain. After they are formed at the ventricular surface, the OPCs migrate away rapidly in all directions to populate the spinal cord.

although the later stages of oligodendrocyte differentiation and myelination are delayed. A similar delay in myelination is observed in mice that lack the high mobility group transcription factor SOX10, which is known to interact with OLIG1 and OLIG2. OLIG2 has also been shown to interact physically with NKX2.2. Therefore, a picture is emerging in which OLIG1/2, NKX2.2, and SOX10 cooperate in controlling myelin gene expression and axon ensheathment. OLIG2 and other members of the SOX family (including SOX8 and SOX9) are required earlier for OPC and/or MN specification, but so far there is no compelling explanation for the neuron–glial switch.

Notch–Delta signaling is clearly involved in the MN/OPC fate choice as in many other binary decisions during development. It has been shown that, in zebra fish, abrogation of Notch signaling results in excess production of MNs at the expense of OPCs. Conversely, constitutive activation of Notch signaling results in excess OPCs at the expense of MNs. A well-established general function of Notch–Delta signaling is to maintain precursor cells in an immature state and to inhibit premature cell cycle exit and differentiation, thus preserving part of the precursor pool for generation of later-born cell types. Since MNs are formed before OPCs, the gain- and loss-of-function experiments in zebra fish spinal cord can be interpreted in those terms – implying that Notch signaling might play a permissive rather than an instructive role in the MN/OPC fate choice.

Control of OPC Proliferation

After they are specified in the embryonic VZ, OPCs proliferate and migrate away from their sites of origin, distributing widely and uniformly throughout the CNS before associating with axons and differentiating into myelin-forming oligodendrocytes (**Figure 3**). Several mitogenic polypeptides have been shown to influence OPC proliferation in culture and/or *in vivo*, including PDGF, FGF, and cytokines. These probably act in concert with one another, possibly in different combinations in different regions of the CNS and/or at different stages of OPC development.

OPCs express receptors for PDGF (PDGFRA subtype), and many neurons and astrocytes synthesize PDGF-A. *PDGF-A* null mice have reduced OPC numbers (approximately 10% normal numbers in the spinal cord and approximately 50% in the cerebral cortex) and correspondingly reduced amounts of myelin. Therefore, homodimeric PDGF-AA is an essential mitogen for OPCs *in vivo*. PDGF-AA is also strongly mitogenic for OPCs in mixed neural cell cultures, such as mixed glial cell cultures derived from perinatal rat optic nerve. However, PDGF-AA is not very mitogenic for pure, immunoselected populations of OPCs cultured on their own in the absence of other cell types. There are other essential mitogens or mitogenic cofactors released into the extracellular medium of mixed cell cultures that are required to cooperate with PDGF-AA. One of these is the CXC cytokine GRO-alpha. Astrocytes are a major source of GRO-alpha in optic nerve cultures and probably also *in vivo*. Cell adhesion molecules, notably integrin family members, also synergize with PDGF and possibly other polypeptide mitogens to drive OPC proliferation at limiting mitogen concentrations.

FGF-2 also enhances the mitogenic effect of PDGF-AA for OPC glial cell cultures. OPCs cultured with both PDGF-AA and FGF-2 can proliferate seemingly indefinitely without differentiating, whereas PDGF-AA in the absence of FGF allows limited OPC proliferation followed by oligodendrocyte differentation. FGF-2 on its own in the absence of PDGF is mitogenic for a late stage of OPC development, when OPCs stop migrating and express the O4 antigen prior to

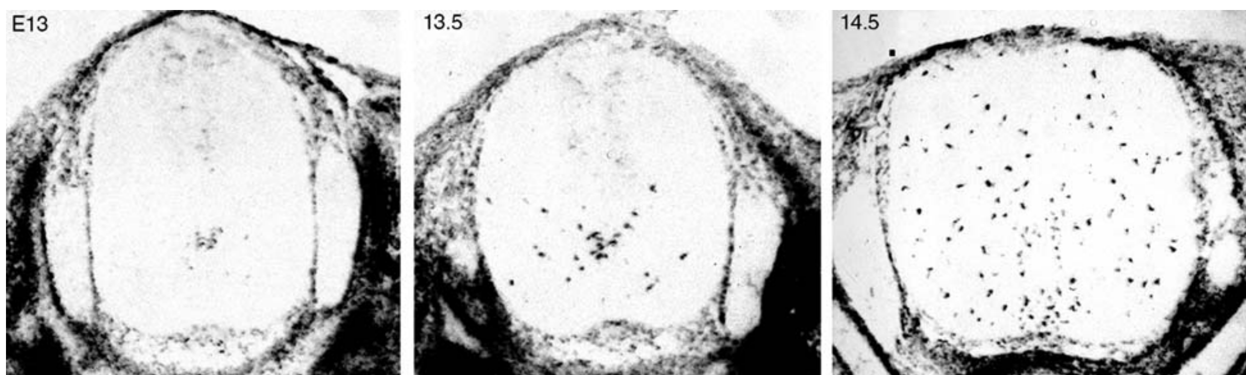

Figure 3 OPCs, visualized by *in situ* hybridization for *Pdgfra* mRNA, in the developing mouse spinal cord. The first OPCs appear in pMN at approximately E12.5, and then they proliferate in response to PDGF-AA and other mitogenic cofactors and migrate throughout the cord, becoming widely distributed by approximately E15. They first start to differentiate into oligodendrocytes in the ventral and dorsal axon tracts soon before birth (E19 in mice).

terminal oligodendrocyte differentiation – so-called 'pro-oligodendrocytes.' Studies on the role of FGF *in vivo* are complicated by the large number of potential ligands (>20 FGF family members) and the fact that OPCs express different FGF receptor subtypes (FGFR1–3) at different stages of their development. Nevertheless, retrovirus-mediated inhibition of FGF-2 activity *in vivo* has shown that FGF-2, acting through FGFR1, negatively regulates OPC terminal differentiation into oligodendrocytes during normal development and after experimental demyelination in adult mice.

Other signaling systems that have been implicated in the control of OPC proliferation include neurotrophin-3, neuregulin-1/glial growth factor (NRG1/GGF), and insulin-like growth factor-1. There is also a growing awareness of the importance of ion channels and neurotransmitters in OPC proliferation and differentiation control.

Control of OPC Differentiation

After OPCs have disseminated throughout the CNS and into the future gray and white matter, something triggers them to differentiate into oligodendrocytes. One key signal seems to be the thyroid hormone triiodothyronine (T3), a constituent of the defined culture medium commonly used for primary nerve cells. When T3 is omitted from the culture medium, OPCs divide for an extended period in response to PDGF-AA, well beyond their normal limit in the presence of T3. It appears that T3 is required for withdrawal from the cell cycle prior to terminal differentiation. This effect of T3 can be mimicked by glucocorticoids or retinoic acid.

There is an established literature on the role of thyroid hormone (TH) in brain development and myelination. The start of myelination is delayed in hypothyroid rats and accelerated in hyperthyroid rats or rats that receive postnatal injections of T3. Moreover, TH normally only becomes available in the rat when the thyroid gland becomes active after birth, which is approximately the time when myelin first starts to appear. Oligodendrocytes and OPCs possess receptors for T3, so it is likely that T3 acts directly on OPCs to control their differentiation *in vivo*.

Notch signaling is also implicated in the control of oligodendrocyte differentiation. OPCs express Notch-1, and adding the Notch ligand Jagged to optic nerve cell cultures inhibits OPC differentiation into oligodendrocytes *in vitro*. Jagged is expressed on optic nerve axons *in vivo*, suggesting that differentiation of OPCs in the optic nerve might be triggered in part by downregulation of Jagged in the retinal ganglion cells that project their axons through the nerve. In support of this general idea, conditional deletion of Notch-1 in OPCs in transgenic mice leads to premature expression of oligodendrocyte differentiation markers such as the myelin-associated glycoprotein.

Control of Myelination by Neuregulin-1/Glial Growth Factor

Not all axons are myelinated: Axons must reach a certain minimum size (diameter) threshold before myelination is triggered. Below this threshold the axon remains naked. This is true in both the CNS and the peripheral nervous system (PNS). In addition, there is a direct relationship between the diameter of a myelinated axon and the thickness of the myelin sheath that develops around that axon (i.e., larger diameter axons have more myelin wraps). The myelin-modulating signal resides on the axonal surface and is interpreted by the myelinating glia (oligodendrocytes or Schwann cells) which adjust their synthesis of myelin membrane accordingly.

Neuregulins are a family of signaling proteins encoded in three loci, *Nrg1–3*, each of which generates alternatively spliced products that can be either secreted or membrane bound. They bind to receptors of the ErbB family (ErbB1–4), which includes the epidermal growth factor receptor ErbB1. NRG1 type III, also known as GGF, is now known to be the main axon-bound regulator of myelination in the PNS, acting through ErbB receptors on Schwann cells. The expectation that one or more NRG isoforms might also regulate myelinogenesis in the CNS is supported by the finding that expressing a dominant negative ErbB transgene in oligodendrocytes results in thinner myelin sheaths and associated physiological deficits, including reduced conduction velocity and, unexpectedly, increased dopaminergic activity of neurons. The precise NRG isoform responsible is not known. These initial results are exciting because of the implied links between myelin deficiency, neuronal activity, and, potentially, psychiatric illness.

OPCs in the Adult CNS

Myelination continues for at least 6 weeks postnatally in mice and rats, peaking 2 or 3 weeks after birth. However, cells with the antigenic phenotype and morphology of OPCs persist in large numbers in the adult CNS (approximately 4% of all cells). Like their perinatal counterparts, they continue to express both PDGFRA and the NG2 proteoglycan and retain the capacity to generate oligodendrocytes in culture or following experimentally induced demyelination. However, because there has been no compelling reason to think that significant numbers of new

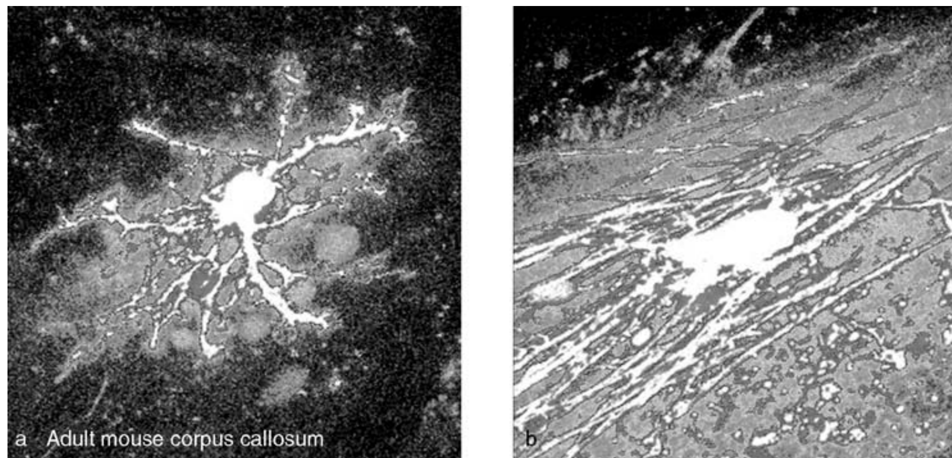

Figure 4 Individual oligodendrocyte lineage cells labeled *in situ* (in 300-μm live sections of postnatal mouse corpus callosum) by microinjection of fluorescent Alexa dye. The myelinating oligodendrocyte (b) was formed between 6 and 8 weeks after birth from a PDGFRA-expressing adult OPC/NG2 cell (a). The long rod-like processes in b are myelin sheaths ('internodes') around nerve axons. Micrographs courtesy of M Rizzi.

oligodendrocytes are needed during normal healthy life, why large numbers of OPCs should survive in the adult has been puzzling.

It has been recognized that OPCs in the adult make contact with nodes of Ranvier (the gaps between adjacent myelin sheaths on an axon), they receive synaptic input from neurons, and they can even fire spontaneous action potentials. This has led to the notion that OPCs participate in the normal physiology of the adult CNS and that their primary role is not necessarily as glial precursors. They have been called 'a fourth glial cell type' (after astrocytes, oligodendrocytes, and microglia), 'synantocytes,' 'polydendrocytes,' or, more commonly, 'NG2 cells.' The physiological role of these cells is under intense scrutiny.

We should not reject the idea that NG2 cells might be needed to generate new or replacement oligodendrocytes throughout life. Cre-lox fate mapping in adult mice (using tamoxifen-inducible *Pdgfra-CreER*^{T2}) shows that they continue to divide and generate significant numbers of new myelinating oligodendrocytes in the corpus callosum of adult animals (**Figure 4**). Whether this ongoing oligodendrogenesis occurs to replace cells that die through natural turnover or to add extra oligodendrocytes to the existing pool is not known. It is now accepted that new neurons are generated in some parts of the adult brain throughout life; for example, new olfactory interneurons are continuously generated from stem cells that reside in the adult subventricular zones of the forebrain. It is conceivable that some adult-born neurons might need to be myelinated, and this could be one important function of the adult NG2 cells. Alternatively, or in addition, the axonal diameters of some previously unmyelinated neurons might increase during life, taking them over the threshold for *de novo* myelination.

Finally, NG2 cells might have more general cell fate potential. It is known that OPCs in perinatal optic nerve cell cultures can give rise to neurons, astrocytes, and oligodendrocytes if cultured in an appropriate manner. It is therefore possible, though not yet demonstrated, that OPCs in the adult CNS might be able to generate new neurons or astrocytes as well as oligodendrocytes. If so, adult OPCs/NG2 cells would qualify as a type of adult neural stem cells.

See also: *Drosophila* Apterous Neurons: from Stem Cell to Unique Neuron; Motor Neuron Specification in Vertebrates.

Further Reading

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Hair Cell Differentiation

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The primary transducers for the sensations of hearing, balance, and acceleration are mechanosensory hair cells located within the auditory and vestibular regions of the inner ear. The auditory region comprises the spiral-shaped cochlea, which contains the organ of Corti, an elongated sensory epithelium with approximately 20 000 mechanosensory hair cells arranged into four or five distinct rows (**Figure 1(a)**). The vestibular system comprises three semicircular canals, each with associated ampullae and two saclike structures, the utricle and the saccule. The ampullae of each semicircular canal contain a sensory crista that detects angular acceleration, and the utricle and saccule each contain a macula for the detection of linear acceleration (**Figure 1(b)**). Regardless of location within the ear, all sensory epithelia are composed of two basic cell types, mechanosensory hair cells and nonsensory supporting cells, arranged in a pseudostratified configuration with hair cells located adjacent to the lumenal surface and single or multiple layers of supporting cells located between the hair cell layer and the basement membrane (**Figure 1(c)**). Because the epithelium is pseudostratified, all supporting cells extend processes that terminate at the lumenal surface. Mechanosensory hair cells derive their name from a group of modified group of microvilli, referred to as stereocilia, that extend from the lumenal surface of each cell. The group of stereocilia appears similar to a tuft of hair. Deflection of the stereociliary bundle in response to fluid movement in the lumenal space results in a depolarization of the resting potential and changes in the rate of neurotransmitter release.

Prosensory Specification

All hair cells are derived from one or two specialized regions, referred to as prosensory patches, which arise in the ventromedial wall of the bilateral otocysts, placodally derived structures that originate adjacent to the hindbrain. With continued development, the otocysts undergo a number of morphogenetic changes to give rise to all of the auditory and vestibular structures of the inner ear. At the same time, cells from these prosensory patches become distributed throughout the ear to form the various sensory epithelia just described. The factors that specify the prosensory patches have not been conclusively identified, but several genes that are known to regulate cellular identity in other systems are expressed at early times in prosensory patch development. These include *Jagged1, Sox2, Lunatic fringe (Lfng), Pax2, Bmp4,* and *FGF10* (**Figure 2**). Functional data concerning the roles of some of these factors are discussed next.

Role of Notch Signaling in Prosensory Specification

The Notch signaling pathway has been highly conserved throughout evolution and has been shown to regulate multiple events in embryonic development. In mammals, four Notch genes code for membrane-bound receptors that are activated on binding with any one of a family of membrane ligands that includes Jagged1, Jagged2, Delta1, and Delta3. Ligand binding results in the cleavage of the Notch protein to yield an intracellular component (Notch-ICD) that is then translocated to the nucleus. Notch1 is expressed throughout the developing inner ear, beginning at the early otic placode stage and persisting throughout development. Jagged1 expression begins about the time of placodal invagination and, although initially diffuse in expression, rapidly resolves to the developing prosensory patches. Historically, the primary role of Notch signaling has been considered to be to mediate lateral inhibitory interactions between cells. However, more recent data suggest that Notch signaling can have a positive, inductive effect on cell fate. The early expression of Notch1 and one of its ligands within developing prosensory patches is consistent with a role in the specification of these patches. And, as would be predicted, the deletion of *Jagged1* within the otocyst leads to a nearly complete loss of hair cells. However, an equivalent deletion of *Notch1* does not result in a similar phenotype. In fact, the loss of *Notch1* results, instead, in an overproduction of hair cells. The bases for these differences are only partially understood, but they strongly suggest that Jagged1 acts through the activation of another Notch gene. The potential role of Notch signaling in the specification of prosensory patches was recently supported by the demonstration that the transient overexpression of a constitutively active Notch1-ICD construct in chick otocysts results in the induction of ectopic sensory patches. Furthermore, the expression of serrate (the chick Jagged1 homolog) was upregulated within ectopic sensory patches, suggesting the existence of a positive feedback loop mediated through Jagged1/Notch activity leading to a lateral induction process.

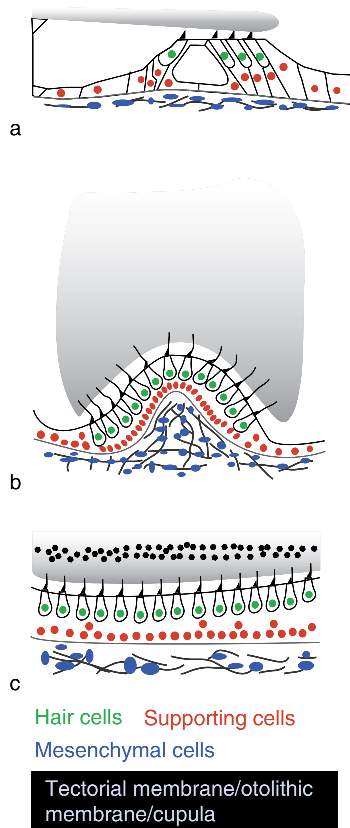

Figure 1 Diagrammatic cross-sections through the sensory organs of the mammalian inner ear: (a) the organ of Corti, the sensory epithelium of the mammalian cochlea; (b) a crista ampullaris, the sensory epithelium within a semicircular canal; (c) a vestibular sensory epithelium such as the sacculus or utriculus. In (a), the sensory epithelium of the organ of Corti is composed of two basic cell types, hair cells (green nuclei) and supporting cells (red nuclei) that lie on a basement membrane referred to as the basal lamina. Beneath the basal lamina is a layer of mesenchymal cells (blue nuclei). Hair cells have a specialized stereociliary bundle (black) on their apical surface that comes in contact with an overlying extracellular matrix called the tectorial membrane (gray). The displacement of this extracellular matrix relative to the hair cells provides the primary stimulus for mechanotransduction through deflection of the stereociliary bundle. In (b), as in the cochlea, the crista ampullaris is composed of hair cells and supporting cells. The hair cell stereocilia come in contact with an overlying extracellular matrix that is similar in composition to the tectorial membrane; it is referred to as a cupula. In (c), hair cells, supporting cells, and mesenchymal cells are present in a pattern similar to that in other hair cell sensory epithelia. Stereocilia project into an overlying extracellular matrix, the otoconial membrane, which also contains individual calcium carbonate-based otoconia (black hexagons).

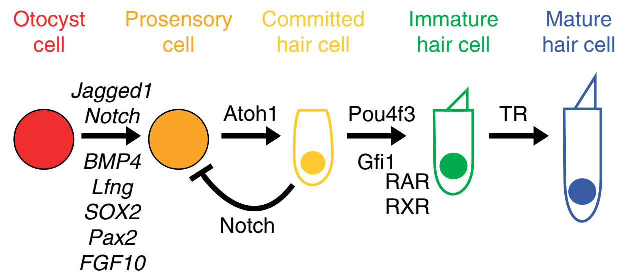

Figure 2 Summary of signaling molecules that are known to mediate hair cell commitment and differentiation. In the first step in hair cell formation, cells within the otocyst become specified to develop as prosensory cells. Although the factors that mediate this transition are not fully understood, a number of genes, including *Jagged1, Notch, Bmp4, Lnfg, Sox2, Pax2,* and *Fgf10,* are expressed in patterns that are consistent with a role in prosensory specification. Next, prosensory cells become committed to develop as hair cells through the expression of *Atoh1*. However, subsequent cell–cell interactions divert some prosensory cells from the hair cell fate through Notch-mediated lateral inhibitory interactions. During the first step of hair cell differentiation, committed hair cells begin to express Pou4f3 and Gfi1. In addition, early hair cell differentiation requires the activation of retinoic acid receptors (RAR and RXR). Finally, later differentiation requires signaling through thyroid hormone receptors (TR).

SOX2

The SRY-related transcription factor SOX2 is also expressed from the early otocyst stage, and its pattern of expression overlaps with Jagged1. However, expression of Sox2 is downregulated in *Jagged1* null mutants, suggesting that it is downstream of Notch signaling. Deletion of *Sox2* specifically within the developing inner ear results in a complete loss of hair cells and hair cell precursors, demonstrating the importance of this gene in hair cell development. A hypomorphic *Sox2* mutant with limited expression within the inner ear shows a partial loss of the prosensory patches, indicating a gene dosage effect.

BMP4

The secreted morphogen BMP4 is considered to be the earliest and most definitive marker for prosensory patches, even though it is expressed in all developing prosensory patches in birds but excluded from the saccular, utricular, and cochlear sensory patches in mammals. Consistent with a role in specification of the prosensory patch, treatment with BMP4 protein *in vitro* results in increased hair cell formation in both chicken otocyst cultures and mammalian cochlear cultures, whereas inhibition of BMP4 signaling using Noggin results in a decrease in the number of hair cells. However, it is important to consider that an additional study actually obtained contradictory results for the role of BMP4 and Noggin in chick otocysts, suggesting that the role of BMP4 may not be straightforward.

Pax2, *Gata-3*, and *Lfng*

As noted, several other molecules with known developmental roles, such as *Pax2*, *Gata3*, and *Lfng* are also expressed in patterns that are consistent with a role in prosensory specification. However, prosensory

patches and hair cells still form in mice with specific deletions in each of these genes, strongly suggesting either that these factors are dispensable for hair cell formation or that compensatory mechanisms exist either normally or in response to loss of gene function.

Exit from the Cell Cycle

Following specification of prosensory patches, individual progenitor cells must become specified to develop as either hair cells or supporting cells. The results of a number of studies have demonstrated a strong link between cell cycle exit and the onset of cellular commitment and differentiation. Within the inner ears of mammals, cell cycle exit occurs in precise patterns that are specific for each sensory epithelium. In the mouse cochlea, cell cycle exit occurs in a gradient that begins in the apex around E12.5 and proceeds toward the base over the next 72 h. In the vestibular epithelia, cell cycle exit typically occurs in a central to peripheral gradient in which cells in the center of the patch are the first to become postmitotic. Cell cycle exit in the inner ear is controlled through the cyclin-dependent kinase (Cdk) inhibitors $p27^{kip1}$ and $p19^{ink4dA}$ and the retinoblastoma tumor suppressor gene pRb, all of which act as negative regulators of proliferation. pRb appears to act as a primary regulator of cell cycle based on a pattern of expression that accurately precedes the pattern of terminal mitosis (except in the cochlea) and the fact that deletion throughout the otocyst results in ongoing proliferation of sensory progenitor cells and an overproduction of hair cells and supporting cells. In the cochlea, the pattern of expression of pRb does not match either the spatial or temporal gradient of terminal mitoses. Instead, cell cycle exit is preceded by expression of $p27^{kip1}$. Moreover, in $p27^{kip1}$ null mice, cell cycle exit is delayed, allowing one or two additional rounds of mitosis, and the pattern of terminal mitosis is altered to follow the pattern of expression of pRb. Finally, deletion of $p19^{Ink4d}$, which is also expressed in hair cells, results in postnatal hair cells reentering the cell cycle, suggesting that hair cells rely on $p19^{Ink4d}$ to maintain a postmitotic state.

Commitment of Hair Cells

As discussed, cellular commitment typically follows closely behind terminal mitosis. Therefore, once cells within the prosensory patches exit the cell cycle, individual progenitor cells must be specified to develop as either hair cells or supporting cells. Based on morphological characteristics and expression of cell-specific markers, hair cells become specified first. The earliest indication of hair cell commitment is the expression of the basic helix–loop–helix transcription factor Atoh1 (formerly, Math1) (**Figure 3**). Atoh1 is initially expressed in a group of cells that appears to include progenitors that ultimately develop as both hair cells and supporting cells. However, as development continues, the expression of Atoh1 becomes restricted to cells that will develop as hair cells. The role of Atoh1 in hair cell development has been examined in a number of different studies. First, targeted deletion of *Atoh1* in a mouse model leads to a complete lack of hair cells in the both the auditory and vestibular sensory epithelia. In addition, the forced expression of Atoh1 in the developing cochlear sensory epithelium is sufficient to induce hair cell formation. And, surprisingly, forced expression of Atoh1 outside of the prosensory patches also leads to hair cell formation. The ability of Atoh1 to induce hair cell formation appears to persist even into the adult because the introduction of an *Atoh1*-expressing adenovirus into the ears of adult guinea pigs results in the formation of ectopic hair cells.

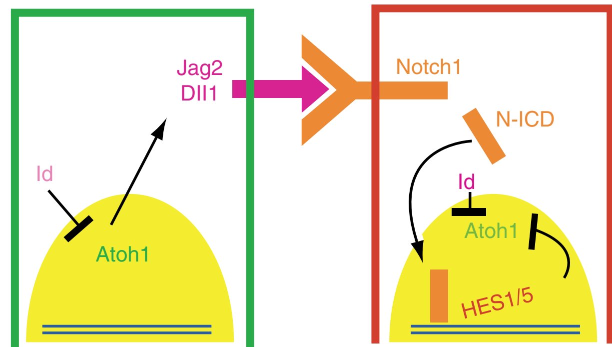

Figure 3 Regulation of hair cell fate through the Notch pathway. Prior to hair cell commitment, cells express Atoh1 along with members of the Id family. In response to downregulation of Ids, some cells are able to increase the expression of Atoh1, leading to the activation of Notch ligands, Jag2 and Dll1. Activated Jag2 and Dll1 bind to Notch1 receptors, leading to the generation of an intracellular fragment of Notch1, referred to as Notch-ICD (N-ICD). N-ICD is translocated to the nucleus, where it activates the expression of the inhibitory bHLH genes, *HES1* and *HES5*. HES1 and HES5 combine with Ids to downregulate the expression of Atoh1, leading to the inhibition of the hair cell fate.

As discussed, Atoh1 is apparently initially expressed in a larger number of cells than ultimately develop as hair cells. This observation suggests that some of the cells that initially become committed to a hair cell fate are subsequently diverted from that fate through cell–cell signaling. In all inner ear sensory epithelia, hair cells and supporting cells are arranged in a mosaic such that hair cells are completely surrounded by supporting cells. However, laser ablation of developing hair cells in either the embryonic mammalian cochlea or in mature amphibian lateral-line

neuromasts results in a change in the fate of neighboring cells, leading to a regenerative response. Based on the mosaic pattern and the observed effects of the removal of existing hair cells, a lateral-inhibitory mechanism was initially proposed to regulate the generation of the cellular mosaic. As previously discussed, the Notch signaling pathway was characterized based on its ability to mediate lateral inhibitory interactions. Therefore, a second role for Notch signaling during inner ear development seemed likely. Consistent with this hypothesis, developing hair cells in different species express one or more Notch ligands, including *Jagged2* and *Delta1*, whereas surrounding supporting cells express *Notch1*. In addition, supporting cells also express the Notch target genes *HES1* and *HES5* Therefore, the patterns of expression for all these genes are consistent with an inhibitory interaction in which developing hair cells use Jagged2 and Delta1 to activate Notch1, and subsequently HES1 and/or HES5, in neighboring cells with the effect of that activation being to inhibit those cells from developing as hair cells. As would be expected based on this hypothesis, the deletion of any of the genes in this pathway leads to an overproduction of hair cells. Similarly, the disruption of the Notch signaling pathway in zebra fish as a result of mutations in the *deltaa* gene or in *mindbomb*, a gene that encodes an E3 ubiquitin ligase that is responsible for targeting Notch for degradation, also leads to an increase in the number of hair cells with a concomitant decrease in the number of supporting cells. In addition, recent work has demonstrated that Atoh1 is a target of Notch1 in the ear and that overexpression of HES1 is sufficient to inhibit the ability of Atoh1 to induce hair cell formation. These results strongly suggest that developing hair cells directly inhibit their neighbors from developing as hair cells through a Notch-mediated inhibition of Atoh1.

One intriguing aspect of the role of Notch signaling in hair cell development is the observation that Jagged2 and Delta1 are not expressed in all Atoh1-positive cells. In many systems in which Notch signaling is used to sort cells into multiple fates, Notch and one or more Notch ligands are initially expressed throughout the population of progenitor cells with subsequent cell–cell interactions leading to some cells increasing the expression of Notch while other cells increase the expression of the Notch ligand. However, in the ear, only developing hair cells appear to express Notch ligands, suggesting that these cells may be biased toward the hair cell fate. Recently, a group of molecules referred to as inhibitors of differentiation (Ids) has been shown to play a role in this bias. Ids are helix–loop–helix molecules that are similar to bHLH molecules, except that they lack the basic domain that is required for DNA binding. As a result, Ids act as negative regulators of bHLH molecules, such as *Atoh1*, through competition for a common dimerization partner. The Id genes, *Id1*, *Id2*, and *Id3*, are expressed in the developing cochlea, but become specifically downregulated in developing hair cells. Moreover, prolonged expression of Id3 in individual cells within the ear leads to the inhibition of hair cell formation. Based on these results, it seems likely that downregulation of Id expression plays key step in the determination of which, and how many, cells within the ear develop as hair cells.

It is also important to consider that, with the exclusion of mammals, all vertebrates classes are able to generate new hair cells throughout the life of the organism, either as a result of normal turnover or as part of a regenerative response. In either case, the generation of new cells requires the specification of progenitors as hair cells. In most cases, the specification of new hair cells in adult animals involves existing cells within the epithelium, presumably supporting cells, reentering the cell cycle to divide and give rise to progenitor cells that then become specified as hair cells or supporting cells through a recapitulation of the developmental processes already described. However, there is also evidence indicating that under some circumstances supporting cells can develop as hair cells through a direct transformation referred to as transdifferentiation. During transdifferentation, supporting cells are presumed to downregulate supporting cell-specific genes and then to upregulate hair cell-specific factors, starting with cAtoh1 (the chick Atoh1 homolog). The subsequent events that lead to the development of these cells as hair cells have not been determined. Moreover, it is not clear how much of the subsequent signaling events described are recapitulated in transdifferentiating cells. For instance, would transdifferentiating hair cells need to activate the Notch signaling pathway to inhibit neighboring cells from developing as hair cells because these cells are already supporting cells? Clearly the answers to these questions, as well as, a more complete understanding of the differences between hair cell development and hair cell regeneration should have significant implications for the development of regenerative strategies.

Differentiation and Survival of Hair Cells

Following the commitment to a hair cell fate, the earliest molecule that is known to be expressed exclusively in differentiating hair cells is the unconventional myosin, myosin-VI. In the developing mammalian cochlea, myosin-VI can be detected in developing hair cells at almost the same time as

Atoh1. However, despite its early onset of expression, myosin-VI does not appear to play a role in hair cell differentiation because mutations in myosin-VI do not result in defects in hair cell development. The same is true for another unconventional myosin, myosin-VIIa, which is expressed soon after myosin-VI but is also not required for hair cell formation. At this point, the early functions, if any, of myosin-VI and myosin-VIIa remain unclear. Both molecules are required for the normal development and maintenance of the stereociliary bundle, but because the bundle does not develop until several days after initial commitment and differentiation, it is assumed that both myosins must play additional roles in early hair cell formation.

POU4f3, Gfi1, and Barhl1

Soon after the onset of expression of myosin-VI and myosin-VIIa, developing hair cells begin to express a group of transcription factors that are required for hair cell survival and possibly differentiation. The first of these factors is the class IV Pou-domain transcription factor, Pou4f3 (also known as Brn-3c or Brn-3.1). Expression of Pou4f3 is first detected approximately 24–48 h after the onset of expression of Atoh1. In cochleae from *Pou4f3*$^{-/-}$ animals, the initial development of hair cells appears normal, as determined by expression of myosin-VI and myosin-VIIa; however, there is a rapid degeneration leading to hair cell death and auditory and vestibular deficits, indicating that Pou4f3 is required for hair cell maintenance. Moreover, late-onset progressive hearing loss occurs in an Israeli Jewish family with an 8 bp deletion in the homeodomain of *POU4F3,* further demonstrating the importance of this gene in hair cell maintenance.

Following the onset of expression of Pou4f3, developing hair cells upregulate Gfi1, a zinc finger transcription factor that has been implicated in development of the hematopoietic system. As is the case for *Pou4f3*, the deletion of *Gfi1* leads to early degeneration of hair cells; however, the expression of Atoh1, myosin-VI, myosin-VIIa, and Pou4f3 is not disrupted, indicating that Gfi1 acts downstream of Pou4f3.

Beginning about the same time as Pou4f3, hair cells also express Barhl1, a member of the Bar family of homeobox genes. In Drosophila, barh1 and barh2 are required for the development of sensory organs. However, the development of hair cells in mice with a targeted deletion of *Barhl1* appears normal, with a full complement of hair cells formed at postnatal day (P)0. Although hair cells develop normally, signs of hair cell degeneration are present beginning at P6 and all hair cells ultimately die, but not until P300. Based on these results, it seems likely that *Barhl1* acts as a hair cell survival gene, whereas *Pou4f3* and *Gfi1* may act as both survival and differentiation genes. Because the hair cells die during the differentiation process in both *Pou4f3* and *Gfi1* mutants, it is not possible to determine whether survival, differentiation, or both are disrupted.

Role of Retinoic Acid Signaling in Hair Cell Differentiation

Another group of transcription factors that are expressed in developing hair cells soon after the onset of cellular differentiation is the nuclear receptors for retinoic acid (RA) and thyroid hormone (TH). Receptors for RA and TH belong to the steroid/thyroid receptor superfamily containing a zinc finger DNA binding domain and a ligand dimerization domain. RA signaling is mediated by two different forms of receptors, RARs (α, β, and γ) and RXRs (α, β, and γ), both of which are expressed in inner ear-sensory epithelia. The treatment of cochlear explant cultures with RA results in supernumerary hair cells and supporting cells, and it similarly induces hair cell differentiation in chicken otocyst cultures. The inhibition of RA synthesis or antagonism of RARα results in a decrease in the number of cells that develop as hair cells. However, Brn3.1 and myosin-VI are still expressed in hair cells, even in the presence of RA inhibitors, suggesting that initial differentiation of hair cells does not require RA. Based on these results, it was suggested that RA signaling through RARα plays a direct role in hair cell differentiation during early stages in development. However, a small number of hair cells are present in RARα/RARγ double-mutant mice (RARα mutant mice are phenotypically normal), suggesting that hair cell differentiation is not completely dependent on RA. However, considering that there are three *RAR* genes and three *RXR* genes, and that functional redundancy of RA receptors has been demonstrated in other systems, it would be informative to inactivate all three RAR genes or all six RA receptor genes within the inner ear.

Role of TH Signaling in Hair Cell Differentiation

Hypothyroidism or iodine deficiencies in pregnant women are known to cause hearing deficits in children, and induced hypothyroidism in animal models results in severe morphological abnormalities in the organ of Corti that appear consistent with a delay in cellular differentiation. Thyroid hormone signaling is mediated through three functional receptors, Trα1, Trβ1, and Trβ2, and a nonligand binding, Trα2.

All of these receptors are expressed within the developing inner ear; however, the expression of *Trβ1* and *Trβ2* is confined to the developing cochlear epithelium. Elimination of TRβ1 and TRβ2 through deletion of the *Trβ* gene results in animals that are deaf as a result of a defect in the maturation of a hair cell ionic conductance that is required for cellular function. However, the overall structure of the organ of Corti is not comparable with hypothryroid animals and, in fact, appears normal, suggesting a possible functional redundancy. Consistent with this hypothesis, the deletion of both *Trα* and *Trβ* results in a more profound hearing loss than in animals lacking just *Trβ*, and the morphology of the organ of Corti in these mice is consistent with the hypothyroid phenotype.

In both hypothyroid animals and *Trα/Trβ* double mutants, the phenotype of the organ of Corti is consistent with a delay or arrest in cellular differentiation. To determine whether this is in fact the case, the expression of a molecule that is turned on fairly late in the development of a subset of hair cells, Prestin, was examined in hypothyroid animals. As expected, Prestin expression is delayed in hypothyroid hair cells. Moreover, an analysis of the promoter region for the *Prestin* gene identified two specific TR binding elements, strongly suggesting that ongoing differentiation of hair cells is dependent on thyroid hormone signaling.

Summary

The specification of hair cells within the inner ear is dependent on the activation of a number of molecular signaling pathways that progressively direct the development of prosensory patches and subsequently direct individual cells within those patches to develop as hair cells. Although the factors that direct the specification of prosensory patches are not fully understood, the Notch signaling pathway and the transcription factor Sox2 clearly play important roles. Within each prosensory patch, the bHLH transcription factor Atoh1 is necessary and sufficient to induce progenitors to develop as hair cells, whereas subsequent Notch-mediated inhibitory interactions actually determine how many cells will develop as mature hair cells. Once committed, developing hair cells activate a number of genes, including *Pou4f3* and *Gfi1,* that are required for their survival and, probably, for differentiation. In addition, developing hair cells become dependent on external signaling factors, such as RA and TH, to stimulate further steps in their differentiation toward functioning hair cells.

See also: Helix–Loop–Helix (bHLH) Proteins: Proneural; Notch Signal Transduction: Molecular and Cellular Mechanisms; Notch Pathway: Lateral Inhibition.

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Retinal Development: An Overview

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Ontogenesis and Organization of the Vertebrate Retina

Development of the vertebrate eye involves the paired bulging of the forebrain to form optic vesicles. Each optic vesicle extends laterally where it contacts the surface ectoderm. Reciprocal inductive signals between the surface ectoderm and optic vesicle result in invagination of the optic vesicle to form the optic cup. The inner layer of the optic cup is then patterned to form the neural retina distally and the ciliary body and iris more proximally (**Figure 1(a)**).

Cell Types of the Retina

The vast majority of cells in the mature neural retina develop from neuroepithelial progenitor cells of the optic cup, the exceptions being vascular cells and some types of immune cells. When fully differentiated, the neural retina is a highly ordered, laminar structure comprising six major neuronal cell types (retinal ganglion cells, amacrine cells, bipolar cells, horizontal cells, and rod and cone photoreceptors) and one intrinsic glial cell type (Müller glial cells) (**Figure 1(b)**). Laminar organization of the retina facilitates the initial steps in processing visual information. Achieving this exquisite cytoarchitecture requires intricate signaling during development. Coordination of proliferation, cell cycle exit, cell type determination, directed cellular migration, and cellular morphogenesis enables the appropriate numbers, correct laminar organization, and proper synaptic connections within the mature retina.

Proliferation of Retinal Progenitor Cells

Interkinetic Nuclear Migration

During development of the vertebrate retina, the optic cup neuroepithelial cells undergo rapid, proliferative expansion. This proliferative phase is marked by several intriguing cellular behaviors that have been hypothesized to provide cell fate information to the multipotent progenitor cells. One remarkable proliferative cell behavior is known as interkinetic nuclear migration (**Figure 2(a)**). This is the process in which the nuclei of neuroepithelial cells migrate in an apical–basal manner and in phase with the cell cycle. M phase and cytokinesis are confined to the apical surface near the retinal pigment epithelium (RPE), whereas G_1, S, and G_2 phases occur at more basal locations. This process is heterogeneous among neuroepithelial cells. Variation has been observed in the time required to progress through one round of interkinetic nuclear migration (which equals the cell cycle period), as well as in the distance of nuclear migration. Studies suggest interkinetic nuclear migration aids in the selection of progenitors that will produce neurons in the next mitosis. This is achieved by establishing asymmetry between neuroepithelia with regard to cell body position during critical 'neurogenic' windows of the cell cycle.

Oriented Cell Divisions

A second heterogeneous cell behavior associated with progenitor cell proliferation is regulated mitotic division plane orientation. Although neuroepithelial cell divisions are confined to the apical ventricular zone near the RPE, the orientation of cleavage axis can vary among progenitors (**Figure 2(b)**). Typically, retinal progenitor cells divide in either an apicobasal or a horizontal manner. Genetic and imaging studies in invertebrates have indicated that mitotic division plane in neural progenitors, and other proliferate cells, is important for symmetric versus asymmetric cell fate outcomes. Symmetric cell divisions create daughter cells that assume the same fates, whereas asymmetric cell divisions produce daughters of different fates. Symmetry in cell fates is subject to definition. For example, symmetry may relate to cell cycle exit and neurogenesis. In this case, symmetric cell divisions produce two daughter cells where both remain proliferative or both leave the cell cycle and become neurons. Alternatively, symmetry can also relate to cell type fate; for example, divisions that produce two ganglion cells or equivalent daughters of other cell types. Studies on cell division orientation in the developing vertebrate retina indicate that this cell behavior influences cell type choice. Mechanistically, it appears that the plane of cell division can equally (symmetrically) or differentially (asymmetrically) partition fate determinants to daughter cells. In the vertebrate retina, a modulator of the Notch signaling pathway, the Numb protein, is the flagship example of this phenomenon. Numb associates with the apical surface of retinal progenitor cells and immunocytochemistry has shown that this protein can differentially segregate between daughter cells. Interestingly, and in contrast to invertebrates, no other examples of vertebrate retinal cell fate determinants have been shown to partition asymmetrically between daughter cells.

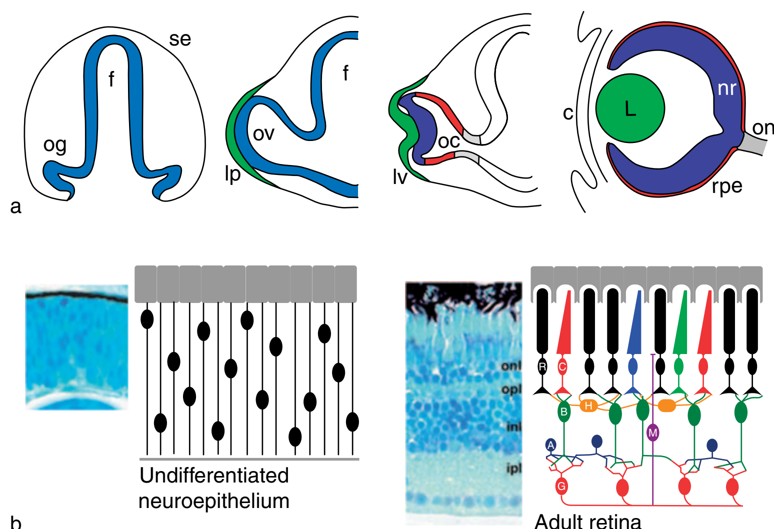

Figure 1 Development of the eye and neural retina. (a) Vertebrate eye development initiates with the paired extension of the forebrain to form optic grooves (og). Each groove extends laterally to form an optic vesicle (ov), which contacts the surface ectoderm and induces the formation of a lens placode (lp). Reciprocal inductive events lead to the invagination of the optic vesicle and lens placode to form the optic cup (oc) and lens vesicle (lv), respectively. c, cornea; L, lens; nr, neural retina; rpe, retinal pigment epithelium; on, optic nerve. (b) Histological cross sections of 24 hpf and adult zebra fish neural retinas with schematic representations of the retina at these stages. At 24 hpf, the retina is a pseudostratified neuroepithelium. The adult, laminated retina can be divided into the outer nuclear layer (onl), which includes rod (R) and cone (C) cell bodies; the inner nuclear layer (inl), which includes horizontal (H), bipolar (B), and amacrine (A) cell bodies; and two plexiform or synaptic layers – the outer plexiform layer (opl), which separates the nuclear layers, and the inner plexiform layer (ipl). M, Müller glia.

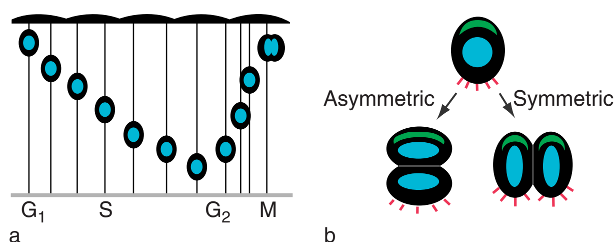

Figure 2 Cell behaviors of the proliferative neuroepithelium. (a) Interkinetic nuclear migration (blue nucleus) is schematized in one retinal neuroepithelial cell over the course of the mitotic cycle. M phase occurs at the apical region adjacent to RPE cells, whereas G_1, S, and G_2 phases take place at more basal locations. (b) Regulated mitotic division plane orientation is modeled for either an asymmetric or a symmetric division. Note that polarized fate determinants associated with either the cell surface (red molecules) or localized intracellularly (green molecules) can be equally or differentially segregated based on the plane of cytokinesis.

Molecular Regulation of Retinal Cell Proliferation

The factors that promote and regulate cell proliferation in the vertebrate retina are beginning to be discovered. Secreted growth factors such as Fibroblast growth factor (FGF), Hedgehog, and Wnt family members, as well as transmembrane proteins such as Notch function in part to control cellular proliferation, but they control other cellular processes as well. Surprisingly, secreted amino acids such as glutamate or nucleotides such as ATP also promote cell proliferation in retinal progenitors cells. In addition to extrinsic influences, specific transcription factors expressed in retinal neuroepithelia regulate parameters of the mitotic cycle. Proliferative roles for both homeodomain- and basic helix–loop–helix (bHLH)-type transcription factors have been demonstrated for retinal progenitor cells. Thus, both extrinsic and intrinsic influences converge to exert an effect on core cell cycle machinery such as the cyclins, cyclin kinases, and cyclin inhibitors. This is a common theme in development: the balance and interactions between intrinsic cell programs and extrinsic influences. This concept of complex signal integration is discussed in more detail later in the context of the competency model for retinogenesis. Specifically for retinal proliferation, however, regulatory molecules influence both

the kinetics of cell cycle progression (how fast cells pass through the various phases of the cell cycle) and neurogenic decisions (whether to remain in the cell cycle or exit mitosis and initiate migration and differentiation).

Spatial Control of Retinogenesis

The overall process of retinogenesis, from cell cycle exit to cellular differentiation, is coordinated in space and time. The first cells to exit the mitotic cycle do so at the center of the optic cup, although dorsal or ventral biases in where this process begins can vary between species. The initiation of retinogenesis, however, appears to always depend on FGF expression in the neuroepithelium. The patch of FGF expression is regulated in part by adjacent midline mesoderm-derived signals. From this central FGF organizing center, cell cycle exit proceeds in a wavelike manner toward the periphery of the optic cup. At any point within the wave, however, only a subset of progenitor cells are selected to produce postmitotic progeny. The remaining cells stay in the cell cycle to produce late-born cell types. Why some cells remain mitotic and others begin neurogenesis is an active area of research.

Lineage and Birth Date Relationships

Experiments *in vivo* and in cell culture from all vertebrate species studied have demonstrated the multipotency of retinal neuroepithelial cells. Lineage-tracing experiments using either tagged replication incompetent retroviruses or microinjection of nontransferable markers have shown that single neuroepithelial cells can divide to produce all six neuron types and Müller glia. Another evolutionarily conserved feature of retinogenesis is the link between birth date and cell type (**Figure 3**). Although subtle differences exist between species in the exact order of cell types generated over developmental time, retinal ganglion cells genesis is normally followed by the differentiation of horizontal cells, cone photoreceptors, and amacrine cells, followed by bipolar cells, rod photoreceptors, and Müller glia. This birth order is also reflected by clonal analyses from lineage studies. Clonal descendents from neuroepithelia labeled at early stages of development ('early' progenitors) are biased to produce ganglion cells, amacrine, horizontal cells, and cone photoreceptor cells. Progeny from 'late' progenitors are biased to produce bipolar cells, rods, photoreceptor cells, and Müller glia. Despite the trend of birth order, different cell types can and do differentiate simultaneously (**Figures 3** and **4**). Finally, lineage studies and cell culture experiments have suggested that the majority of retinal neuroepithelia remain multipotent until the last cell cycle prior to terminal mitosis or, for some cell types, just following cell cycle exit. Overall, observations from lineage and birth order studies, as well as data from other experiments, suggest that the timing of cell cycle exit, intrinsic lineage bias, and influences from adjacent cells all function in cell fate determination.

Competency Model for Retinogenesis

The competency model has emerged as a general paradigm for retinogenesis and posits that retinal neuroepithelia progress through temporal states in which the progenitor is competent to generate only a subset of retinal cell types (**Figure 4**). This bias in cell type competency is influenced by both determinant intrinsic and modulatory extrinsic factors, each of which are dynamic in nature. In other words, within the developing retina, the type, levels, and expression domain of secreted factors change with time and exert differential influences depending on the changing intrinsic state of the neuroepithelial cell. The compentency model is applicable to two interrelated cell fate decisions that neuroepithelia make during their maturation process: (1) when to exit the cell cycle and (2) what cell type to become. Although

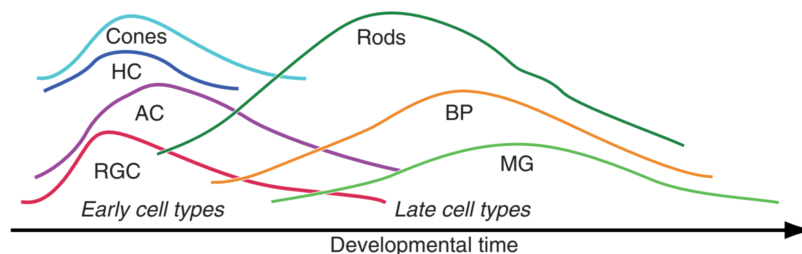

Figure 3 Birth orders for cell types of the mammalian retina; schematic showing the timing of cell cycle exit (birth date) for classes of retinal cells. The areas under the curves represent the relative proportions of cell types typically found in nocturnal (rod-dominated) rodents. Subtle differences exist in the exact birth orders between various species. Note the overlapping timing for genesis of multiple cell types and the relative clustering of 'early' and 'late' cell types.

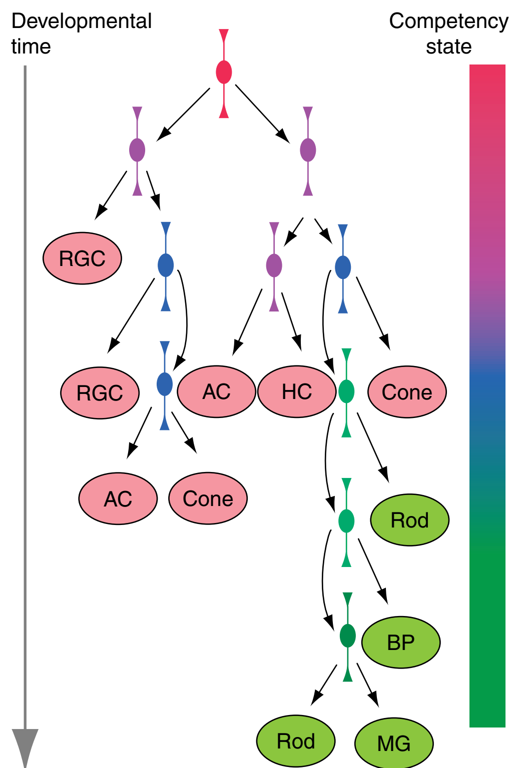

Figure 4 Competency model for retinal cell fates. As retinal progenitor cells divide and give rise to other proliferative progenitor cells (colored, elongated neuroepithelial cells), the intrinsic cellular competency to differentiate to particular classes of postmitotic cells (labeled ovals) changes. The changing colors represent changes in cellular competence. Postmitotic cells have been grouped into early cell types (pink ovals) or late cell types (green ovals). Note that multiple cell types can be generated at the same time of development and that single cells give rise to complex combinations of differentiated cell types.

general in nature, some of the molecules and signaling pathways underlying intrinsic and extrinsic components of the competency model have been discovered.

Atoh7 and Regulation of Cellular Competence

The bHLH transcription factor Atoh7 (previously known as Ath5) serves as a specific example of the molecular underpinnings of cellular competence for cell cycle exit and cell type fate determination. Atoh7 was first discovered based on its striking expression pattern and loss-of-function phenotype. The expression of *atoh7* is specific to the neural retina and commences in neuroepithelial cells in their last cell cycle preceding a neurogenic cell division (where one or both daughter cells exit the cell cycle and differentiate as neurons). Disruption of *atoh7* in mouse, frog, and fish results in a dramatic loss of retinal ganglion cells. Careful analysis of *atoh7* mutants showed that in addition to the loss of retinal ganglion cells, there was a marked decrease in the proportion of progenitor cells leaving the cell cycle. Importantly, *atoh7* linage-marking studies demonstrated that *atoh7* is expressed transiently, just before cell cycle exit in multiple cell types, but not all cell types. These data suggest that Atoh7 is required for cell cycle exit and biases cells toward particular cell type fates. The exact cell type depends on the co-expression of other transcription factors. Interestingly, ectopic expression of *atho7* alone does not drive cell cycle exit. This is consistent with Atoh7 as important for neurogenic competency, while also providing influence to eventual cell type fate. The initial expression of *atoh7* appears to be established at a time much earlier than its actual expression. This 'clock-like' mechanism is probably set by signals provided at the earliest stages of optic vesicle formation by cells associated with the embryonic midline axis. However, termination of *atoh7* expression is regulated in part by extrinsic proteins. GDF11, a secreted growth factor, has been shown to extrinsically influence the expression of *atoh7*. Mice with deletions in *gdf11* show an extended expression of *atoh7* and a concomitant increase in early born retinal cell types. Therefore, GDF11 regulates the temporal window during which retinal progenitors retain competence to produce distinct neural progeny. Other factors, both autonomous and non-autonomous, interact to provide the complete regulatory network that enable the appropriate numbers and cell types to be generated during retinal development.

Postmitotic Cell Migration

Modes of Migration

Following terminal mitosis at the apical surface, newborn retinal cells migrate to their appropriate laminar position within the retina. Classic histological and more recent time-lapse experiments have demonstrated that the various cell types of the retina employ multiple strategies during postmitotic cell migration. The modes of cell migration can be classified as guided cell migration, somal translocation, and unconstrained migration (**Figure 5**). These are not necessarily mutually exclusive forms of motility, and some cells may use multiple strategies while migrating. Direct evidence exists for the latter two modes of cell positioning. Within the retina, somal translocation has been demonstrated for retinal ganglion cell and bipolar cell precursors. Some newborn amacrine cells have been shown to move using unconstrained migration, in which dynamic and nonpolarized processes appear to sample the local environment

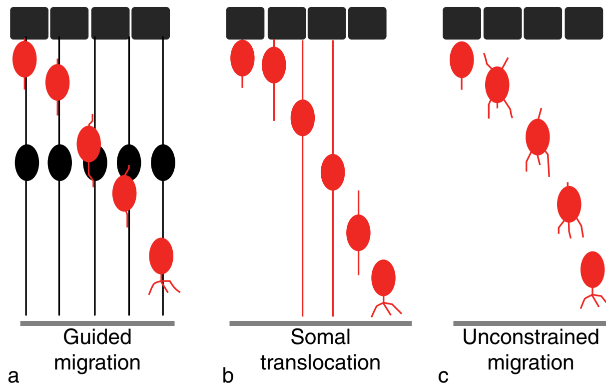

Figure 5 Modes of cell migration. Postmitotic cells (red) can migrate to their appropriate laminar position in the retina in one of three ways. (a) In guided migration, postmitotic cells use adjacent neuroepithelial cells as a scaffold to move from the apical surface toward their final destination. (b) Somal translocation occurs independently of adjacent cells. After its terminal mitosis a newly formed neuron extends processes to both the apical and the basal cell surfaces. Subsequent to this, the cell body translocates along this process until reaching its appropriate position within the retina, where it then retracts its processes. (c) In unconstrained migration, newly formed neurons migrate independently of other cells or cell processes, often extending neuritis in an 'exploratory' manner.

en route to the inner nuclear layer. It is likely that neuroepithelial-guided cell migration is utilized, to some extent, for both nuclear translocation and free migration. This form of guided cell migration is similar to that in the developing cerebral cortex, where glial cells, instead of adjacent neuroepithelia, provide the substrate and cues for cell positioning.

Within the retina, all forms of migration are restricted primarily to the radial direction along the apical–basal axis. However, clonal analysis using various progenitor cell marking techniques has demonstrated subtle lateral (or tangential) displacements of ganglion cells, horizontal cells, cone photoreceptors, and amacrine cells. This tangential displacement is thought to ensure an even 'planar' distribution of specific classes of cell subtypes and facilitate appropriate synaptic partnering. Although migration is largely restricted to the radial direction, migration is not restricted to one direction because some postmitotic cells show bidirectional movements. For example, some horizontal cells migrate first to the basal surface and then return to their appropriate outer retinal position.

Importance of Cell Polarity and Basement Membranes

Although much has yet to be discovered regarding the mechanisms and regulation of cellular migration in the retina, some important molecules and signaling pathways have been identified. One important class of molecules are those associated with apical cell junctions of neuroepithelia. The apical junctional complex includes the proteins Pard3, Pard6, Prkci, Mpp5, and Crb. Mutations in genes encoding these proteins result in cell positioning defects. Interestingly, studies *in vivo* have demonstrated that the apical junctional complex functions noncell autonomously in the retina for cell migration. This suggests that this signaling complex either regulates the adhesion between cells or facilitates polarized secretion of guidance molecules. In addition, some of these mutants result in disruptions to the RPE, exposing Bruch's membrane, which is the basement membrane of RPE cells. Basement membranes are distinct, thin sheets of extracellular matrix, rich in specific isoforms of collagens and laminins. In these mutants, ganglion cells become misdirected and migrate apically rather than basally. Guidance cues associated with the exposed Bruch's membrane may lead to these migration errors. Direct experiments have demonstrated the importance of the retinal basement membrane in directed cell migration. For example, transient disruption of the basement membrane in chick embryos results in lamination defects. Similarly, mutations in laminin α_1 in zebra fish cause migration defects for cone photoreceptors. Detailed characterizations for how the basement membrane translates into intracellular responses are limited. However, it is likely, based on cell culture work, that membrane-bound receptors such as dystroglycan and integrins, as well as their intracellular associated signaling molecules such as integrin-linked kinases and focal adhesion kinases, mediate directed cell migration.

Regulation of Cellular Differentiation

Coordination of Differentiation

Once a retinal cell reaches its appropriate laminar position, differentiation and morphogenesis begins. However, overt cellular differentiation does not always commence immediately. In addition to the spatial–temporal waves of cell cycle exit, cellular morphogenesis also progresses from central locations to the periphery in a wavelike manner. This wavelike progression of cellular morphogenesis is not a simple consequence of birth date. For example, in mice, expression of rhodopsin and other markers of rod photoreceptor differentiation are independent of birth date. Because rod genesis is very protracted over the course of retinogenesis, adjacent photoreceptor cells can have birth dates that differ by days. Despite differences in the time of terminal mitosis, differentiation and morphogenesis still occurs at approximately

the same time for adjacent cells. Two studies suggest signaling pathways important for coordinated differentiation. The SOCS3 protein (suppressor of cytokine signaling 3) has been shown to regulate and coordinate the timing of photoreceptor signaling by modulating the STAT signaling pathway. More generally, downregulation of the Notch pathway is important for multiple retinal cell types to begin morphogenesis.

Chromatin Remodeling

Chromatin remodeling factors are a diverse class of molecules that may be regulated by differentiation transducers such as the STAT and Notch pathways to coordinate differentiation programs within cells. Genetic analysis of the Brahma chromatin remodeling complex has revealed an essential role in cellular differentiation for all retinal neurons. The Brahma complex is part of the SWI/SNF group of chromatin remodeling complexes. These are large macromolecular complexes that use the energy of ATP to slide nucleosomes and create local alterations in chromatin. In addition to opening chromatin, Brahma subunits can also bind to gene-specific transcription factors and coordinate ocular differentiation programs. Other chromatin remodeling factors with demonstrated roles in global retinal cell differentiation are Dnmt1 (a DNA methyl transferase), Suv39h1 (a histone methyl transferase), and Hdac1 (a histone deacetylase).

Cell Type-Specific Differentiation

In addition to these global coordinators of retinal cell differentiation, a multitude of cell type-specific transcription factors has been shown to regulate particular differentiation programs. Many of these 'cell type-specific' transcription factors, however, are dynamic in expression and function in combination with other factors to control multiple steps of retinogenesis. The homeodomain transcription factor Ceh10 (also known as Chx10) serves as a prime example of the pleiotropy found in retinal differentiation factors. At the optic cup stages, *ceh10* is expressed in all proliferative neuroepithelia. This protein is required for normal proliferation. Following cell cycle exit, *ceh10* is downregulated in most cells but upregulated in differentiating bipolar cells. When *ceh10* is deleted, progression through the mitotic cell cycle is very slow and bipolar cells are missing. In wild-type bipolar cells, Ceh10 binds promoters of bipolar cell-specific target genes to positively regulate gene transcription. Within these same cells, Ceh10 also binds rod photoreceptor cell-specific promoters to inhibit their expression. Genetic complementation using Ceh10 pathway mutants demonstrated that the functions of Ceh10 in proliferation and differentiation are distinct. Interestingly, ectopic expression of *ceh10* in retinal progenitors promotes bipolar cell fates at the expense of rod photoreceptor cells. Cumulatively, these studies demonstrate that Ceh10 functions not only in cellular differentiation but also in cell proliferation and cell type fate decisions. Other factors that regulate specific cell differentiation pathways show similar complexity in function.

Protein Trafficking and Cellular Differentiation

In addition to transcriptional regulation during cell differentiation, protein complexes that drive cell differentiation and morphogenesis are beginning to be resolved. One of the most dramatic examples of cellular morphogenesis is that of retinal photoreceptors. Newly postmitotic photoreceptors are nearly spherical in shape and reside at the apical surface of the retina. However, as photoreceptors begin to differentiate, significant shape changes take place, including the dramatic growth of outer segments. Outer segments are elongated stacks of membranous disks and folds that contain components for phototransduction. Among the first features of photoreceptor morphogenesis is the establishment of the connecting cilium. The connecting cilium is a primary cilium composed of microtubules arranged in a $9 + 0$ array. Because no protein synthesis occurs in the outer segment, an elaborate protein/membrane transport mechanism exists to both build and maintain photoreceptor outer segments. Trafficking of outer segment components occurs through the connecting cilium and along the backbone microtubules. The transport process is shared with other cilia-based cell appendages and is known as intraflagellar transport (IFT). IFT was discovered through genetic and biochemical studies of *Chlamydomonas* (green alga) and found to be the mechanism responsible for establishing and maintaining flagella. In photoreceptors, IFT particles composed of two macro-multiprotein complexes nucleate at the base of the connecting cilium where the basal body resides. IFT particles docked at the basal body and loaded with outer segment cargo are assembled onto kinesins, plus end-directed microtubule-based motors, to enable outward movement of the building blocks for the outer segment. Dyneins, minus end-directed microtubule-based motors, return the cargoless IFT particles, as well as the kinesin motors, to the basal body. Many questions remain regarding the details of IFT in photoreceptors, but it is certain that this process is essential for photoreceptor outer segment differentiation.

Neuronal Process Outgrowth and Synaptogenesis

Another obvious consequence of cell differentiation in the retina is the formation and elaboration of neuronal processes. Much of our knowledge of axon and dendrite development in the retina has been derived from serial electron microscopy analyses. Now, time-lapse experiments in mammalian slice culture and in zebra fish are revealing much of the complexity of process outgrowth and synaptogenesis. Multiple approaches have revealed that even before postmitotic neurons reach their final laminar position, process outgrowth initiates. Like retinogenesis as a whole, elaboration of axons and dendrites is dependent on both intrinsic and extrinsic cues. The initial orientation of ganglion cell axons, for example, relies on positional information provided by the retinal basal lamina. Retinal ganglion cells also depend on intrinsic factors such as Brn3b, a POU-domain transcription factor, which is essential for retinal ganglion cells to extend axons. Deletion of *brn3b* in mice results in retinal ganglion cells that prefer to generate dendrites at the expense of axons. Although analysis of process outgrowth and differentiation of individual cell types is ongoing, a general model is supported in which retinal neurons first extend processes in fields more extensive than their final arbors (**Figure 6**). In the synapse-refinement phase of process differentiation, cell–cell and cell–matrix communication appears to be very important. Similar to organization of neuronal cell bodies into lamina, final axon and dendrite arbors are stereotyped and highly specific. Retinal plexiform layers are organized so that specific subtypes of neurons synapse with each other in specific 'sublamina' or zones. For example, cells comprising the 'on-center' or 'off-center' pathways to light stimulus show sublaminar synaptic organization.

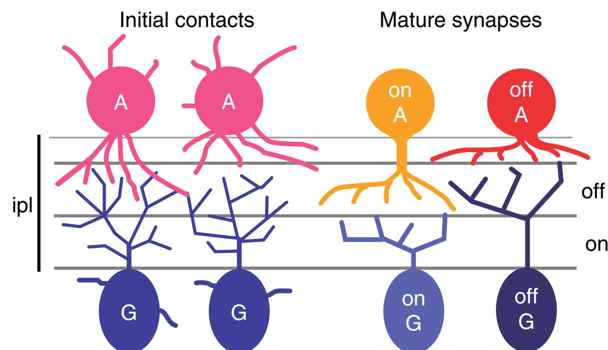

Figure 6 Sublamination of the inner plexiform layer. Early in development, as amacrine cell (A) and ganglion cell (G) dendrites first interact, process outgrowth is broad and relatively unstratified (left synaptic partners). Amacrine cells appear to guide 'on' and 'off' sublamination of the inner plexiform layer (ipl), which becomes strikingly polarized and stratified in the mature retina (right synaptic partners).

The on-center and off-center pathways can be distinguished by bipolar cells, which depolarize or hyperpolarize, respectively, when the photoreceptors from which they receive input are stimulated. On and off bipolar cells synapse with on and off ganglion cells in different substrata of the inner plexiform layer. Modulatory amacrine cells also specifically target their dendrites to either the on or off sublamina. In fact, amacrine cells play a central role in directing sublamination of the on and off circuits. Initially in development, the contacts between on and off cell processes are distributed throughout the inner plexiform layer. Over time, these synapses segregate so that off cells interact in a very discrete region apical to the synapses of on cells. This remodeling of ganglion cell synapses requires synaptic activity from amacrine cells. Interestingly, amacrine cell sublamination does not require ganglion cells or their dendrites at all and instead relies in part on cues from adjacent amacrine cells. Other sublamina, in addition to the on and off zones, exist in both the inner and the outer plexiform layers.

Cell Adhesion and Synapse Partnering

In addition to the activity-based mechanisms of synapse refinement, initial targeting of axons and dendrites to the appropriate lamina also depends on the expression of specific cell-associated guidance molecules. These molecules mediate both attractive and repulsive activities to potential synaptic partners. Based on gene expression and results from the spinal cord, various forms of the Protocadherin and Sidekick families of cell adhesion molecules have been proposed to facilitate initial axon/dendrite targeting within the retina. As with other processes of retinal development, our understanding of neuronal process differentiation and synaptogenesis is rudimentary, and much more research is required for a deeper understanding.

Conclusions

Normal retinal development occurs through a series of continuous refinements in which a simple pseudostratified epithelium is specified and patterned to form a highly organized sensory neuroepithelium. The underlying genetics and cellular interactions that drive retinogenesis are necessarily intricate and subjected to complicated feedback regulatory mechanisms. Current models of retinal development are still general, but our understanding of the gene networks and signaling factors that facilitate retinogenesis is increasing. Many important questions remain to be solved. For example, how is the cell cycle precisely controlled in progenitors, and how are cell type fate determinants integrated with regulation of

cell cycle exit? What are the cues that guide postmitotic cell migration in the retina, and what cues inform cells to stop migrating and begin morphogenesis? What are the factors that mediate the necessary cytoskeletal rearrangements required for both cell migration and morphogenesis? How are the spatial waves of neurogenesis and cell differentiation coordinated? What mechanisms work to allow neurons to find each other and form functional circuits? These and other questions are motivating hard work, driving technological advances, and pushing the creativity of many inspired retinal biologists.

See also: Retinal Development: Cell Type Specification.

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Retinal Development: Cell Type Specification

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The vertebrate retina is a thin layer of light-sensitive neural tissue lining the back of the eye. It comprises seven major cell classes harmoniously organized in three cellular layers (**Figure 1**). The outer nuclear layer (ONL) contains the cell bodies of rod and cone photoreceptors, whereas the inner nuclear layer (INL) contains the cell bodies of bipolar cells, amacrine cells, horizontal cells, and Müller glial cells. Finally, the ganglion cell layer (GCL) contains the cell bodies of retinal ganglion cells (RGCs), the projection neurons of the retina, as well as those of displaced amacrine cells. Finally, the inner and outer plexiform layers are synaptic layers located between the GCL and the INL, and between the INL and ONL respectively.

The seven major classes of retinal cells are generated in an evolutionary conserved, overlapping birth order. Generally, in vertebrates, the RGCs, cones, and horizontal cells are among the first cells to be generated, followed closely by amacrine cells, and finally by rods, Müller glia, and bipolar cells, which are among the last cell types to be generated (**Figure 2**).

Multipotency of Retinal Progenitor Cells

Pioneering lineage studies used retroviral vectors or injection of tracers to label retinal progenitor cells (RPCs), which made possible the identification of the entire progeny of individual RPCs, often referred to as their 'clone' (**Figure 3**). Some of these clones were composed of all the major retinal cell types, when the ancestor cell was labeled early during retinogenesis, indicating that at least some RPCs are multipotent at some point during retinal development. However, this does not preclude the possibility that a subset of progenitor cells may have restricted fate choices. Consistent with this idea is the finding that certain clones were composed of only one cell type, even when the RPC was labeled early in retinogenesis, suggesting that some RPCs might be constrained to generate one cell type. Whether such restricted progenitors have a characteristic 'molecular fingerprint' remains unknown, but recent gene expression profiling of RPCs should help address this issue. Interestingly, the homeodomain transcription factor Pax6 was recently shown to be required to confer the multipotent state of RPCs. Indeed, specific inactivation of Pax6 in RPCs, at the beginning of retinogenesis, prevented the generation of all retinal cell types except amacrine cells. Thus, it appears that Pax6 function in RPCs, at least early in development, is required to confer the multipotent character to RPCs.

To try to reconcile the finding that at least some RPCs are multipotent, together with the finding that different retinal cell types are generated in a strict chronological order, it has been proposed that RPCs undergo changes in their competence to generate particular cell types as development proceeds. Thus, early embryonic RPCs are competent to generate early-born retinal cell types, whereas more mature, postnatal RPCs have lost the competence to generate early-born cell types and gained the competence to generate late-born cell types. These changes in competence appear to be largely intrinsically driven. This was first suggested by the fact that the proliferative capacity and the timing of differentiation of early embryonic RPCs cultured *in vitro* do not change, even in the presence of a 50-fold excess of older postnatal retinal cells. In these mixed-age cultures, postnatal RPCs proliferated less and gave rise to rods much sooner than did embryonic RPCs, suggesting that early and late RPCs are intrinsically different. In addition, when early embryonic RPCs are cultured *in vitro* they can generate RGCs, whereas older postnatal RPCs, placed in the same *in vitro* conditions, generate mostly rods, even when mixed with an excess of early retinal cells. Moreover, isolated late RPCs, cultured at clonal density in serum-free and extract-free medium, can give rise to clones of the same general birth order, size, and composition as clones generated *in vivo*. The presence of a particular retinal cell type within such isolated clones did not appear to be required for the generation of any other retinal cell type, suggesting that instructive environmental signals from previously differentiated cells are not necessary for normal cell fate decisions, at least in late RPCs. Nonetheless, negative environmental signals most likely operate in such clonal density cultures, as well as *in vivo* (see below).

Coordinating Cell Fate and Proliferation

Of particular importance during retinal development is the coordination of proliferation and cell fate specification. Indeed, if RPCs exit the cell cycle too early during retinal development, the number of early-born cells increases at the expense of late-born cells. Thus, the decision to withdraw from the cell cycle must be coordinated with cell fate specification to guarantee that the correct proportion of each neuron and glia

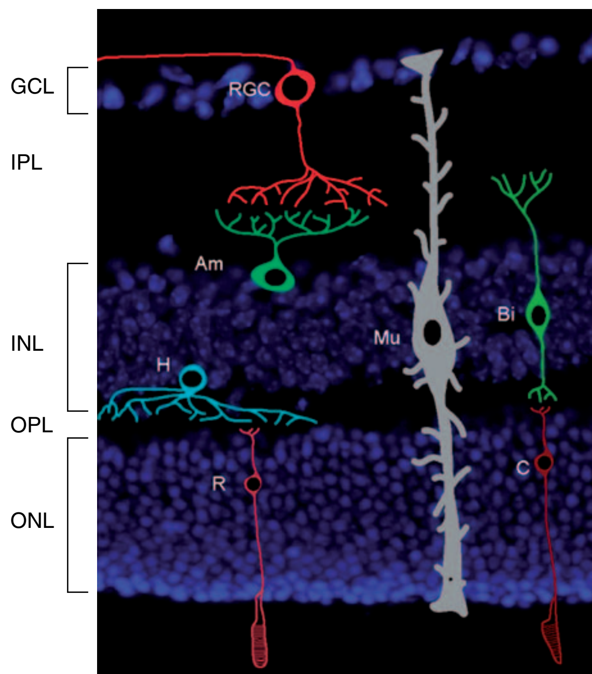

Figure 1 Organization of the vertebrate retina. The seven major retinal cell types are drawn on top of an actual histological section of rat retina (blue nuclei). Retinal ganglion cells (RGC) are found in the ganglion cell layer (GCL). Amacrine (Am), Müller (Mu), horizontal (H), and bipolar (Bi) cells are located in the inner nuclear layer (INL). Rod (R) and cone (C) photoreceptors are located in the outer nuclear layer (ONL). The inner plexiform layer (IPL) and outer plexiform layer (OPL) form the synaptic layers.

cell type is generated. But how does a cell know when to exit the cell cycle? Recent evidence suggests that there are mechanisms that connect cell cycle to fate determination. For example, subsets of RPCs express different types of cyclin-dependent kinase (CDK) inhibitors, suggesting that subpopulations of RPCs might use different components of the cell cycle machinery to control cell cycle exit. Such factors that direct cell cycle arrest may also specifically regulate cell fate determination pathways. Indeed, recent work has shown that the cyclin kinase inhibitor p27Xic1 promotes both cell cycle exit and Müller cell fate through distinct domains of the p27Xic1 protein.

Environmental Factors in Retinal Cell Fate Decisions

As retinal development proceeds, the microenvironment changes as a result of various retinal cell types being generated, raising the possibility that signals secreted by differentiated cell types act on progenitor cells to influence their fate. While the evidence for environmental signals directly instructing RPCs to produce particular retinal cell types *in vivo* is weak, the evidence for feedback inhibition signals that act to prevent the production of certain cell types is convincing. Perhaps the best-studied case of feedback inhibition concerns the RGCs. Young embryonic chick progenitors are inhibited in their ability to produce RGCs when cultured adjacent to old retinal cells, and depletion of the ganglion cells from the old retinal cell population abolishes this inhibition, indicating that signals from RGCs prevent the generation of too many RGCs. Since RGCs are generated first, one possibility is that they might also secrete signals that are required for progenitors to generate the other retinal cell types (instructive signals). This question was recently addressed using a transgenic mouse that expresses diphtheria toxin under the regulatory elements of Brn3b, a key transcription factor required for generation of RGCs. In this mouse, the toxin rapidly kills RGCs as they are born, thereby producing an RGC-depleted retina. Although the retinas of these animals showed reduced proliferation, there were no differences in the relative proportions of the various retinal cell types generated. Taken together, these results suggest that although environmental signals secreted from RGCs are important for normal RPC proliferation and to prevent RPCs from generating too many RGCs, they do not appear to be necessary to positively instruct the generation of other retinal cell types.

To determine how negative feedback signals could influence the production of RGCs in the retina, progenitors expressing green fluorescent protein (GFP) under the regulatory elements of ath5, a transcription factor required for the generation of RGCs, were imaged. In a wild-type retina, the ath5-positive cells systematically divided once to generate a RGC and another cell type (see below). However, when transplanted into a mutant retina, which lacks RGCs, these same progenitors often generated two RGCs, directly demonstrating that the lineage of RPCs can be modified by feedback inhibitory environmental signals.

What might these feedback signals be? Recent studies show that the conditional ablation of Sonic Hedgehog (Shh), which is expressed by RGCs in the mouse retina, causes a rapid depletion of the progenitor pool due to precocious cell cycle exit and neuronal differentiation. Moreover, progenitors are apparently biased to generate RGCs in the Shh knockout mouse, which contains increased number of RGCs. These results suggest that Shh provides feedback inhibition signal in the developing mouse retina. In addition, there is evidence that Shh signaling from RGCs is required for the normal laminar organization in the vertebrate retina, most likely by promoting normal Müller glial cell development. Negative feedback

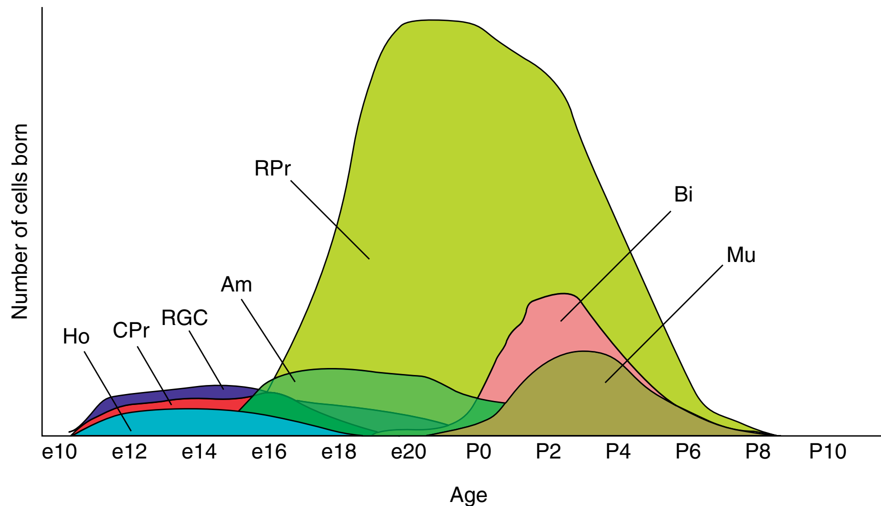

Figure 2 Chronological order of cell birth in the rat retina. The different retinal cell types are born in a strict but overlapping chronological order from around embryonic (e) day 10 to postnatal (P) day 10. Ho, horizontal cells; CPr, cone photoreceptors; RGC, retinal ganglion cells; Am, amacrine cells; RPr, rod photoreceptors; Bi, bipolar cells; Mu, Müller cells. This diagram is based on published results from Rapaport DH, Wong LL, Wood ED, Yasumura D, and Lavail MM (2004) Timing and topography of cell genesis in the rat retina. *Journal of Comparative Neurology* 474: 304–324.

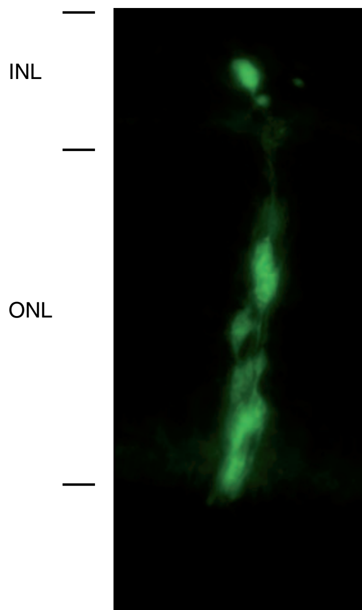

Figure 3 An example of a clone obtained by labeling a retinal progenitor cell at P0 with a GFP-expressing retroviral vector. The clone contains several photoreceptors, which are located in the outer nuclear layer (ONL), and a bipolar cell, which is located in the inner nuclear layer (INL).

signals may also affect the timing of differentiation. In the mouse retina, the growth differentiation factor 11 (GDF11) is a key regulator of RGC genesis. Evidence suggests that GDF11 acts by regulating the temporal window during which progenitors are competent to produce RGCs.

Another interesting group of environmental cues are the ligands of Notch. Constitutive stimulation of the Notch signaling pathway in rat RPCs biases the development of Müller glia at the expense of neurons, probably by interfering with the function of proneural bHLH transcription factors. At the same time, Notch clearly affects cell cycle exit. Experiments in *Xenopus* retina indicated that the Notch signaling pathway controls when a progenitor can differentiate. When cells are released from the inhibition mediated by the Notch pathway, basic helix–loop–helix (bHLH) transcription factors act as intrinsic factors that bias neuroblasts toward particular fates and histogenically appropriate cell cycle exit (see below).

Intrinsic Factors in Retinal Cell Fate Decisions

bHLH and Homeodomain Transcription Factors

As in other regions of the central nervous system, there are a number of transcription factors that intrinsically influence several facets of cell fate specification in the retina. The two major classes of transcription factors involved in cell fate identity comprise the bHLH and the homeodomain (HD). A bHLH motif consists of a short α helix connected by a loop to a second, longer α helix, whereas HD transcription factors constitute a special family of helix-turn-helix DNA-binding proteins, constructed from two α helices connected by a short extended chain of amino acids, which constitute the turn. These two classes of transcriptions factors bind to regulatory DNA sequence

either alone or via a complex with other transcriptions factors. In some cases binding triggers or enhances transcription, while in other cases it reduces or blocks transcription of developmental genes.

There are two functionally distinct groups of bHLH genes: repressors and activators. bHLH repressors (e.g., Hes1 and Hes5) are expressed in progenitors and inhibit neuronal differentiation. These repressors help maintain the undifferentiated state in the embryonic phase of retinogenesis, whereas they appear to promote gliogenesis in the postnatal retina. Misexpression of Hes1 in embryonic retina prevents differentiation and maintains the RPC fate. On the other hand, in Hes1 knockouts, progenitors show decreased proliferation, resulting in small-eye phenotype. In mice, the lack of Hes1 upregulates the expression of Mash1 (bHLH activator) resulting in increase of early-born types of neurons at the expenses of late-born cell types. Double mutation experiments have shown that another member of the Hes family, Hes5, acts cooperatively with Hes1 to regulate the maintenance of RPCs. Upon stimulation by Notch ligand from adjacent cells, Hes1 and Hes5 expression is increased, leading to the repression of bHLH activators genes, which in turns inhibits neuronal differentiation.

The role of bHLH and homeodomain transcription factors has been extensively studied in the retina. Here, we summarize the general results for the different retinal cell types, but it is beyond the scope of this article to describe in detail the function of these genes in retinal cell specification.

Müller glial cells In the postnatal retina, Hes1 and Hes5 are expressed in differentiating Müller glial cells and in RPCs. Misexpression of either Hes1 or Hes5 in postnatal RPCs increases Müller glia cell fate. Because both Hes1 and Hes5 are Notch effectors, these data indicate that activation of Notch signaling induces glial fate determination at the expense of other fates. Consistent with these results, constitutive activation of Notch signaling in postnatal RPCs increases the generation of Müller glial cells.

Interestingly, several lines of evidences suggest that progenitors and Müller glia cells are more closely related to each other than was previously thought. First, both cell types express the same transcriptions factors (Hes1, Hes5, and rax). Second, they are morphologically very similar, and lastly, Müller glia cells have been shown to have progenitor-like potency such as the capacity to self-renew and the ability to give rise to neurons and glia cells after retinal injury.

Bipolar cells It has been demonstrated that for bipolar cell fate specification two bHLH activators genes, Mash1 and Math3, and the HD factor Chx10 are required. Misexpression in RPCs of either Mash1 or Math3 alone mostly increases photoreceptor generation, whereas misexpression of Chx10 alone increases generation of Müller glia or undifferentiated cells, but not mature bipolar cells. In contrast, co-expression of Mash1 or Math3 with Chx10 in RPCs predominantly forces the generation of bipolar cells. Knockout mouse experiments have shown that Mash1/Math3 double mutants have no bipolar cells, whereas the number of Müller glial cells is increased. Loss of Chx10 function also prevents bipolar cell genesis but, in contrast to the Mash1/Math3 double mutant, is not accompanied by an increase of Müller glia cells, suggesting that Chx10 and Mash1/Math3 have distinct roles in specification of the bipolar cell fate.

Amacrine cells Two bHLH genes, NeuroD and Math3, are transiently expressed in differentiating amacrine cells. A mutation in either NeuroD or Math3 alone does not significantly affect amacrine cell development, but mutation in both NeuroD and Math3 severely decreases amacrine cell genesis, while increasing ganglion cell production.

Although NeuroD and Math3 are expressed during amacrine cell development, misexpression of each gene alone does not increase amacrine cell genesis, but significantly increases photoreceptor cell production. These results indicate that other genes may be required for amacrine cell development. Two homeodomain genes, Pax6 and Six3, are also expressed in amacrine cells. Although misexpression of each gene alone does not lead to increased amacrine cell production, concomitant expression of Pax6 or Six3 with NeuroD and Math3 significantly increases amacrine cell numbers. Thus, as in bipolar cell genesis, the co-expression of the bHLH and homeodomain genes influences the fate of amacrine cells.

Ganglion cells Math5, another member of the atonal bHLH activator gene, plays an important role in ganglion cell genesis. Math5 appears to directly activate the expression of the POU domain transcription factor Brn3b, which in turn is required for terminal differentiation of RGCs. In mice, it has been shown that a Math5 null mutation causes a dramatic decrease in RGC production and an increase of amacrine cells. These results, as stated earlier, are contrary to those of NeuroD/Math3 double mutants, which favored ganglion cell genesis at the expenses of amacrine cell production. It is plausible that NeuroD/Math3 and Math5 antagonistically control each other for amacrine versus ganglion cell fate specification. However, the mechanism for this regulation remains unknown.

Photoreceptor cells The homeodomain genes Crx and Otx2 have been shown to be required for photoreceptor cell development. In humans, mutations in Crx are associated with retinal diseases leading to photoreceptor degeneration and vision loss, such as cone–rod dystrophy-2, retinitis pigmentosa, and Leber's congenital amaurosis. Crx is expressed specifically in photoreceptor cells, where it is thought to regulate the expression of photoreceptor-specific genes such as rhodopsin and cone opsins. Overexpression of Crx in RPCs results in an increased generation of photoreceptor cells, and Crx knockout mouse photoreceptor cells do not elaborate outer segments and lack normal electrophysiological activity.

While Crx activity regulates the differentiation of both rods and cones, other factors specifically control the production of rod photoreceptors. The protein neural retina leucine zipper (Nrl) is a basic motif–leucine zipper transcription factor that is preferentially expressed in rod photoreceptors, where it is thought to act synergistically with Crx to regulate rhodopsin expression. Mutations in human NRL have been associated with autosomal dominant retinitis pigmentosa, which is characterized by rod photoreceptor degeneration. Interestingly, deletion of Nrl in mice results in the complete loss of rod function and an increase in cone function. Histological analysis of the Nrl knockout retina revealed that rod photoreceptors do not develop, whereas cone photoreceptors are overproduced. Thus, it appears that Nrl acts as a binary molecular switch during rod photoreceptor development by modulating rod-specific genes and inhibiting the cone differentiation pathway.

Another important regulator of rod photoreceptor development is the retinoblastoma (Rb) gene. During postnatal retinal development, Rb is expressed in proliferating RPCs and in differentiating rod photoreceptors. In the absence of Rb, RPCs continue to divide, and rods do not differentiate normally, as judged by abnormal inner and outer segments and disrupted pedicles. Based on these results, it has been proposed that Rb functions to regulate cell cycle exit of RPCs and rod differentiation.

Horizontal cell It was recently shown that co-expression of the homeodomain genes Pax6 or Six3 with the bHLH gene Math3 promotes primarily horizontal cell formation, in addition to amacrine cells. However, Math3 null mice develop horizontal cells normally, indicating that other genes may be compensating for Math3 function in horizontal cells.

The HD Prox1 is another essential gene for horizontal cell development. During retina development, Prox1 is expressed in differentiating horizontal cells, bipolar cells, and a subtype of amacrine cells. RPCs lacking Prox1 are less likely to stop dividing, and ectopic expression of Prox1 forces RPCs out of the cell cycle. In Prox1 knockout mice, horizontal cells are absent and misexpression of Prox1 in postnatal RPCs promotes horizontal cell production. Thus, it appears that Prox1 is both necessary and sufficient for RPC proliferation and horizontal cell fate determination in the vertebrate retina.

Asymmetric Cell Divisions in Retinal Cell Fate Decisions

Increasing evidence suggests that the asymmetric inheritance of cell fate determinants during an RPC division is an important mechanism regulating cell fate specification. In vertebrates, a number of studies have now provided evidence that the orientation of cell division is correlated to symmetric and asymmetric outcomes in various organs in different animals (**Figure 4**). Specifically, in the developing retina, the process of symmetric and asymmetric division has been most studied in terminal divisions that generate two postmitotic daughter cells. Upon terminal division, most RPCs divide with their mitotic spindle oriented parallel (horizontal) to the plane of the neuroepithelium, but a substantial minority divides with their spindle oriented perpendicular (vertical) to the plane of the neuroepithelium, along the apico-basal axis. A mammalian homolog of Numb (m-Numb), a natural antagonist of Notch signaling, is asymmetrically localized at the apical pole of the dividing RPCs and thus would be presumably asymmetrically inherited by the apical daughter cell of vertical divisions, whereas it would be symmetrically inherited by both daughter cells of horizontal divisions. Live imaging experiments of retrovirally labeled rat RPCs demonstrated that the two daughter cells of terminal divisions with a horizontal spindle tend to become the same cell type, whereas the two daughter cells of divisions with a vertical spindle tend to produce daughters that become different cell types. Moreover, overexpression of m-Numb in RPCs resulted in more daughter cell pairs of the same cell type and fewer daughter cell pairs of different cell types. Thus, the plane of cell division can apparently influence cell fate choice in the developing rat retina, presumably by controlling the asymmetric segregation of cell fate determinants such as m-Numb.

In the zebra fish retina, the orientation of cell division also appears to be correlated with cell fate. Although no apical–basal divisions are found in the zebra fish retina, some RPCs reorient their mitotic spindle within the plane of the neuroepithelium and divide either along the central to peripheral axis or along the circumferential axis. As development proceeds, the orientation of RPC divisions switches from

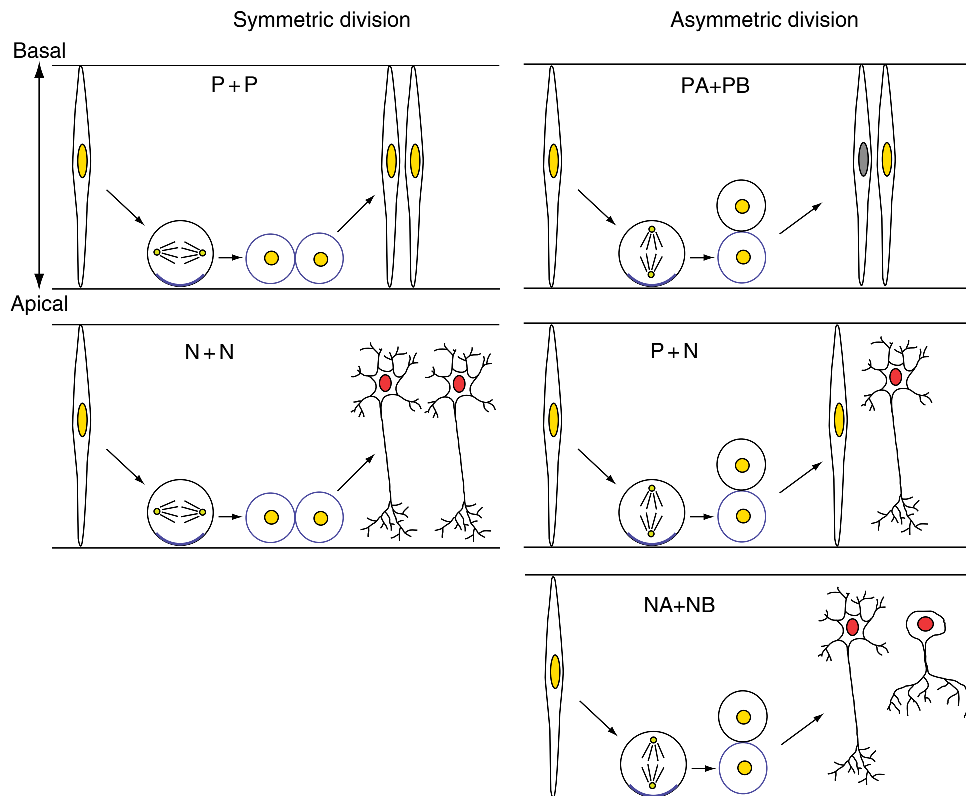

Figure 4 Different possible outcomes of symmetric and asymmetric cell divisions. RPCs undergo mitosis at the apical side of the neuroepithelium and segregate cell fate determinants (blue) asymmetrically at the apical pole of the cell cortex. In a symmetric division (left panels), the RPC divide parallel to the plane of the neuroepithelium, and cell fate determinants are inherited symmetrically by both daughter cells. The outcome of the division is two daughter cells of the same type, which can be two progenitors (P + P) or two neurons (N + N). In an asymmetric cell division (right panels), the RPC reorients its mitotic spindle to divide perpendicular to the asymmetric concentration of cell fate determinants at the apical pole of the cell cortex. This leads to asymmetric inheritance of cell fate determinants by the daughter cells. The outcome of the division is two daughter cells of different types, which can be two progenitors with different intrinsic potential (PA + PB), a progenitor and a neuron (P + N), or two neurons of different types (NA + NB).

being mostly central-peripheral to being mostly circumferential, suggesting that changing the orientation of cell division may contribute to particular cell fate decisions. Recent live imaging studies in the zebra fish retina support this possibility. In these experiments, it was shown that ath5:GFP RPCs in a wild-type retina divide along the circumferential axis to give rise to a RGC and another cell type, most likely a photoreceptor, whereas when these ath5:GFP RPCs are grafted in a RGC-depleted environment, they tend to divide along the central-peripheral axis to give rise to two RGCs, indicating that the orientation of cell division can predict the outcome in terms of the fate adopted by the daughter cells. It remains to be determined whether cell fate determining proteins are asymmetrically inherited by the daughter cells of circumferential divisions in the fish retina, but proteins involved in the establishment of planar cell polarity are certainly good candidates.

See also: *Drosophila* Apterous Neurons: from Stem Cell to Unique Neuron; Helix–Loop–Helix (bHLH) Proteins: Hes Family; Helix–Loop–Helix (bHLH) Proteins: Proneural.

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Neural Stem Cells: Ocular

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Introduction

Most of the neurons in the mature central nervous system (CNS) are generated during the embryonic or neonatal periods of development. In a few 'neurogenic' regions of the mature CNS, new neurons continue to be generated throughout life. The subventricular zone and hippocampal progenitor zone are the two neurogenic regions of the mammalian brain that have been best characterized, whereas in nonmammalian vertebrates, other neurogenic regions include the HVC of the songbird and, most important for this article, the ciliary margin of the retina. In all these regions, it is thought that a region-specific 'neural stem cell' maintains the progenitors that produce the new neurons and glia. Several lines of evidence indicate that a 'retinal stem cell' exists throughout life in nonmammalian vertebrates. Evidence from both rodents and primates, including humans, indicates that multipotential, mitotically competent cells exist at the retinal margin and can be activated *in vitro* to generate neuronal cells. This article discusses the literature on retinal development and the developmental origins of the retinal stem cells in nonmammalian vertebrates, describes their function in retinal growth and regeneration, and details efforts to identify and manipulate the remnants of this zone in mammals. Finally, common features among neural stem cell zones throughout the mature nervous system are discussed.

Retinal Progenitors in Normal Development

Each retina is initially derived from one of two evaginations of the ventral diencephalon of the neural tube, known as optic vesicles. Soon after they form, the optic vesicles undergo a further morphological transformation to form the optic cups. The cells of the optic cup resemble neural progenitors from other regions of the CNS in that they have a simple bipolar morphology and span the width of the neuroepithelium. Clonal analysis of the progeny of the mitotically active progenitor cells shows that they can give rise to all the different types of retinal neurons, and that the clones have mixed neuronal and glial lineages (**Figure 1(a)**). Birthdating studies have demonstrated that the different types of retinal neurons are generated by the progenitor cells in a sequence that is conserved among vertebrates. Ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells are generated during early stages of development, and most rod photoreceptors, bipolar cells, and Müller glia are generated in the latter half of the period of retinogenesis.

Progenitor cells in the retina have gene expression profiles that are similar to those of progenitors from other regions of the CNS. They express many of the same homeodomain transcription factors. Several homeodomain transcription factors, such as Pax6, Chx10, Prox1, Sox2, and Six3, are expressed in retinal progenitors and are sometimes collectively called the eye field transcription factors (EFTFs) since their early expression defines the presumptive eye-forming region of the neural tube. However, these genes are also expressed in progenitors in other anterior regions of the CNS. The retinal progenitors also express some unique transcription homeodomain transcription factors, such as Rx and Crx. A similar pattern is observed for proneural transcription factors, such as Mash1, Neurogenin2, and Math3; these genes are expressed in progenitors from retina as well as other regions of the CNS. In addition, retinal progenitors express at least one unique member of this family of transcription factors, Math5. Presumably, it is through unique combinations of the common CNS factors and the retinal-specific factors that the unique retinal cell types, such as photoreceptors and retinal ganglion cells, are specified.

In addition to the complex intrinsic factors regulating retinal progenitors, these cells are also influenced by extrinsic signaling factors. Most of these factors have similar effects on the retinal progenitors as they would elsewhere in the CNS. For example, epidermal growth factor (EGF) was first described as a mitogen for retinal progenitor cells prior to its identification as a mitogen for brain-derived neural stem cells. Fibroblast growth factors (FGFs) and sonic hedgehog are additional mitogens for retinal progenitor cells that have also been demonstrated to have mitogenic effects in other regions of the CNS. Negative regulators of proliferation are also present in the developing retina to control the extent and duration of neurogenesis. Transforming growth factor-β2 is the most well-described inhibitor of proliferation in the retina, as it is in other regions of the CNS, and it likely functions through the regulation of the cell cyclin-dependent kinase inhibitor p27kip.

Another key regulator of progenitor cells in the retina is the Notch pathway. At least two Notch

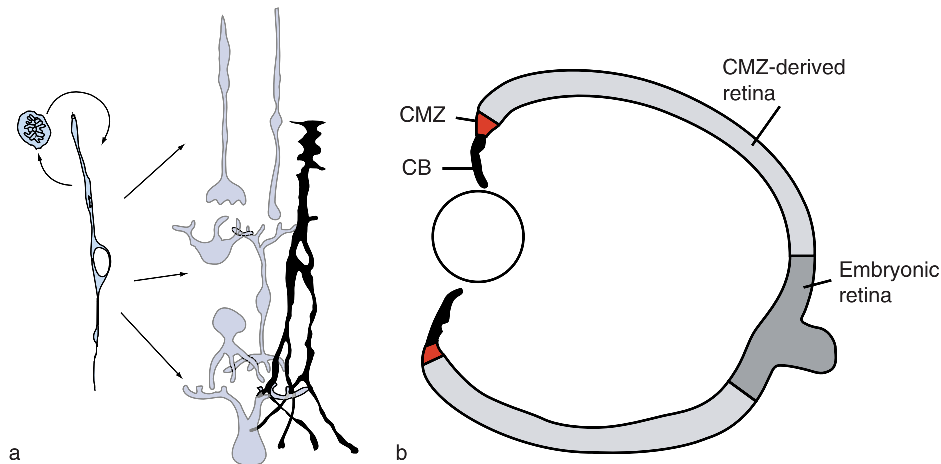

Figure 1 (a) Diagram of the multipotent progenitor that generates neurons (pink) and Muller glia (black) during embryonic development. (b) These cells persist at the retinal margin, in the 'ciliary marginal zone' (CMZ (red)) and are then called retinal stem cells, since they can persist the lifetime of the animal. The CMZ is located between the ciliary epithelium of the ciliary body (CB). The relative contributions of the retina derived from the embryonic progenitors (dark gray) and the CMZ (light gray) in a frog is also shown in this figure.

receptors and several Notch ligands are expressed by the retinal progenitor cells. Loss-of-function and gain-of-function studies have shown that Notch activity regulates progenitor differentiation. If the Notch receptor is activated in progenitors, they remain as progenitors, whereas inactivation of the Notch receptor occurs just prior to differentiation into one of the retinal neuronal types. The downstream effectors of the Notch pathway are the Hes genes. Hes1 and Hes5 are both expressed by subsets of the mitotically active cells in the developing retina, and both respond to Notch activation. Knockout and overexpression studies of the Hes genes indicate that they are necessary for both maintenance of the undifferentiated state of the progenitors and the development of the Müller glia.

In summary, the mitotically active cells in the developing retina have many characteristics of neural/glial progenitors in many other regions of the CNS. They share many of the same transcription factors, signaling molecules, and cell biology. In the retina, these cells have been called multipotent progenitors because at early stages of retinal development, these cells are competent to generate the entire complement of retinal neurons and glia. Thus, these cells have many characteristics of neural stem cells that have been isolated from other regions of the CNS. In addition, several groups have shown that the early progenitors can be cultured as 'neurospheres' (i.e., nonadherent aggregate cultures of neural stem and progenitor cells that can be passaged in EGF and/or FGF-containing medium). As described later, the adult retina of some vertebrates continues to add new neurons and glia at the peripheral margin, and thus true retinal stem cells exist. Presumably, these cells were derived from a population of similar cells in the developing retina, but there is currently no definitive way to distinguish the stem cells from the progenitors during retinogenesis.

Retinal Stem Cells in the Ciliary Marginal Zone

In the mammalian retina, the neurons and glia are generated in a continuous period of time from fetal to neonatal stages. In the mouse, for example, retinal neurons and glia become postmitotic starting at embryonic day 12.5 and ending approximately 1 week after birth. In all vertebrates, there is a central-to-peripheral pattern to histogenesis and differentiation in the retina. The first neurons are produced in the central retina and the last are produced at the periphery.

In contrast to the mammalian retina, in the amphibian, fish, or avian retina, histogenesis continues after the embryonic or neonatal period. In these animals, the retina continues to add new neurons into adulthood and, in some, throughout life. Teleost fish, for example, have a dramatic growth of the eye during their lifetime of up to 100-fold. The growth of the retina is accomplished by the continued production of new retinal neurons from a zone of cells at the peripheral margin, adjacent to the nonneuronal ciliary body, called the ciliary marginal zone (CMZ; **Figure 1**(**b**)). This zone is similar to neural stem cell zones in other regions of the CNS, such as the hippocampal progenitor zone or the subventricular zone. However, in contrast to these other zones, which typically generate only one type of neuron, the CMZ cells generate all types of retinal neurons

and Müller glia. In fact, most of cells in the retina of the mature frog or fish are generated by the cells of the CMZ. Lineage tracing studies of CMZ cells have shown that these cells can give rise to clones that contain all types of retinal neurons. The CMZ is thought to be organized with the most primitive cells (true stem cells (?)) in the most peripheral part (possibly mixed with the cells of the ciliary epithelium) and progressively more mature progenitor cells (transit amplifying cells (?)) closer to the differentiated retina.

The CMZ is highly productive in fish and some amphibians, but in birds it is greatly reduced. In birds, most of the retina is generated during embryonic development and only a small number of retinal neurons are generated by the CMZ. It is not known whether this zone persists throughout the lifetime of the bird, but new retinal neurons are generated at the peripheral edge of the retina in chickens up to 1 month of age and in the quail eye for up to 1 year. Like those of fish and amphibians, the CMZ cells of birds resemble the early progenitor cells of the retina, in terms of both their gene expression and their response to mitogenic factors.

The CMZ is greatly reduced or absent in the eyes of mammals that have been examined to date, including rodents and nonhuman primates. However, there is evidence that a CMZ-like zone can form in rodents under certain conditions. For example, mice with a single functional allele of the patched gene, a negative regulator of Shh signaling, retain a small number of proliferating, nestin-expressing cells at the retinal margin into adulthood. Moreover, when these mice are bred onto a background in which photoreceptors degenerate, the proliferation increases, reminiscent of the response to retinal damage observed in the CMZ cells of lower vertebrates. Studies have found that the normal period of proliferation of retinal progenitors can be extended by the injection of specific growth factors, but no study has successfully ‘resuscitated’ cells at the retinal margin of an adult mammal *in vivo*.

The CMZ in fish, amphibians, and birds can provide a source of regeneration following damage to the retina. In fish and amphibians, destruction of retinal neurons with neurotoxins or by surgery causes an increase in the proliferation of the CMZ cells. The cells produced by the CMZ then differentiate into neurons and in some cases migrate considerable distances to replace the cells lost by the experimental injury. In birds, neurotoxin damage does not by itself stimulate the proliferation of the CMZ cells, but destruction of retinal ganglion cells, followed by insulin-like growth factor and FGF injections, causes the CMZ cells to produce new ganglion cells, replacing the lost ganglion cells in the peripheral retina.

Additional Sources of Retinal Stem and Progenitor Cells

The Pigmented Epithelium

One of the most striking examples of regeneration in vertebrates is that of the amphibian retina. Following complete removal of the neural retina in larval frogs and adult urodeles (newts and salamanders), the adjacent pigmented epithelial (RPE) cells reenter the cell cycle, lose their pigmentation, and begin to express markers of retinal progenitors. The de-differentiated RPE cells go on to generate new retinal neurons in a recapitulation of normal retinal histogenesis. During a period of just a few weeks, histogenesis is complete and new retinal ganglion cells reconnect with visual centers in the brain. This process of RPE transdifferentiation into retinal progenitors does not occur in fish, adult birds, or adult mammals. However, a similar process occurs in birds and mammals at very early stages of eye development in embryos. The molecular mechanisms underlying the transition of the pigmented epithelial cells to retinal progenitors are only beginning to be understood. The de-differentiating pigment cells begin to resemble retinal progenitors soon after the retinal injury and express many of the EFTFs. FGF is a key stimulus for retinal regeneration from the RPE cells in both amphibians and chick embryos. When FGF is added to cultures of RPE cells or implanted into the vitreous *in ovo*, the RPE cells adopt a retinal progenitor identity and new, laminated retina is generated. Additional factors, such as activin and Shh, antagonize the trans-differentiation of RPE into regenerated retina, and blocking these factors enhances the retinal regeneration from the RPE that is induced by FGF.

The Ciliary Epithelium

The ciliary body is also considered a possible source of retinal stem cells or progenitors. The ciliary body is composed of a neural tube-derived part, called the ciliary epithelium, as well as a neural crest-derived part. The ciliary epithelium has two layers (**Figure 1**) – a pigmented layer that is continuous with the RPE and a nonpigmented layer that is continuous with the neural retina. The ciliary epithelium is anatomically and developmentally analogous to the choroid plexus in the rest of the CNS. The nonpigmented layer of the ciliary epithelium can generate neurons under certain conditions *in vivo* in birds. Intraocular injection of growth factors (insulin, FGF2, and EGF) stimulates the proliferation and neuronal differentiation of a subset of ciliary epithelial cells. Like the CMZ, the cells of the nonpigmented ciliary epithelium also express at least some of the EFTFs. The neurons

generated in this region following intraocular growth factor injections resemble amacrine cells and ganglion cells but not bipolar cells or photoreceptors. Thus, these cells may not actually be true multipotent progenitors but, rather, retain only some of their characteristics.

The mammalian ciliary epithelium has also received attention as a potential source of new neurons in adult animals. Several groups have found that cells can be isolated from the rodent ciliary epithelium that display characteristics of neural stem cells isolated from the mature brain. Although the cells appear initially pigmented, suggesting they are resident in the pigmented layer of the ciliary epithelium, they give rise to spheres of both pigmented and nonpigmented cells. Some of the spheres derived from the ciliary epithelium can be passaged to form new spheres, and although they appear to have a more limited life *in vitro* than neurospheres derived from the brain, they have been labeled retinal stem cells by the authors of these studies. The spheres are also capable of differentiation into cells that express markers of retinal neurons, including the rod photoreceptor-specific protein, rhodopsin. Human eyes also contain these cells, and they can be grown *in vitro* for extended periods of time and transplanted. However, the cells derived from the spheres typically express markers of specific neuronal types without taking on the morphological characteristics of the neurons. This is particularly clear in the case of photoreceptors, which have a distinct morphology when derived from retinal progenitors during normal development but appear flat and glial-like when derived from ciliary epithelial-derived cells. Thus, it is unclear whether the situation for these cells is more like what was previously described for the bird ciliary epithelium – that is, the cells take on some, but not all, of the characteristics of retinal progenitors. The nonpigmented epithelial cells from mammalian eyes can also be maintained as dissociated cell cultures grown on adherent substrates. Under these conditions, the cells are also capable of expressing neuronal markers, but again only some of the normal markers of retinal progenitors are expressed.

Intrinsic Stem Cells, Rod Precursors, and Müller Glia

In fish, there are several sources of new retinal neurons within the differentiated retina. In teleosts, new rod photoreceptors are added throughout the retina as it grows. The addition of new rods to the circuitry allows the fish to maintain a constant rod density as the retina stretches with ocular growth. Thus, sensitivity to light is maintained with increasing size. To accomplish this feat, the fish has a specific progenitor, called the rod precursor, which is normally restricted to generating new rods. These cells are found in association with the Müller glia and divide in the outer nuclear layer. In addition to the rod precursors, fish also have a relatively quiescent stem cell in the inner nuclear layer. Like the cells of the ciliary epithelium and those of the CMZ, this cell has also been labeled the retinal stem cell. The role of this slow-cycling retinal stem cell in normal retina is not clear, but when a small patch of central retina is surgically removed, both the rod precursor and the intrinsic retinal stem cell proliferate and form a blastema. The blastemal cells also resemble retinal progenitors, and over a few weeks they fill in the excised patch of retina.

The final source of retinal progenitors that have potential to replace lost neurons from within the retina are the Müller glia. Müller glia are the only glial cells in the retina produced by the multipotent progenitor. Müller glial cells are among the last cell types produced by the retinal progenitors during the normal period of histogenesis, and gene expression profiling studies have shown a high degree of overlap in the genes expressed by Müller glial cells and retinal progenitors. Despite their similarity, Müller glial cells in mammals and birds do not normally express many of the critical transcription factors that define progenitors in the retina and elsewhere in the CNS, such as Neurogenin2, Pax6, or Mash1. However, in posthatch chickens, damage to the retina by neurotoxins causes some of the Müller glia to reenter the cell cycle and reexpress both proneural genes, such as Mash1, and EFTFs, such as Pax6 and Chx10. In addition, after neurotoxic damage to the retina, some of the proliferating Müller glia go on to generate cells that express markers and morphology of neurons. The regenerative process initiated by retinal damage in young birds is largely abortive, however, and the majority of the Müller glial progeny remain as undifferentiated cells.

A similar process of regeneration from Müller glial cells may occur in teleost fish. As noted previously, regeneration of the teleost retina is particularly robust, and both rod precursors and intrinsic retinal stem cells are thought to contribute to the process. However, lesions to the retina of mature zebra fish also lead to Müller glial proliferation, and these cells may also contribute to the process, as in the chick. Attempts to stimulate regeneration from Müller cells in mammalian retina are under way and initial findings are promising, although there are still major hurdles to overcome.

See also: Neural Stem Cells: Adult Neurogenesis; Retinal Development: An Overview; Stem Cells and CNS Repair; Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging.

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Neural Crest

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Introduction

The development of the nervous system begins overtly with the formation of the neural plate in the surface ectoderm. Soon after the neural plate forms, it folds in on itself to form neural folds. The dorsal lips of the neural folds subsequently fuse to form the neural tube, a process called primary neurulation. Just before, during, and slightly after the fusion of the neural folds, a population of neuroepithelial cells in the dorsal lips of the neural fold and the dorsal region of the neural tube undergo an epithelial–mesenchyme transition (EMT) and emigrate away from neural tube; this cell population is the neural crest (**Figures 1(a)** and **1(b)**). The formation of the lumbosacral neural tube in birds and mammals, and the entire neural tube in fish, occurs by a different process, known as secondary neurulation. Here, a compact mass of cells forms and then cavitates to form a neural tube. Neural crest cells also form at the dorsal regions of the neural tube, adjacent to the epidermis, during secondary neurulation. The neural crest is a transient structure that only exists around the time of formation of the neural tube. Neural crest cells migrate along particular pathways before settling in many different locations. They give rise to most of the facial skeleton, the peripheral nervous system, melanocytes, and many other derivatives.

The neural crest was first described and recognized as a migratory population in the 1860s. From the 1920s on, many methods have been used to experimentally probe its development, including ablation (removal) of neural crest in living embryos and later observations of deficits, and application of various stains to neural crest cells to follow them. However, the method that has been pivotal in studying the migration pathways and fates of neural crest cells is the quail–chick chimera technique. This method was devised in the late 1960s by Nicole Le Douarin, who saw that the nuclei of quail cells can be distinguished from those of chick cells using histological or immunohistochemical techniques (**Figure 2(c)**). Segments of neural primordium containing premigratory neural crest cells from specific regions of the neural axis in quails are transplanted into chick embryos (**Figures 2(a)** and **2(b)**), and the location of the quail cells within the chick host embryos can be determined later, thus revealing the migratory pathways and fate of neural crest cells from different axial levels.

The neural crest can be subdivided into three main axial populations – cranial, vagal, and trunk – although the vagal population includes the most caudal cranial and the most rostral trunk neural crest cells (**Figure 3**). The different axial populations migrate along different pathways and each gives rise to a range of cell types. Some of these cell types are common to several axial levels, whereas some are unique. For example, all neural crest levels give rise to sensory neurons. In contrast, cranial neural crest cells give rise to most of the bone and cartilage of the head and neck, whereas trunk neural crest cells have minimal ability to form skeletal elements. This article discusses the induction of the neural crest and the migratory pathways and derivatives of cranial, vagal, and trunk neural crest cells.

Induction and EMT

The first portents that the neural crest is different from the more medial ectoderm of the neural plate (which produces the central nervous system) and the more lateral epidermis (which produces the skin) are seen in gastrulation, even before neurulation. At this stage, a restricted region of epiblast (surface cell layer) fated to contribute to the neural crest expresses the paired-box transcription factor gene *Pax-7*. This region of epiblast is able to produce cells with neural crest markers when isolated from all inductive influences, and suppression of Pax-7 prevents this.

Later, important signaling processes occur across the border between the neural and epidermal ectoderm to mark this zone as different from the adjacent but contiguous epithelia. Important in this process are secreted growth factors of the Wnt family and of the transforming growth factor family, especially bone morphogenetic proteins such as BMP-4. The lineage-specifying action of BMP-4 in particular has a quantitative nature; high levels specify epidermal lineage and moderate levels promote neural crest characters, whereas low levels permit neural plate character. BMP-4 gene expression is homogenetically induced in the dorsal neural ectoderm, giving a positive feedback capacity. A gradient of BMP-4 activity is sharpened by a countergradient of its secreted inhibitor Noggin. These signals from the epidermis act within the ectodermal layer on the edge (later dorsal) cells of the neural ectoderm. In addition,

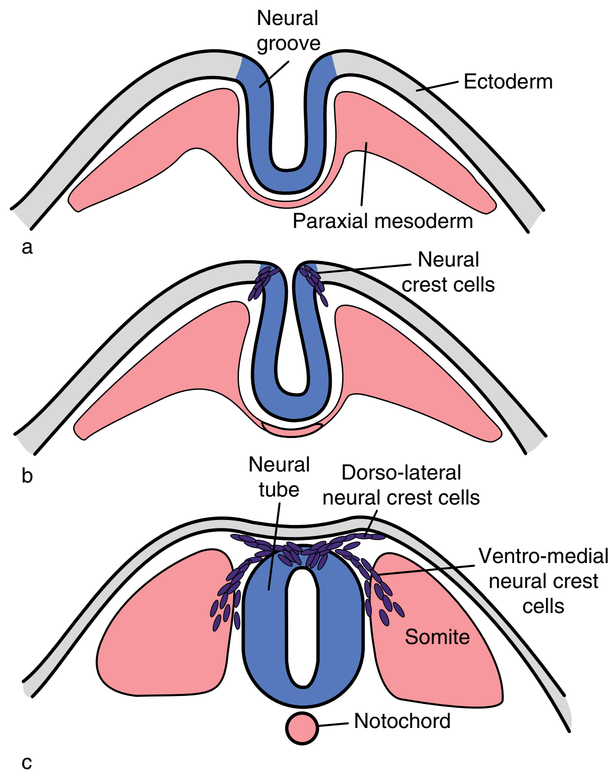

Figure 1 (a, b) Primary neurulation, which involves the formation of the neural plate and folds, and subsequent closure of the folds to form the neural tube. Neural crest cells delaminate from the dorsal regions of the neural folds and neural tube. (c) In the trunk region, neural crest cells migrate along two main pathways; first, they migrate along a ventral pathway through and between the somites to form sympathetic ganglia, Schwann cells, and dorsal root ganglia. Later, emigrating cells migrate along a dorsolateral pathway to form melanocytes.

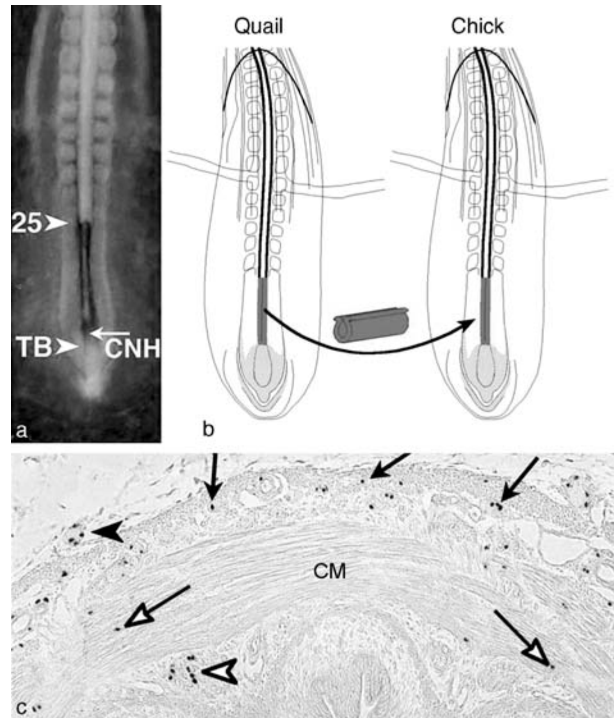

Figure 2 Quail–chick grafting technique devised by Nicole Le Douarin and colleagues. A defined region of the neural tube containing premigratory neural crest cells, in this case sacral neural tube, is removed from chick embryos (a) and replaced with transplanted neural tube from quail embryos (b). Following subsequent development, the location of the quail cells within the chick host embryos can be determined using immunohistochemical methods. CNH, chordoneural hinge; TB, tail bud. (c) Transverse section through the hindgut following quail–chick sacral neural crest grafting. Quail cells, which are stained brown, are found within nerve bundles outside the gut (arrowheads), external (arrows) and internal (open arrowhead) to the circular muscle layer (CM) of the gut, as well as within the circular muscle layer (open arrows). These experiments show that sacral neural crest cells give rise to derivatives both within and outside the hindgut. Reproduced from Burns AJ and Douarin NM (1998) The sacral neural crest contributes neurons and glia to the post-umbilical gut: Spatiotemporal analysis of the development of the enteric nervous system. *Development* 125: 4335–4347, from The Company of Biologists.

fibroblast growth factor signaling from another layer, the underlying mesoderm, is also important for neural crest identity.

The outcome of this signaling is the expression of specific genes in the lateral neural plate border region which includes the dorsal neural tube as well as cells fated for neural crest; these ‘border genes’ include *Zic3* and *Pax-3*. Expression of other genes such as *Sox-2* in the medial region act to limit the neural crest zone to the neural plate border. On the lateral epidermal side, the high levels of BMP-4 signaling induce genes such as *Dlx* and *Msx* family members, whose products repress a variety of proneural genes such as *Zic3* and *Sox-2*, while promoting expression of epidermis-defining keratins.

Experimental manipulation of these border genes indicates that they control expression of genes that are closely related to neural crest fate and to neural crest behavior such as EMT and migration. Included in this gene repertoire are the zinc finger transcription factors *Snail-1* or *Snail-2* (formerly called *Slug*). These genes have an interesting evolutionary history: the neural crest of fish, amphibians, and mammals expresses *Snail-1*, whereas that of reptiles and birds employs *Snail-2*. In a very short period of time, expression of the forkhead gene *FoxD3*, and the Sry HMG-box genes of the *Sox-E* subfamily (*Sox-8*, *-9*, and *-10*), is induced in the neural crest region. When overexpressed (or suppressed), these genes can induce (or inhibit) neural crestlike behavior such as migration in nonneural crest neural ectoderm, as shown first by Angela Nieto for *Snail2*. These genes are sharply temporally regulated, and with the exception of *Sox-10*, their mRNA levels decline around or just after the time when neural crest migration commences. *Sox-10*, however, continues to be expressed

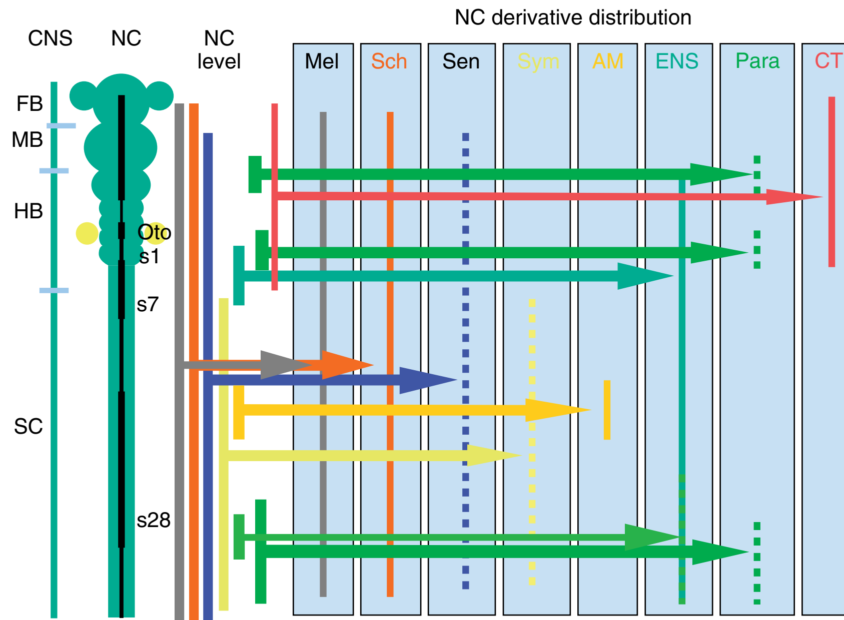

Figure 3 Axial level of origin of neural crest derivatives in chick embryos. Melanocytes arise from all levels of the neural axis, whereas only cranial neural crest contributes to skeletal elements (the cranial skeleton). Cardiac neural crest, which arises adjacent to somites 1–3 and contributes to the outflow tracts of the heart, is not included in the diagram. AM, adrenomedullary; CNS, central nervous system; CT, connective tissue; ENS, enteric nervous system; FB, forebrain; HB, hindbrain; MB, midbrain; Mel, melanocyte; NC, neural crest; oto, otic vesicle; Para, parasympathetic ganglion; s, somite; Sch, Schwann cell; Sen, sensory neuron; SC, spinal cord; Sym, sympathetic ganglia.

especially in vagal and trunk neural crest and has other functions; including maintenance of an undifferentiated state. *Dlx* and *Sox-9* expression reappears later in postmigratory cranial neural crest-derived cells prior to their formation of skeletal tissues. Work in many species by many authors, particularly Marianne Bronner-Fraser and colleagues, has opened up this field of neural crest induction. Yet the regulatory circuits between these genes are complex and not fully elucidated (**Figure 4**). Moreover, although these cells are specified as neural crest (since they express many appropriate genes), they are not necessarily committed to this fate. The morphogenetic event of EMT might be thought of as marking a watershed in neural crest cell lineage commitment, yet experimental transplantation back into the neural tube reveals that at least some can still contribute to the neural tube.

The neural crest at any one level produces several different types of differentiated cells, and different axial levels produce some unique types. At any one level, during the premigratory phase, some cells are broadly multipotent within the neural repertoire and differentiation is guided by exposure to instructive cues they encounter later. Others become more restricted; the last cells to commence emigration are more likely to be restricted to a melanocyte lineage, and this is related to prolonged exposure to the neural tube. The intrinsic differences between axial levels of the neural crest are tied to the general positional identity of axial structures. Broadly stated, the cranial neural crest has a less restricted range of potentiality, whereas more caudal neural crest lacks some potentials. This is most notably demonstrated by the inability of trunk neural crest to produce connective tissue derivatives such as cartilage. A similar but less complete restriction is also seen in the limited ability of trunk neural crest to generate an enteric nervous system. This seems to involve the nested expression of *Hox* genes, and there is genetic cross-modulation between these axial position genes and some to the genes previously mentioned that specify the neural crest.

The outcome of this is the EMT and onset of migration of neural crest cells, sometimes termed delamination. This is the archetypal example of a process repeatedly seen in development and which seems to be replicated pathologically in carcinoma invasion. This involves relocation and reduction of the molecules that hold the neural epithelial cells together, most prominently the classic adherens junction molecule, N-cadherin. Experiments indicate that N-cadherin inactivation can trigger migration in neural epithelial cells. Other nonclassic cadherins are induced, and these may be important in maintaining the transient cell–cell adhesions seen between migrating

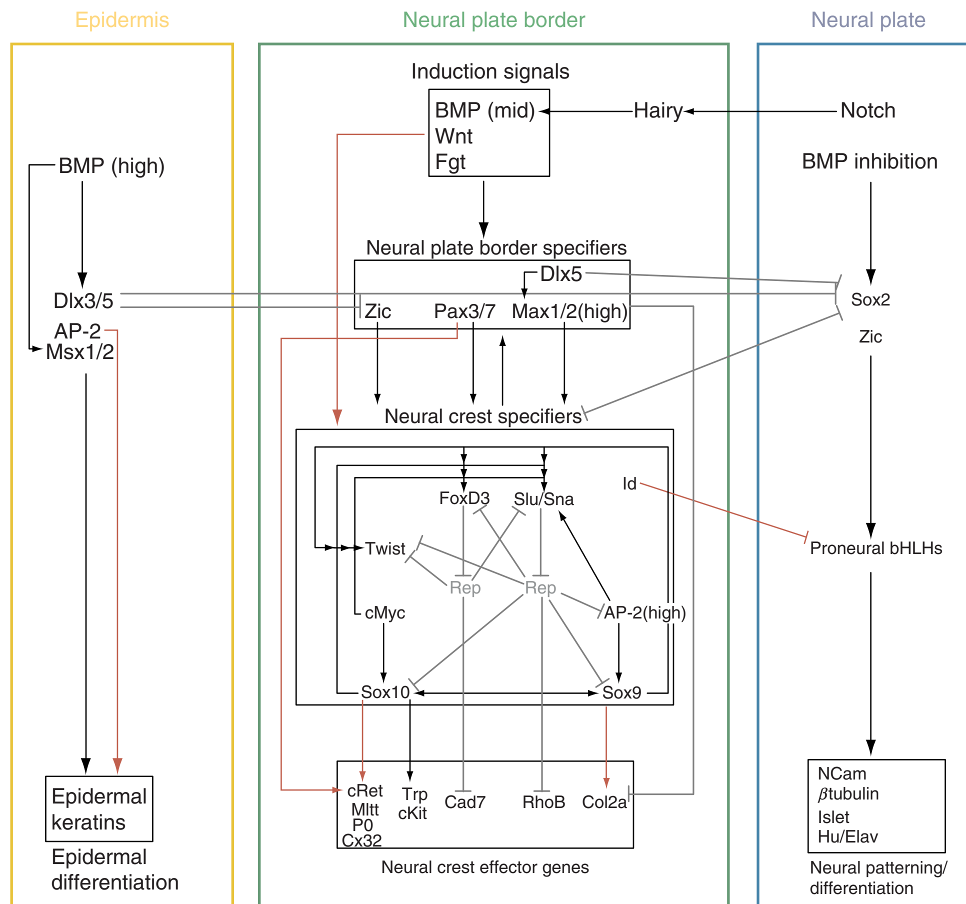

Figure 4 Putative gene regulatory and signaling interactions in action at the neural plate border of vertebrates. Red arrows, proven direct regulatory interactions; black arrows, genetic interactions suggested by gain- and loss-of-function analyses largely in *Xenopus*; gray lines, repression. Reproduced from Meulemans D and Bronner-Fraser M (2004) Gene-regulatory interactions in neural crest evolution and development. *Developmental Cell* 7: 291–299, with permission from Cell Press, Elsevier.

neural crest cells. In addition, there are changes in the suite of integrins expressed on the cell surface. The integrins allow new adhesive contacts to develop with extracellular matrix (ECM) molecules, which form the substrate for the first steps of neural crest cell migration. Like other migrating cells, neural crest cells modify their surrounding ECM via expression of several matrix metalloproteases (MMPs). Inhibition of MMPs can prevent neural crest EMT and subsequent migration, and in particular may be required to penetrate the neural tube basal lamina, as seen at the cranial level in rodents. In addition, the actin-based cytoskeleton of these cells is reorganized from the apico-basal polarity typical of neural epithelial cells to the more flexible network arrangement which allows cell motility. The transient expression of the *RhoB* gene immediately prior to EMT may be involved in this.

The timing of neural crest EMT is stereotyped but not precisely related to other morphogenetic events in the neural epithelium. Sometimes, the newly mesenchymal neural crest cells individually crawl out of the neural epithelium before neural tube closure (cranial level of rodents), but this event occurs after neural tube closure in birds and in the trunk of rodents. In amphibians, the neural crest separates as a coherent population from the neural tube and only commences migration later, when permissive ECM changes occur.

Cranial Neural Crest

Cranial neural crest cells contribute to much of the bone, cartilage, and connective tissue in the head, including most of the head skeleton and parts of the teeth. Many congenital craniofacial deformations are due to defects in the development of cranial neural crest cells. Cranial neural crest cells also give rise to some smooth muscle in the head, melanocytes, all cranial parasympathetic neurons and their support

cells, and all glia and some, but not all, neurons in cranial sensory ganglia (**Figure 3**). Sensory neurons in cranial ganglia that do not arise from the neural crest arise from placodes, which are specialized regions of the ectoderm. Some cells within the thyroid, parathyroid, and thymus glands also arise from the cranial neural crest.

In the hindbrain, the segmental rhombomeres not only play a vital role in the patterning of branchiomotor nerves and cranial ganglia but also influence neural crest cell migratory pathways in this region. Hindbrain neural crest cells migrate in three distinct streams adjacent to rhombomeres 2, 4, and 6 and populate the first, second, and third branchial arches, respectively (**Figures 5(a)** and **5(b)**). The regions adjacent to rhombomeres 3 and 5 are therefore neural crest cell-free zones. Some studies attributed the absence of neural crest cells adjacent to rhombomeres 3 and 5 to apoptosis of premigratory neural crest cells at these axial levels induced by signals derived from the even-numbered rhombomeres. However, subsequent studies by Peter Farlie, Paul Kulesa, Scott Fraser, Paul Trainor, and Rob Krumlauf have shown that neural crest cells are generated at odd-numbered segments but migrate anteriorly and posteriorly to join the streams of neural crest cells migrating from the even-numbered rhombomeres. The authors of these studies have proposed that the environment adjacent to the neural tube determines the behavior of cranial neural crest cells – inhibitory signals present adjacent to rhombomere 3 induce neural crest cells at this level to migrate anteriorly and posteriorly and limit their generation, whereas the otic vesicle provides a physical barrier to neural crest cells generated at rhombomere 5.

Cranial neural crest cells, through their interactions with facial epithelium and neuroectoderm, contribute to the development of most craniofacial structures in vertebrates (**Figure 6**). Different species have different facial morphologies. To examine whether species-specific facial patterning is intrinsic to neural crest cells or determined by cues from the facial ectoderm, neural crest cells destined to form the upper beak from duck were transplanted into quail embryos and vice versa. The resultant chimeric ‘qucks’ (duck embryos containing quail frontonasal neural crest cells) had short beaks typical of quail, and ‘duails’

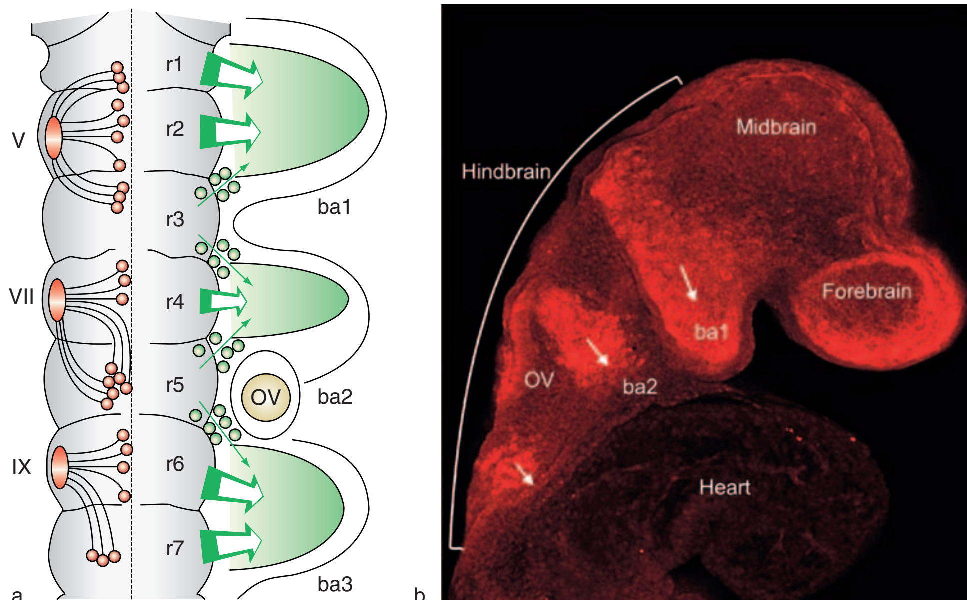

Figure 5 (a) Diagram showing migration of hindbrain neural crest cells in three streams. Neural crest cells from rhombomeres 1 and 2 (r1, r2) colonize branchial arch 1 (ba1). Only a small number of neural crest cells are generated in rhombomere 3 (r3), and those that are generated migrate anteriorly or posteriorly to join the streams lateral to r2 and rhombomere (r4), respectively. Neural crest cells from r4 colonize branchial arch 2 (ba2). The otic vesicle (OV) forms a physical barrier to neural crest cells from rhombomere 5 (r5) and they consequently migrate anteriorly or posteriorly to join the streams lateral to r4 and rhombomere 6 (r6), respectively. Neural crest cells from rhombomeres 6 and 7 colonize branchial 3 (ba3). Cranial motor nerves are shown on the left-hand side: V, trigeminal; VII, facial; IX, glossopharyngeal. (b) Head of an 8.5-day-old mouse embryo that had been processed for immunohistochemistry using an antibody to the neurotrophin receptor p75, which labels neural crest cells; p75-immunoreactive cells are stained red. There are three streams of neural crest cells (arrows) migrating from the hindbrain into the branchial arches (ba). At this developmental stage, crest cells from r1 and r2 are already colonizing ba1, but migration of neural crest cells caudal to the OV from r6 has just commenced. (a) Reproduced from Trainor PA, Sobieszczuk D, Wilkinson D, and Krumlauf R (2002) Signalling between the hindbrain and paraxial tissues dictates neural crest migration pathways. *Development* 129: 433–442, from The Company of Biologists. (b) Adapted from Anderson RB, Stewart AL, and Young HM (2006) The phenotypes of neural crest-derived cells in vagal and sacral pathways. *Cell Tissue Research* 323: 11–25, from Springer-Verlag.

(quail embryos containing duck neural crest cells) possessed flattened ducklike beaks (**Figure** 7). These transplantation experiments showed that neural crest cells intrinsically contain species-specific information, which is a major determinant of facial morphology. The genes involved in facial patterning are well conserved between species, and different facial morphologies are probably in part generated by differences in the temporal patterns of expression of these genes. Quail cranial neural crest cells transplanted into duck not only retained their own temporal pattern of gene expression but also altered the pattern of gene expression in nonneural crest-derived host tissue.

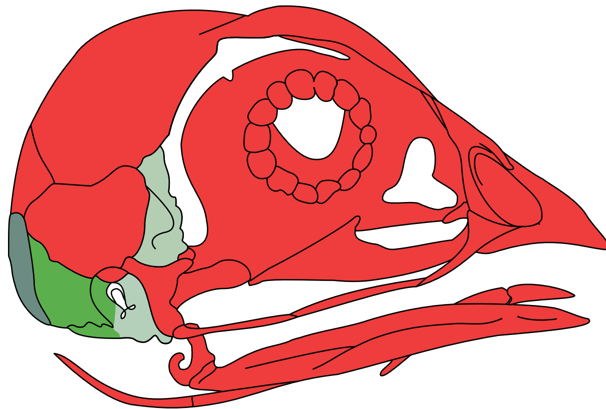

Figure 6 Lateral view of an avian skull showing the contribution of cranial neural crest (red), cephalic mesenchyme (blue), and somitic mesoderm (green). Adapted from Le Douarin NM and Kalcheim C (1999) *The Neural Crest*, 2nd edn. Cambridge, UK: Cambridge University Press.

Studies have also examined whether cranial neural crest cells at different rostrocaudal levels are prespecified to a particular fate prior to emigration from the neural tube. For example, neural crest cells destined to give rise to first branchial arch skeletal derivatives have been transplanted to a more posterior location in the hindbrain. In the 1970s, Drew Noden found that such embryos possessed duplicate first arch derivatives, such as two upper jaws, suggesting that cranial neural crest cells are prespecified prior to emigration. Studies by Paul Trainor and colleagues have shown that if the transplanted tissue excludes the mid-hindbrain isthmus, the transplanted neural crest cells show considerable plasticity. Because the isthmus was included in Noden's experiments, and because it expresses high levels of fibroblast growth factor 8 (fgf-8), it appears to act as a patterning center. Our current understanding is that cranial neural crest cells are not preprogrammed to a particular fate prior to emigration, nor is their fate determined solely by the environment into which they migrate but, rather, cranial neural crest cell fate depends on

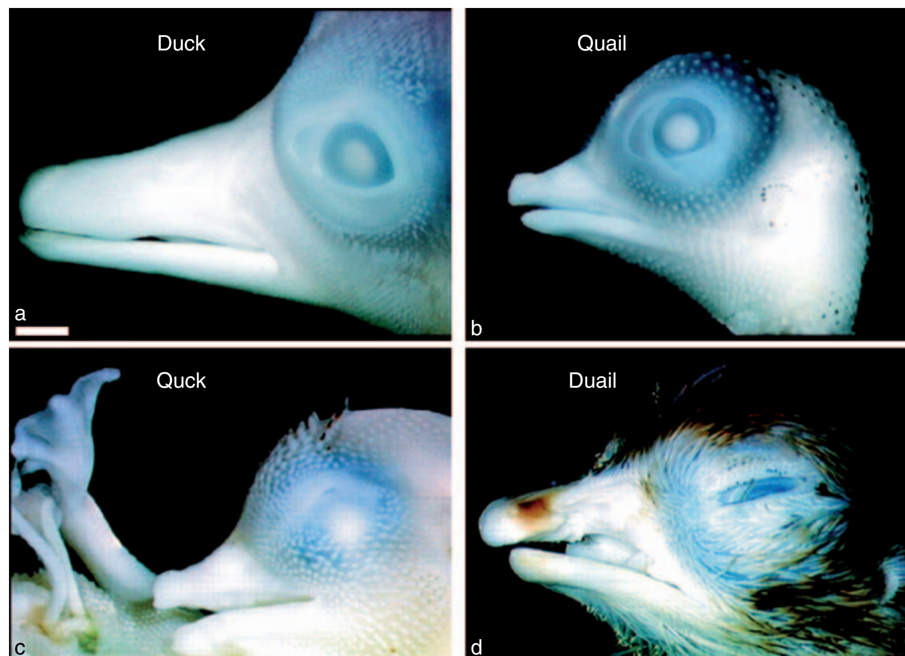

Figure 7 Differences in morphology of duck (a) and quail (b) beaks; the duck beak is flat and broad, whereas the quail beak is short and narrow. When neural crest cells of the presumptive beak region from duck embryos are removed and replaced with equivalent tissue from quail embryos, the 'quck' embryo has a beak that resembles a quail beak (c). Conversely, when neural crest cells of the presumptive beak region from ducks are transplanted into quail embryos ('duail'), the embryo has a beak that resembles a duck beak (d). Adapted from Schneider RA and Helms JA (2003) The cellular and molecular origins of beak morphology. *Science* 299: 565–568, from the American Association for the Advancement of Science.

a combination of signals from the hindbrain and from the branchial arch environment, as well as interactions between neural crest cells.

Vagal Neural Crest

Vagal neural crest cells are defined as neural crest cells that originate adjacent to somites 1–7 (**Figure 3**). The head–neck boundary corresponds approximately to somite 5, and thus vagal neural crest cells include the most caudal cranial neural crest cells and the most rostral trunk crest cells. Vagal neural crest cells give rise to two well-characterized derivatives – the cardiac outflow tracts and the enteric nervous system.

Cardiac Neural Crest

In the 1980s, using the quail–chick transplantation method described previously, Margaret Kirby showed that neural crest cells that emigrate adjacent to somites 1–3 contribute to the aorticopulmonary septation complex within the cardiac outflow tract of the heart and to smooth muscle (tunica media) of the aortic arch and its major branches; thus, these neural crest cells are known as the cardiac neural crest. Studies using a number of different strains of transgenic mice have largely confirmed the cardiac neural crest derivatives first identified in birds (**Figure 8**). However, some extra cardiovascular derivatives have also been reported in transgenic mice, including a neural crest contribution to the epicardium, but it is unclear whether these represent ectopic transgene expression or actual neural crest contribution to the cardiovascular system in mice.

A large number of genes and signaling pathways are now known to be involved in cardiac neural crest development in mice. The molecules include growth factors including fgf-8, transforming growth factor-β2, bone morphogenetic proteins, neurotrophin-3, Wnt1, and vascular endothelial growth factor; adhesion molecules including NCam, N-cadherin, and connexin-43; transcription factors including Pax3, Nkx2.6, FoxD3, Tbx1, Sox9 and Snail-1/Snail-2; and guidance cues including semaphorin 3C and notch signaling. Many of the aforementioned genes were identified from mouse models of congenital heart defects, but only a small number (e.g., Notch ligands and Tbx1) have been implicated in congenital heart anomalies in humans.

Enteric Nervous System

Within the wall of the gastrointestinal tract there is a vast network of neurons and glial cells called the enteric nervous system. Pioneering ablation experiments performed by Chester Yntema and Warner Hammond in the 1950s, and subsequent chick–quail transplantation studies performed by Nicole Le Douarin and colleagues in the 1970s, showed that all enteric neurons and glial cells arise from the neural crest, and the vast majority arise from vagal neural crest cells. Vagal neural crest cells migrate toward the gut along a pathway that is later followed by the vagus nerve (**Figure 9**). After

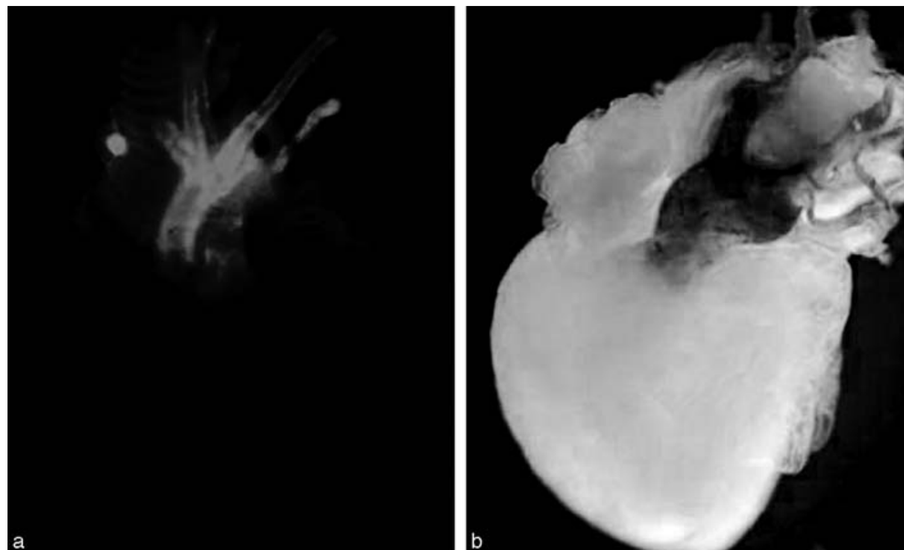

Figure 8 The heart and nearby blood vessels of transgenic mice in which cardiac neural crest cells express green fluorescent protein (GFP) (a) or the reporter gene, *lacZ*, and thus appear blue after histochemical processing (b). Cranial neural crest cells contribute to the aortic arch and great vessels. Reproduced from Stoller JZ and Epstein JA (2005) Cardiac neural crest. *Seminars in Cell and Developmental Biology* 16: 704–715, with permission from Elsevier.

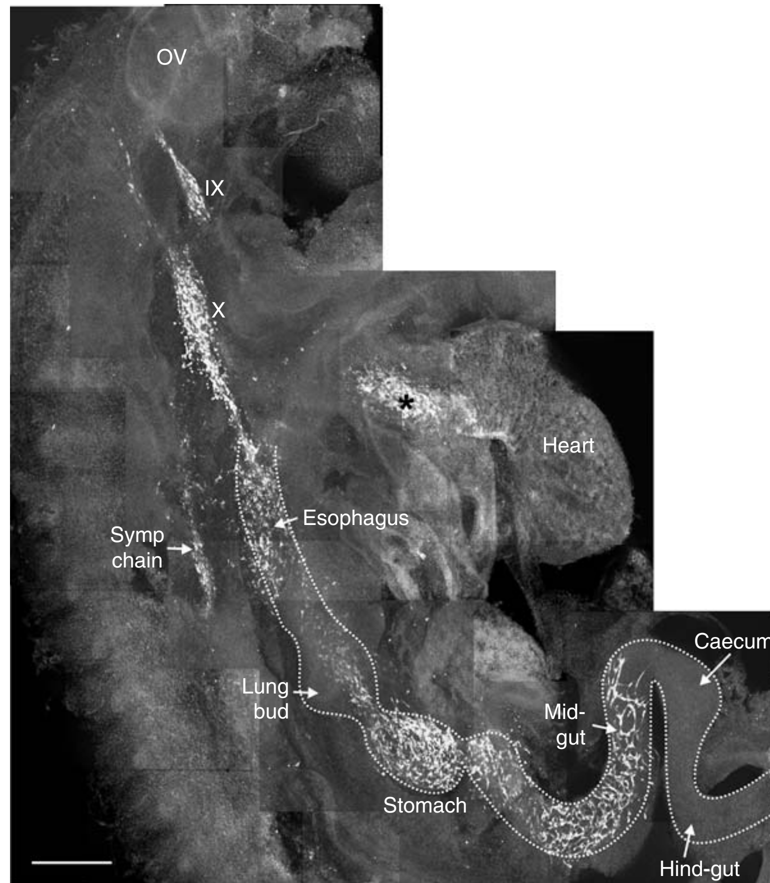

Figure 9 Lateral view of a 10.5-day-old transgenic mouse embryo in which cells expressing the receptor tyrosine kinase, Ret, also express green fluorescent protein (GFP). The epithelium had been removed to reveal the developing organs. Ret (and thus GFP) is expressed by vagal (including cardiac) and trunk neural crest cells. There is a stream of GFP+ cells that migrate from the hindbrain caudal to the otic vesicle (OV), adjacent to somites 1–7, into the gut along the pathway that will later be followed by the vagus nerve (X). At this developmental stage, GFP+ vagal neural crest cells have colonized the stomach and part of the midgut but not the caecum or hindgut; the neural crest cells within the gut will form the enteric nervous system. Vagal neural crest cells also give rise to ganglia of cranial nerves IX and X, and those arising from somites 1–3 contribute to the cardiac outflow tracts (asterisk). Trunk neural crest cells forming sympathetic chain ganglia (symp chain) also express GFP+. Reproduced from Anderson RB, Stewart AL, and Young HM (2006) The phenotypes of neural crest-derived cells in vagal and sacral pathways. *Cell Tissue Research* 323: 11–25, from Springer-Verlag.

entering the foregut, they migrate caudally through the gut mesenchyme to colonize the entire gastrointestinal tract.

A congenital disease called Hirschsprung's disease, or congenital megacolon, occurs in humans when neural crest cells fail to colonize the most distal regions of the gastrointestinal tract. Genetic studies in humans with Hirschsprung's disease and a variety of transgenic or mutant mice with defects in the enteric nervous system have been pivotal in identifying the genes required for the development of the enteric nervous system. Mutations in the genes encoding members of two signaling pathways account for approximately 50% of the cases of Hirschsprung's disease – these are glial cell line-derived neurotrophic factor acting through the receptor tyrosine kinase, Ret, and endothelin-3 acting through endothelin receptor B (EDNRB). Other signaling pathways involving neurotrophin-3, BMPs, hedgehog proteins, netrins/DCC (deleted in colorectal cancer), Slit/Robo, the cell adhesion molecule L1, and a number of transcription factors, including Sox8 and 10, Phox2b, Mash1, and Sip1, are also involved in the development of the enteric nervous system.

Trunk Neural Crest

Trunk neural crest cells give rise to dorsal root (sensory or spinal) ganglia, sympathetic postganglionic neurons, Schwann cells, adrenal chromaffin cells, and melanocytes. After emigrating from the neural tube, trunk neural crest cells follow two main pathways: neural crest cells destined to give rise to dorsal root ganglia, sympathetic ganglia, Schwann cells, and adrenal chromaffin cells follow a ventromedial pathway between the somites and the neural tube, within

the rostal half of each somite and in the intersomitic spaces, whereas melanocyte precursors follow a dorsolateral pathway, immediately under the ectoderm (**Figure 1(c)**). Neural crest cells that follow a ventral pathway emigrate from the neural tube prior to the cells that follow a dorsolateral pathway. Migration pathways are defined as a complex spatial array of guidance molecules, both attractive and repulsive. The substrates for migration include both extracellular matrix and cell surfaces.

Ventral Pathway

Early migrating neural crest cells follow a ventral pathway and appear to be prevented from entering a dorsolateral pathway by repulsive cues (**Figure 10(a)**). Ephrins and Slits are expressed in the dorsal regions of the developing somites (the dermamyotome), and their receptors – EphBs and Robos, respectively – are expressed by early migrating trunk neural crest cells; these guidance cues appear to repel early emigrating neural crest cells from the dorsolateral pathway. As they move ventrally, the crest cells diverge laterally due to repulsive signals from the medially placed notochord. These signals are mediated in part by the lectican (large chondroitin sulfate proteoglycan) aggrecan.

Neural crest cells migrate through the rostral halves of the somites and between somites but avoid the caudal half of each somite (**Figure 10a**). The development of segmental dorsal root ganglia is linked to the segmentation of the mesoderm. In particular, the selective migration of neural crest cells through the rostral half of each somite is essential for establishing the segmental organization of dorsal root ganglia; if the caudal halves of somites are replaced with rostral halves prior to the emigration of neural crest cells from the neural tube, a single continuous 'polyganglion' develops instead of segmental ganglia (**Figure 11**). A number of molecules are differentially expressed by the rostral and caudal halves of somites. Some molecules that were initially identified as repulsive axon guidance cues, including semaphorin 3A (Sema3A) and ephrin-B1, are expressed by the caudal, but not rostral, half of each somite, and the receptors for Sema3A and ephrin-B1 are expressed by migrating neural crest cells. *In vivo* and *in vitro* experiments have shown that Sema3A and ephrin-B1 are repulsive to migratory trunk neural crest cells, and thus trunk neural crest cells are channeled into the rostral half of each somite by the presence of repulsive cues in the caudal regions of the somites (**Figure 10**). Some ECM molecules such as F-spondin, tenascin-C, and lecticans that are selectively expressed in the caudal half of each somite are inhibitory to migrating crest cells and most likely

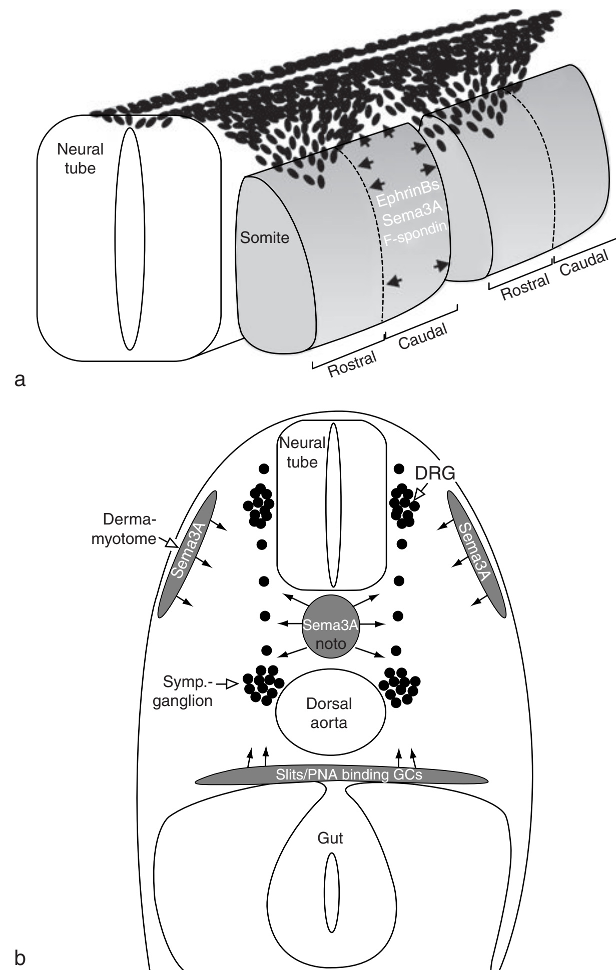

Figure 10 Pathway of trunk neural crest cells that migrate along a ventral pathway and the molecules that influence their pathways. Neural crest cells in the dorsolateral pathway are not shown. (a) Neural crest cells delaminate all along the neural tube and are then channeled into streams of cells through the rostral half of each somite by repulsive cues expressed exclusively in the caudal half of each somite; the repulsive cues include Ephrin-Bs, semaphorin 3A (Sema3A), and F-spondin. (b) The migration of cells to form sympathetic ganglia is influenced by repulsive cues in the dermamyotome and notochord and in tissue dorsal to the gut which prevents trunk neural crest cells from entering the gut. DRG, dorsal root ganglia. Reproduced from Young HM, Anderson RB, and Anderson CR (2004) Guidance cues involved in the development of the peripheral autonomic nervous system. *Autonomic Neuroscience* 112: 1–14, with permission from Elsevier.

contribute to the exclusion of neural crest cells from the caudal half of each somite.

Some trunk neural crest cells coalesce to form dorsal root ganglia adjacent to the neural tube, whereas others migrate further ventrally and coalesce into sympathetic ganglia adjacent to the dorsal aorta (**Figure 10(b)**). In fact, the first trunk neural crest cells that emigrate from the neural tube migrate directly to the dorsal aorta and form sympathetic ganglia, whereas dorsal root ganglia arises from crest cells

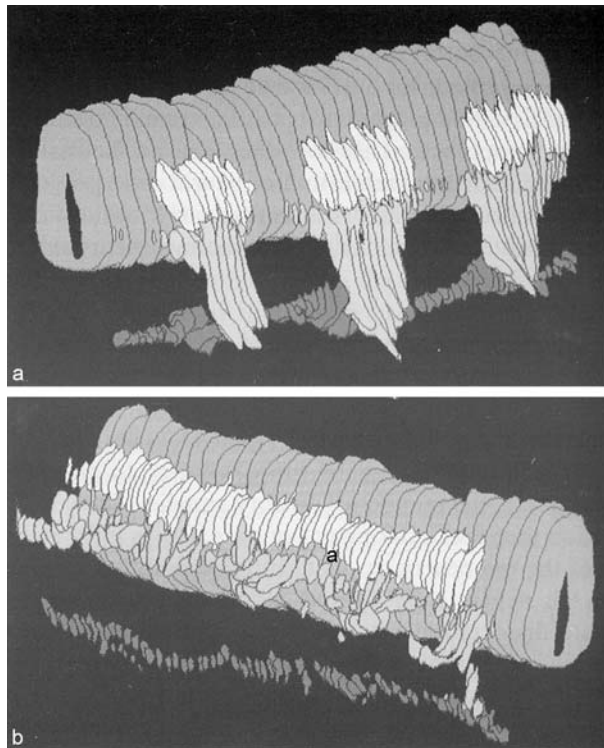

Figure 11 Three-dimensional reconstructions of the neural tube and dorsal root ganglia (DRG) from an embryo in which the caudal halves of each somite were replaced with rostral halves on one side of the embryo only. (a) On the control side of the embryo, DRG are segmental and the location of each ganglion corresponds to the rostral half of each somite. (b) On the experimental side, which contains only rostral halves of somites, there is a 'polyganglion' instead of individual segmental ganglia. Reproduced from Le Douarin NM and Kalcheim C (1999) *The Neural Crest*, 2nd edn., Cambridge, UK: Cambridge University Press.

that migrate slightly later. The general rule seems to be that for any locus of the neural crest, the first cells to migrate tend to disperse the farthest. Neuregulin, a growth factor whose effects are mediated by members of the ErbB family of receptor tyrosine kinases, is required for the migration of cells ventral to the neural tube to form sympathetic ganglia but not for the formation of dorsal root ganglia. Guidance cues expressed by the dermamyotome, notochord, and tissue dorsal to the gut are also thought to influence the migration of sympathetic ganglion precursors.

Dorsal root ganglion neurons and sympathetic postganglionic neurons have different phenotypes and use different neurotransmitters. Sympathetic postganglionic neurons are induced to express the appropriate phenotype by bone morphogenetic proteins produced by the dorsal aorta.

Dorsolateral Pathway

The last neural crest cells that emigrate from the neural tube follow a dorsolateral pathway, just beneath the ectoderm, and give rise to melanocytes (pigment cells) (**Figure 1(c)**). Emigration of neural crest cells along a dorsolateral pathway commences as migration along a ventromedial pathway is ceasing – late migrating neural crest cells seem to express different molecules from the early migrating cells that enable them to overcome the repulsive cues in the dermamyotomes. Melanocytes probably arise from all axial levels of the neural crest, but their development has been most extensively studied from trunk neural crest cells.

Two signaling pathways are known to be essential for the development of melanocytes. Endothelin-3 acting at EDNRB receptors on neural crest cells promotes the proliferation and migration, and delays the differentiation, of melanocyte precursors. Stem cell factor is produced by the dermamyotome and acts at Kit tyrosine kinase receptors on neural crest cells. This signaling pathway is required for the survival of melanocyte precursors until the late stages of differentiation in the skin, and it may direct their migration dorsolaterally. The transcription factor Mitf (microphthalmia-associated transcription factor) is also required for melanocyte development. Mitf has been shown to regulate genes required for pigmentation. However, since melanocyte precursors undergo apoptosis or adopt an alternative cell fate in the absence of Mitf protein, Mitf must also regulate genes required for the survival and proliferation of melanocyte precursors and the maintenance of melanocyte identity.

Sacral Neural Crest Cells

The location of the sacral region of the neural axis varies between species. Sacral neural crest cells are those that emigrate caudal to somite 28 in chick embryos and caudal to somite 24 in embryonic mice. Sacral neural crest cells give rise to neurons and glial cells in pelvic parasympathetic ganglia and to some enteric neurons, mostly in the hindgut (**Figures 2** and **12**). They are also thought to give rise to melanocytes and Schwann cells.

Plasticity of Trunk Neural Crest Cells

Like cranial neural crest cells, a large number of studies have examined whether trunk neural crest cells are predetermined or biased to a particular fate prior to emigration from the neural tube. Studies by Le Douarin and colleagues, in which premigratory neural crest cells from one axial level were transplanted to different axial levels, showed that at least some neural crest cells show considerable plasticity, and their fate was largely dependent on the environment. Experiments by Marianne Bronner-Fraser, Scott Fraser, and colleagues, in which individual premigratory neural crest cells were labeled, showed

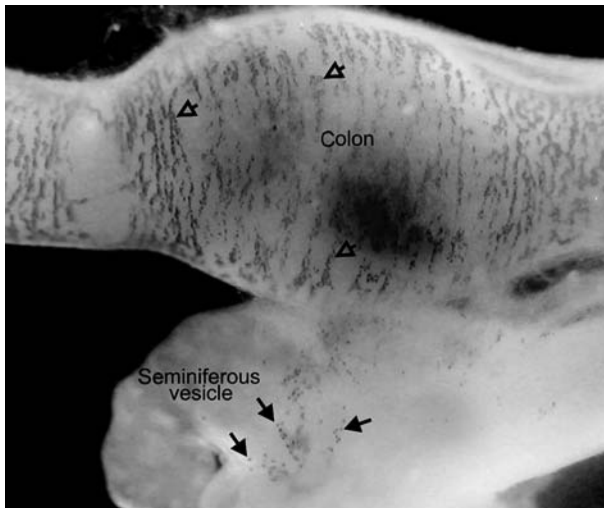

Figure 12 Colon and nearby reproductive organs from a 4-day-old transgenic mouse in which many vagal, trunk, and sacral neural crest cell derivatives express the reporter gene, *lacZ*, and so appear blue after histochemical processing. A dense network of stained neurons is present in the gut (open arrows) – most of these will have arisen from vagal neural crest cells, but some sacral crest cells also colonize the hindgut (**Figure 2(c)**). There are also stained cells (closed arrows) associated with the seminiferous vesicles – these are pelvic ganglion neuron precursors.

that individual premigratory neural crest cells could give rise to a variety of derivatives. For example, one labeled crest cell gave rise to melanocytes and Schwann cells as well as cells in dorsal root ganglia and the adrenal medulla. However, other labeled cells gave rise to only a single derivative. There is also evidence, particularly for melanocyte precursors, that some neural crest cells are prespecified, or at least biased to a particular fate, prior to emigrating from the neural tube. For example, melanocyte precursors express cell type-specific markers while still within the dorsal neural tube. Evidence suggests that some premigratory trunk neural crest cells are multipotent and their fate is probably determined by the environment through which they migrate or by tissue they colonize, whereas other premigratory trunk neural crest cells are biased or perhaps even prespecified prior to emigration. That the early migrating cohort includes multipotent cells and the late emigrating cohort comprises determined cells may indicate that restrictive lineage decisions are time regulated without regard to the morphogenetic decision of migration onset.

Conclusion

The neural crest is a transitory, migratory population of cells that gives rise to a remarkable diversity of cell types. It only occurs in vertebrates, and because of its role in craniofacial development, it is thought to have played a pivotal role in the evolution of vertebrates.

See also: Autonomic Neuroplasticity: Development; Autonomic Nervous System Development; Enteric Nervous System Development; Neural Crest Cell Diversification and Specification: ErbB Role; Neural Crest Cell Diversification and Specification: Melanocytes; Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation.

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Schwann Cell Development

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Introduction

Schwann cells, found in association with axons in peripheral nerves, are the main glial cell type in the peripheral nervous system (PNS). They exist in two quite distinct forms, either making myelin sheaths round large axons to speed impulse conduction or enclosing groups of smaller axons, holding them together in tight bundles. A mature peripheral nerve therefore consists of a mixture of myelinated and unmyelinated axon–Schwann cell units, usually referred to as nerve fibers. This article provides an overview of the development of Schwann cells from their origins in the neural crest. The generation of PNS glia parallels the formation of glial cells in the central nervous system (CNS). Nevertheless, many of the key molecules that control gliogenesis differ between the two systems.

The Schwann Cell Lineage

The neural crest is a transient group of cells that segregates from the dorsal neural tube and gives rise to a variety of derivatives, including autonomic and sensory neurons, chromaffin cells, glial cells including Schwann cells and satellite cells of the autonomic and sensory ganglia, fibroblastic cells, smooth muscle cells, and melanocytes (**Figure 1**). The majority of Schwann cells are derived from the group of neural crest cells that migrate ventrally through the anterior part of the somites, although it has been shown that Schwann cells in the dorsal roots are derived from a specialized group of cells called boundary cap cells that also give rise to some neurons and satellite glial cells in dorsal root sensory ganglia (DRG).

Mature myelinating and nonmyelinating Schwann cells arise from the neural crest via two intermediate stages, the Schwann cell precursor and the immature Schwann cell (**Figure 2**). Schwann cell precursors are the glial cells found in embryonic day (E) 14 or 15 rat nerves (mouse E12 or E13). Immature Schwann cells are generated from Schwann cell precursors and populate rat nerves from E17 or E18 (mouse E15 or E16) to approximately the time of birth, when myelination starts. At the immature Schwann cell stage, Schwann cells that by chance come to be associated with large axons receive axon-associated signals that instruct them to make myelin sheaths, and they adopt the specific gene expression profile appropriate for myelination. Mature nonmyelinating Schwann cells only appear approximately 2 weeks after myelination starts. These cells also express genes that differentiate them from myelinating cells and typically hold several small-diameter axons individually in troughs that run along the cell surface, forming unmyelinated (Remak) fibers.

Three major developmental steps define the lineage – that is, the transition from migrating neural crest cells to axon-associated Schwann cell precursors, the transition from precursors to immature Schwann cells, and finally the divergence of this population to form the two mature Schwann cell types found in adult nerves. At all stages, there is a continuous close association between these cells and axons. Also, it is a striking feature of all the cell types that they are highly dependent on survival factors, mitogens, and differentiation signals from axons. Another notable feature is plasticity, because much of the developmental sequence is readily reversible. For example, mature myelinating and nonmyelinating Schwann cells respond to nerve injury by reverting to a phenotype similar to that of immature Schwann cells, and Schwann cell precursors can be diverted, at least *in vitro*, to other neural crest derivatives. Only the transition from precursors to immature Schwann cells appears to be irreversible.

In vivo, rapid proliferation is a characteristic of all early stages of the lineage – namely, neural crest cells, Schwann cell precursors, and immature Schwann cells. In contrast, the onset of myelination is clearly linked with cell cycle exit, and both myelinating and nonmyelinating cells are quiescent in normal adult nerves. However, the cells retain the potential to proliferate because they reenter the cell cycle when they dedifferentiate in response to nerve injury. Apoptotic cell death is also a feature of developing cells in early nerves, whereas myelinating and mature nonmyelinating cells are resistant to apoptosis.

Markers of Schwann Cell Development

Each cell stage in the embryonic phase of the lineage can be defined by a distinct combination of differentiation markers (**Figure 3**). Additional criteria, such as morphology, relationships to other cells and tissues, and response to extrinsic signals, can also be used to define the cell types and to study the signals that are important in controlling progression from one stage to the next.

The markers can be divided into five different categories depending on expression patterns (**Figure 3**).

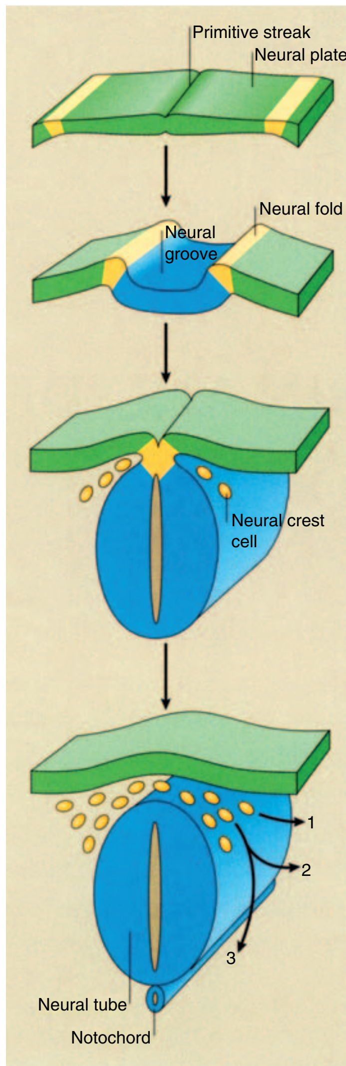

Figure 1 The neural crest. In a process known as neurulation, the neural plate, which is found along the dorsal surface of an embryo, gradually folds in on itself to form the neural groove. As the neural folds fuse to form the neural tube, the neural crest cells segregate from the tips of the folds. After taking up an initial position at the dorsal surface of the tube, the crest cells in the trunk region soon migrate along one of two major streams: in a lateral direction (1) to give rise to melanocytes in the skin, and in a ventral direction (2 and 3) to give rise to neurons in dorsal root sensory ganglia and glia (2) or glia, autonomic neurons, and chromaffin cells (3). Neural crest cells in the most anterior part of the trunk, the cardiac crest, also generate fibroblasts and smooth muscle cells, and the cephalic crest in the head region also forms the cells of cartilage and bone. The mechanisms that allow the apparently homogeneous population of crest cells to generate such diversity have been intensively studied. It is now considered likely that some neural crest cells are already committed to certain fates, whereas others are multipotent. Although some cells may enter lineages in a stochastic and undirected manner, a combination of positive and negative instructive signals probably plays an important part in directing neural crest cell differentiation. How migrating neural crest cells, which initially move through immature connective tissue on each side of the neural tube, end up as Schwann cell precursors in tight association with axons in early embryonic nerves is not clear, either in terms of their detailed migratory route or in terms of the signals that cause these cells to adopt an early glial phenotype. Reproduced from Jessen KR and Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* 6: 671–682.

The first category, exemplified by the transcription factor SRY (sex determining region Y) box 10 (SOX10), includes molecules that are present at all developmental stages; the second, exemplified by the transcription factor activator protein 2α (AP2α), includes genes/proteins present at high levels in neural crest cells and Schwann cell precursors but which are downregulated in immature Schwann cells; the third category, of which cadherin 19 is the only known example, represents genes expressed only in Schwann cell precursors; and the fourth category represents genes/proteins expressed by Schwann cell precursors and immature Schwann cells but not by neural crest cells, such as brain fatty acid-binding protein, myelin protein zero (P_0), and connexin 29. The last category represents molecules such as S100 or glial fibrillary acidic protein (GFAP) that are present in high levels on immature Schwann cells but are low or absent from neural crest cells or Schwann cell precursors. It should be emphasized that the use of technologies such as Affymatrix that allow comparative analysis across the genome reveals larger sets of different genes, but which of these will be useful in the study of mechanisms that control development of the lineage remains to be determined.

Several additional criteria provide crucial information in analyzing Schwann cell development. Schwann cell precursors and immature Schwann cells, but not neural crest cells, share a feature characteristic of glial cells in both the PNS and the CNS, namely their close physical apposition with axons (neurons). Schwann cell precursors also differ from migrating crest cells in their response to survival factors and in being relatively insensitive to the neurogenic actions of bone morphogenetic protein (BMP) 2. They are also more sensitive to the actions of Notch and are strongly biased toward the generation of Schwann cells rather than other neural crest derivatives.

A striking difference between Schwann cells and Schwann cell precursors is the ability of Schwann cells to support their own survival in the absence of axons using autocrine survival circuits. The cytoarchitecture of the nerves at the stage when they contain Schwann cell precursors (E14 or E15) is also distinctly different from that of nerves containing immature Schwann cells (E17 or E18).

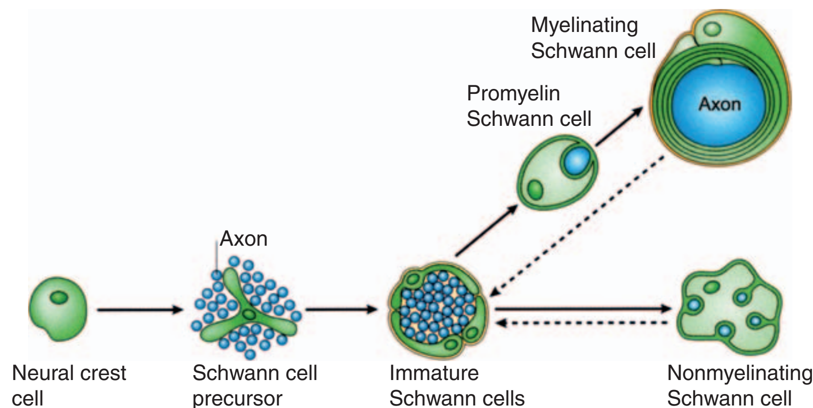

Figure 2 The Schwann cell lineage. Schematic illustration of the main cell types and developmental transitions involved in Schwann cell development. Dashed arrows indicate the reversibility of the final, largely postnatal transition during which mature myelinating and nonmyelinating Schwann cells are generated. The embryonic phase of Schwann cell development involves three transient cell populations. First are migrating neural crest cells, which are discussed further in the legend to **Figure 1**. Second are Schwann cell precursors. These cells express various differentiation markers that are not found in neural crest cells, including brain fatty acid-binding protein (BFABP), protein zero (P_0), and desert hedgehog (DHH) (**Figure 3**). At any one time, a rapidly developing population of cells, such as the glia of embryonic nerves, will contain some cells that are rather more advanced than others. Third are immature Schwann cells. All immature Schwann cells are considered to have the same developmental potential, and their fate is determined by axons with which they associate. Myelination occurs only in Schwann cells that by chance envelop large-diameter axons; Schwann cells that ensheathe small-diameter axons progress to become mature nonmyelinating cells. Reproduced from Jessen KR and Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* 6: 671–682.

Gliogenesis from the Neural Crest

The generation of more differentiated cells from stem cells or cells that share some stem cell properties, including the neural crest, is a topic that continues to generate widespread interest. The consensus is that cell specification involves interplay between cell-autonomous intracellular signaling and extracellular cues arising from the niche in which the stem cell resides. The best evidence is that generation of Schwann cells and other glia from the neural crest involves both of these mechanisms.

The Role of SOX10 in PNS Gliogenesis

In the generation of glial cells from the neural crest, the transcription factor SOX10 is the most important player known so far, because it is the only gene known to be essential for this process. Initially expressed by all migrating neural crest cells, its expression persists in the developing satellite glial cells of the DRG, in Schwann cell precursors, and in Schwann cells of peripheral nerves. However, SOX10 is downregulated very early in neurogenesis. In line with this, neurons are initially generated in normal numbers in mice in which SOX10 has been inactivated. In contrast, satellite cells and Schwann cells fail to develop in these animals. In place of satellite cells (i.e., glia), the DRG contain a population of neural crest-like cells and nerve trunks also contain a few neural crest-like cells but no Schwann cell precursors, indicating that in the absence of SOX10 glial specification is blocked. Experiments using cultured cells also suggest that SOX10 plays a role in specifying and maintaining the glial phenotype. It may act in part by upregulating levels of the neuregulin receptor ErbB3, thus increasing the responsiveness of neural crest cells to the growth factor β-neuregulin-1. There is evidence that later in development, it acts in conjunction with other transcription factors such as KROX20 (EGR2) to regulate the promoters of genes that are crucial in myelination, such as P_0 and connexin 32.

β-Neuregulin-1 and the Neural Crest

β-Neuregulin-1, signaling through its receptors ErbB2 and ErbB3, is a growth factor that has multiple important functions throughout Schwann cell development. In neural crest cell cultures, it inhibits the development of neurons, a function that might lead indirectly to increased development of glia. Overproduction of neurons, however, has not been noted in mutants that lack components of the neuregulin signaling pathway, so it is not clear that β-neureglin-1 regulates neuronal numbers *in vivo*. When β-neuregulin-1 is added to migrating neural crest cell cultures, it increases the proportion of Schwann cells generated. Nevertheless, these cultures generate Schwann cells even in the absence of added neuregulin, and the same is true when the appearance of Schwann cell

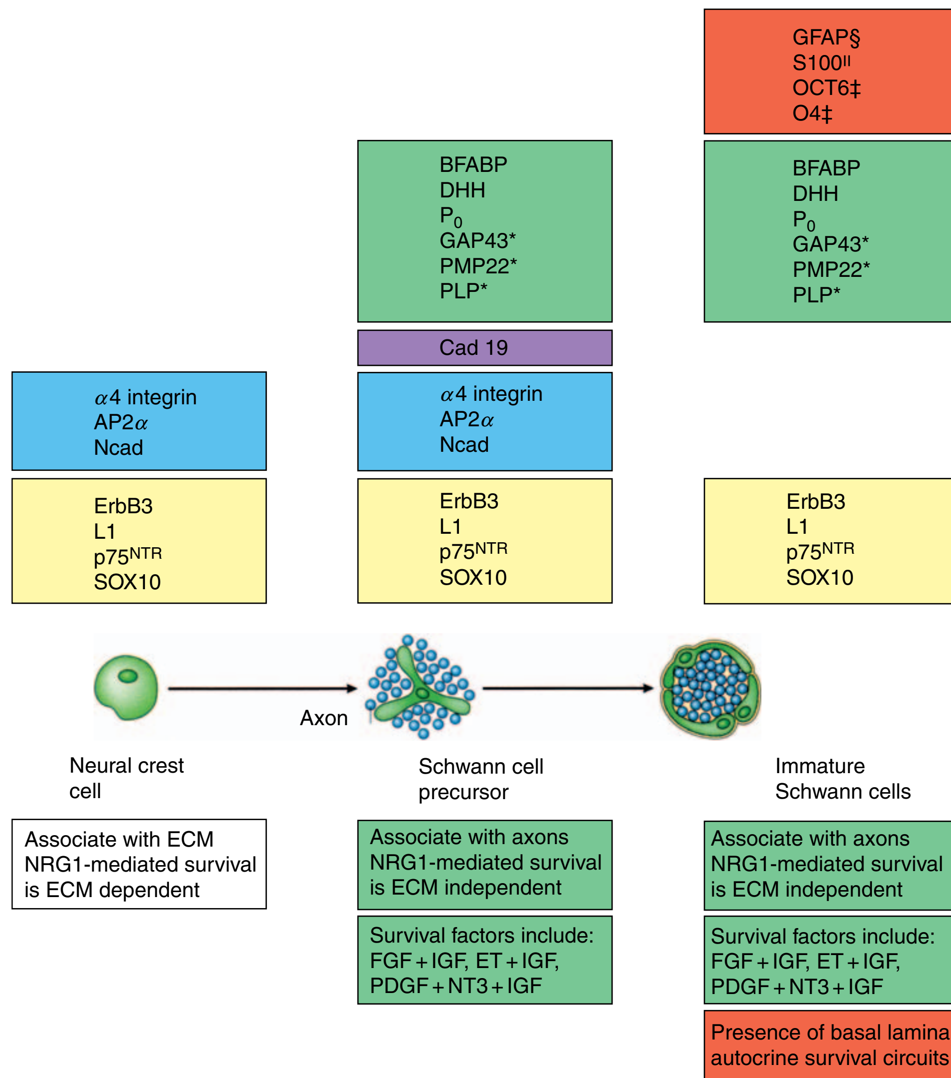

Figure 3 Changes in phenotypic profile as cells progress through the embryonic Schwann cell lineage. Shared profiles are indicated by distinct colors. The boxes above indicate the changes in gene expression that take place during embryonic Schwann cell development. The gene expression shown here is based on observations of endogenous genes rather than on observations of reporter genes in transgenic animals. Note that Cadherin 19 (Cad 19) is exclusively expressed in Schwann cell precursors. Each developmental stage also involves characteristic relationships with surrounding tissues and distinctive cell signaling properties (boxes below lineage drawing). For instance, neural crest cells migrate through extracellular matrix. By contrast, Schwann cell precursors and Schwann cells are embedded among axons with minimal extracellular spaces separating them from nerve cell membranes, a characteristic of glial cells in the CNS and PNS. Basal lamina is absent from migrating crest cells and Schwann cell precursors but appears on Schwann cells. *In vitro*, β-neuregulin-1 (NRG1) only supports neural crest survival in the presence of extracellular matrix (ECM), although this is not required for the β-neuregulin-1-mediated survival of Schwann cell precursors and Schwann cells. Migrating neural crest cells also fail to survive in the presence of several factors that support the survival of Schwann cell precursors and Schwann cells, including combinations such as fibroblast growth factor (FGF) plus insulin-like growth factor (IGF), endothelin (ET) plus IGF, and platelet-derived growth factor (PDGF) plus neurotrophin (NT)-3 and IGF. Schwann cells also have autocrine survival circuits that are absent from Schwann cell precursors. [*]Proteins that also appear on neuroblasts/early neurons. [‡]Markers that are acutely dependent on axons for expression. [§]Glial fibrillary acidic protein (GFAP) is a late marker of Schwann cell generation since significant expression is not seen until approximately the time of birth. GFAP is reversibly suppressed in myelinating cells. The early expression of GFAP has not been examined carefully in mice. [‖]Schwann cell precursors have been shown to be S100 calcium-binding protein (S100)-negative and Schwann cells S100-positive using routine immunohistochemical methods; however, low levels of S100 are detectable in many mouse Schwann cell precursors when the sensitivity of the assay is significantly increased. AP2α, activator protein 2α; BFABP, brain fatty acid-binding protein; DHH, desert hedgehog; ErbB3, neuregulin receptor; GAP43, growth-associated protein 43; L1, L1 adhesion molecule; N-cad, N-cadherin; OCT6, octamer-binding transcription factor 6; 04, lipid antigen; PLP, proteolipid protein; PMP22, peripheral myelin protein 22 kDa; P_0, protein zero; $p75^{NTR}$, p75 neurotrophin receptor; SOX10, SRY (sex determining region Y) box 10. Reproduced from Jessen KR and Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* 6: 671–682.

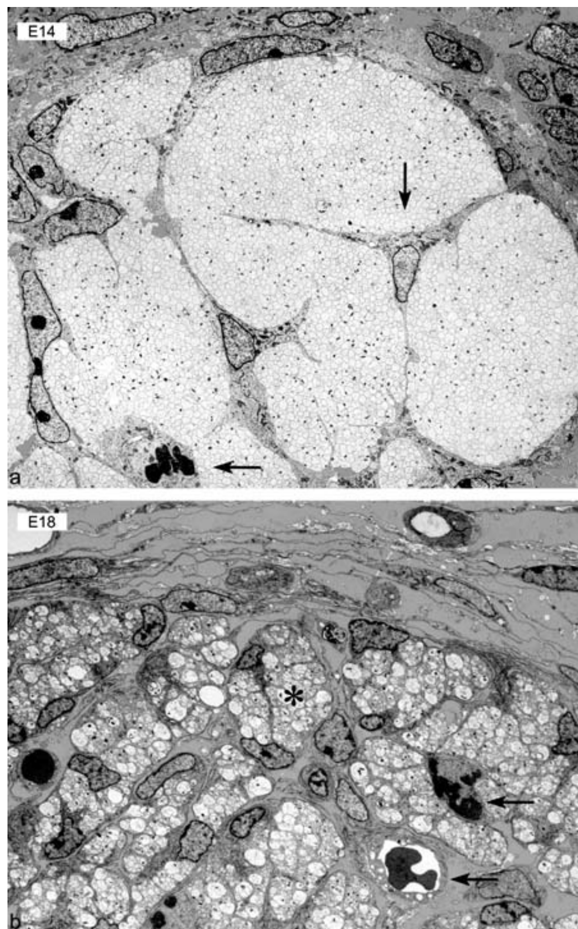

Figure 4 The appearance of early cells in the Schwann cell lineage. (a) An electron microscopic image of a transverse section of a nerve in the hindlimb of a rat embryo at embryonic day (E) 14. Schwann cell precursors branch among the axons inside the nerve (large arrow) and are also found in close apposition to axons at the nerve surface. One precursor cell is undergoing mitosis (small arrow). Extracellular connective tissue space (turquoise), which contains mesenchymal cells, surrounds the nerve but is essentially absent from the nerve. These nerves are also free of blood vessels, and the axons are of smaller and more uniform diameter than those seen in mature nerves. Magnification, ×2000. (b) Schwann cells in a transverse section of the sciatic nerve of a rat embryo at E18, shown at the same magnification. In marked contrast to the nerve at E14, connective tissue spaces now branch through the nerve among compact bundles of immature Schwann cells and their associated axons (Schwann cell families; for example, see asterisk). Blood vessels (small arrow) and fibroblasts (e.g., directly above the vessel) have also appeared inside the nerve. One Schwann cell is undergoing mitosis (large arrow). Outside the nerve (in the uppermost part of the figure), connective tissue, which contains flattened fibroblasts of the early developing perineurium and two blood vessels, can be seen. Reproduced from Jessen KR and Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* 6: 671–682.

precursors from neural crest cell cultures is monitored. Furthermore, in mouse mutants in which neuregulin signaling has been abrogated *in vivo*, satellite glia in the DRG are generated, although Schwann cell precursors are severely depleted. In these mutants, most neural crest cells fail to migrate ventrally below the level of the DRG to the sites where sympathetic ganglia are formed, resulting in underdeveloped (hypoplastic) ganglia. In summary, these data indicate that, in principle, β-neuregulin-1 is not required for the generation of glia from the neural crest, although it modulates this process by suppressing neurogenesis and by promoting migration of neural crest cells or early crest derivatives, particularly those that will form sympathetic ganglia. There is also evidence that β-neuregulin-1 accelerates the transition of Schwann cell precursors to Schwann cells.

Schwann Cell Precursors

In peripheral nerve trunks, Schwann cell precursors represent the first clearly defined stage of glial differentiation and are the cells from which the immature Schwann cells are derived. They represent the large majority of cells in the limb nerves of E14/E15 rats (E12/E13 mice). They are initially seen at the edge of early embryonic nerves but are later seen inside them as well. They communally surround large groups of axons with their sheetlike processes and divide the nerves into territories. At this stage, the nerves are compact structures that contain essentially no connective tissue and are not vascularized (**Figure 4**).

β-Neuregulin-1 and Schwann Cell Precursors

A vital role for β-neuregulin-1 in the Schwann cell lineage is indicated by the observation, mentioned previously, that Schwann cell precursors, and later Schwann cells, are absent or severely depleted in the peripheral nerves of mouse mutants that lack neuregulin signaling. This probably reflects the major role of β-neuregulin-1 as an essential survival factor and mitogen for Schwann cell precursors, although impaired migration might also have a role. Axonally derived β-neuregulin-1 is an essential survival factor and mitogen for Schwann cell precursors *in vitro* and it has been shown that the most important isoform expressed by axons is the membrane-bound β-neuregulin-1 type III (**Figure 5**). It is the major isoform present in DRG and motor neurons *in vivo*. In mice in which this isoform has been selectively eliminated, although peripheral nerves are initially populated by Schwann cell precursors, few remain after E14. *In vivo*, Schwann cell precursors die after nerve injury and this can be prevented by externally applied β-neuregulin-1. Taken together, this indicates that axonal β-neuregulin-1 type III is a key survival signal in embryonic Schwann cell development, being an essential survival factor for Schwann cell precursors.

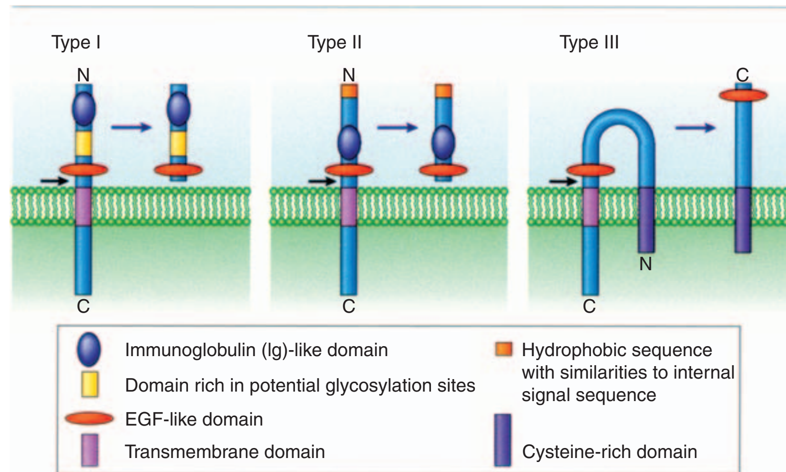

Figure 5 Neuregulin-1. β-Neuregulin-1 seems to have exceptionally numerous and varied functions in Schwann cell biology. It is involved in neural crest migration and has been implicated in the specification of neural crest stem cells and shown to be essential for the survival of Schwann cell precursors. It is also involved in Schwann cell generation, proliferation, and survival. In postnatal nerves, β-neuregulin-1 is a positive regulator of myelin sheath thickness, but paradoxically, at least in experiments, it appears to drive the dedifferentiation of Schwann cells in injured nerve fibers. No other molecule has been proposed to be so comprehensively involved in the control of Schwann cell development. There are a surprising number (>15) of neuregulin-1 α and β protein isoforms. The schematic structures of the main isoforms found in the nervous system are shown here. Although splice variants without the transmembrane domain exist for all these isoforms, transmembrane isoforms (as shown here together with the products of a proteolytic cleavage in the juxtamembrane area) predominate in the nervous system. The epidermal growth factor (EGF) domain is found in all bioactive forms of neuregulin-1 and is sufficient for activation of ErbB receptor kinase. The type III β-neuregulin-1 isoform is expressed in axons and is the main regulator of survival of Schwann cell precursors and myelin sheath thickness. It is thought to have two membrane-spanning domains and to undergo proteolytic cleavage that generates a membrane-attached protein carrying the EGF domain. β-Neuregulin-1 shows high-affinity binding to two receptors, ErbB3 and ErbB4, whereas a related protein, ErbB2, acts as a coreceptor in ErbB3–ErbB2 and ErbB4–ErbB2 complexes. The former is the main receptor in peripheral glial cells. The action of axonal β-neuregulin-1 type III on ErbB3–ErbB2 in developing Schwann cells is probably the best established molecular signaling pathway between neurons and glia in the PNS. Reproduced from Jessen KR and Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* 6: 671–682.

In vitro experiments show that in addition to its survival function, β-neuregulin-1 accelerates the transition from Schwann cell precursors to immature Schwann cells. This transition is also controlled by another signaling system present in embryonic nerves – endothelin. In contrast to neuregulin, endothelins negatively regulate Schwann cell generation, as shown by the observation that Schwann cells are generated prematurely in rats in which the endothelin B receptor is inactivated.

Notch and Schwann Cell Precursors

Notch transmembrane receptors and their ligands delta, jagged, and serrate are known to influence glial cell fate choices in the developing nervous system. In the CNS, the classical view of Notch signaling is that it acts to maintain neural stem cells in an undifferentiated state, but evidence suggests that it can promote the appearance of radial glia, astrocytes, and Muller cells. It also stimulates the formation of oligodendrocyte precursors while inhibiting their progression to myelinating cells.

The question of whether Notch instructively promotes gliogenesis from neural crest cells is still controversial. Notch activation in neural crest cells inhibits the generation of neurons *in vivo* and *in vitro*, whereas in cultures derived from a subpopulation of cells from E14 sciatic nerves it increases the number of GFAP-positive Schwann cells and the rate at which they are generated. There is also evidence that Notch, like β-neuregulin-1, acts on early glial cells, stimulating Schwann cell precursors to generate Schwann cells. Notch also promotes the proliferation of immature Schwann cells *in vitro* and *in vivo*. Although it is not settled whether the only actions of Notch are to accelerate lineage progression and stimulate proliferation, or whether Notch also directs crest cells to the glial lineage, there are strong parallels between the actions of Notch and β-neuregulin-1 in this system. Both signals suppress neurogenesis in

crest cells and stimulate Schwann cell generation from precursors and Schwann cell proliferation. It will be interesting to explore the significance of this in future studies.

Immature Schwann Cells

The switch from Schwann cell precursor to immature Schwann cell between E15 and E17 in rat (mouse E13–E15) involves a coordinated change in molecular expression and in the response of the cells to survival signals and mitogens. This is accompanied by architectural reorganization that involves the appearance of blood vessels and connective tissue in the nerve, eventually leading to the formation of basal laminae on the surface of individual Schwann cells. A distinct layer of perineurium also starts to appear at the nerve surface (**Figure 4**).

The mechanisms of cell survival also change with this transition. Schwann cell precursors are acutely dependent on β-neuregulin-1 for survival, whereas immature Schwann cells acquire autocrine survival mechanisms. This enables Schwann cells to support their own survival in the absence of axons, a mechanism that is likely to be important for axonal regeneration after injury. The autocrine survival factors that have been identified include a cocktail of insulin growth factor (IGF)-2, platelet-derived growth factor-BB, and neurotrophin (NT)-3, potentiated by laminin, and lysophosphatidic acid and leukemia inhibitory factor. This switch in survival strategy makes biological sense. Precursor survival is completely dependent on neuronally derived β-neuregulin-1, a property that may serve to match axon and Schwann cell precursor numbers and to keep precursors confined to developing nerves as they grow through body tissues to reach their targets. In contrast, the fact that Schwann cells can survive in the absence of axons ensures that if axonal injury occurs the surviving Schwann cells can provide essential factors to support axonal regrowth.

Little is known about the transcription factors that control the precursor to Schwann cell transition. Levels of activator protein 2α (AP2α) are high in precursors and decrease sharply in immature Schwann cells, and enforced expression of AP2α *in vitro* retards the transition. Endothelin also regulates the transition in a negative fashion, as mentioned previously.

As the cells transit from Schwann cell precursors to Schwann cells, developmental options narrow. Dedifferentiation of immature Schwann cells leading to the emergence of Schwann cell precursors has not been observed definitively, and Schwann cells in culture are resistant to signals such as BMPs and fibroblast growth factor 2 that can induce the generation of other neural crest derivatives from Schwann cell precursors.

Boundary Cap Cells Give Rise to Schwann Cells in Spinal Roots

Schwann cells in spinal roots have a different origin from the majority of Schwann cells in peripheral nerves. They are derived from boundary cap cells, a specialized set of cells that originate from the neural crest. These cells are found during embryonic development clustered in groups where dorsal and ventral roots enter and exit the spinal cord. They can be identified by the expression of the transcription factor KROX20 (EGR2) long before this gene appears in myelinating Schwann cells. Lineage tracing studies of the fate of these cells *in vivo* have revealed that they give rise not only to the Schwann cells of the dorsal and ventral roots but also to a small subset of nociceptive neurons and some satellite cells within the DRG. Few boundary cap cell-derived glia were detected in spinal nerves. Therefore, these findings do not affect the classical view that Schwann cells of limb nerves originate from migrating neural crest cells.

Functions of Schwann Cell Precursors and Schwann Cells

Trophic Support of Neuronal Survival

A major function of glial cells is to provide trophic support for developing neurons. In the case of Schwann cell precursors and immature Schwann cells, persuasive support for this view comes from experiments in which these cells have been deleted from peripheral nerves. In mouse mutants that lack SOX10, β-neuregulin-1 isoform III, or the neuregulin receptors ErbB2 or ErbB3, Schwann cell precursors and Schwann cells are depleted or absent owing to the importance of these molecules in gliogenesis and glial survival. Importantly, in these mutants most of the DRG and motor neurons that project into limb nerves die by E14 and E18, respectively, although they are initially generated in normal numbers. This suggests that an important function of precursors and immature Schwann cells is to provide essential survival signals for developing neurons. Impaired axon-target contacts may also contribute to sensory and motor death in the β-neuregulin-1 isoform III mutants.

Together with the evidence that axons provide essential β-neuregulin-1-mediated survival support for Schwann cell precursors that was discussed previously, these studies indicate that there is a discrete early phase of nerve development in which neurons and glia depend on each other for survival.

Morphogenesis: Maintaining the Normal Structure of the Spinal Cord, Nerve Trunks, and Neuromasts

An important morphogenetic role for the early glia of ventral roots has been revealed by studies using several mouse mutants, all of which have in common the absence of glial cells from nerve roots. In all of these mice, the cell bodies of motor neurons are displaced into the ventral roots. Comparable observations have been made in the chick. This indicates that an important function of boundary cap cells, or the Schwann cell precursors that are derived from them, is to maintain the normal location of motor neurons within the spinal cord.

Another morphogenetic function for immature Schwann cells and their precursors is that of holding peripheral nerve trunks together in a unified structure since in mouse mutants that lack these cells, large nerves separate into a number of smaller bundles. Remarkably, however, even without accompanying glial cells, the nerves initially grow out into limbs more or less correctly and find their way to their target areas. A morphogenetic function for neural crest-derived glia is also seen in the lateral line nerve of zebra fish, where developing glial cells (immature Schwann cells or their precursors) control nerve fasciculation and the formation of secondary neuromasts – organs that are specialized to detect water movements.

The Generation of Immature Schwann Cells and Endoneurial Fibroblasts

Lineage tracing studies have revealed an unexpected role for Schwann cell precursors. They show that the relatively small population of fibroblasts found within the nerve at birth (5–10% of total cells) arises from cells in the nerve that express desert hedgehog and p75 neurotrophin receptor ($p75^{NTR}$) and are therefore presumably Schwann cell precursors. This accords with the observation that not only Schwann cells but also fibroblasts appear in the nerve at the precursor–Schwann cell transition. It is also consistent with earlier experiments that demonstrated that, in principle, early PNS glia from rodents and bird nerves have the potential to generate cells other than Schwann cells since they can be induced to generate melanocytes in cell culture. A finding that provides an additional example of the unexpected developmental potential of early PNS glia concerns the boundary cap cells mentioned previously. These cells are tightly associated with axons and express glial-associated genes such as P_0 and KROX20, and they can therefore be regarded as having the phenotype of early glial cells. Nevertheless, these cells give rise not only to glia but also to some DRG neurons during normal development. This is reminiscent of the observation that early CNS glia, namely radial glia, generate both astrocytic glial cells and neurons, and it is in accord with the emerging concept that early glia can act as multipotent progenitors in both the developing PNS and the CNS.

The Transition from Immature Schwann Cells to Myelination

The transition from Schwann cell precursors to immature Schwann cells is essentially complete by E18, and at that time nerves have also acquired the basic tissue architecture known from postnatal nerves since they now contain relatively large extracellular spaces, connective tissue, fibroblasts, and blood vessels. At this stage, the Schwann cells, which have started to assemble a basal lamina, still ensheathe large groups of axons communally (**Figure 4**). Myelination starts approximately 3 days later, at approximately birth. This requires radial sorting, a process of radical change in the configuration of Schwann cells and axons that allows the larger diameter axons that will become myelinated to acquire a 1:1 relationship with individual Schwann cells, a configuration that is a prerequisite for myelin formation. At the same time as radial sorting is taking place, Schwann cell numbers are adjusted to the number of axons by controlling proliferation and survival. During this period, a number of signaling systems probably act together to prevent premature myelination.

Radial Sorting

This process, which allows individual Schwann cells to become associated with single large-diameter axons, is rapidly being dissected at the molecular level, although many aspects remain unclear. Defects in radial sorting are seen in the absence of laminin isoforms and in the absence of β_1 integrin. Radial sorting also fails in the clawpaw mutant mouse, which has a mutation in Lgi4, a secreted molecule of unknown function that belongs to a family of molecules, another member of which, Lgi1, complexes with Kv potassium channels and causes certain forms of epilepsy. Lastly, lack of a disintegrin and metalloprotease protein (ADAM)22 also causes sorting defects and severe hypomyelination. In the absence of laminin, or the laminin receptor

dystroglycan, Schwann cell proliferation is also impaired and apoptosis increased. Interestingly, these functions are unaffected by the absence of β_1 integrin.

Cell movement is a crucial component of radial sorting, and it is possible that factors that control Schwann cell migration in culture could also affect radial sorting. They include the growth factors β-neuregulin-1, IGFs, and NT-3, all of which promote migration, and brain-derived neurotrophic factor, which inhibits it. Levels of activation of the small GTPase Rac also appear to regulate Schwann cell–axon interactions, and in other cell types low levels of rac activation promote directionally persistent cell migration, whereas high levels promote random migration. There is also evidence that the p38 mitogen-activated protein kinase pathway is required just prior to myelination, perhaps for the correct alignment of axons and Schwann cells.

Control of Schwann Cell Numbers

During the process of sorting and myelination that occurs at approximately birth, it becomes important to match the numbers of Schwann cells and axons. By then, the period of neuronal cell death is largely over. This process is therefore controlled by a balance between rates of Schwann cell proliferation and death.

Experiments using cultured cells suggest that axons are the major stimulators of Schwann cell proliferation, an idea supported by the observation that *in vivo* Schwann cell proliferation decreases as Schwann cells lose contact with axons in transected nerves of newborn animals. In co-cultures of Schwann cells and DRG neurons, β-neuregulin-1 acts as a major axonal mitogen, but this has not been confirmed *in vivo*. Transforming growth factor (TGF)-βs are also potential mitogens that are present in developing nerves. Studies show that mouse Schwann cells that lack TGF-β type II receptor proliferate more slowly *in vivo* than normal Schwann cells, demonstrating that TGF-β is normally involved in regulating Schwann cell proliferation during nerve development.

Schwann cell survival in developing nerves is likely controlled by a balance between factors that support survival and those that promote cell death. Axonally derived β-neuregulin-1 and autocrine circuits provide survival support, together with laminin acting through dystroglycan receptors. Two signals that promote cell death have been identified *in vivo*, including TGF-β. Deletion of TGF-β type II receptors suppresses developmental death in E18 to newborn nerves, and cell death after newborn nerve transection is also lower in these mutant nerves. Signals acting through $p75^{NTR}$, possibly nerve growth factor, are required for the cell death that occurs after neonatal nerve transection but not for normally occurring developmental death.

The Onset of Myelination

Schwann cell myelination is a remarkable example of cell–cell interaction in which the association of an immature Schwann cell with a large-diameter axon induces a radical change in morphological and molecular phenotype. This leads to the formation of the myelin sheath and reciprocal changes in axonal membrane proteins, ion channels, and cytoskeleton that enable saltatory conduction along large nerves. The role of the basal lamina in promoting myelination is well established, although the molecular mechanisms involved are just beginning to be revealed.

The Role of β-Neuregulin-1 in Myelination

It has been shown that β-neuregulin-1 type III is one of the crucial axonal signals involved in controlling Schwann cell myelination. Mutant mice heterozygous for this isoform have thinner myelin sheaths, whereas overexpression results in thicker myelin sheaths and induces myelination of axons that would not normally be myelinated. β-Neuregulin-1 activates signaling pathways that positively regulate myelination, including the phosphatidyl inositol-3 kinase and protein kinase A pathways.

Myelin-Related Transcription Factors

The most important transcription factor involved in myelination is KROX20, which acts together with its partners, the NGF1-A-binding proteins 1 and 2 (NAB1/2), to upregulate a large number of myelin genes and proteins. The absence of KROX20 or, alternatively, the combined absence of NAB1/2, both of which are upregulated by KROX20, results in Schwann cell arrest at the 1:1 promyelin stage and failure to myelinate, indicating the central importance of the KROX20/NAB1/2 complex for gene activation and myelination. In humans, KROX20 (EGR2) mutations are associated with hereditary sensory and motor neuropathies. The octamer-binding transcription factor 6 (OCT6) and, to a lesser extent, brain 2 class III POU-domain protein (BRN2) control the timing of myelination. In mice that lack OCT6, myelination is severely delayed, probably because OCT6 is required for upregulation of KROX20 at the appropriate time in development, whereas BRN2 can partially compensate for loss of OCT6. *In vitro*, the transcription factor NF-κB and the Sloan–Kettering Institute proto-oncogene (SKI) are expressed prior to myelination, and in Schwann cell–neuron co-cultures lack of NF-κB or SKI in Schwann cells results in failure to myelinate.

Negative Regulation of Myelination

Although the onset of myelination is characterized by activation of promyelin pathways, studies indicate that signaling pathways that inhibit myelination also play a role in the timing of myelination. One of these inhibitory signals is the c-Jun N-terminal kinase (JNK) pathway. This pathway is active in Schwann cells of E10 to newborn nerves, where it is required for β-neuregulin-1 and TGF-β-induced signaling, at least in cultured cells. The pathway is inactivated as individual cells start to myelinate using a mechanism that depends in part on KROX20. In mice in which KROX20 has been inactivated, the JNK pathway is still active, proliferation remains high, and the cells arrest at the promyelin stage of development. When expression of JNK pathway constituents is enforced in cultured Schwann cells, myelination in neuron–Schwann cell cultures is blocked and myelin gene expression that would normally result from promyelin signals such as KROX20 is also blocked. The transcription factor SOX2 also promotes proliferation, inhibits myelination when overexpressed in Schwann cells in neuron–Schwann cell co-cultures, and remains high in mutant mice expressing low levels of KROX20 (EGR2). Similarly, Notch signaling, which, as mentioned previously, promotes proliferation in immature Schwann cells, is downregulated as cells start to myelinate *in vivo*, and in neuron–Schwann cell co-cultures enforced expression of Notch inhibits myelination. It is unclear how these apparently diverse inhibitory pathways are interlinked, and the details of how they interact with promyelin signals are just starting to be revealed.

See also: Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation; Schwann Cells and Axon Relationship; Schwann Cells and Plasticity of the Neuromuscular Junction.

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Schwann Cells and Axon Relationship

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Introduction

Vertebrates owe their evolutionary success to myelin. This multilayered wrap of electrical insulation speeds the conduction of impulses through slender axons. Without myelin, axons would need to be much thicker to carry impulses at the same rate. Evolution of myelin allowed miniaturization of nerve fibers and thus compaction of the nervous system into a brain of incomparable complexity – accelerating information processing in the nervous system of animals with backbones by magnitudes beyond the cerebral capabilities of the most evolutionarily advanced invertebrates. On a cellular level, myelin is perhaps the most intricate of all interactions between cells. Myelination requires cell–cell recognition, intercellular signaling, adhesion, motility, coordinated regulation of cell proliferation, differentiation, and synthesis of enormous sheets of insulating membrane, compacted into thin spiral layers in a manner unlike any other and with fine structural intricacies at the nodes of Ranvier to a molecular, crystalline level of precision, discernable only with the electron microscope. Myelin is unlike any other cell–cell interaction.

The cells that form myelin in the nerves of our limbs and trunk (peripheral nerves) are Schwann cells. In the brain, an entirely different type of cell, oligodendrocyte, with a distinct embryological origin, forms myelin. Schwann cells were first described in 1838 by the same pioneering biologist who conceived the concept of the cell as the basic building block of all life, Theodore Schwann. However, the purpose and origin of these peculiar cells were a mystery to him. Schwann cells coat axons like flattened pearls on a string, inspiring Schwann to speculate that they might form the nerve axon during fetal development by aligning and coalescing into a tube. In retrospect, this quaint idea appears naive, but the intimate interdependence of both axon and Schwann cell is now deeply appreciated.

Communication between Axons and Schwann Cells

Signals from axons are essential in regulating most Schwann cell functions, including proliferation, adhesion, migration, differentiation, and myelination. After an axon is damaged, Schwann cells respond to the loss of axon contact by de-differentiating. They lose their myelin and begin to proliferate and secrete growth factors to help guide the regenerating axon back to its target. The axon, in turn, is dependent on Schwann cells for its own integrity, structural development, and survival. In addition to providing structural support and electrical insulation, Schwann cells provide growth factors to axons, and they organize the arrangement of macromolecular ion channels into proper spatial domains along the axon membrane to support saltatatory conduction. Saltatory conduction describes the way an electrical impulse skips from node to node down the full length of an axon, speeding the arrival of the impulse at the nerve terminal in comparison with the slower continuous progression of depolarization spreading down an unmyelinated axon. Each node, situated between adjacent Schwann cells along the axon, is a bare segment of axon with specialized membrane properties for generating action potentials.

Embryological Origin of Schwann Cells

Schwann cells derive embryologically from the neural crest, which comprises multipotent cells migrating away from the dorsal neural tube. Neural crest cells differentiate into Schwann cell precursors, which migrate and proliferate along tracts of axons that have already extended into the periphery. The Schwann cell precursors then embark on a process of cellular differentiation into an immature Schwann cell to eventually become either a myelinating or a nonmyelinating Schwann cell. Only large-diameter axons, which conduct impulses at the highest speed, become myelinated. The slender, slow conducting fibers become bundled together and engulfed by massive, globular nonmyelinating Schwann cells.

Axon Signals Sustaining Schwann Cell Precursors

Early Schwann cell precursors are critically dependent on axon-derived signals for maintenance, proliferation, and differentiation. Axon contact stimulates proliferation of Schwann cell precursors, and neuregulin-1 (NRG-1) is recognized as one of the most potent axon-derived mitogens. Neuregulins are a family of growth factors that act as mitogens by binding the ErbB family of receptor kinases. The primary receptor for NRG-1 in Schwann cells is a heterodimer composed of ErbB2 and ErbB3, and mice genetically engineered to lack these receptors lose all Schwann cells on their peripheral axons. ErbB signaling is also

required to maintain the directed migration of Schwann cell precursors along axons. Genetic deletion experiments show that premigratory Schwann cells near the dorsal root ganglia fail to migrate out along the axons in the absence of neuregulin signaling.

The progenitors must proliferate as they migrate out along axons to produce sufficient numbers of cells to string the entire length of the axon, but precisely the correct number of cells is essential because this determines the number of nodes of Ranvier along the fiber. This is one of the important elements determining the conduction velocity and safety factor that will ensure the nodes are spaced closely enough for the wave of electrical depolarization to sustain a sufficient distance along the axon to activate the next node in sequence. As a limiting survival factor for Schwann cell precursors, NRG-1 helps establish the appropriate total number of Schwann cells on each axon. In a similar manner, transforming growth factor-β (TGF-β) also acts as a mitogen on Schwann cells at this stage to control the total number of cells.

Secreted factors inhibiting development are equally important to coordinate the timing of different development steps. Endothelins, AP2α, and bone morphogenic protein (BMP)-2 and -4 are examples of factors inhibiting the formation of Schwann cell precursors. Schwann cell precursors can also give rise to other types of cells, including neurons and fibroblasts *in vitro*. The remarkable plasticity of Schwann cells, recapitulated after nerve injury, aids in recovery from axon injury.

Differentiation to Immature Schwann Cells

Formation of immature Schwann cells from Schwann cell precursors is promoted by insulin-derived growth factor-2 (IGF-2), neurotrophin-3 (NT-3), (platelet-derived growth factor-β (PDGF-β), fibroblast growth factor-2 (FGF-2), Notch, and leukemia inhibitory factor (LIF) (**Table 1**). These factors can be produced by the Schwann cells and act in a autocrine manner when the axon signals are lost following injury.

TBF-β and the p75 neurotrophic factor induce programmed death of immature Schwann cells, and extracellular adenosine 5-triphosphate (ATP) and adenosine, acting on two different types of purinergic receptors (P2 and P1, respectively), inhibit Schwann cell proliferation and differentiation beyond the immature stage. ATP is released by axons firing action potentials, and adenosine is the end product of ATP degradation. This activity-dependent axon–Schwann cell signaling provides a mechanism for regulating the development of Schwann cells according to the state of functional activity in developing neural circuits.

The Schwann cell precursors are vitally dependent on axon survival signals, but mature Schwann cells can self-generate many of the essential survival factors in the absence of axons. Thus, these cells survive after the axon dies from injury. Indeed, the axon depends on the chain of surviving Schwann cells and the growth factors they secrete to grow back to its proper target. As discussed later, however, prolonged separation of Schwann cells from axons leads to detrimental changes in Schwann cells.

Myelination

Myelination begins after Schwann cells exit the cell cycle. In the promyelinating stage, just before beginning to form myelin, Schwann cells begin to increase expression of several myelin proteins, including myelin-associated glycoprotein (MAG) and myelin protein zero. The latter is the most abundant protein in peripheral nerve, and it is responsible for maintaining the compact layers of myelin together. A different protein, proteolipid protein, performs this function in central nervous system myelin.

The process of myelination begins by a Schwann cell first engulfing several axon segments at once (**Figure 1(a)**). These are sorted out until the cell selects only one axon segment to wrap with myelin (**Figure 1 (b)**). Formation of extracellular matrix is an essential early step in the process, and in cell culture, myelination will not commence until ascorbic acid is added to

Table 1 Growth and transcription factors affecting Schwann cell differentiation

	Schwann cell precursor	*Immature Schwann cell*	*Myelinating Schwann cell*
Promotes differentiation to this stage	NRG-1, Notch, FGF-2	Adenosine, ATP, β_1 integrin, c-Jun, Claw paw, Ets transcription factors, IGF, laminin, NRG-1, NT-3, SOX-2, p38, p75, TGF-β	BDNF, BRN-2, cAMP, GDNF, IGF, Krox20/NAB1/2, Erg2, laminin, NF-κB, NRG-1, Oct6, progesterone, PI3K-Akt, SKI
Inhibits differentiation to this stage	Endothelin, BMP2/4, AP2α		ATP, c-Jun, Notch, NT-3, PAX-3, SOX-2, TGF-β

Data from Jessen KR and Mirsky R (2005) The origin and development of glial cells in peripheral nerves. *Nature Reviews Neuroscience* 6: 671–682.

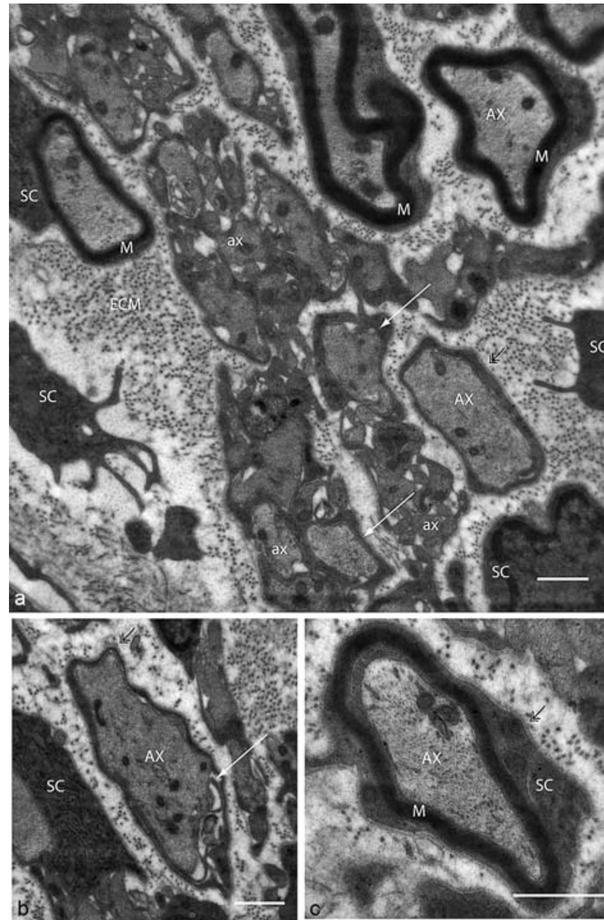

Figure 1 Schwann cell–axon relations during remyelination of regenerated axons in rat sciatic nerve. After axotomy, Schwann cells (SC) provide trophic factors and extracellular matrix components (ECM) to guide regenerating axons to form appropriate connections. (a) Early events in remyelination by SCs can be seen in sciatic nerve regenerating after being severed. Cellular processes extending from SCs (white arrows) sort out unmyelinated axons (ax) from larger diameter axons (AX). The small-diameter axons will become engulfed together in bundles inside nonmyelinating SCs and remain unmyelinated. A single SC will become associated with one segment of a large-diameter axon and begin synthesizing multiple layers of myelin membrane around a segment of the axon (M). This will provide rapid, saltatory impulse conduction by insulating the axon and organizing the fiber into internodal and nodal domains having distinct types of ion channels. Basal lamina, synthesized by SCs (double black arrows), is necessary for sorting out large-diameter axons and initiating myelination. (b) A large-diameter axon in the earliest stages of myelination is shown ensheathed by a single wrap of SC membrane. (c) Another myelinated axon shows a later stage in which compact myelin is formed by wrapping multiple layers of membrane around the axon and squeezing the cytoplasm out from between the stacks of insulating membrane. Scale bar = 500 nm.

the medium to promote formation of the basal lamina (**Figure 1**, black arrows). Laminin-2 is a major component of basement membrane, and mutations in α_2 laminin cause dysmyelination. β_1 integrin is one of the laminin receptors on Schwann cells, and animals lacking this receptor fail to establish the one-to-one relation with axons. Dystroglycan, another laminin receptor on Schwann cells, is not vital for early stages of myelination, but it participates in organizing the Schwann cell structure at the node.

The factors initiating myelination are not fully understood, nor is it clear why Schwann cells only myelinate large-diameter axons. Just before Schwann cells begin to myelinate, nuclear factor-kappa B (NF-κB) is upregulated, and its activation and translocation to the nucleus is necessary to regulate transcription of genes essential for myelination. A number of transcription factors are involved in initiating myelination, including Krox20/EGR2, NGFI-1-binding proteins, Oct6, and BRN2. As might be expected, cell adhesion molecules have an important role in axon–Schwann cell adhesion and myelination. The cell adhesion molecules L1 and NCAM are expressed on nonmyelinated axons, and they are downregulated during early myelination. MAG, a cell adhesion molecule that promotes association between axon and myelinating Schwann cells, is upregulated immediately after the initial layer of myelin is wrapped around the axon (**Figure 1(b)**). Synthesis of myelin proteins increases, and the mRNAs for myelin proteins, such as myelin basic protein (MBP),

become concentrated at distal sites in Schwann cells to provide local synthesis of myelin membrane.

Wrapping the layers of myelin around axons and squeezing the cytoplasm out from between the leaflets requires extensive cell extension and motility, which are regulated by actin–myosin cytoskeletal dynamics (**Figure 1(c)**). The Rho kinase (ROCK) regulates actin–myosin mechanical transduction by activating Rho, and in the absence of ROCK activity, the single myelinating process of a Schwann cell splits abnormally to form many smaller internodes, with resulting deleterious consequences for nerve conduction.

Myelin is promoted by many growth factors, including glial-derived neurotrophic factor (GDNF), NRG-1, myelin basic protein (IGFs), brain-derived neurotrophic factor (BDNF), as well as the sex hormone progesterone and the extracellular matrix molecule laminin. Multiple intracellular signaling pathways are involved, notably PI3K-Akt and cAMP. Myelination by Schwann cells is blocked by Notch activation, the neurotrophin NT-3, as well as by extracellular ATP. The latter is released by axons firing bursts of action potentials. TGF-β also inhibits myelination. Neurotrophins such as nerve growth factor (NGF) promote neuronal survival, but NGF stimulates myelination by Schwann cells through an indirect effect on axons. Indirect signals from the axons must be involved because experiments have shown that Schwann cell myelination of types of axons that are not depend on NGF for survival is unaffected by experimental manipulation of NGF.

The thickness of the myelin layer is precisely regulated in proportion to the axon diameter. Axons of larger caliber have thicker myelin, but the ratio of axon diameter to total diameter of axons including the myelin sheath falls within a range of 0.6 to 0.7 (g-ratio), regardless of the diameter of a myelinated axon. How the Schwann cell maintains this strict proportionate myelin thickness to varying axon caliber is unknown, but the thickness can be altered experimentally. BDNF, p75 neurotrophin receptor, or neuregulin overexpression results in abnormally thick myelin. Conversely, if ErbB receptors in Schwann cells are eliminated after myelination has begun, the sheath does not develop to its normal thickness. Myelin on axons regenerated after injury is typically thinner than normal, however.

Node of Ranvier

Formation of the node of Ranvier involves forming adhesive junctions between the axon and the extreme edges of the Schwann cell membrane abutting the node. At this junction, the multiple layers of myelin wrapped around the axon each seal against the axon membrane stacking in a staggered sequence at the edge of the node, thus forming the paranodal domain. In dividing the axon into nodal, paranodal, and internodal domains, the Schwann cell also affects the precise localization of ion channels and adhesion molecules along the axon, which are essential for salutatory conduction.

An important adhesion complex in forming and maintaining the paranode involves the axonal protein Caspr (contactin-associated protein, also known as paranodin), together with another protein contactin, binding to the glial partner neurofascicn 155 (Nfasc-155) at the paranodal junction. Cross-links between membrane proteins and the cytoskeleton maintain the appropriate ion channels in the proper axonal domains. Sodium channel localization at the node depends on association with Nfasc-186/NrCAM together with AnkyrinG/betaIV spectrin to anchor the channels to the actin cytoskeleton at the node. The node forms by nucleation of macromolecules in the nodal membrane, which then act as anchors for clustering additional sodium channels. Potassium channels become localized lateral to the paranode (the juxtaparanodal region), anchored by Caspr2, an axonal protein similar to Caspr, which localizes sodium channels at the node.

Myelinated Schwann cells are postmitotic, so as the axon grows, the Schwann cells must elongate at the same time, and the distance between nodes increases as the animal grows. Cytoplasmic channels beneath the outer layer of the myelin sheath, called Cajal bands, are an essential route for mRNA translocation to distal regions of the Schwann cell. When these bands are disrupted by disrupting a complex of molecules linking basement membrane to the Schwann cell plasma membrane (the L-periaxin-DRP2–dystroglycan complex), Schwann cells are impaired in their ability to elongate during nerve growth. The internodal length is reduced and conduction velocity slows.

Axon Dependence on Schwann Cells

Most of the axons die in limbs of mice genetically modified to lack Schwann cells, indicating definitively that axons and immature Schwann cells depend on each other for survival at crucial points in development. Long-term disabilities in patients with demyelinating diseases, such as Charcot–Marie–Tooth disease, can lead to axon degeneration. Myelinating Schwann cells have been shown to affect the extent of neurofilament phosphorylation in axons. Through this regulation, myelinating glia reduce the rate of slow axonal transport, in turn shrinking the caliber of the axon.

Regeneration

Axon damage results in Schwann cell responses that reflect the close interdependence of these two cells. After axotomy, the portion of the axon distal to the damage degenerates, but the Schwann cells in the distal stump begin to proliferate and synthesize growth factors that sustain the survival of the Schwann cells and the axons. Survival of neurons and elongation of sprouting axons from the proximal stump of severed axons is sustained by factors secreted by the Schwann cells and the extracellular matrix that they form. These factors include NGF, BDNF, and LIF. These beneficial properties of Schwann cells have been exploited to promote regeneration of axons in the central nervous system in Schwann cell transplantation experiments.

Schwann cells that are chronically isolated from their axons, however, begin to lose the capacity to support axon survival and outgrowth. This is one of the major reasons for permanent paralysis and failure to restore function in patients with damage to long nerves. Axon regeneration proceeds relatively slowly, at a rate of approximately 1 mm per day, and the Schwann cells in the extreme regions of limbs lose their ability to sustain axon regeneration before the regenerating axons can reach them.

Terminal Schwann Cells

At the distal end of motor neuron axons, a specialized form of Schwann cell ensheaths the neuromuscular junction. Functionally, these perisynaptic Schwann cells resemble astrocytes in the brain. Terminal Schwann cells help maintain the physical integrity of the synaptic junction, but they also have receptors for neurotransmitters that allow them to respond to neurotransmission. In turn, they release neuroactive substances that regulate the strength of synaptic transmission. These Schwann cells are also intimately associated with the axon and respond dynamically to axon injury. After axotomy, the terminal Schwann cells become highly branched and provide a route for axon sprouts from surviving junctions to reinnervate the vacated synapse.

Conclusion

Myelin appeared suddenly in the earliest vertebrates, fully formed and equally developed in both the central and the peripheral nervous system, even though the axon sheathing is made by completely different types of cells in the peripheral and central nervous system. This sudden evolutionary advance is puzzling because comparative anatomy reveals no intermediate forms. Considering the intricate choreography of cellular differentiation, recognition, mutual dependence on survival, motility, and membrane synthesis involved in myelination, this situation is especially surprising. What is clear is that the evolutionary advantage of myelin catapults nervous system function in vertebrates far beyond the limits of the invertebrate nervous system, which shares with vertebrates very similar neuronal structure and function. This success of vertebrate organisms derives from the highly regulated and complicated cellular interdependence of axon and glia.

See also: Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation; Schwann Cell Development; Schwann Cells and Plasticity of the Neuromuscular Junction.

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Neural Crest Cell Diversification and Specification: ErbB Role

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The peripheral nervous system (PNS) mainly derives from the neural crest and develops in a process of highly organized migration of pluripotent precursor cells that become progressively committed to fully differentiated cell types, including neurons and glia cells. The main glial cell type in the PNS are two types of Schwann cells (SCs) that serve distinct functions. Remak (or nonmyelinating) SCs engulf several small caliber C-fiber axons without enwrapping them. This ensheathment is nevertheless required for long-term axonal integrity. Myelinating SCs form compact myelin sheaths around single axons of large-caliber motor and sensory neurons, an essential prerequisite for rapid and precise impulse conduction. Myelination critically depends on reciprocal molecular interactions between neurons and glia cells. In the PNS, signaling of a neuronal growth factor, Neuregulin-1 (NRG1), to glial receptor tyrosine kinases of the ErbB family has emerged as a central regulator of SC development and myelination. This is in addition to other presumed roles of NRG1–ErbB signaling in the mammalian nervous system, such as neuronal migration, synaptogenesis, and synaptic plasticity.

Components of the NRG–ErbB Signaling System

Neuregulins (NRGs) are a family of membrane-associated growth factors with an extracellular epidermal growth factor (EGF)-like signaling domain. The term neuregulin derives from neu differentiation factor (NDF) and heregulin, the first cloned ligands for the oncogene ErbB2 (a.k.a., neu and HER2). Four genes (*Nrg1*–*Nrg4*) are present in mammals, but only *Nrg1* has been studied in detail. Due to alternative promoter usage and mRNA splicing, multiple (>16) NRG1 isoforms are produced that are subgrouped by their distinct N-terminal domains (**Figure 1**(**a**)). NRG1 type I (also known as heregulin, NDF, or acetylcholine receptor-inducing activity) and NRG1 type II (also known as glial growth factor (GGF)) have N-terminal immunoglobulin-like domains. Transmembrane forms of NRG1 undergo proteolytic cleavage by metalloproteinases, including tumor necrosis factor-α converting enzyme and BACE1 (β-site amyloid precursor protein–cleaving enzyme 1), a type I transmembrane aspartyl protease. As a consequence, NRG1 types I and II are shed from the neuronal cell surface and act as paracrine signaling molecules. NRG1 type III is defined by its cysteine-rich domain, which functions as a second transmembrane domain. Consequently, type III is thought to remain tethered to the cell surface after cleavage and to act as a juxtacrine signal (**Figure 1**(**a**)). In addition, exons encoding shorter amino termini of NRG1 have been identified (referred to as types IV–VI), but these isoforms have not been further characterized.

The EGF-like domain (contained in all NRG1 isoforms) is necessary and sufficient for binding and activation of transmembrane receptor tyrosine kinases of the ErbB family. The term ErbB originally derived from its homology to the erythroblastoma viral gene product, v-erbB. The basic structure of all four members of the ErbB receptor family (ErbB1–4) includes two extracellular cysteine-rich ligand binding domains, a transmembrane region, the tyrosine kinase domain (inactive in ErbB3), and a C-terminal domain (**Figure 1**(**b**)). NRG1 directly binds to ErbB3 and ErbB4. ErbB2, which is devoid of an activating ligand binding site, is the preferred dimerization partner. ErbB receptors dimerize not by virtue of a bridging effect of NRG1 but, rather, following a ligand-activated conformational change in the ectodomain of ErbB3 or ErbB4. Crystallographic data indicate that ErbB2 constitutively exposes a dimerization loop and can form heterodimers with ligand-activated ErbB3 or ErbB4 receptors. NRG1 binding induces homodimer (ErbB4/ErbB4) or heterodimer formation (ErbB2/ErbB3, ErbB2/ErbB4, and ErbB3/ErbB4), which leads to receptor cross-phosphorylation, recruitment of SH3-containing adapter molecules, and activation of downstream signaling pathways. Complexity of NRG1–ErbB signaling emerges from isoform-specific receptor affinity and dimer-dependent stimulation of signaling pathways. Moreover, several observations suggest that NRG1–ErbB signaling is bidirectional. Upon binding to an ErbB receptor and/or following neuronal depolarization, the C-terminal intracellular domain (ICD) of NRG1 is proteolytically cleaved off in a γ-secretase-dependent process and subsequently translocated to the cell nucleus. Here, depending on cell type and interactions with other transcription factors, the ICD might act as an enhancer or repressor of gene expression, resulting, for instance, in increased neuronal survival or enhanced synaptic plasticity. ErbB receptors have also been identified to be translocated to the nucleus, either the entire protein or its cytoplasmic domain. In particular, γ-secretase-dependent proteolysis (after NRG1 binding) and nuclear translocation have been observed for the cytoplasmic domain of

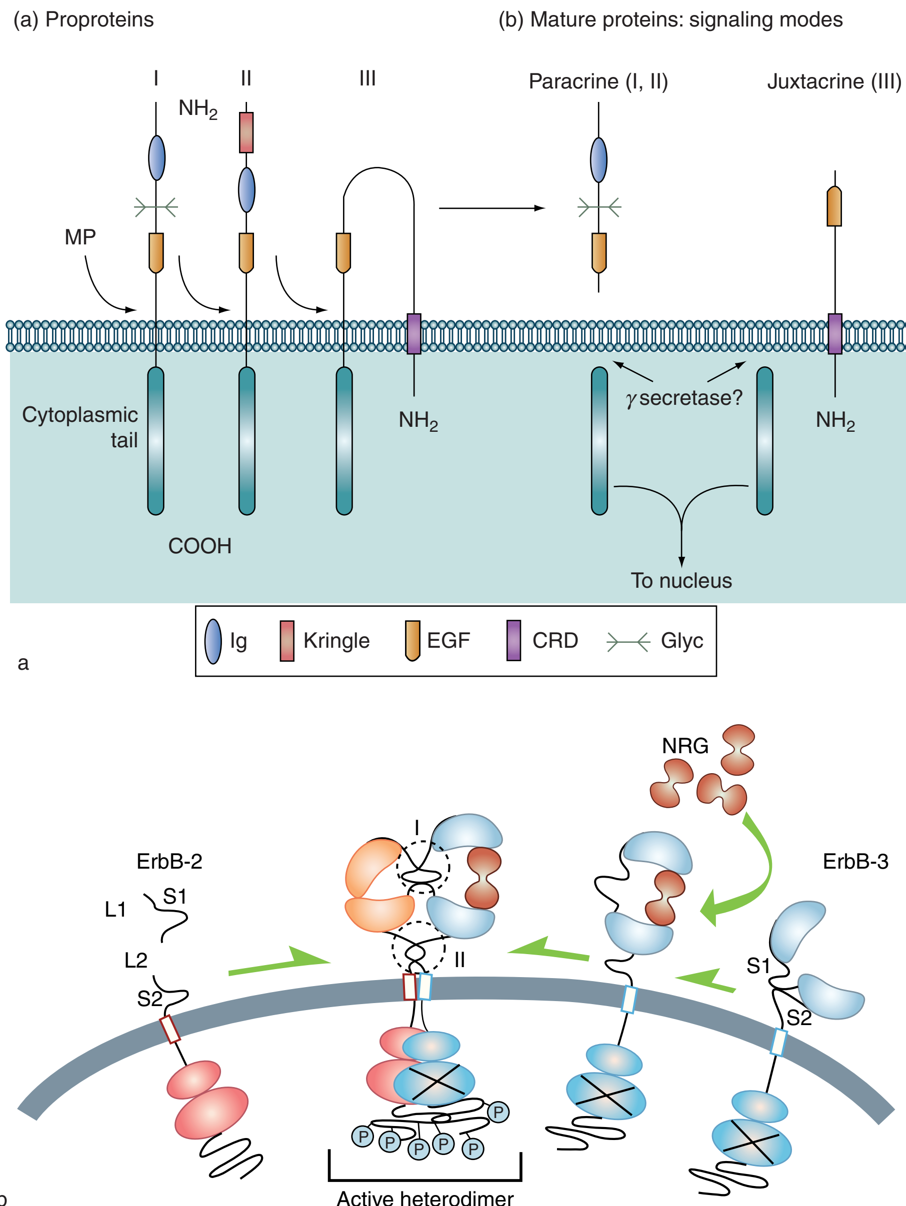

Figure 1 Structure of NRG1/ErbB signaling components. (a) NRG1 types I and II are synthesized as single pass transmembrane proteins; type III has two transmembrane domains. With metalloproteinase (MP) cleavage, types I and II are shed as paracrine signals, type III remains tethered through its cysteine-rich domain (CRD) and is a juxtacrine signal. The cytoplasmic domain might undergo further cleavage stimulated by binding of ErbB receptors to NRG1, followed by translocation to the nucleus. (b) Proposed mechanism of NRG1-induced ErbB2/ErbB3 heterodimer activation. Extracellular ErbB receptor domains are represented by two CRDs (S1 and S2) and two cysteine-free, ligand-binding domains (L1 and L2). ErbB3 (blue) lacks a functional intracellular tyrosine kinase domain and exists on the cell surface in an autoinhibited conformation (resulting from the interactions between the S1 and S2 domains). Bivalent binding of NRG1 (NRG, red dumbbell) to the L1 and L2 domains of ErbB3 rearranges the conformation of the extracellular domain, leading to heterodimer formation with ErbB2, which is devoid of a functional ligand binding site. Dimerization between a ligand-bound ErbB3 and an ErbB2 molecule results in receptor auto-phosphorylation and activation of downstream signaling pathways. (a) Reproduced from Nave KA and Salzer JL (2006) Axonal regulation of myelination by neuregulin1. *Current Opinion in Neurobiology* 16: 492–500. (b) Reproduced from Citri A and Yarden Y (2006) EGF–ERBB signalling: Towards the systems level. *Nature Reviews Molecular Cell Biology* 7: 506–516.

ErbB4, suggesting that both ErbB receptors and their ligands can function as regulators of gene expression.

NRG1–ErbB Signaling Regulates SC Lineage Development

In rodents, development of SCs from pluripotent neural crest cells progresses through two intermediate cell types, SC precursors (SCPs) and immature SCs, which proliferate and migrate along preexisting axons. Early migratory neural crest cells and mesenchymal cells express NRG1, ErbB2, and ErbB3, but not ErbB4. Before embryonic day (E) 10, only type I isoforms of NRG1 are expressed. Later, NRG1 type III becomes the predominant isoform in spinal cord motor neurons and

sensory neurons of dorsal root ganglia (DRG). In contrast, ErbB2 and ErbB3 are strongly expressed in SCPs and immature SCs but are mostly absent from sensory and motor neurons, at least during embryogenesis.

Multiple studies in which a recombinant EGF domain (derived from NRG1) was added to cultured SCs or injected into rodent sciatic nerves have demonstrated that ErbB signaling operates during all stages of SC development and has potent effects on SC survival, proliferation, migration, differentiation, and myelination.

The essential requirement of NRG1–ErbB signaling for the development of the SC lineage *in vivo* was demonstrated through gene targeting in mice. Mouse mutants lacking NRG1, ErbB2, or ErbB4 die at midgestation (E10.5) due to heart malformation. Most ErbB3 null mutants die between E11.5 and E13.5 (also due to defective heart development), but some embryos develop to term. In NRG1, ErbB2, and ErbB3 (but not ErbB4) null mutants, the number of SCPs that line peripheral axons is severely diminished already at E10.5, as evidenced by the reduced expression of several SC markers (e.g., ErbB3, p75, Sox10, and S100). The severity of SCP loss, however, depends on the axial level. Likewise, glial cells in the enteric nervous system are absent in the mutants. Analysis of ErbB3-deficient mice at later embryonic stages revealed not only loss of SCPs along peripheral nerves but also a reduction of up to 80% of motor and sensory neurons, again depending on the axial level. Neuronal death is likely to be a secondary effect and may be attributed to the absence of SC-derived neurotrophic factors (e.g., nerve growth factor, ciliary neurotrophic factor, or glial cell line-derived neurotrophic factor) that support neuronal survival.

The importance of ErbB2 signaling at later stages of prenatal SC development became apparent through transgenic rescue of the heart defect. Rescued ErbB2 mutants die at birth and completely lack SCPs and immature SCs in peripheral nerves at this time. In contrast, SCPs are present, albeit at reduced numbers, in DRG (at E12). This observation was attributed to the compromised ability of mutant SCPs to migrate out of the DRG and into peripheral nerves. However, the true lineage relationship between SCs in dorsal and ventral nerve roots, DRG, and peripheral nerves is not fully understood. Thus, the presence of some SCs in DRG of ErbB2 (and ErbB3) mutants could also reflect a differential dependency on ErbB2 signaling of certain SCP subpopulations, such as satellite SCs in DRG.

Do different NRG1 isoforms serve distinct functions during PNS development? Several lines of evidence strongly suggest that signaling of a membrane-tethered type III isoform of NRG1 is most critical for SC maturation (and myelination). Mouse mutants selectively lacking NRG1 isoforms with an Ig-like domain (affecting types I and II) die at E10.5 and display abnormalities in heart and cranial ganglia, which are very similar to those observed in pan NRG1 mutants. In contrast, SCPs that line spinal nerves are unaffected. The generation of mice that specifically lack NRG1 type III variants allowed researchers to directly address the role of this isoform in SC development. NRG1 type III mutants die at birth due to an inability to breathe. Interestingly, the consequences of type III-restricted gene targeting on SC development appear milder than those in ErbB2 or ErbB3 mutants. SCPs (defined by ErbB3 expression) are present along peripheral nerves already at E11.5, albeit at reduced numbers. At E14.5, immature SCs (Sox10/ErbB3/S100+), although dramatically reduced, are still present close to dorsal and ventral roots and along peripheral nerves. Nevertheless, S100+ cells are completely absent from nerve terminals. DRG explants from type III mutants prepared at E14.5 display a marked SC deficiency at first but are partially repopulated by endogenous SCs during the course of several weeks. However, many of these SCs fail to attach and ensheathe neurites properly. Thus, NRG1 type III isoforms are essential for the peripheral migration and/or survival of SCs, but signaling by other NRG1 isoforms is sufficient to also allow the initial generation and early differentiation of SCs.

Taken together, neuronal NRG1 type III signaling to SCs is mediated through an ErbB2/3 heterodimer and critically important for multiple aspects of SCP and premature SC development, including migration, differentiation, and survival.

NRG1–ErbB Signaling as a Master Regulator of Myelination in the Peripheral Nervous System

Immature SCs, which are generated from SCPs (between E13 and E15 in late embryonic nerves), first communally ensheathe large groups of axons. Subsequently, by radial sorting, individual SCs associate either with large-diameter axons in a 1:1 ratio or with several small-diameter (<1 μm) axons. During this process, the emergence of SC polarity, reflected by the asymmetric distribution of molecules (e.g., the polarity protein Par-3), is a prerequisite for focal deposition of myelin components. Early postnatally, SCs engulfing a large-diameter axon are selectively activated to differentiate into myelinating SCs that wrap multiple layers of myelin membrane around this axon. In contrast, SCs associated with small-diameter axons mature into 'nonmyelinating' SCs that ensheathe 5–25 (up to 50) small-caliber axons (in a so-called Remak bundle) without forming compact myelin sheaths. Since SC proliferation and survival both depend on axonal signals, limited axonal contact provides a

means by which the number of SCs and axons are matched.

In contrast to the control of myelination, the role of NRG1–ErbB signaling in radial sorting, SC polarity, timing of myelination onset, and the survival of SCs in postnatal nerves has not been determined *in vivo*. Several studies, however, support involvement of NRG1 in some of these processes. For instance, rescue of axotomy-induced SC death by exogenous NRG1 suggests that it is a component of an axon-derived survival signal at perinatal stages. The role of NRG1 in timing the onset of myelination through a phosphatidylinositol 3-kinase (PI3K)-dependent pathway is supported by the observation that treatment of rat SC cultures with recombinant NRG1 (similar to axonal contact) activates PI3K and mitogen-activated protein kinase (MAPK). In early SC–DRG neuron cocultures, elongation of SCs and subsequent myelination can be blocked by inhibition of PI3K (but not MAPK), whereas PI3K stimulation promotes myelination. In contrast, the maintenance of myelin sheaths in mature cocultures does not critically depend on PI3K activity. In agreement with this observation, pharmacologic inhibitors of ErbB receptors demonstrate a requirement of NRG1 signaling for the initiation of myelination also in zebra fish.

The first demonstration that NRG1–ErbB signaling plays a direct role in myelination came from the analysis of mouse mutants with a conditional deletion of the *ErbB2* gene in myelinating SCs. ErbB2 deficiency causes abnormally thin myelin sheaths, containing fewer myelin wraps. Similarly, mice that overexpress a dominant negative ErbB receptor in SCs (under control of the CNPase promoter) are hypomyelinated.

The level of NRG1 type III on the axonal surface, rather than axon diameter *per se*, has been identified to act as an instructive signal to induce myelination *in vitro*. Expression of NRG1 type III on the axon correlates with the ensheathment fate of axons *in vivo*: Unmyelinated, autonomic neurons express low levels of NRG1 type III on the axon surface, whereas BDNF/NT3-dependent DRG neurons, whose axons are heavily myelinated, express high levels. DRG axons from NRG1 type III null mice are not myelinated by SCs in cocultures and do not induce myelin-specific structural proteins or transcription factors, thus demonstrating that axonal NRG1 type III is essential for myelination (**Figure 2(A)**). Furthermore, mice haploinsufficient for NRG1 type III have a significantly higher proportion of axons that are persistently unmyelinated. In contrast, forced expression of NRG1 type III in the postganglionic fibers of sympathetic neurons converts these normally unmyelinated fibers to myelination *in vitro* (**Figure 2(B)**). Importantly, and despite addition of a soluble NRG1 type III ectodomain, cocultures of DRG neurons from type III null mutants and wild-type SCs fail to myelinate. Also, juxtacrine signaling (from heterologous cells stably expressing NRG1 type III) is not sufficient to induce myelin protein expression in cocultured SCs, demonstrating that NRG1 type III requires additional signals present on the axon to induce SC myelination. Together, these results suggest that threshold levels of axonal NRG1 type III provide the long-sought instructive signal that triggers SC myelination, at least *in vitro* (**Figure 3**).

Finally, NRG1–ErbB signaling has also been recruited for the regulation of the final stage of SC differentiation: the quantitative control of myelin sheath thickness (**Figures 2(C)** and **2(D)**). Rapid impulse propagation is a function of axon caliber and myelination, and the optimal myelin thickness is reached when the 'g-ratio' is close to 0.68 (i.e., the numeric ratio between the diameter of the axon cylinder and that of the myelinated axon), a ratio remarkably well maintained for peripheral axons. Thus, axon size is monitored by myelinating SCs in order to assemble the correct number of myelin wraps. Mouse mutants with altered expression levels of NRG1 demonstrated that this information is encoded, at least in part, by the amount of membrane-associated NRG1 type III on the axon surface. Heterozygous NRG1 type III null mutant mice, which display approximately 50% NRG1, are hypomyelinated and exhibit reduced nerve conduction velocity. In contrast, transgenic mice that overexpress NRG1 type III in DRG sensory neurons and spinal cord motor neurons (under control of the neuronal Thy1 promoter) become hypermyelinated (**Figures 2(C)** and **2(D)**). This effect appears to be specific to NRG1 type III because transgenic mice that overexpress the secreted NRG1 type I are not hypermyelinated. NRG1 has emerged as the rate-limiting factor of myelin growth control but not SC survival since SC numbers are unaltered in NRG1 +/− sciatic nerves. ErbB2 and ErbB3 are expressed by SCs at saturating levels. Only a dramatic reduction in number disrupts myelination (i.e., in a conditional mouse mutant with an SC-specific null mutation of the ErbB2 gene).

Whereas the requirement of the type III isoform for myelination is well established, the role of posttranslational modifications of NRG1 during myelination is only beginning to be addressed. First insight into the importance of proteolytic processing came from mouse mutants expressing only 'noncleavable' NRG1 variants. These mice display a phenotype analogous to NRG1 null mice (impaired heart development),

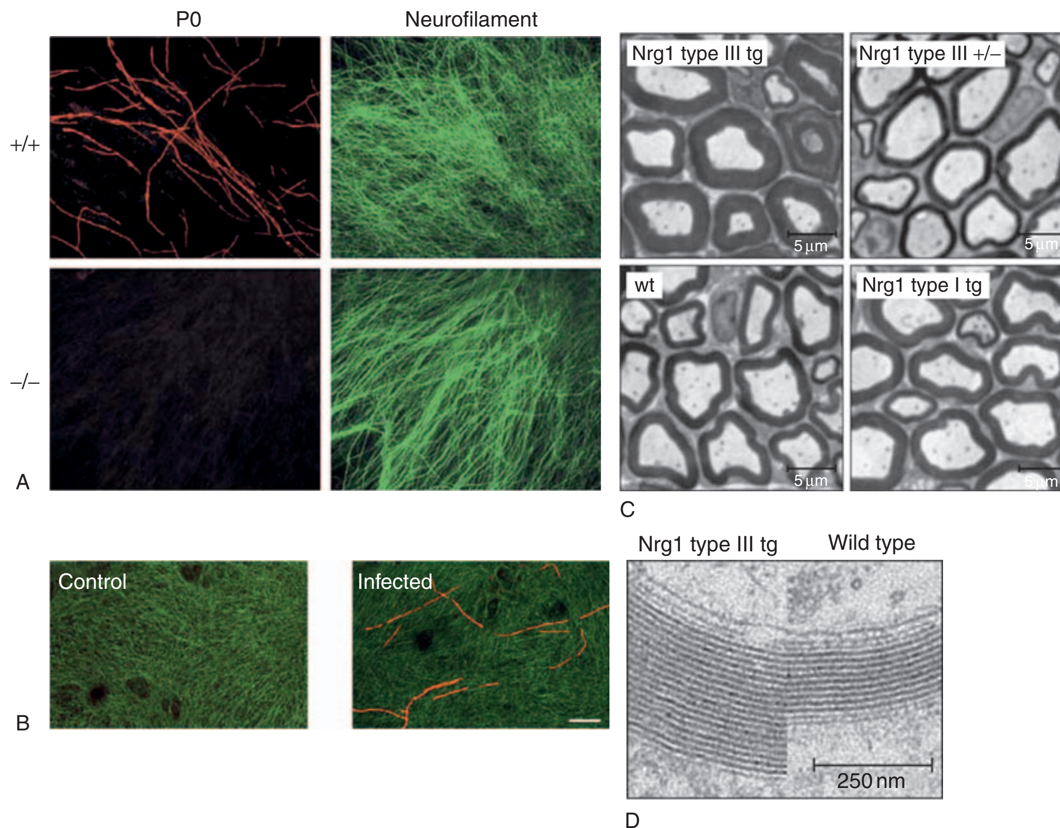

Figure 2 NRG1 type III is a master regulator of peripheral myelination. (A) Myelination by Schwann cells is completely blocked in the absence of NRG1 type III. Co-cultures of wild-type rat Schwann cells and sensory neurons from wild-type mice (+/+) or NRG1 type III null mutants (–/–) were maintained *in vitro* for 3 weeks, fixed, and stained for myelin (protein P0, red) and axons (neurofilament, green). Numerous myelin segments are evident in wild-type co-cultures, in contrast, no myelin is formed in co-cultures containing sensory neurons derived from NRG1 type III mutants. (B) Ectopic expression of NRG1 type III induces myelination of normally unmyelinated sympathetic neurons. Co-cultures of Schwann cells and control (left) or superior servical ganglion (SCG) neurons infected with NRG1 type III lentivirus (right) were stained for MBP (red) and neurofilament (green). (C) The level of axonal NRG1 type III determines myelin sheath thickness. Semithin cross sections (0.5 μm) of the sciatic nerve comparing transgenic NRG1 overexpressing mice with a wild-type control at age 5 months. Type III transgenics are hypermyelinated (Nrg1 type III tg), but not the type I transgenics (Nrg1 type I tg). The specific function of NRG1 type III is confirmed by a corresponding hypomyelination of mice with a type III isoform-specific null allele (Nrg1 type III +/–). (D) The ultrastructure and periodicity of compact myelin in hypermyelinated fibers, prepared from NRG1 type III transgenic mice (left), is indistinguishable from myelin prepared from wild-type mice (right). This demonstrates that hypermyelination is a consequence of additional membrane wraps. The two axons shown are of about equal diameter. Scale bar = 100 μm (B). (A, B) Reproduced from Tavegia C, Zanazze G, petrylak A, et al. (2005) Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron* 47: 681–694. (C, D) Reproduced from Michailov GV, Sereda MW, et al. (2004) Axonal neuregulin-1 regulates myelin sheath thickness. *Science* 304: 700–703.

indicating that cleavage of NRG1 is critical for its function. Studies have identified BACE1 as essential for normal peripheral (and central) myelination. BACE1 is coexpressed with NRG1 in motor and sensory neurons at postnatal stages. BACE1 deficiency in mouse mutants results in the accumulation of unprocessed NRG1, hypomyelination of peripheral nerves, and aberrant segregation of small-diameter axons by SCs. The exact number and sequence of proteolytic cuts, the participation of other proteases, and the mechanisms that regulate proteolysis remain to be determined.

Taken together, the published studies support a model in which a threshold level of NRG1 type III is required to trigger myelination. At higher levels, the amount of myelin formed is a function of the amount of NRG1 type III presented by the axon to the SCs. This model also suggests a mechanism by which axons, via differing levels of NRG1 type III, coordinate SC numbers as they adopt alternative phenotypes. For example, axons expressing higher levels of NRG1 type III generate the additional SCs required to establish the one-to-one relationship characteristic of myelinated fibers. Open questions include the regulation of

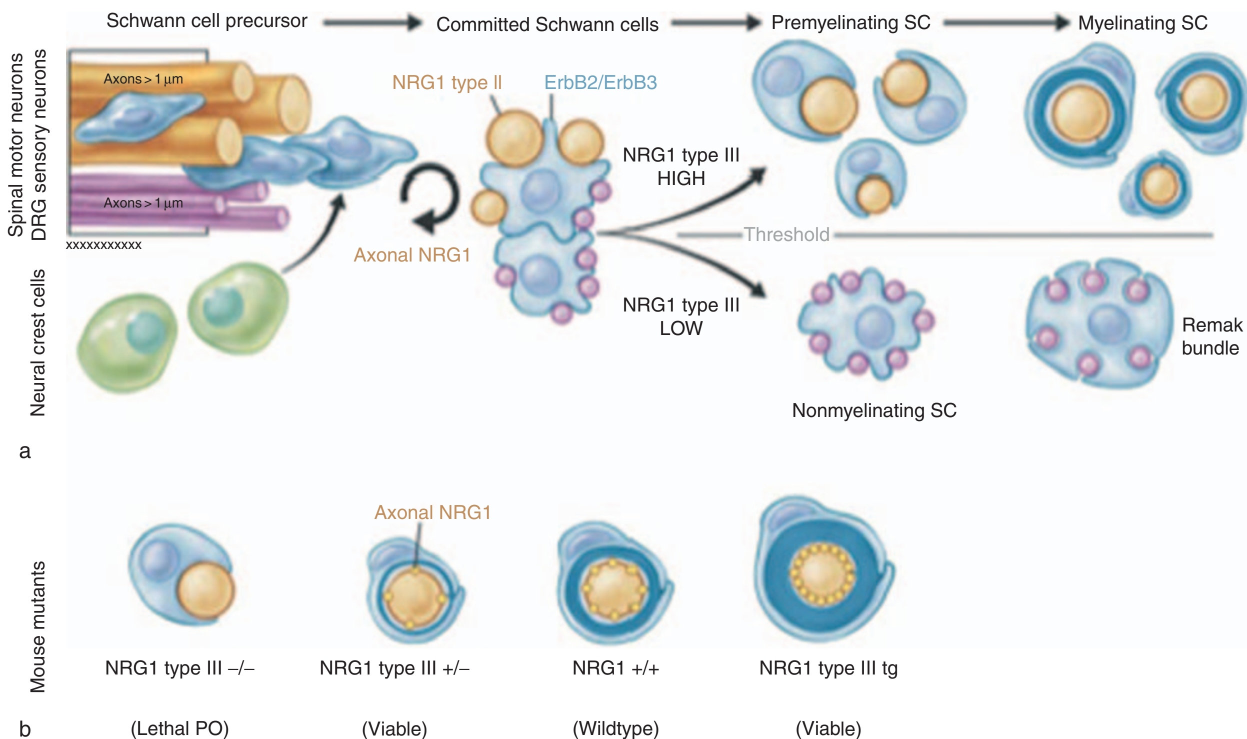

Figure 3 Axonal NRG1 regulates successive steps of Schwann cell differentiation. (a) Schwann cells (in blue) arise from neural crest precursor cells (in green) and interact with both large and small caliber axons of spinal motor and sensory neurons. During embryogenesis, NRG1 on the axon regulates Schwann cell development by activating ErbB signaling cascades, thereby promoting Schwann cell differentiation and expansion. The amount of NRG1 type III on the axon detected by committed Schwann cells, which is a function of axon size and NRG1 levels, then drives them either into segregating single axons and myelination (top), or into a nonmyelinating phenotype and formation of a Remak bundle (bottom). Above threshold levels, NRG1 type III signals axon size to Schwann cells to optimize myelin sheath thickness. (b) In mouse mutants lacking NRG1 type III (–/–), heterozygous NRG1 type III mutants (+/–) and transgenic NRG1 type III overexpressing mice, the amount of myelin made by Schwann cells varies directly as a function of axonal NRG1 type III levels (indicated by yellow dots) rather than as a function of axon diameter. Reproduced from Nave KA and Salzer JL (2006) Axonal regulation of Mylination by neuregulin 1. *Current Opinion in Neurobiology* 16: 492–500.

NRG1 expression in individual neurons to match NRG1 steady-state levels to axon caliber and length.

ErbB Signaling in the Mature Peripheral Nervous System and in Pathological Conditions

Adult sensory (DRG) and spinal motor neurons continue to express NRG1 type III. Likewise, expression of ErbB2 and ErbB3 is maintained in mature SCs, albeit at reduced levels. Moreover, differentiated SCs, at least in culture, express several NRG1 isoforms, and autocrine NRG1 signaling in SCs may explain why SCs become progressively less dependent on neuronal NRG1 signaling during development. Indeed, expression of ErbB receptors has also been reported in adult motor and sensory neurons. Thus, NRG1-expressing SCs might signal to neurons that they myelinate. Moreover, ErbB2 expression is induced in SCs upon sciatic nerve transection in adult rats. SC proliferation in Wallerian degeneration follows a fixed time course, and the expression of NRG1 and ErbB2 is coordinately induced, implicating this pathway in SC mitogenesis and remyelination after axotomy.

Surprisingly, conditional inactivation of ErbB2 in adult myelinating SCs (induced at 2.5 months of age) does not impair normal myelin sheath maintenance or the proliferation of SCs after nerve axotomy. Whether remyelination is unaffected in these mutants awaits further analysis. Thus, the molecular control of SC function differs significantly between development and regeneration.

In contrast to myelinated motor axons, sustained ErbB signaling is required for nonmyelinating SCs and small axons (C-fibers) in the mature nerve. Disruption of ErbB signaling in adult nonmyelinating SCs by expression of a dominant negative ErbB receptor causes cell death and proliferation of nonmyelinating SCs and, indirectly, loss of unmyelinated axons in transgenic mice.

Aberrant ErbB receptor activity contributes to the development of many human cancers. Receptor

overexpression, kinase domain mutations, and autocrine ligand production can cause ErbB activation in tumors. Since SCs express various forms of NRG1 and autocrine NRG1–ErbB signaling has been implicated in their proliferation, it is plausible that NRG1/ErbB2 may be involved in the pathogenesis of human nerve sheath tumors and Schwannomas. This is also supported by the development of nerve sheath tumors in transgenic mice that overexpress a soluble NRG1 type II variant (GGFβ3) in myelinating SCs. Overexpression of ErbB2 and ErbB3 by SCs has been noted in nerves from patients with Charcot–Marie–Tooth disease type 1A, the most common hereditary peripheral neuropathy. The upregulation of these receptors may correlate with the generation of supernumerary SCs ('onion bulbs') or play a role in the inhibition of myelination or demyelination.

Aberrant signaling through the ErbB2 receptor is also a cause of demyelination in leprosy. *Mycobacterium leprae*, the causative agent of leprosy, directly binds and activates ErbB2 on the surface of myelinating SCs without the usual dimerization with ErbB3. The subsequent activation of MAPK1/2 by phosphorylation results in demyelination. This process can be blocked at the receptor activation stage by Herceptin, a monoclonal antibody directed against the ectodomain of ErbB2 (**Figure 4**).

Taken together, these studies have demonstrated that ligand-independent ErbB activation does not necessarily recapitulate NRG1 signaling but, rather, orchestrates a distinct spectrum of potentially pathological cellular responses.

Future Research

Although principal mechanisms of myelination control by NRG1–ErbB signaling have been defined, a number of gaps remain in our understanding of the molecular details. For example, the relevance of intracellular processing, transport, and localization for isoform-specific functions (e.g., myelination versus neuromuscular junction formation) remains poorly defined, and the exact composition and localization along the axon of the core NRG1 type III–ErbB2/3

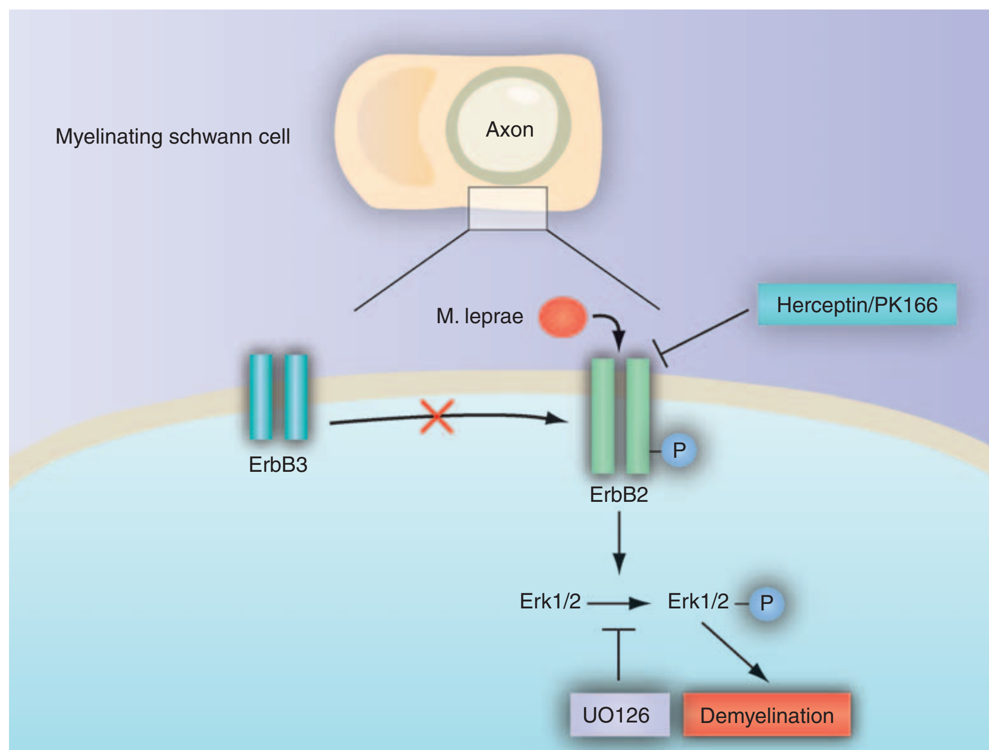

Figure 4 Leprosy induced peripheral demyelination is mediated by ErbB2, *Mycobacterium leprae,* the causative agent of leprosy, activates ErbB2 receptors on the surface of myelinating Schwann cells. This leads to their activation by phosphorylation (P) without the usual dimerization with ErbB3. The subsequent activation of the extracellular signal-regulated kinases (Erk) 1/2 by phosphorylation, a mitogen-activated protein kinase kinase (MAPKK)-dependent event, results in demyelination. The process can be blocked at the receptor activation stage by Herceptin (a monoclonal antibody directed against ErbB2) or at the Erk1/2 phosphorylation stage using a MAPKK inhibitor (UO126). Reproduced from Franklin RJ and Zhad C (2006) Tyrosine kinases: Maining in leprosy. *Nature Medicine* 12: 889–890.

signaling complex are unknown. It will be of particular interest to determine whether the C-terminal fragment of NRG1, after proteolytic cleavage by BACE1 (or other proteases), remains associated with the N-terminal EGFL domain-containing fragment or whether, upon γ-secretase cleavage, it mediates back-signaling by nuclear translocation. The N-terminal fragment might be further proteolytically processed such that the EGFL domain is released into the adaxonal space. Because the activity of the core NRG1 type III–ErbB2/3 signaling complex is most likely modulated by multiple interactions with other molecules on the axonal and glial surface and the extracellular matrix, the identification of interacting partner molecules is another important task. Finally, the downstream signaling pathways that transmit information provided by the activated ErbB2/3 heterodimer from the growing myelin membrane to the SC nucleus and the set of target genes which is transcriptionally regulated by those pathways have yet to be identified.

See also: *Drosophila* Apterous Neurons: from Stem Cell to Unique Neuron; Neural Crest; Neural Crest Cell Diversification and Specification: Melanocytes; Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation; Schwann Cells and Axon Relationship.

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Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation

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The Role of Transcription Factors in the Schwann Cell Lineage

Schwann cells and satellite glia are the two main glial cell types of the peripheral nervous system (PNS). Whereas satellite glia are found within ganglia in close association with neuronal somata, Schwann cells are found in close contact with axons in the peripheral nerves. Both Schwann cells and satellite glia arise from neural crest stem cells. Following their specification, Schwann cells go through several consecutive developmental stages, including the Schwann cell precursor and the immature Schwann cell, before they finally undergo terminal differentiation and become myelinating or nonmyelinating Schwann cells of the adult PNS. Each developmental stage is characterized by specific patterns of gene expression, and progression from one stage into the next requires corresponding changes. Taking the role of transcription factors as regulators of gene expression into account, each stage of Schwann cell development and each transition from one stage into the next must be brought about by changes in transcription factor occurrence or activities.

Transcriptional Control of Schwann Cell Specification

Transcription factors with importance for Schwann cell development are already present in pluripotent neural crest stem cells. This includes the α isoform of transcription factor AP2, the forkhead protein FoxD3, the paired domain protein Pax3, and the high-mobility-group domain containing protein Sox10. Because all these transcription factors are expressed during the specification event, their presence must at least be compatible with the transformation of a neural crest stem cell into a Schwann cell precursor. Except for Sox10, whether these transcription factors are also required for specification has not been rigorously tested.

In neural crest stem cells, Sox10 is important for survival and for the maintenance of pluripotency; trunk neural crest stem cells show a reduced survival rate in mice deficient for Sox10. However, enough neural crest stem cells survive to aggregate to dorsal root ganglia and populate roots and proximal portions of peripheral nerves. Whereas neuronal specification in the dorsal root ganglia is initially undisturbed in the absence of Sox10, neural crest cells fail to undergo specification to glia. These loss-of-function studies show that Sox10 is indeed required for the specification of Schwann cell precursors as well as satellite glia precursors, both of which can be distinguished from neural crest stem cells by the expression of B-FABP. Schwann cell precursors also start to express low levels of the myelin protein zero (MPZ) as a marker. This basal level of MPZ expression is probably due to a the direct activation of the MPZ promoter by Sox10 and is orders of magnitude below the level attained later during active myelination.

Because the specification of both Schwann cells and satellite glia is under control of Sox10, Sox10 alone cannot account for the difference between both glial cell types. Rather, different transcription factors must be present in presumptive glia along nerves and in dorsal root ganglia, so that cooperation of these transcription factors with Sox10 leads to different glia, depending on their localization. This model fits the general perception of Sox proteins as transcription factors that function mainly in combination with partner proteins. Although this limits the intrinsic impact of Sox proteins on developmental processes, it also endows them with functional versatility. Sox10, for example, exerts many additional functions at later stages of Schwann cell development with changing partner proteins.

Hooked on Neuregulins: The Schwann Cell Precursor

Newly formed Schwann cell precursors depend for survival and proliferation on neuregulin 1 signaling. In particular, the membrane-bound isoform III of neuregulin 1 is strongly expressed on axons at this time, thus making survival of Schwann cell precursors dependent on axonal contact. Schwann cells recognize the neuregulin 1 signal with a heterodimeric receptor consisting of ErbB2 and ErbB3. In the absence of Sox10, the expression of ErbB3 is strongly reduced in neural crest-derived cells along the nerve, proving that Sox10 is genetically upstream of ErbB3 expression. Ectopic expression of Sox10 in a neural cell line, furthermore, leads to a rapid induction of ErbB3 expression, arguing that ErbB3 transcription may be

directly dependent on Sox10 in neural cells. Sox10 may, thus, also ensure the survival of Schwann cell precursors by making them responsive to neuregulin 1.

Neuregulin 1 continues to be important for Schwann cells and promotes both the transition of Schwann cell precursors to immature Schwann cells and myelin formation. Neuregulin 1 signals seem predominantly transduced through the mitogen-activated protein kinase (MAPK) pathway in Schwann cells and through Ets proteins as their nuclear effectors. Accordingly, several Ets proteins, in particular Net and GA binding protein (GABP-)α, are prominently expressed in Schwann cell precursors, immature Schwann cells, and throughout myelination.

Neuregulin 1 also stimulates the expression of the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and induces sustained phosphorylation of CREB on its serine residue 133 in Schwann cells. Signaling through CREB appears to contribute to Schwann cell proliferation during the early stages of lineage development.

Molting into an Immature Schwann Cell

Several days after specification, Schwann cell precursors develop further into immature Schwann cells. In the mouse, this transition occurs around 13 days postcoitum, requires continued neuregulin signaling, and is characterized by changes in gene expression. Most important, from this stage onward Schwann cells establish an autocrine signaling mechanism that makes them less dependent on axonal signals for survival and proliferation. It is not yet known which transcription factor or which combination of transcription factors is needed to drive Schwann cell precursors into the immature Schwann cell stage. However, evidence exists to implicate AP2-α as a negative regulator of this process. As already mentioned, AP2-α expression has continued in Schwann cell precursors from the neural crest cell stage. Immature Schwann cells normally arise once AP2-α expression is extinguished. Reciprocally, prolonged expression of AP2-α interferes with the timely progression from the Schwann cell precursor to the immature Schwann cell stage. Taking into account that endothelins exert a similar effect on Schwann cell development through the endothelin B receptor, a link may exist between AP2-α and endothelin signaling in Schwann cell precursors.

Maintaining the Immature State

In contrast to AP2-α, Sox10 continues to be expressed in immature Schwann cells, possibly ensuring continued ErbB3 expression during this stage of Schwann cell development. The same continued expression from neural crest stem cells into the immature Schwann cell stage is also observed for FoxD3 and Pax3.

In addition, immature Schwann cells start to express transcription factors that are undetectable at earlier stages of their development. This includes the two related POU homeodomain transcription factors – Oct6 (also known as SCIP and Tst-1) and Brn2 – as well as the zinc finger protein Krox20 (also known as Egr2). Initial expression of these transcription factors in immature Schwann cells is at a low basal level, orders of magnitudes below the maximal levels achieved at later times of Schwann cell development. Expression of Krox20 is, furthermore, inversely correlated to the expression of its close relative Krox24 (also known as Egr1), which is expressed at highest levels in Krox20-negative Schwann cell precursors and is completely absent later in myelinating Schwann cells which exhibit maximal Krox20 levels.

More prominently expressed than Oct6 and Krox20 in immature Schwann cells is the high-mobility-group domain protein Sox2. In contrast to its distant relative Sox10, it is specifically turned on in the Schwann cell lineage and not expressed in neural crest stem cells. In fact, forced expression of Sox2 in the presumptive neural crest at the time of neurulation leads to a fate switch and causes these cells to become part of the neuroepithelium. Sox2 expression is important to maintaining Schwann cells in the immature state. Similarly, this appears to be the case for Pax3 and FoxD3, as well as for the bZip transcription factor cJun. Similar to phosphorylated CREB, these transcription factors all stimulate Schwann cell proliferation. They also appear to repress terminal differentiation.

Schwann cell proliferation is also enhanced by glucocorticoids, indicating that immature Schwann cells express the glucocorticoid receptor. Glucocorticoid receptor is not the only nuclear hormone receptor in Schwann cells. Thyroid hormone receptor and progesterone receptor have also been detected, as well as several members of the p160 coactivator family of steroid receptor coactivators (SRC1–SRC3) as crucial mediators of nuclear hormone receptor effects. Interestingly, Schwann cells also have the capacity to synthesize progesterone, indicating that progesterone signaling may be part of the autocrine loop that exists in Schwann cells from the immature state onward.

Choosing between Two Alternative Fates during Terminal Differentiation

Progesterone and thyroid hormone have also been reported to promote myelin gene expression in Schwann cells and may thus be involved in triggering

terminal differentiation at the end of embryogenesis. During terminal differentiation, Schwann cells choose between two mutually exclusive pathways. Schwann cells in contact with small-caliber axons ensheath these axons as nonmyelinating Schwann cells. These cells no longer actively divide, but are not permanently withdrawn from the cell cycle. They, furthermore, continue to express many of the same transcription factors that were already expressed in the immature Schwann cells. Myelinating Schwann cells, on the other hand, exit the cell cycle, establish a one-to-one relationship with large-caliber axons and form myelin sheaths around an axonal segment. Myelin formation, in addition, requires dramatic changes in the expression pattern of myelinating Schwann cells as lipid biosynthesis and the production of myelin proteins (such as MPZ, MBP, PMP-22, and Connexin-32) and the synthesis of specific glycolipids are steeply increased.

Triggering Myelination by Axonal Signals

Of all the steps in Schwann cell development, transition from the immature Schwann cell to the myelinating Schwann cell is best understood. It is critically dependent on at least three groups of transcription factors, the first being nuclear factor (NF)-κB proteins, in particular p65/relA. NF-κB is already present in Schwann cells before myelination, and it is mainly localized during this stage in a complex with I-κB proteins in the cytoplasm and, thus, in an inactive state. At the onset of terminal differentiation, an axonal signal activates the low-affinity nerve growth factor (NGF) receptor p75 and thereby leads to nuclear translocation of NF-κB and induction of its transcriptional activity. In the mouse, blockade of NF-κB activity or the genetic deletion of p65 attenuates peripheral myelination significantly.

NF-κB also stimulates Oct6 expression in Schwann cells and is thus part of the strong axon-dependent upregulation of Oct6 gene expression immediately prior to myelination. Axonal signals influence Oct6 gene expression also through increased intracellular cAMP levels. This inducibility by cAMP has, in fact, led to the designation of Oct6 as suppressed cAMP-inducible POU (SCIP) protein.

Escorting Schwann Cells into the Myelinating Stage

Maximal Oct6 levels coincide with the phase during which Schwann cells in contact with large-diameter axons, establish a one-to-one relationship, and prepare for myelination. Oct6 is, therefore, strongly associated with this promyelinating stage. Schwann cells in Oct6-deficient mice become arrested in their development at the promyelinating stage, supporting the notion that Oct6 mediates the progression from the promyelinating into the myelinating stage. However, the arrest is only transient, so that Schwann cells eventually overcome this block in Oct6-deficient mice and form myelin sheaths with a delay of approximately 2 weeks.

The transient nature of this developmental defect can probably be attributed to the closely related POU protein Brn2, which is co-expressed with Oct6 in Schwann cells. During normal development, Brn2 is expressed at lower levels than Oct6 in Schwann cells. As a result, the loss of Brn2 does not lead to a noticeable change in pattern or timing of Schwann cell differentiation. Once Oct6 is experimentally deleted, a compensatory upregulation of Brn2 occurs, and these increased Brn2 levels may now be able to overcome the arrest of Schwann cell development in Oct6-deficient mice.

In agreement with functional redundancy between Oct6 and Brn2, the overexpression of a Brn2 transgene rescues the developmental delay of Oct6-deficient Schwann cells. Simultaneous loss of Oct6 and Brn2, on the other hand, causes affected Schwann cells to persist in the promyelinating stage much longer than if they were only Oct6-deficient. Accordingly, compound mutant mice show a myelination delay of more than 8 weeks, compared to the 2 weeks in Oct6-deficient mice. Nevertheless, some residual capacity to enter the myelination stage is retained. At present, it is unclear why that is, but Oct-1, a ubiquitously expressed POU homeodomain protein distantly related to Oct6 and Brn2, could be responsible. In contrast, Brn-5, another POU homeodomain protein that is expressed in the Schwann cell lineage, was excluded as a compensating factor because it is predominantly expressed in myelinating Schwann cells and was unable to rescue the Oct6 phenotype when overexpressed as a transgene in Oct6-deficient Schwann cells.

It has long been controversial whether Oct6 (and by analogy Brn2) functions as a transcriptional activator or repressor. Differential display studies on the sciatic nerves of wild-type and Oct6-deficient mice have preferentially identified genes that are downregulated in the absence of Oct6, thus pointing to a primarily activating role of Oct6 in Schwann cells, although it has not been established that the identified genes (including the genes for the fatty acid transporter P2, the amino acid transporter tramdorin1, and the LIM domain protein cysteine-rich protein (CRP)2) are directly activated by Oct6.

There is very good evidence that the Krox20 transcription factor is under the direct positive control of Oct6. Studies in transgenic mice have identified an

enhancer 35 kb upstream of the transcriptional start site of the mouse Krox20 gene that is responsible for Krox20 expression in Schwann cells and that at the same time depends for its activation on Oct6 binding. Interestingly, Oct6 is not the only transcription factor that binds to the enhancer. There also are several recognition elements for Sox10, which is still strongly expressed in Schwann cells during the promyelinating stage. The enhancer is cooperatively activated by Oct6 and Sox10, and it is likely that *in vivo* cooperative activation is responsible for the dramatic upregulation of Krox20 expression in promyelinating Schwann cells.

It is also assumed that Oct6 is not only needed for the progression from the promyelinating to the myelinating stage but has additional, possibly repressive, functions during the early phases of myelination. This assumption is supported by the continued Oct6 expression in young myelinating Schwann cells. However, proof of these additional functions in myelinating Schwann cells is difficult to obtain in the currently available mouse models. In older myelinating Schwann cells, Oct6 is eventually downregulated. Interestingly, Oct6 itself is involved in its downregulation, pointing to the existence of a negative autoregulatory loop.

Finalizing Myelination

The dramatic upregulation of Krox20 in promyelinating Schwann cells follows that of Oct6, in accord with the epistatic relationship between both transcription factors. One of the functions of Krox20 is to suppress the immature state. It inactivates the proliferative response of Schwann cells toward their major axonal mitogen neuregulin 1. At the same time, Krox20 makes the cells less susceptible to cell death. To achieve this, Krox20 prevents the c-Jun N-terminal kinase (JNK)-dependent phosphorylation of cJun which is required for both the proliferative and the apopotic response of immature Schwann cells. As a consequence, Krox20-deficient Schwann cells are arrested in the promyelinating stage, but in contrast to Oct6-deficient Schwann cells, the block is permanent. There is additional evidence that Krox20 is involved in the downregulation of its activator Oct6 because Oct6 expression continues in Krox20-deficient Schwann cells.

Krox20 is also actively involved in establishing the myelinating stage. As is evident from gene expression profiling, most myelin genes and lipid biosynthetic enzymes are under direct or indirect control of Krox20. Confirming the link between Krox20 and myelin gene expression, several human patients have been identified with heterozygous mutations in Krox20. These heterozygous mutations manifest themselves as congenital hypomyelinating neuropathies or peripheral neuropathies of the Charcot–Marie–Tooth type 1 and Dejerine-Sottas syndrome, which are more frequently observed for genetic disturbances of myelin genes, including MPZ, PMP22, and Connexin-32. The patients' phenotype is thus compatible with a function of Krox20 during terminal differentiation of myelinating Schwann cells.

Regulating Myelin Gene Expression

In myelinating Schwann cells, Krox20 directly binds to the Nab1 and Nab2 cofactors. This cofactor interaction is crucially important for Krox20 function because mice deficient for Nab1 and Nab2 show defects in peripheral myelination similar to Krox20-deficient mice. Although Nab1 and Nab2 have been shown to function as repressors of Krox20 *in vitro*, the observed phenotype in Nab1/Nab2-deficient mice suggests that *in vivo* they are coactivators as well as corepressors.

It is unlikely that the Krox20–Nab1/Nab2 complex alone is responsible for the activation of all myelin genes and key enzymes in lipid metabolism. Thus, Schwann cells express transcription factors that have been associated with the transcriptional regulation of lipid metabolism in other tissues, including sterol regulatory element binding protein (SREBP)1 and SREBP2, the δ isoform of C/EBP, and the δ isoform of peroxisome proliferator-activated receptor (PPAR). Their increasing expression levels during myelination is compatible with a similar role in Schwann cells. Several SREBP target genes have been shown to be synergistically activated by SREBPs and Krox20 in tissue culture.

Another transcription factor with potential relevance for the myelinating stage of Schwann cells is the still expressed Sox10. Although a role of Sox10 during peripheral myelination has not been formally proven *in vivo*, there are several lines of evidence to implicate Sox10 in the process. The first comes again from the analysis of myelin gene expression in tissue culture. Thus, Schwann-cell specific regulatory regions of the MPZ, MBP, and Connexin-32 genes are all activated by Sox10. In case of the MPZ gene, an intronic enhancer appears to mediate this activation and is synergistically activated by Sox10 and Krox20. The Connexin-32 promoter is likewise synergistically activated by Sox10 and Krox20. Sox10 and Krox20 may thus cooperate in the activation of many genes during myelination, just as Sox10 cooperated during earlier phases of Schwann cell development with other transcription factors such as Oct6 during Krox20 activation in promyelinating Schwann cells.

The second piece of evidence for a role of Sox10 in peripheral myelination comes from patients with peripheral myelinopaties. Some carry heterozygous mutations in Sox10. Others have been found to carry mutations in a region of the Connexin-32 promoter that binds Sox10 and mediates Sox10-dependent activation. A role in myelination has already been proven for Sox10 in the oligodendrocytes of the central nervous system. Similar to Schwann cells, oligodendrocytes express Sox10 from earliest time, but in contrast to Schwann cells, they do not depend on Sox10 until terminal differentiation because of the additional presence of the closely related and functionally redundant Sox9. When Sox9 is downregulated in oligodendrocytes immediately before terminal differentiation, Sox10 function becomes evident and myelination is disrupted in the central nervous system of Sox10-deficient mice.

Open Questions

Although many transcription factors have been identified over the last years that contribute to Schwann cell development, there are still obvious gaps in our knowledge. Transcription factors that drive the transition from the Schwann cell precursor to the immature Schwann cell are, for instance, still unknown. In addition, several transcription factor families with important roles as developmental regulators in other neural cell types are missing from the list of Schwann cell regulators. The most conspicuous example concerns cell-specific (class B) basic helix-loop-helix (bHLH) proteins which, as proneural proteins, influence fate decisions in central and peripheral neurons as well as central glia. Because both Id proteins as the negative regulators of bHLH proteins and class A proteins as the heterodimerization partners for class B bHLH proteins have already been detected in Schwann cells, it is only a matter of time before a Schwann-cell-specific bHLH protein is identified. Many transcription factors thus remain to be discovered to complete the picture.

More and more findings point to functional interactions between already identified transcription factors, as exemplified by the cooperative activation of myelin genes by Sox10 and Krox20. Such interactions and cross-regulatory activities are probably essential for Schwann cell development. It is highly unlikely that the regulation of Schwann cell development can be explained by the isolated actions of a handful of cell- and stage-specific transcription factors; rather, their combination and cross-talk with more widely expressed transcription factors drive Schwann cell development. A second challenge thus is to find out how Schwann cell transcription factors influence one another's activity and to establish an integrated picture of the transcriptional network in Schwann cells.

Given the fact that lineage progression in other cell types is strongly associated with changes in chromatin structure, it can be safely assumed that such epigenetic changes are also relevant to Schwann cell development. Up until now, the question of how transcription factors influence chromatin structure has been barely addressed in Schwann cells and will certainly be an issue in the future.

Last, transcriptional regulation of Schwann cell development is thought to reflect many of the principles that are active during remyelination following nerve injury. Several transcription factors have indeed been shown to contribute to both processes. However, whether transcriptional control of remyelination is indeed a recapitulation of the regulatory events during Schwann cell development still requires rigorous testing.

See also: Neural Crest; Neural Crest Cell Diversification and Specification: ErbB Role; Neural Crest Cell Diversification and Specification: Melanocytes; Peripheral Nerve Regeneration: An Overview; Schwann Cells and Axon Relationship; Schwann Cells and Plasticity of the Neuromuscular Junction.

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Neural Crest Cell Diversification and Specification: Melanocytes

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Introduction

Melanocytes are pigment-producing cells of vertebrates. They are found in the integument, the eye, the inner ear, and, depending on the species, also in a variety of other inner organs, such as the lung, the heart, and around the aorta. Their pigment, termed melanin, is a biopolymer that is synthesized through a series of catalytic steps starting with the conversion of the amino acid tyrosine to dihydroxyphenylalanine (DOPA) by a copper-binding enzyme called tyrosinase. There are two types of melanin : eumelanin, which is black, and pheomelanin, which is yellow. Both types are made in membrane-bound intracellular organelles, called melanosomes, in which they can be found in pure form or mixed. Not every cell that contains melanosomes is a melanocyte, however, because melanocytes can transfer their melanosomes to other cell types that are not capable of producing melanin. In human skin, for instance, melanosomes are transferred from melanocytes to keratinocytes and into hair shafts, and in birds they are found in feathers. Clearly, a major role of melanocytes is to provide a body's visible pigmentation and so to aid in camouflage, display, and sun protection.

However, melanocytes are also critical for other organs. In the eye, for instance, they are found in the iris, which serves as an aperture that controls the depth of focus and regulates the amount of light that enters the eye. They are also found in the choroid behind the retina, where they serve as a light screen, blocking the passage of light to deeper tissues. In the inner ear of mammals, they are found in a portion of the wall of the cochlear duct, called the stria vascularis, where they regulate the ionic composition of the endolymph and ensure that auditory hair cells function properly. In fact, diminished numbers of strial melanocytes can lead to hearing impairment, as is likely the case in human Waardenburg syndromes, which are characterized by pigmentary abnormalities in skin, hair, and eyes and by varying degrees of deafness. Importantly, the function of melanocytes in hearing is independent of their pigmentation because mammals that lack melanin but retain melanocytes usually have normal hearing. Hence, melanocytes have important roles beyond fashioning the variety and beauty of an organism's coloration.

Development of Melanocytes

Melanocytes are generated from the neural crest, which is an embryonic population of cells that forms at the border between the neural plate and the adjacent surface ectoderm. Neural crest cells are induced by extracellular signals such as bone morphogenic proteins (BMPs), the Wingless/Int family of proteins (WNTs), and fibroblast growth factors (FGFs). These ligands induce transcription factors that hierarchically serve to specify the neural plate border, the neural crest, and, finally, a variety of cellular derivatives. In addition to melanocytes, these include the glial cells and neurons of the peripheral nervous system, smooth muscle cells, and bone and cartilage of the head.

The precursors to melanocytes, called melanoblasts, emerge at all axial levels between the region of the diencephalon and the tail end of the neural tube. This is in contrast to other crest derivatives, which are usually made at more restricted sites. Soon after their generation, neural crest cells accumulate in an area, called the migration staging area, close to the dorsal lip of the neural tube. From there, they migrate to their ultimate destinations, first leaving from the head region and then progressively from regions further toward the tail. In many vertebrates, melanoblasts migrate only after other crest derivatives have left. In regions where somites are present, melanoblasts travel between the somites and the surface ectoderm, with a major direction from back to belly but also longitudinally from head to tail and tail to head. This pathway is called the dorsolateral pathway and is different from the pathway taken by nonmelanoblastic crest derivatives, which migrate between the somites and the neural tube. In regions where no somites are present, particularly in the area of the otic vesicle, melanoblasts travel close to the neural tube as well as laterally toward the surface ectoderm. The majority of melanoblasts enter the dermis and then cross the basal membrane to take up residence in the epidermis, feather buds, or hair follicles. Others migrate toward the developing inner ear, the choroid, the ciliary body, the iris, and other inner organs. While traveling, melanoblasts begin to express a series of proteins, including the melanin-synthetic enzymes dopachrome tautomerase (DCT), tyrosinase-related protein-1, and tyrosinase, and the melanosomal protein pMEL17. In mice, expression of these proteins follows a characteristic sequence over several days. This sequence culminates with the induction of tyrosinase, which is the rate-limiting enzyme in melanin synthesis. Importantly, both cell proliferation and migration can continue even after

melanin has accumulated in the cells. The principal stages of melanoblast development in mouse embryos are shown in **Figure 1**.

This brief description of melanocyte development leaves us with a number of specific questions. What molecular signals specify melanoblasts and segregate them from other crest derivatives? Is specification irreversible, and if so, when does irreversibility occur – before migration or only later when the cells are closer to their final destination? How many cells are specified as melanoblasts, and how much do they have to proliferate to populate an entire body? What are the mechanisms that control melanoblast proliferation, guide them to their destinations, and control the progression toward their differentiation? What are the mechanisms that replenish melanocytes that are lost in adults, for instance, during hair and feather molting, when there is no recourse to the embryonic neural crest? We briefly discuss our current knowledge on these questions.

Melanoblast Specification: The Interplay between Signaling Pathways and Transcription Regulation

The analysis of both *in vivo* genetic models and *in vitro* culture systems made it possible to identify a number of factors involved in melanoblast specification. Three common notions have emerged from these studies. First, many of the factors involved in

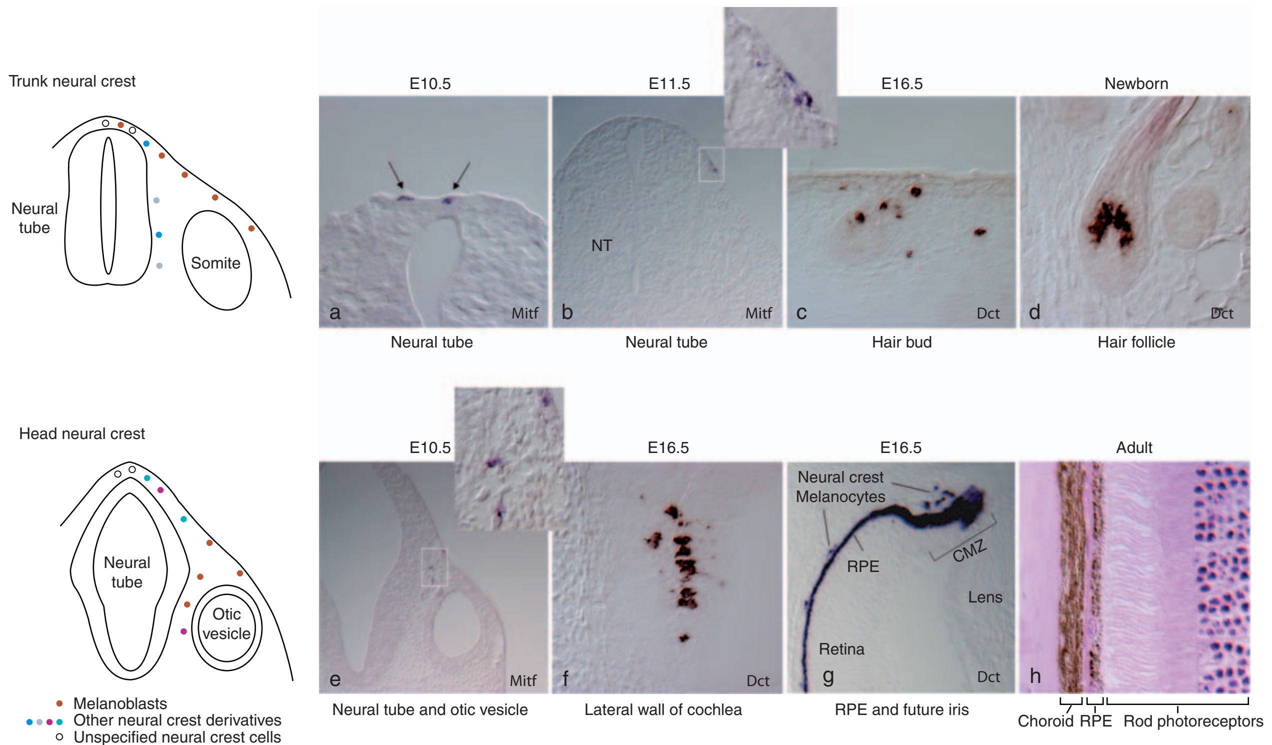

Figure 1 Melanoblast development and migration from the neural crest in mouse embryos. The left side shows schematic drawings of sections through the trunk and head areas as well as the respective migration pathways of melanoblasts and other neural crest derivatives. (a–g) Represent cryostat sections prepared from albino embryos or newborns that were labeled by nonradioactive *in situ* hybridization using riboprobes. Hence, the label marks gene expression, not pigmentation. (a, b, and e) Expression of the transcription factor gene *Mitf* is, together with KIT expression (not shown), one of the first signs of melanoblast specification in the neural crest. In the trunk area, *Mitf*-expressing cells are first found in the region of the roof plate of the neural tube (arrows in a). Then they migrate on a dorsolateral pathway underneath the surface ectoderm (b, and magnification of boxed area in b). (c and d) At later stages, the *Mitf* marker becomes progressively weaker and melanoblasts can best be labeled by expression of the melanogenic gene *Dct*. (c) Shows *Dct*-positive cells that have entered the epidermis and developing hair buds, and (d) shows *Dct*-positive cells in hair follicles of newborns. (e) In the head region, neural crest cells express *Mitf* only after they have migrated away from the crest. They are found underneath the surface ectoderm and on a pathway toward the otic vesicle. (f) *Dct*-positive melanoblasts have entered the lateral wall of the cochlear duct at E16.5. (g) Other melanoblasts have migrated close to the optic cup and start to populate the region behind the retinal pigment epithelium (RPE). This region will form the pigmented choroid. Neural crest-derived melanoblasts have also migrated close to the ciliary margin zone (CMZ), that will form the ciliary body and the iris of the eye. Note that the RPE is also positive for *Dct* but is derived directly from the optic neuroepithelium, not the neural crest. (h) H&E-stained section through the choroid and RPE of an adult eye of a pigmented mouse. Only the choroidal pigment cells are neural crest derived.

early specification operate not at a single stage but during several phases of melanocyte development. Second, many of the factors can either promote or inhibit melanocyte formation, depending on the conditions. Third, many of these factors also operate in other organ systems, and their mutations can lead to disorders unrelated to melanocyte function, including embryonic or adult lethality.

A list of factors currently implicated in melanocyte development is shown in **Table 1**. The factors are arranged by functional groups and alphabetically within groups, starting with a group of factors operating from outside the cells and ending with a group functioning inside. Not listed are the typical differentiation (pigmentation) genes, and we refer the reader to other reviews for more detailed information on the factors not specifically covered here.

Numerous genetic and expression studies as well as biochemical and cell-biological analyses have demonstrated that among the early effectors of melanoblast development, the cell surface receptor KIT and the transcription factor MITF mark melanoblasts selectively in the neural crest and are the ones most specifically involved in their development. KIT is a receptor tyrosine kinase that binds a single ligand, KIT ligand (KITL), and activates the mitogen-activated protein (MAP) kinase pathway and the phosphoinositide-3 (PI3) kinase pathway. Based on natural and genetically engineered mouse mutants, the MAP kinase pathway is critical for melanocyte development, but the PI3 kinase pathway is dispensable. MITF is a basic–helix–loop–helix–leucine zipper transcription factor that was originally identified in mice with transgenic insertional mutations at the microphthalmia locus (hence its name, microphthalmia-associated transcription factor). Such mice are entirely white and deaf because their melanoblasts do not develop properly, and they have small eyes (microphthalmia) because the development of the pigmented epithelium of the retina, a neuroepithelial derivative, also depends on MITF. MITF has several isoforms, one of which, the M-MITF isoform, is under the control of its own specific promoter, the M-promoter, and is the major isoform of melanoblasts.

There is *in vitro* evidence that KIT and MITF engage in cross-talk. The KIT-mediated activation of the MAP kinase pathway leads to serine phosphorylation of MITF, which regulates MITF's transcriptional activity and stability. Conversely, MITF is needed for KIT to accumulate in melanoblasts. KIT and MITF are thus part of a feedback loop. This feedback loop intersects with a second signaling pathway that also acts early in melanoblast development and involves the ligand endothelin-3 (EDN3) and the G-coupled endothelin receptor-B (EDNRB). Consistent with the observation that EDNRB signaling stimulates melanoblast proliferation *in vitro*, EDNRB-deficient mouse embryos have few melanoblasts, and after birth melanocytes are largely missing except in spots around the neck and the base of the tail. Although EDNRB is expressed in melanoblasts where it acts cell autonomously, it is also expressed in other cell types, notably in cells of the neural tube. Interestingly, co-cultured, EDNRB wild-type neural tube or nonmelanoblastic neural crest cells can rescue the proliferation of EDNRB-deficient melanoblasts and let them develop at least to the tyrosinase-positive stage, but similar cells genetically deficient in KITL cannot. Because KITL alone can rescue EDNRB-deficient melanoblasts *in vitro*, it is conceivable that EDNRB signaling also helps melanoblast development in a cell nonautonomous manner by stimulating KITL production or secretion in other cells. Hence, it appears that all three factors – EDNRB, KIT, and MITF – are functionally linked during the early stages of melanoblast development.

What, then, induces these key factors? Much work has gone into the identification of transcription factors that directly stimulate the M-MITF promoter. They include SOX10, a member of the SRY-box-containing high-mobility group of transcription factors; PAX3, a paired domain transcription factor; LEF1/TCF, another high-mobility group protein; and CREB, a basic–leucine zipper protein. None of these are specific to melanoblasts, however. Thus, much as with many other promoters, it is likely the combinatorial network of these and other factors, each with its own specific transcriptional activities, that regulates the M-MITF promoter. Similar reasoning applies to KIT and EDNRB even though much less is known about their transcriptional regulation in melanoblasts.

On the next level up, extracellular ligands have been identified that regulate the regulators of MITF. Among them are WNT proteins, which are cysteine-rich secreted proteins that bind members of the G-coupled receptor family called Frizzled receptors (FZD) and receptors related to low-density lipoprotein receptor (LPR; particularly LPR5 and LPR6). The coactivation of these two types of receptors induces the so-called canonical WNT signaling pathway, whose hallmark is the accumulation of β-catenin and its transfer to the cell nucleus, where it interacts with LEF/TCF to stimulate the transcription of specific target genes. Together with BMPs and FGFs, WNTs are critical in specifying the neural crest by operating upstream of a series of transcription factors, including PAX3, SOX9, SOX10, SLUG/SNAIL, AP-2, and FOXD3 (**Table 1**). Many of these factors are also engaged in extensive yet species-specific cross-regulatory networks. Interestingly, after its early role in neural

Table 1 Factors implicated in melanocyte specification and development

Functional group	*Symbol*	*Name*	*Function*	*Human neural crest-related disorders (OMIM No.)*[a]
Extracellular matrix and cell adhesion	ADAMTS20	Adamts20 metalloprotease	Involved in ECM remodeling	
	ECM	Extracellular matrix (collagen, fibronectin, laminin)	Migration of melanoblasts	
	EFNB	Ephrin B	Early migration of melanoblasts	
	EPHB	Ephrin B receptor	Early migration of melanoblasts	
	INT	Integrins	Migration of melanoblasts	
Growth factors and growth factor receptors	BMP	Bone morphogenic protein	Promotes or inhibits melanoblast specification depending on conditions	
	CDH	Cadherins	Migration of melanoblasts regulated by various combinations of cadherin family members	
	EDN3	Endothelin-3 (a 20-residue peptide)	Melanoblast proliferation and survival	Waardenburg syndrome IV (277580), Hirschsprung disease and related disorders (142623, 209880)
	EDNRB	G-coupled endothelin receptor B	Melanoblast proliferation and survival	Waardenburg syndrome IV (277580), Hirschsprung disease and related disorders (142623, 600501)
	FZD	Frizzled family of G-coupled receptors for WNT	Activation of canoncial WNT signaling, induction of neural crest and promotion of melanoblast development	
	KITL	KIT ligand	Melanoblast survival, proliferation, migration	
	KIT	KIT receptor tyrosine kinase	Activates Ras/Raf/MAP kinase pathway, melanoblast survival, proliferation, migration	Piebaldism (172800)
	LRP	Family of receptors related to low-density lipoprotein receptors	Co-receptor for FZD, activation of canoncial WNT signaling, induction of neural crest and promotion of melanoblast development	
	NOTCH	Family receptors related to *Drosophila* Notch	Melanoblast survial and melanocyte stem cell maintenance	
	WNT	Wingless-related/MMTV integration site	Induction of neural crest, promotion of melanobalst development	
Transcription factors and cofactors	AP-2	AP-2 transcription factor	Melanoblast differentiation	
	CATNβ	β-Catenin	One of the effectors of WNT signaling	
	FOXD3	Forkhead box D3	Neural crest induction, can inhibit melanoblast specification	
	LEF1/TCF	Lymphoid enhancer factor/T cell-specific transcription factor	One of the effectors of WNT signaling	
	MITF	Microphthalmia-associated transcription factor, basic helix–loop–helix–leucine zipper family	Melanoblast specification, survival, proliferation, migration	Waardenburg syndrome IIa (193510)
	PAX3	Paired domain protein-3	Melanoblast specification, survival, and proliferation; regulation of adult melanocyte stem cells	Waardenburg syndrome I, II, III (193500, 193510, 148820)
	SNAI2/ SLUG	Snail homolog protein-2, zinc finger protein	Melanoblast specification downstream of MITF?	
	SOX9	SRY-box containing protein-9	Induction and differentiation of the neural crest	
	SOX10	SRY-box containing protein-10	Melanoblast specification, survival, proliferation, and differentiation	Waardenburg syndrome IV (277580)

[a] *OMIM, Online Mendelian Inheritance in Man.*

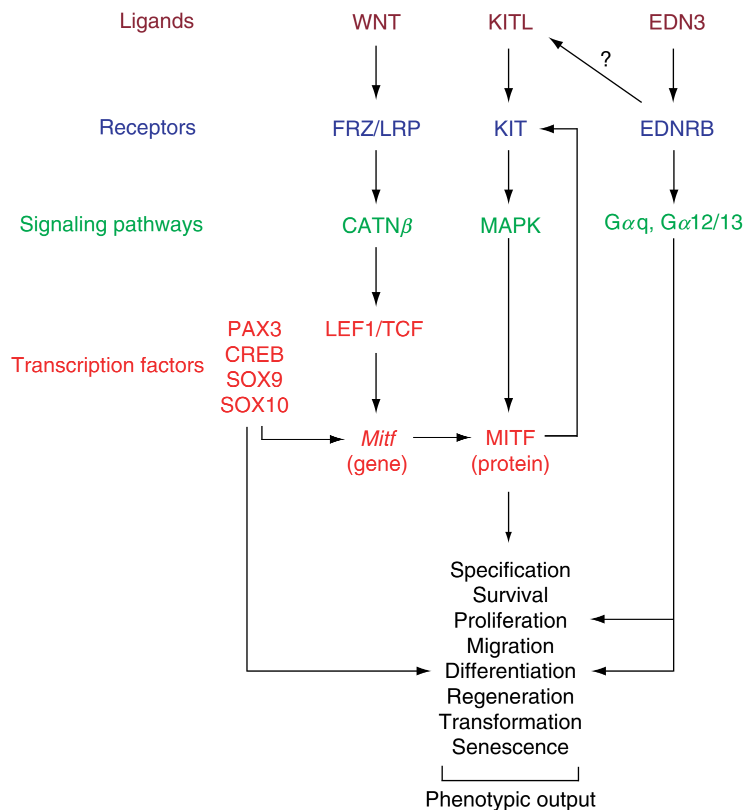

Figure 2 Simplified schematic representation of the interplay between signaling pathways and transcription factors involved in melanocyte development. For abbreviations, see **Table 1**. Biochemical evidence shows that the MAPK pathway regulates MITF activity and stability through phosphorylation. Genetic evidence suggests that EDNRB signals through Gnaq and Gna11, two members of the Gαq family of G-proteins, as well as through Gα12/13.

crest specification, canonical WNT signaling is also critical in promoting the melanoblast fate. For instance, in mouse embryos, elimination of β-catenin in neural crest stem cells leads to a lack of melanoblasts and sensory neuronal lineages but not other crest derivatives. In zebra fish, overexpression of β-catenin in premigratory neural crest cells leads to excess pigment cell formation through the stimulation of MITF. In transgenic mice, however, stimulation of MITF by stably active, nuclear β-catenin can also lead to inhibition of melanoblast proliferation, illustrating that potent factors such as β-catenin need to be finely tuned with respect to their levels and specific time points of expression. This is all the more important because β-catenin also acts downstream of cadherins, which together with integrins regulate cell adhesion and migration. A simplified scheme of the interactions of the extracellular ligands and transcription factors involved in melanoblast development is shown in **Figure 2**.

Melanoblast Specification: Cell Number and Location

In previous decades, the existence of melanoblasts could be assumed only indirectly from the existence of their descendents, the melanocytes. Using pigmentation as the readout, two types of studies have suggested that in mice, melanocytes must arise from a very small number of precursors. Mintz produced chimeras of embryos of pigmented and unpigmented, tyrosinase-deficient (albino) mice. From the width and arrangement of the pigmentation stripes that appear in adults, she concluded that there are precisely 34 melanocyte precursors, 17 equally spaced on either side of the embryo – three for the head, six for the body, and eight for the tail. Support for a small number of melanocyte precursors, although not exactly 34, came from the infection of albino embryos with retroviral vectors expressing wild-type tyrosinase. The resulting mice had small pigmented stripes whose widths were reminiscent of those observed in Mintz's chimeras. In later studies, the use of *in situ* hybridization or antibody probes specific for melanoblasts indicated that the number of initially specified melanoblasts is indeed small, particularly in the trunk region. These studies showed, however, that melanoblasts are unevenly distributed between head and tail. The use of a line of transgenic mice in which the bacterial *lacZ* gene is expressed under control of the promoter of the early melanogenic gene *Dct*

also showed an uneven head-to-tail distribution of melanoblasts. However, the *Dct–lacZ* marked melanoblasts were found to mix extensively along the head-to-tail axis and to be considerably more numerous than suggested from the earlier studies. Each study has to be considered with caution, however. The first assumes that one stripe represents a clone derived from a single cell, which is only true if the cells are thoroughly mixed early in the chimeric embryos but their descendents do not mix later in development. The second may not reflect the marking of the earliest melanoblasts because cells could be infected at different stages during lineage development. The third is limited by the sensitivity of the *in situ* probes, and the fourth may overestimate the number of melanoblasts because it cannot be excluded that *lacZ*-marked cells may give rise to other cells besides melanocytes. Thus, the truth may well lie somewhere in between: more than 34 melanoblasts in mice but less than 1000.

The question of where and when melanoblasts are committed to the melanocyte lineage has also not been answered definitively. On the one hand, the coexpression of KIT and MITF in premigratory mouse neural crest cells strongly argues for an early commitment of melanoblasts. On the other hand, there is *in vitro* evidence that *Dct*-positive neural crest cells, usually considered to be melanoblasts, are capable of generating both melanocytes and smooth muscle cells. In the quail, even epidermal melanocytes have been observed to switch their fate and become Schwann cells, the myelin-forming cells of the peripheral nervous system (and vice versa, Schwann cells have been seen to become melanocytes). In fact, the question of commitment cannot be answered universally for all vertebrate species, and probably not even universally for all axial levels in the same species. In the head region of mouse embryos, for instance, *Kit*-positive cells, identified by expression of a knock-in Kit^{lacZ} allele, are first observed at the dorsal midline of the neural tube, but MITF expression, detected by antibody labeling or *in situ* hybridization, is usually seen only in postmigratory melanoblasts. In contrast, at the trunk level, both KIT and MITF are first expressed together in cells at the top of the neural tube (**Figures 1(a)** and **1(e)**).

Although there are thus arguments for both premigratory and postmigratory commitment, the proof of a true restriction in a melanoblast's developmental potential requires systematic challenges with conditions permissive for alternative fates: Fate maps are not indicative of fate restrictions. For instance, it is not known whether MITF/KIT double-positive melanoblasts of mice, which normally develop only into melanocytes, are entirely incapable *in vivo* of developing into other cell types. This point is not of mere academic interest because a prolonged retention of a broader developmental potential in one cell lineage may provide an embryo with the means to correct developmental aberrations in another, and thus still yield a healthy adult.

Melanoblast Survival, Proliferation, and Migration

The developing population of melanoblasts is regulated by the parameters of cell survival, cell proliferation, and cell migration. The molecular dissection of these parameters has indicated a series of important players. Recent evidence suggests, for instance, that Notch signaling, which is involved in many developmental processes, is also critical for melanoblast survival. Notch receptors represent a family of conserved transmembrane proteins that are activated by specific ligands, Delta and Jagged, through direct cell–cell interactions. In fact, Notch signaling, acting through the downstream target gene *Hes1*, plays a critical role in preventing apoptosis of melanoblasts. Moreover, the previously mentioned extracellular factors WNT, EDN3, and KITL, are critical for cell survival and for promoting cell proliferation. As alluded to previously, the actions of these factors converge on MITF, the all-purpose melanoblast transcription factor, which has been shown to regulate cell survival and proliferation. *In vitro* experiments in melanoma cells suggest, for instance, that MITF stimulates the expression of the antiapoptotic genes *Bcl2* and hypoxia-inducible factor-1α (*Hif1α*), but a C-terminal fragment of MITF, liberated by the proapoptotic, activated caspase-3, enhances apoptosis. MITF also seems to have both positive and negative effects on cell proliferation. It promotes cell proliferation by stimulating the transcription of the gene encoding cyclin-dependent kinase-2 (CDK2), which promotes the G_1-to-S transition during the cell cycle. MITF also stimulates the transcription factor TBX2, which represses the expression of p21, a protein inducing growth arrest. Furthermore, MITF appears to be essential, and in fact is increasingly expressed, in highly aggressive, metastasizing melanoma cells, earning it the name 'lineage addiction oncogen.' On the other hand, MITF is also clearly antiproliferative: it activates the gene encoding INK4a, which promotes cell cycle exit, and it directly stimulates the gene encoding p21, which induces G_1 arrest. What controls the net result of these opposing actions is not entirely clear but may rest in part on the balance of expression of distinct MITF isoforms. For instance, when expressed *in vitro*, isoforms that lack certain subexons are less antiproliferative, or may even promote proliferation, compared to isoforms

that contain these subexons. Hypothetically, then, distinct MITF isoforms might be expressed at different developmental time points or during different phases of the cell cycle and may regulate different subsets of cell cycle regulators. This might occur either at the transcriptional level or by direct interactions at the protein level. MITF and cell cycle regulators may hence be integrated at multiple levels.

While melanoblasts proliferate, they also migrate, but proliferation and migration are not synchronized because there are spurts of growth that alternate with spurts of migration. Migration is controlled in part by cell adhesion and the extracellular matrix. Cadherins form a family of glycoproteins involved in calcium-dependent cell adhesion, regulation of the cytoskeleton, and cell signaling. Melanoblasts express distinct cadherins very dynamically during development. For instance, when neural crest cells delaminate from the epithelium, they express cadherin 6 but only low levels of N-cadherin. In migrating melanoblasts, E- and P-cadherins are weakly upregulated, but upon arrival in the dermis, neither is expressed. E-cadherin increases 200-fold when the cells enter the epidermis and interact with E-cadherin-expressing keratinocytes. Melanoblasts that enter hair follicles, however, downregulate E-cadherin again and exclusively express P-cadherin. The few melanocytes remaining in the epidermis continue to express E-cadherin, and those in the dermis express N-cadherin. These dynamic changes in cadherin expression seem to adapt the cells to their local environment, a hypothesis that has been tested by both gain-of-function and loss-of-function mutations in select cadherins. Because cadherins have wide-ranging actions in other tissues, however, specific effects on melanoblasts are not always easy to discern.

The parameters of cell migration have also been explored in heterologous cell grafting experiments using unrelated cell types, such as tumor cells or embryonic stem cells. These experiments point out the importance of β_1 integrin, a member of a family of transmembrane proteins that assemble in heterodimers and serve as major binding partners for extracellular matrix components, but their relevance to melanoblast migration is unclear. Support for the importance of the extracellular matrix in melanoblast migration comes from genetic observations on the role of proteases known to remodel the extracellular matrix. The mouse coat color mutation *belted*, for instance, is due to a mutation in the metalloprotease gene *Adamts20*. This gene is homologous to *Gon-1* of *Caenorhabditis elegans*, which encodes a metalloprotease required for cell migration. In summary, the regulation of cell migration involves a variety of partners that all collaborate to ensure that the cells end up on the right tracks and in the right locations.

Melanoblast Differentiation

Melanoblast differentiation, characterized by melanization and the acquisition of a dendritic phenotype, relies heavily on MITF, whose targets include many of the genes involved in melanin biosynthesis (such as the previously mentioned *Dct*, tyrosinase-related protein-1, and tyrosinase). A common *cis* element in the promoters of these genes is the so-called M-box, which in its core contains an Ephrussi (E) box of the sequence 5'CATGTG3'. E boxes are widespread in the genome and bound by a wide array of basic–leucine zipper, basic–helix–loop–helix, and basic–helix–loop–helix–leucine zipper transcription factors. Although flanking sequences influence the efficiency with which specific factors bind a given E box, they are insufficient to explain the restriction of the melanogenic genes to the melanocyte lineage. Again, it is likely the combination of several distinct transcription factors that is critical for this regulation. As alluded to previously, the activity of MITF is also regulated by post-translational modifications such as phosphorylation. Moreover, it has been described that MITF is dynamically modified by small ubiquitin-like modifier (SUMO) side chains, which are proteins of approximately 100 residues that are added to two lysines of MITF. Sumoylation, as the modification is called, reduces MITF's activity on promoters with multiple E boxes, but not on those with single E boxes, and thus may influence target gene specificity. Furthermore, both *in vitro* and *in vivo* tests show that some melanogenic genes are stimulated not only by MITF but also directly by SOX10, a regulator of MITF, suggesting the existence of feed-forward loops between SOX10 and MITF target genes. By regulating genes involved in the dynamics of the actin cytoskeleton, MITF also modulates cell shape. Nevertheless, although MITF is expressed at high levels in melanoblasts as they differentiate into melanocytes, it soon fades away in most differentiated cells; thus, it is conceivable that maintenance transcription of melanogenic genes in mature melanocytes, and possibly the maintenance of cell shape, is controlled by other E-box binding factors expressed in mature melanocytes. Without functional MITF, however, melanocytes can never mature and become pigmented, but when they remain unpigmented because they have mutations in the melanin biosynthetic genes, they stay alive and thrive.

Adult Melanocyte Stem Cells

Tissue injury, be it the result of physical or metabolic processes, is a common feature of life, and hence the mechanisms of tissue repair are of great importance for the maintenance of health. Much excitement has

been created by the recognition that adult mammals contain stem cells capable of regenerating lesioned tissues. Stem cells are usually defined as undifferentiated cells capable of self-renewal and of generating a variety of differentiated cells. Many stem cells reside in a particular compartment, called the niche, that provides the necessary environment for their maintenance. An example of such a niche is the bulge region of hair follicles which lies between the hair bulb and the sebaceous gland. As the hair goes through its cycles of loss and regrowth (technically, the phases are know as catagen, telogen, and anagen), multipotent stem cells from the bulge replenish all types of epithelial cells of the hair structure. A particular slow-cycling cell, marked in mouse transgenic studies by the previously mentioned *lacZ* reporter under the control of the *Dct* promoter, is capable of self-renewal and returning to a quiescent stage; of populating neighboring, vacant bulges; and of giving rise to melanocytes. These cells share many features with developmental melanoblasts but have been dubbed melanocyte stem cells. However, the usual stem cell feature, multipotentiality, has not been shown to be among their traits, and their relationship to multipotent stem cells in the niche is unclear.

Major questions in stem cell biology concern the mechanisms of stem cell maintenance and the distinction between self-renewal and differentiation. Interestingly, several of the signaling pathways and transcription factors mentioned previously in the context of melanoblast development are also involved in melanocyte stem cell regulation. For instance, Notch signaling is required for the maintenance of melanocyte stem cells in the hair follicle, and PAX3, SOX10, and MITF, together with WNT signaling, are involved in regulating differentiation. Whereas SOX10 and MITF collaborate to stimulate *Dct* transcription, PAX3 represses *Dct* transcription. It does so by recruiting the Groucho corepressor GRG4 and by interacting with LEF/TCF, whose binding to the *Dct* promoter competes with MITF binding, resulting in low *Dct* expression. What can flip the switch is extracellular signaling, particularly the activation of the canonical WNT signaling pathway and its concomitant β-catenin activation. Activated β-catenin interferes with the binding of PAX3 to LEF/TCF and GRG4 and dislodges PAX3 from the *Dct* promoter. This relieves repression of the *Dct* promoter and frees PAX3 to stimulate MITF expression to higher levels, which in turn activates the *Dct* promoter and sends the cells on the way to differentiation. Of course, this process is not allowed to act indiscriminately in all melanocyte stem cells, lest there would be no stem cell left for the next hair cycle. How some cells of the niche escape the effects of WNT signaling is not yet known, however. Intriguingly, premature differentiation and concomitant loss of stem cells seem to be a feature of a mutant allele of *Mitf*, $Mitf^{vit}$, which leads to premature graying. This and other mutants in MITF and in other factors underscore the importance of the mentioned pathways not only for development but also for tissue regeneration.

In summary, the biology of melanocytes and their regulation from birth to death has provided us with many mechanistic insights that are not readily attainable in other systems. Melanocytes, in fact, are highly suitable to elucidate basic principles of cell lineage determination in the neural crest.

See also: Macroglial Lineages; Neural Crest; Neural Crest Cell Diversification and Specification: ErbB Role; Neural Crest Diversification and Specification: Transcriptional Control of Schwann Cell Differentiation; Neural Stem Cells: Adult Neurogenesis.

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Autonomic Nervous System Development

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Origin of the Autonomic Nervous System from the Neural Crest

All neurons and glia of the autonomic nervous system – the sympathetic, parasympathetic, and enteric nervous systems – are derived from the neural crest (NC). The NC, a migratory cell population unique to vertebrates, arises during early embryogenesis, becoming microscopically distinguishable in a rostral-to-caudal wave in human embryos approximately from embryonic (E) days E18 to E40, in mice from E8.5 to E11, and in chicks from E1.3 to E3.5. The induction of the NC has been elucidated by, among others, Marianne Bronner-Fraser's group (bird embryos) and Robert Mayor's group (frog embryos). This cell population is induced by earlier signals from neighboring tissues, and it consists of epithelial cells at the border between the medial neural ectoderm, which gives rise to the central nervous system (CNS), and the more lateral epidermal ectoderm, which contributes to the skin. This involves signaling via secreted growth factors: fibroblast growth factors (FGFs), Wnts, and transforming growth factor (TGF)-β family members, especially the bone morphogenetic proteins (BMPs). This sets up a pattern of gene activity that specifies the NC cells as different from the ectodermal cells on each side. This involves expression in the nascent NC cells of genes such as *Pax7* and *Zic3* (in mice) for transcription factors (which control the expression of other genes). In a positive feedback, the gene for the inducer BMP-4 is also activated in the NC. This is rapidly followed by the transient expression of transcription factor genes such as *Snail1* (in mice; *SNAI2/SLUG* in birds and reptiles), the forkhead gene *Foxd3* (mice), and the Sox group E genes (in mice, *Sox8*, *Sox9*, and *Sox10*).

The transcription factors encoded by these genes promote the onset of cell migration, a classic example of the epithelial–mesenchymal transition. NC cells lose the homophilic cell–cell adhesion molecule N-cadherin first at translational then at transcriptional levels. This is accompanied by reorganization of the cytoskeleton into a motile form and by the ability to adhere to and also digest extracellular matrix involving, among others, expression of genes for RhoA (actin modulator), integrin (matrix adhesion molecule), and matrix metalloproteases (MMPs). This allows the formerly coherent epithelial cells to assume a quasi-individualistic migratory behavior.

The fate of the NC cells after migration has been demonstrated in most detail by Nicole Le Douarin and colleagues using chick–quail orthotopic transplantation as a device for labeling and tracking cells. These form not only the peripheral autonomic nervous system, but also many other neural and related endocrine cells, and even connective tissues. These fates can be mapped to position of origin along the neuraxis (**Figure 1**). The differentiation competence of NC cells has been tested by heterotopic transplantation. These experiments indicate that competence is broader than the fate, and the particular lines of differentiation are controlled by the tissues with which the NC cells interact. However, superimposed on this are also some early-imposed restrictions on differentiation. More rostrally originating cells have a wider range of options; this is epitomized by the restriction of connective tissue competence to cranial NC cells. This early spatial restriction also extends to some autonomic competences such as enteric neurogenesis capacity. These cells can be elicited when NC cells are combined with intestinal tissues, but only cranial NC cells are competent to produce large numbers of neurons. In contrast, sympathetic neurons can differentiate from all of the NC, including cranial levels that are not fated to do so.

The nascent NC cells, like the forming CNS, express multiple homeobox genes in a nested rostro-to-caudal pattern. There are four clusters of these genes (A, B, C, and D), each cluster being variously numbered (from 1 to 13). The equivalently numbered genes of each cluster are similar and tend to be co-expressed. The most rostral and earliest formed neural tissues including NC, at forebrain and midbrain levels, express no homeobox genes. Progressively higher number homeobox genes are expressed in the NC at progressively more caudal levels. Thus, *HOXB1* is expressed maximally at hindbrain levels rostral to the ear, *HOXB4* in the hindbrain caudal to the ear, and *HOXB9* at the level of the neck. A combinatorial code of homeobox gene expression is important in specifying an early positional memory that influences later differentiation processes. It is possible that the rostral-to-caudal patterned restrictions on later differentiation in early NC cells, noted earlier, are regulated by the homeobox code.

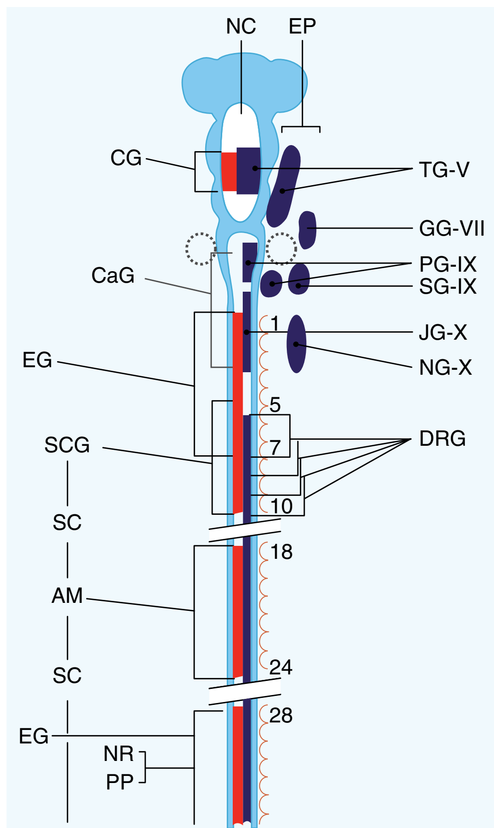

Figure 1 Scheme of neural crest (NC) derivatives mapped onto the neuraxis (light blue). Autonomic nervous system derivatives (red) include ciliary ganglion (CG), cardiac ganglion (CaG), enteric ganglia (EG), superior cervical ganglia (SCG), sympathetic ganglia (SC), nerve of Remak (NR; avian only), pelvic ganglia (PP), and adrenomedullary (AM) gland ganglia. Related sensory neural structures (dark blue), dorsal root sensory ganglia (DRG), and epibranchial placodes (EP) give rise to cranial sensory ganglia. The somites are numbered, and the otic (ear) vesicle position is circled. Adapted from Newgreen DF and Erickson CA (1986) The migration of neural crest cells. *International Review of Cytology* 103: 89–145.

Migratory Morphogenesis of Neural Crest Cells

The outstanding characteristic of NC cells is their migratory morphogenesis. The sympathetic and parasympathetic cells require a migration of only several 100 μms from their NC origin, and in mouse and chick embryos this takes about half a day. Trunk NC cells that form the sympathetic system migrate in lateroventral chains through the mesenchymal cell mass of the rostral half of the adjacent sclerotome (a subregion of the somitic body segments) to reach the dorsal aorta (**Figure 2**). Recent live cell imaging *in vivo* has shown that these cells then turn rostrally and caudally parallel to the aorta, so that cells from one segmental level become distributed over four or more segments.

The enteric nervous system is by far largest division of the autonomic nervous system (ANS) in cell numbers and is much more widely distributed. Yet it arises from more restricted NC regions, mostly stemming from the vagal neuraxis (in the caudal hindbrain and slightly overlapping the trunk) with a numerically minor component from lumbosacral levels (**Figure 1**). The vagal cells migrate over and through the vagal-level somites to the nearby foregut (esophageal, gastric, and duodenal primordia), then migrate through the dense gut mesenchyme of the midgut (future small intestine and cecum) and hindgut (future colon). This colonization takes about 4 weeks in humans and 4–5 days in mouse and chick embryos. Live cell imaging from Heather Young and Miles Epstein and their colleagues has shown that these cells also migrate as dynamic chains (**Figure 3**). Biomathematical modeling and experimental studies by Kerry Landman and co-workers have indicated that proliferation in the NC wavefront is a major driver of colonization of the gastrointestinal tract. However, completion of colonization to the distal colon is made more difficult, since the gut is simultaneously elongating. Failure to complete colonization leads to Hirschsprung's disease, in which the distal gut lacks neural ganglia, cannot perform peristalsis, and, after birth, becomes massively distended with fecal contents proximal to the aganglionic region. Mutations in many genes predispose to neural dysplasias of the intestine, and many of these defects are Hirschsprung-like (**Table 1**). Many of these genes control NC cell numbers by affecting cell proliferation or survival; this may lead to an apparent migration defect.

Molecular Control of Migration

Migration routes of NC cells are controlled by receptor-mediated interactions with their surroundings, including extracellular matrix molecules, growth factors, and molecules on the surface of non-NC cells. Initial migration is into extracellular matrix that forms a migratory substrate. Important matrix molecules include fibronectin, laminins, and collagens, for which NC cells possess multiple integrin class adhesion and signaling receptors. Treatment with peptides and antibodies that block matrix interactions can cause NC cell migration to stall *in vitro* and *in vivo*. Chemoattraction of NC cells may also occur: the glial cell line-derived neurotrophic factor (GDNF) appears to attract enteric NC cells in *in vitro* tests, and is produced by intestinal mesenchyme cells (**Figure 4**). However, analysis is complicated, because growth factors often have several functions; in this

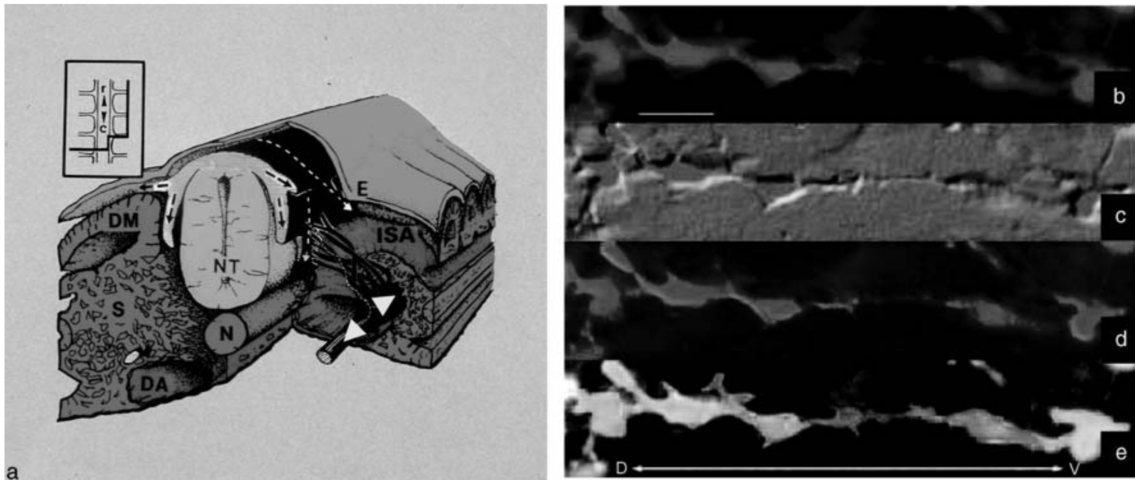

Figure 2 (a) Cutaway scheme of neural crest cells migrating as chains through the rostral sclerotome (S) to the dorsal aorta (DA), where they turn rostrally and caudally (arrows; see inset: r, rostral; c, caudal). Other areas depicted are the demomyotome (DM), epidermal ectoderm (E), intersegmental artery (ISA), notochord (N), and neural tube (NT). (b–e) Single neural crest cell chain, extending from neural tube to dorsal aorta. (b) Green fluorescent protein-labeled neural crest cells. Scale bar = 20 μm. (c) Embossed image of chain. (d) Fluorescence and embossed overlay. (e) Individual cells within chain colored separately; D↔V, dorsal to ventral. (b–e) From Kasemeier-Kulesa JC, Kulesa PM, and Lefcort F (2005) Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia. *Development* 132: 235–245.

example, as well as a chemotactic role, GDNF is a required survival factor, a potent mitogen, and a differentiation driver for NC cells possessing its receptor, Ret (the product of *ret proto-oncogene*).

Negative regulators of migration are of equal importance to positive regulators. These molecules repel NC cells from regions such as the perinotochordal zone, the caudal half of the somites, parts of the gut mesenchyme, and elsewhere. These repulsive molecules include ephrinB1 (receptor on NC cells: EphB3), semaphorins 3A and 3F (receptors: neuropilins 1/2), and slit 2 (receptor: Robo), the large chondroitin sulfate proteoglycan aggrecan, the glycoprotein tenascin-C, and the growth factor sonic hedgehog (Shh).

Migrating NC cells contact each other, and these contacts are important for migration and directional choice. Isolated NC cells show little translocation. NC cells at the forefront of migration, and at certain 'decision points,' extend filopodia simultaneously in many directions, and move erratically, but NC cells in contact in chains move more consistently. Major cell–cell adhesion molecules N-cadherin and N-CAM are reduced on NC cells in the migratory phase, but lower affinity cadherins may maintain the transient adhesions observed. Interference with the cell adhesion molecule L1CAM on mouse NC cells disrupts the chainlike connections of these cells in the intestine and delays migration. Interestingly, *L1CAM* is a modifier gene for Hirschsprung's disease genes (see **Table 1**), and since it is located on the X chromosome, it may contribute to the 4:1 male:female ratio of Hirschsprung's disease.

Gangliogenesis

Less is known about gangliogenesis in the ANS than about the process of migration, but upregulation of the homophilic cell adhesion molecules N-cadherin and N-CAM often occurs, and genetic disruption of these systems leads to less compact aggregates. In the forming of sympathetic ganglia, initially hemisegmental NC cells migrate longitudinally across segmental boundaries to form a relatively uniform chain (**Figure 2(a)**). This chain then, via N-cadherin upregulation, forms a string of aggregates. These aggregates assemble at the anterior half of each hemisegment, and this is based on avoidance of repulsive interactions with ephrinB1 that is increasingly expressed in the alternate posterior hemisegment.

In the enteric nervous system the ganglia are uniformly small and regularly spaced in two dimensions, in two layers in the gut mesenchyme. These ganglionated plexuses form at a distance from the gut endoderm. When Shh expression is reduced, neurons are found closer to the endoderm, suggesting that Shh is involved in establishing a domain that excludes NC-derived cells. The cell behavior in ganglion formation, visualized by time-lapse microscopy, suggests that the uniformity of size and spacing could result from a balance between contact-mediated NC cell cohesion and repulsion-at-a-distance between

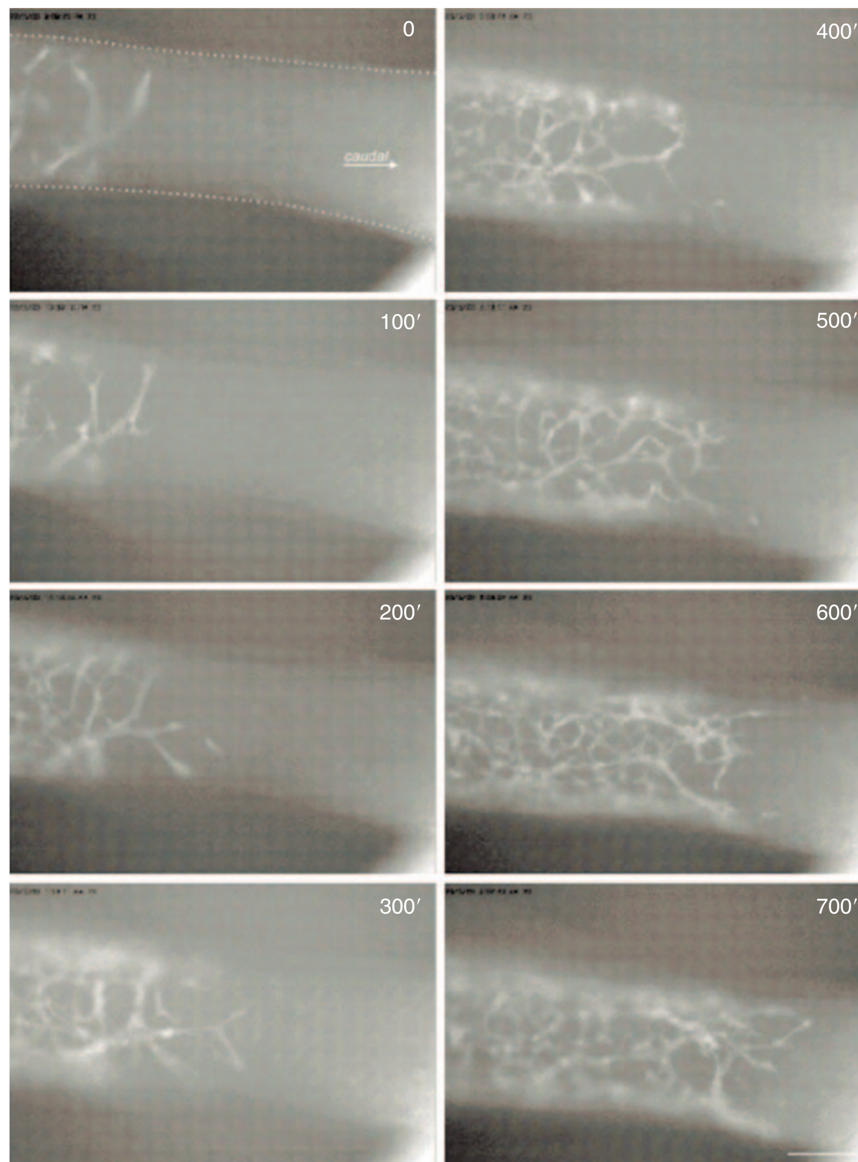

Figure 3 Selected time-lapse frames of the hindgut of an E12.5 mouse showing the caudal progression of green fluorescent protein-expressing neural crest cells. Time is noted in minutes. Most of the cells are present in intersecting chains that follow a variety of trajectories. Scale bar = 100 μm. From Young HM, Bergner AJ, Anderson RB, et al. (2004) Dynamics of neural crest-derived cell migration in the embryonic mouse gut. *Developmental Biology* 270: 455–473.

like cells. However, the molecules responsible for this reorganization are not yet known.

Sympathetic Ganglia: Neuronal Differentiation and Connections

The sympathetic chain ganglia, derived from trunk NC (**Figure 1**), innervate all of the organs, smooth muscle, skeletal muscle, and glands, and serve to modulate their function. Innervation proceeds in a rostral-to-caudal pattern, with more rostral neurons innervating rostral structures and caudal neurons innervating caudal organs.

The preganglionic motor neurons that innervate sympathetic chain ganglia arise at thoracic levels of the spinal cord. Their axons leave the spinal cord via the ventral roots and project both rostrally and caudally. The rostral-to-caudal projection pattern is determined by the location of the cell bodies along the neural axis. Several factors contribute to motor column-specific motor neuron identity as well as generation of a segment-specific projection pattern. The generation of neurons in the Column of Terni (designation for preganglionic motor neurons in the chick) depends upon loss of expression of the homeodomain

Table 1 Genes implicated in enteric nervous system formation and dysplasias

Gene	*Human chromosomal location*	*Phenotype of ENS in mice in which the gene is homozygously inactivated*
RET	10q11.2	Absence of neurons from small and large intestines
GDNF	5p12-p13.1	Absence of neurons from small and large intestines
GFRAI	10q25	Absence of neurons from small and large intestines
EDNRB	13q22	Absence of neurons from distalmost large intestines
EDN3	20q13.2-q13.3	Absence of neurons from distalmost large intestines
ECE1	1p36.1	Absence of neurons from distalmost large intestines
PHOX2B	4p12	Absence of neurons from entire gastrointestinal tract
SOX10	22q13	Absence of neurons from entire gastrointestinal tract
PAX3	2q37	Absence of neurons from small and large intestines
ASCL1	12q22-q23	Absence of neurons from esophagus
IHH	2q33-q35	Absence of neurons from parts of the small intestine and colon
SHH	7q36	Ectopic neurons within mucosa
ZEB2 (*SIP1*)	2q22	Absence of neurons from distalmost large intestine
TLX2	2p13.1	ENS hyperplasia in colon and hypoplasia in small intestine

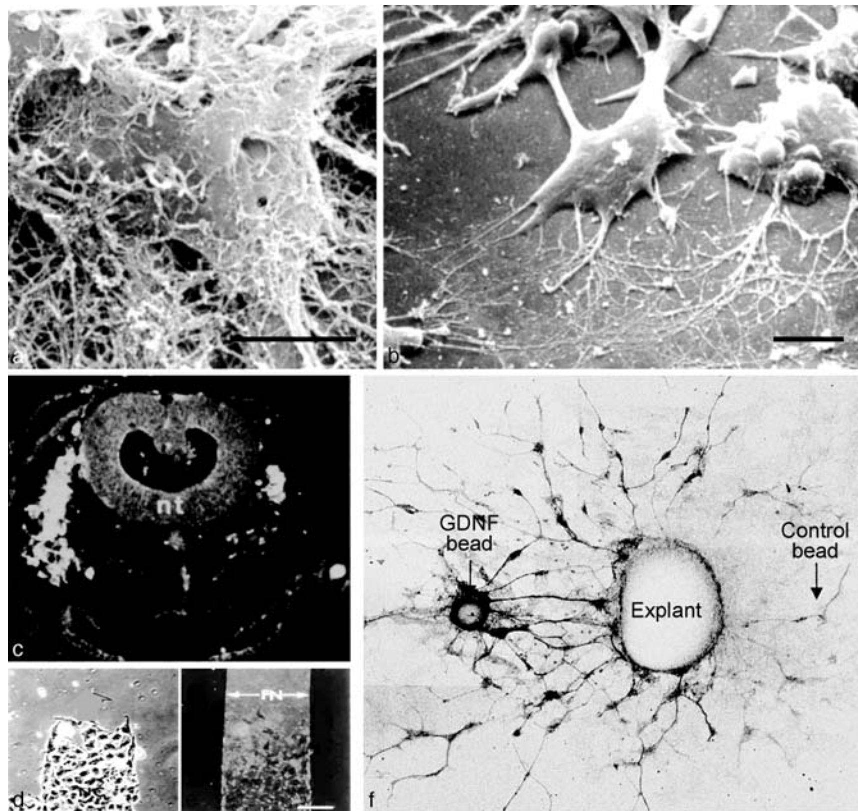

Figure 4 The microenvironment influences neural crest cell migration. (a) Scanning electron micrograph of neural crest cells *in vivo*, migrating in a fibrillar fibronectin extracellular matrix. (b) Fibrillar fibronectin strongly promotes neural crest cell migration in *in vitro* assays. (c) Transverse section of the head of a chick embryo injected on the right side (*) with a function-blocking antibody to the fibronectin receptor. The neural tube (nt) is labeled. Neural crest outgrowth (white area) labeled with the HNK-1 antibody to avian neural crest cells is reduced on the injected side. (d, e) Neural crest (NC) cells migrating *in vitro* (d, arrow) accurately follow stripes of fibronectin (FN); (e) shows same field shown in (d), but is immunolabeled (red) for fibronectin. (f) Enteric neural crest cells labeled with PGP9.5 antibody migrate *in vitro* from a gut segment explanted onto a collagen gel. This migration is strongly biased toward a bead loaded with glial-derived neuronal factor (GDNF), compared to migration toward a control bead. Scale bars = 20 μm (a), 20 μm (b), 50 μm (c). (a) From Newgreen DF (1985) Control of the timing of commencement of migration of embryonic neural crest cells. *Experimental Biology and Medicine* 10: 209–221; (c) from Bronner-Fraser M (1985) Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *Journal of Cell Biology* 101: 610–617; (f) adapted from Young HM, Hearn CJ, Farlie PG, et al. (2001) GDNF is a chemoattractant for enteric neural cells. *Developmental Biology* 229: 503–516.

DNA-binding proteins MNR2, Lim3, and HB9. Few molecular markers that distinguish visceral from somatic motor neurons have been identified, but expression of BMP-5 appears to be specific for neurons in the Column of Terni. Although mechanisms underlying the segment-specific projection pattern observed in sympathetic preganglionic fibers are not completely understood, it appears that soluble signals present in the somitic mesoderm contribute to segment-specific identity. Correct patterning is necessary in order to ensure that preganglionic input is received by the appropriate postganglionic neurons. Following early phases of development, preganglionic inputs are necessary for regulation of neurotransmitter biosynthesis in postganglionic neurons.

Postganglionic sympathetic neurons are located in three anatomically distinct sets of ganglia. The majority of the principal sympathetic neurons are located in the paravertebral ganglia that are bilaterally distributed along each side of the spinal cord. The prevertebral ganglia are located at the midline; these cells lie anterior (ventral) to the dorsal aorta. The previsceral (terminal) ganglia, in a pattern more similar to parasympathetic ganglia, are situated in close proximity to several organs in the pelvis, mainly the bladder and rectum. Interestingly, although noradrenaline (norepinephrine) is the major neurotransmitter utilized by principal sympathetic ganglion neurons, similar to that found in the enteric nervous system, there is chemical coding of the neurotransmitters and neuropeptides expressed by sympathetic ganglion neurons. This chemical template is dependent upon the ganglia in which the cell bodies reside and the identity of their target. Neurons that innervate sweat glands (sudomotor) and the periosteum of bone are cholinergic; these represent 10–15% of sympathetic ganglion neurons. There is now general agreement that for some rather specialized neurons that neurotransmitter identity is plastic and changes with development once target innervation is complete. In addition to the now classic case of the rodent footpad, where the neurons are initially noradrenergic and become cholinergic in response to target-derived factors, it appears that the expression of neuropeptides in the prevertebral and previsceral ganglia also depends upon target for their expression. Neuropeptides, including neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP), have been co-localized primarily with noradrenaline (NPY) and acetylcholine (VIP), and additional molecules such as substance P, dopamine, and serotonin have been found co-localized with these neurotransmitters in neurons of the previsceral and prevertebral chains.

Choice of neurotransmitter is an attribute that is acquired early in development and is dependent upon instructive cues encountered by the NC-derived precursor cells during their migration from the neural tube, as well as at sites along the dorsal aorta where these cells segregate into ganglia (**Figure 5**). There are two sources of instructive soluble signals required for appropriate migration and differentiation of NC-derived precursor cells as sympathetic ganglion neurons. The neural tube synthesizes and secretes an as yet unidentified member of the TGF-β family that is necessary for migration and differentiation as sympathetic neurons. BMP is a second required instructive factor synthesized and secreted by the dorsal aorta. BMP is an essential determinant of the noradrenergic phenotype. BMP is a proximal signal that induces expression of a network of DNA-binding proteins required for both neurogenesis and cell type-specific expression of noradrenergic marker genes. The core network of DNA-binding proteins that support differentiation of NC-derived precursor cells as sympathetic ganglion neurons includes the homeodomain (HD) proteins Phox2b and Phox2a, the basic helix–loop-helix (bHLH) DNA-binding proteins achaete-scute homolog 1 (MASH1 in mouse, CASH1 in chick), and HAND2, and the zinc finger protein GATA3 (GATA2 in chick). These proteins function together for both cell determination and differentiation; it is common to find that bHLH and HD proteins function together in networks of cross-regulated DNA-binding proteins. An essential function for both Phox2b and HAND2 has been demonstrated by gene knockout in mice. Deletion of Phox2b results in loss of autonomic neurons in each branch of the ANS; this DNA-binding protein is a master regulator of specification for the ANS. Deletion of HAND2 results in loss of sympathetic ganglion neurons with no apparent effect on parasympathetic ganglion neurons. Interestingly, loss of HAND2 affects migration and differentiation of NC-derived precursors that will contribute to the enteric nervous system. The differential effects on HAND2 in the generation of sympathetic, compared to enteric, neurons suggests that additional instructive cues necessary for specification of neurotransmitter phenotypic characteristics remain to be elucidated. The roles of various molecules in sympathetic, parasympathetic, enteric, and sensory neuron differentiation are illustrated in **Figure 6**.

Although neurotransmitter specification and expression are early developmental events, this aspect of phenotypic choice and expression is remarkably plastic. The adult neurotransmitter phenotype for some neurons is not achieved prenatally. Sympathetic ganglion neurons innervating the eccrine sweat glands and periosteum are noradrenergic prior to birth. Expression of noradrenergic characteristics does not depend upon target innervation; this sets

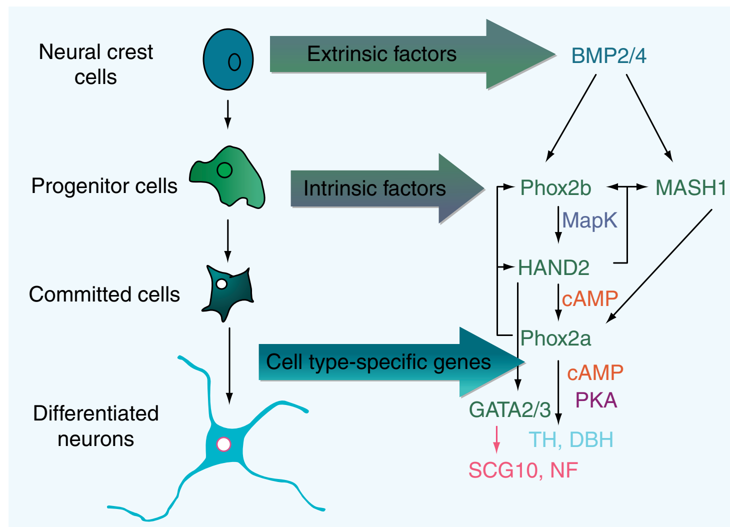

Figure 5 Specification and differentiation of peripheral autonomic neurons are dependent upon the interplay between cell extrinsic and cell intrinsic factors. Initial instructive cues from the neural tube influence neural crest cells that then respond to bone morphogenetic proteins (BMP2/4) derived from the dorsa aorta or retro-orbital mesenchyme. Induction of paired-like homeobox 2b (Phox2b) and mammalian achaete-scute homolog 1 (MASH1) proteins is followed by the induction of heart and neural crest derivatives-expressed protein 2 (HAND2) and Phox2a, resulting in expression of genes encoding pan-neuronal proteins (SCG10, superior cervical ganglion-10 protein (also called stathmin-like 2 protein); NF, neurofilament protein) and cell type-specific proteins (TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; choline acetyltransferase and vesicular acetylcholine transporter, not shown). The recognized transcription factors required for the differentiation of noradrenergic sympathetic and cholinergic parasympathetic neurons are the same, with the notable exception of HAND2. Consequently, it is possible that this diversity could be in part the result of exclusive expression of HAND2 in precursors of noradrenergic sympathetic ganglion neurons. Cross-regulation of transcription factor expression suggests patterns of regulation based on generation of local gradients. MapK, mitogen-activated protein kinase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; GATA2/3, GATA transcription factors 2 and 3. From Howard MJ (2005) Mechanisms and perspectives on differentiation of autonomic neurons. *Developmental Biology* 277: 271–286.

these neurons apart from other cholinergic neurons whose neurotransmitter phenotype is determined early in development and does not depend upon instructive target-derived signals. Interestingly, in the adult, the neurotransmitter phenotype of these neurons is altered following target innervation. This switch in neurotransmitter expression from noradrenergic to cholinergic depends upon retrograde transport of a target-derived factor that affects expression of a number of genes; the cholinergic differentiation factor has not been definitively identified, but is likely cardiotrophin 1. In addition to expression of choline acetyltransferase (ChAT), these neurons also express the vesicular acetylcholine transporter (VAChT), the choline transporter (ChT), as well as VIP. The expression of each of these molecules can be increased (induced) in sympathetic ganglion neurons by a variety of cytokines signaling through gp130. Expression of ChAT and VAChT is coordinately regulated and these two gene products comprise the cholinergic gene locus. Expression of both the cholinergic gene locus and VIP is regulated, in part, by the zinc finger DNA-binding protein REST. Although expression of ChAT, VAChT, ChT, and VIP appear to increase in parallel, relatively little is known about the underlying transcriptional mediators or how expression of these genes is coordinately regulated.

As development proceeds, sympathetic ganglion neurons become dependent upon nerve growth factor (NGF) for their maturation and survival. Precursor cells and young neurons express the neurotrophin receptor TrkC and have some dependence upon neurotrophin-3 (NT-3) for their survival. Mature postmitotic sympathetic ganglion neurons express TrkA and later acquire dependence upon NGF. These trophic factors are target derived and are retrogradely transported to the neuron somata, where they affect many cellular processes involved in cell death, cell survival, and maturation. Adult sympathetic ganglion neurons depend upon both NGF and NT-3. Together these trophic factors affect neuron survival. In addition, NGF influences axon targeting, the size and extent of dendritic arbors, and expression of the biosynthetic enzymes required for synthesis of noradrenaline. A little appreciated aspect of aging, dependent in part upon NGF and NT-3, is a decrease in levels of noradrenaline as well as a decrease in the innervation of many sympathetic ganglion target tissues, including heart, spleen, and cerebral blood vessels. Although not well understood, it appears that there are

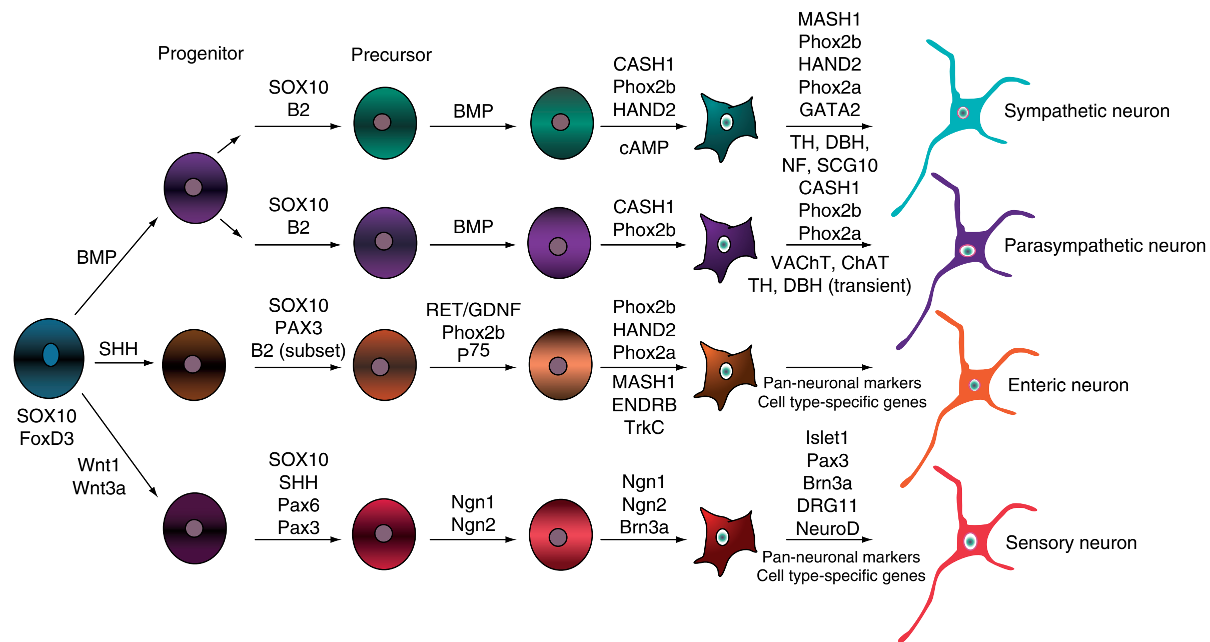

Figure 6 Analyses *in vitro* and *in vivo* have identified a series of growth factors and transcriptional regulators (markers) affecting different stages of neurogenesis of neural crest-derived progenitor cells. This schematic diagram summarizes work from many laboratories and includes some information not explicitly described in the body of the text. The compiled data provide a roadmap for following important hallmark events in the development of autonomic, enteric, and sensory neurons. Neural crest cells segregated from the neuroepithelium can be identified by the expression of FoxD3 and Sox10 (among other markers); cells expressing FoxD3 give rise to neurons and not melanocytes. Sox10 maintains multipotency in neural crest-derived cells as well as neurogenic potential. In the enteric nervous system, Sox10 and Pax3 together regulate Ret, which is required for normal development of these neurons. Progenitor cells differentiate into sympathetic, parasympathetic, enteric, or sensory neurons, in part dependent upon instructive signals encountered early at or near the time of egress from the neural tube. Additionally, extrinsic cues encountered during migration or at sites where neural crest-derived cells differentiate influence patterns of gene expression. In autonomic ganglia, expression of HAND2 appears to select cells as noradrenergic sympathetic ganglion neurons, as well as functioning in cell type-specific gene expression. In the sensory neuron lineage, the POU domain transcription factor Brn3a, expressed downstream of neurogenins 1 and 2 (Ngn1, Ngn2), regulates a large array of genes influencing cell death, neurotransmitter expression, and axon guidance. The signaling molecule sonic hedgehog is necessary for the expression of neurogenin. In the enteric nervous system, HAND2 is expressed downstream of Phox2b in all segments of the developing gut; the function of HAND2 in development of enteric neurons is unknown. The neurotrophin receptor TrkC is expressed early by neural crest-derived cells, the potential of which is restricted to neuronal or glial lineages as well as a subset of enteric neurons. From Howard MJ (2005) Mechanisms and perspectives on differentiation of autonomic neurons. *Developmental Biology* 277: 271–286.

alterations in the transcriptional and translational regulation of expression of both NGF and NT-3 coincident with the changes in function and survival of sympathetic ganglion neurons with increased age.

Parasympathetic Ganglia: Neuronal Differentiation and Connections

Of the components of the ANS, the least is known about parasympathetic ganglia because they are small, diffusely structured ganglia embedded within the tissues that they innervate (**Figure 7**). Parasympathetic ganglia are derived from the NC; however, the detailed axial origins of many of the ganglia are not known. The cranial parasympathetic ganglia, including the ciliary, lacrimal, otic, and sphenopalatine, arise from the cranial NC rostral to the otic (ear) placode. Caudal to this, the cardiac ganglion originates from the NC near the otic placode to the third somite, overlapping the region giving rise to the enteric nervous system (see later). Most of the trunk NC contributes the sympathetic ganglia (as previously mentioned), but the lumbosacral NC also gives rise to the parasympathetic pelvic ganglia and, in birds, the ganglion of Remak near the hindgut that is thought to be an extension of the pelvic ganglia (these origins are diagrammed in **Figure 1**).

The specification and differentiation of parasympathetic ganglia have many pathways in common with sympathetic ganglia (see **Figure 6**): both rely on signaling by BMP and require the expression of the transcription factors MASH1, Phox2a, and Phox2b. Specification of the cholinergic phenotype typical of parasympathetic neurons may occur prior to migration and is reinforced by suppression of the

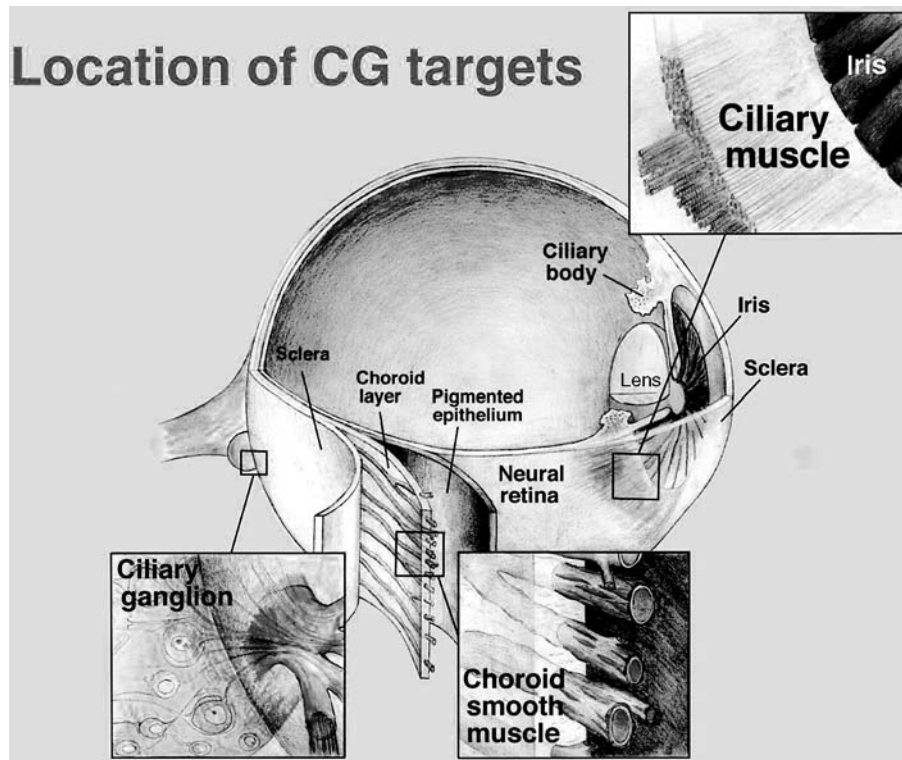

Figure 7 The ciliary ganglion (CG) and its targets of innervation, illustrating the relatively close relationship between parasympathetic ganglia and their innervation targets. The schematic shows the location of the ciliary ganglion and its target tissues, the vascular smooth muscle of the choroid layer, and the striated muscle of the iris and ciliary body. The enlargements show the details of the histological structures of the tissues. The ciliary ganglion contains small, unmyelinated neurons that innervate the vascular smooth muscle (choroid neurons) and large, myelinated neurons that innervate the iris and ciliary muscle (ciliary neurons).

transcription factor HAND2 (dHAND), which is necessary for the expression of catecholamines. In the examples that have been best studied (ciliary ganglion, cardiac ganglion, and submandibular ganglion), the precursors of parasympathetic ganglia migrate to their targets of innervation prior to the onset of organogenesis of the particular organ. As morphogenesis commences, neuronal differentiation occurs. For example, as the salivary gland epithelium branches and forms ducts, many of the neurons associated at the base of the organ rudiment are already postmitotic and extend axons as epithelial buds form (**Figure 8**). Likewise, cardiac ganglion neurons undergo neurogenesis within the developing heart, and ciliary ganglion neurons elaborate axons as the optic vesicle forms the eye and its associated structures.

This intimate differentiation of parasympathetic neurons along with their targets of innervation suggests that trophic interactions reciprocally guide development. In fact, the expression of GDNF and the closely related molecule neurturin in target tissues is essential for the differentiation and development of a number of parasympathetic ganglia. These include ciliary, otic, sphenopalatine, submandibular, lacrimal, penile, and pancreatic islet neurons. In many instances, differentiation of parasympathetic ganglia appears to require sequential action of GDNF, followed by neurturin.

By far the best-studied parasympathetic ganglion is the avian ciliary ganglion (see **Figure 7**). This ganglion contains two populations of principal neurons: ciliary neurons, which innervate the iris and ciliary muscle, and choroid neurons, which innervate the arterial smooth muscle in the choroid layer. Both populations differentiate, innervate their respective target tissues, and undergo programmed cell death in midgestation (between E6 and E14 of chick development). Target tissues control the degree of programmed cell death that occurs as well as other aspects of neuronal differentiation, such as the expression of the neuromodulatory neuropeptide somatostatin, and the expression of ion channels, such as the calcium-activated potassium channel and the nicotinic acetylcholine receptor (**Table 2**). Afferents from the accessory oculomotor nucleus of the midbrain also regulate differentiation

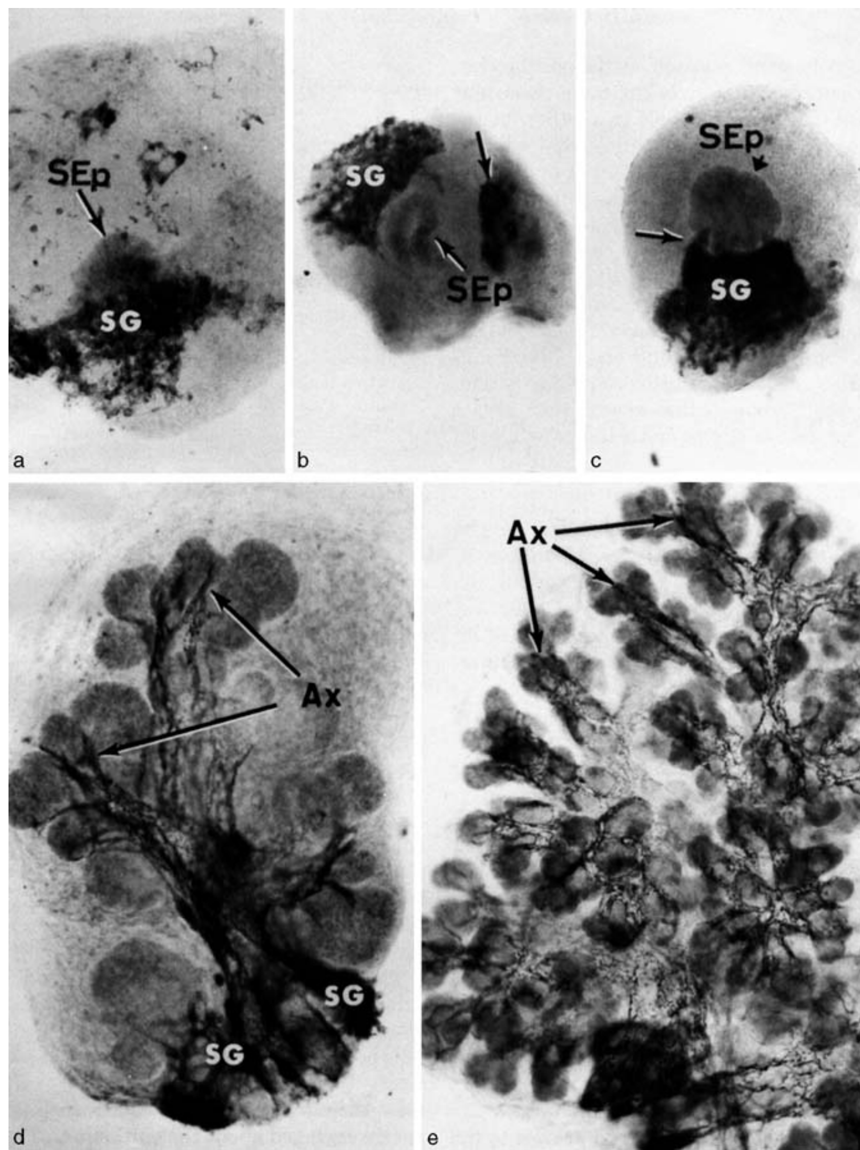

Figure 8 Early development of parasympathetic nerves matches the morphogenesis of the target, shown in whole mounts of the submandibular salivary gland stained for acetylcholinesterase activity. (a) At E12, the salivary gland epithelial bud (SEp) is surrounded by darkly staining salivary ganglion cells (SG); (b) E12, looking down on the preparation shown in a; (c) early E13, when the lobule of the SEp has just begun to form clefts (arrowhead) and axons begin to extend into the epithelium (arrow); (d) E14 gland; the epithelium has grown and has begun to branch extensively, and axons (Ax) course over the epithelium and travel in the clefts between the buds; (e) a portion of an E15 gland; axon outgrowth continues to parallel the growth of the epithelium. From Coughlin MD (1975) Early development of parasympathetic nerves in the mouse submandibular gland. *Developmental Biology* 43: 123–139.

of the ciliary ganglion neurons. These inputs influence cell death and differentiation through nicotinic cholinergic activation of ciliary neurons. Recently, nicotinic activation has been tied to the expression of a chloride transporter, which changes the magnitude of the chloride gradient across the plasma membrane, thereby influencing γ-aminobutyric acid (GABA)ergic signaling in neurons as well as axonal morphology. Whether the principles of development uncovered by studies of the ciliary ganglion apply to other parasympathetic ganglia is a matter of ongoing investigation.

Enteric Ganglia: Neuronal Differentiation and Connections

The enteric nervous system (ENS) is by far the largest division of the ANS, and the most complex. The enteric nervous system consists of numerous small ganglia placed as nodes in a lattice of interconnections. Two

Table 2 Anterograde and retrograde influences of development in the avian ciliary ganglion

Effector molecule	*Mode*	*Neuronal property*	*Effect*
ACh (all nAChRs)	Anterograde	Chloride transporter	Increase
Neuregulin	Anterograde	Ca^{2+}-activated K^+ channels	Increase
Neuregulin	Anterograde	nAChRs	Increase
ACh (α7 nAChRs)	Anterograde	Neuronal survival	Decrease
ACh (all nAChRs)	Anterograde	Neuronal survival	Increase
CNTF	Retrograde	Neuronal survival	Increase
GDNF	Retrograde	Neuronal survival	Increase
TGF-β4	Retrograde	Ca^{2+}-activated K^+ channels	Increase
TGF-β3	Retrograde	Ca^{2+}-activated K^+ channels	Decrease
Activin	Retrograde	Somatostatin	Increase
Unknown	Retrograde	nAChRs	Increase

such layers occur, the myenteric plexus and the submucosal plexus, with extensive radial connections. The ENS has a full reflex circuitry with sensory, motor, and interneurons, and to a degree can function without CNS input, which derives from visceral motor neurons in vagal and sacral levels of the CNS (the same levels from which the enteric NC precursors arise). At least 15 neurotransmitters (but not noradrenaline) and neuromodulatory peptides occur in mature enteric ganglia, and each small ganglion has several neuron types differentiated by neurochemical code, morphology, and projection pattern. How so many different neuronal types are specified in each ganglion is not well understood. As explored by Michael Gershon and co-workers, differences in timing of differentiation of different neuron classes could be involved, and neuron type-related differences in median birth days does occur, as determined by ascertaining neuronal birth days using pulse-delivered tritiated thymidine or bromodeoxyuridine (BrdU). However, neurons of each class are born over a time range, and the ranges for different classes overlap strongly. Moreover, some neuron types, such as 5-hydroxytryptamine neurons, are specified prior to or early in NC migration. As with other parts of the ANS, Phox2b is a decisive requirement, and deletion of HAND2 has revealed its essential role in the specification and differentiation of enteric nervous system neurons that express VIP.

Differentiation of enteric neurons has similarities to that of the neurons of the rest of the ANS (**Figure 6**). For example, it is marked by the loss of the transcription factor Sox10 and the appearance of the neuron-specific transcription factor family member HuC/D. Sox10 remains present in enteric precursors and enteric glia, and the latter also express glial fibrillary acidic protein (GFAP) like CNS glia, but unlike glia of the other ANS divisions. One of the first neuron classes to appear is nitric oxide synthase-expressing neurons, and in mice these cells are initially transiently catecholaminergic, showing dopamine (DOPA) metabolism such as DOPA decarboxylase and dopamine-β-hydroxylase. These early differentiating neurons cease to migrate but extend axons distally, in parallel with the migration of vagal enteric NC cells.

Unlike the rest of the ANS and CNS, the enteric neuron population size does not normally overshoot and then undergo apoptotic pruning. Instead, the population proliferates up to a density set by the gut tissue. Instrumental in this so-called logistical growth is GDNF. This factor, via its receptor Ret on NC cells and its co-receptor GDNF family receptor-α (GFR-α) has a complex role, since it is not only a mitogen for enteric NC cells, but also induces differentiation into postmitotic neurons. *In vitro* experiments suggest that the small peptide endothelin-3, via its receptor EdnrB on NC cells, retards the differentiation effect of GDNF while preserving or even increasing its mitogenic function. Mutation of all the genes for these can cause Hirshsprung's disease (**Table 1**) either by directly decreasing GDNF mitogenic signaling (for mutations in *RET*, *GFRA1*, and *GDNF*) or by removal of the brake on GDNF-stimulated mitotic withdrawal (for mutations in *EDN3*, *EDNRB*, and *ECE1*).

See also: Enteric Nervous System Development; Neural Crest.

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Autonomic Neuroplasticity: Development

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Introduction

The concept that the nervous system can undergo substantial reorganization has been appreciated for over a century. In 1909, in *Modern Problems in Psychiatry,* Ernesto Lugaro used the term ‘plasticity’ in the context of the nervous system to convey the idea that chemical interactions responsible for the developmental patterning of the nervous system also provide adaptive mechanisms for behavioral modification and nervous system repair.

While plasticity can occur throughout the nervous system, the autonomic nervous system arguably has been most instructive in revealing the underlying principles. One reason is that the peripheral autonomic nervous system provides a particularly tractable system for studying neuroplasticity. Because its principal neurons are located outside of the central nervous system, it is relatively easy to isolate and remove relatively pure neuronal populations, either for cell culture under regimented conditions or to examine the effects of extirpation or transposition. A second reason is that sympathetic neurons are responsive to the prototypic neurotrophic factor, nerve growth factor (NGF); thus the role of target-derived proteins and autonomic neuronal survival and growth has been recognized and manipulated in this system for over half a century. Moreover, this system has proved not only to be highly complex, with multiple factors controlling development, but also very amenable to investigations using genetic manipulation to elucidate the role of various genes and proteins. Accordingly, the autonomic nervous system has served as a primary model system for advancing our knowledge of cellular and molecular processes regulating nervous system development. Perturbations to these processes have allowed us to more fully appreciate the repertoire of responses that comprise developmental neuroplasticity.

The process of nervous system development is itself inherently plastic. During development, undifferentiated cells undergo variable degrees of migration and cell division, giving rise to pools of precursor cells which provide the anatomical substrate of the nervous system. Precursor cells differentiate, taking on specific properties and becoming responsive to chemical cues in their environment, and eventually elaborate axons which find their way to appropriate targets. Neuron numbers are further refined through apoptosis in response to limitations in available target-derived survival factors, thus matching neuronal numbers to target mass and cell type. Target innervation density is then established through the actions of both propulsive and repulsive signals, and retrogradely transported proteins contribute to final determination of transmitter phenotype. In the perinatal period, hormonal factors play increasingly important roles in refining nerve–target relationships. Perturbations to any of these dynamic processes can lead to significant alterations in nervous system properties, which are appropriately defined as ‘developmental neuroplasticity.’

Developmental autonomic neuroplasticity is defined here broadly to include pre- and postnatal developmental processes associated with sympathetic and parasympathetic nervous system formation and those perturbations that affect structure, phenotype, and function. These include (1) molecular signaling events regulating autonomic neuronal ontogeny and the impact of their disruption through genetic manipulation and transpositioning or removing individual structures, (2) the establishment and maintenance of autonomic projections to peripheral targets, and (3) establishment, maintenance, and alterations of neurochemical phenotype during development or in response to injury.

Autonomic Gangliogenesis

A defining event in the ontogeny of the autonomic nervous system is the formation of motor ganglia. These consist of aggregates of sympathetic and parasympathetic postganglionic neurons, located in the periphery, which receive excitatory cholinergic synaptic input from preganglionic neurons located in the central nervous system. Autonomic postganglionic neurons derive from the neural crest, a transient structure that originates from cells migrating rostrocaudally from the neural tube shortly after closure. Neural crest precursor cells give rise to a variety of cell types, including melanocytes, peripheral glia, cartilage, smooth muscle, adrenomedullary chromaffin cells, and sensory and autonomic neurons. Those destined to become autonomic ganglion neurons of the peripheral nervous system migrate ventrally through the rostral somitic mesoderm to populate sympathetic and parasympathetic ganglia. Any perturbation that disrupts normal ganglion formation will severely impact autonomic nervous system organization and function.

The generation of autonomic neuronal precursors is complex, involving a variety of transcription factors and signaling proteins (**Figure 1**, **Table 1**). A pool of

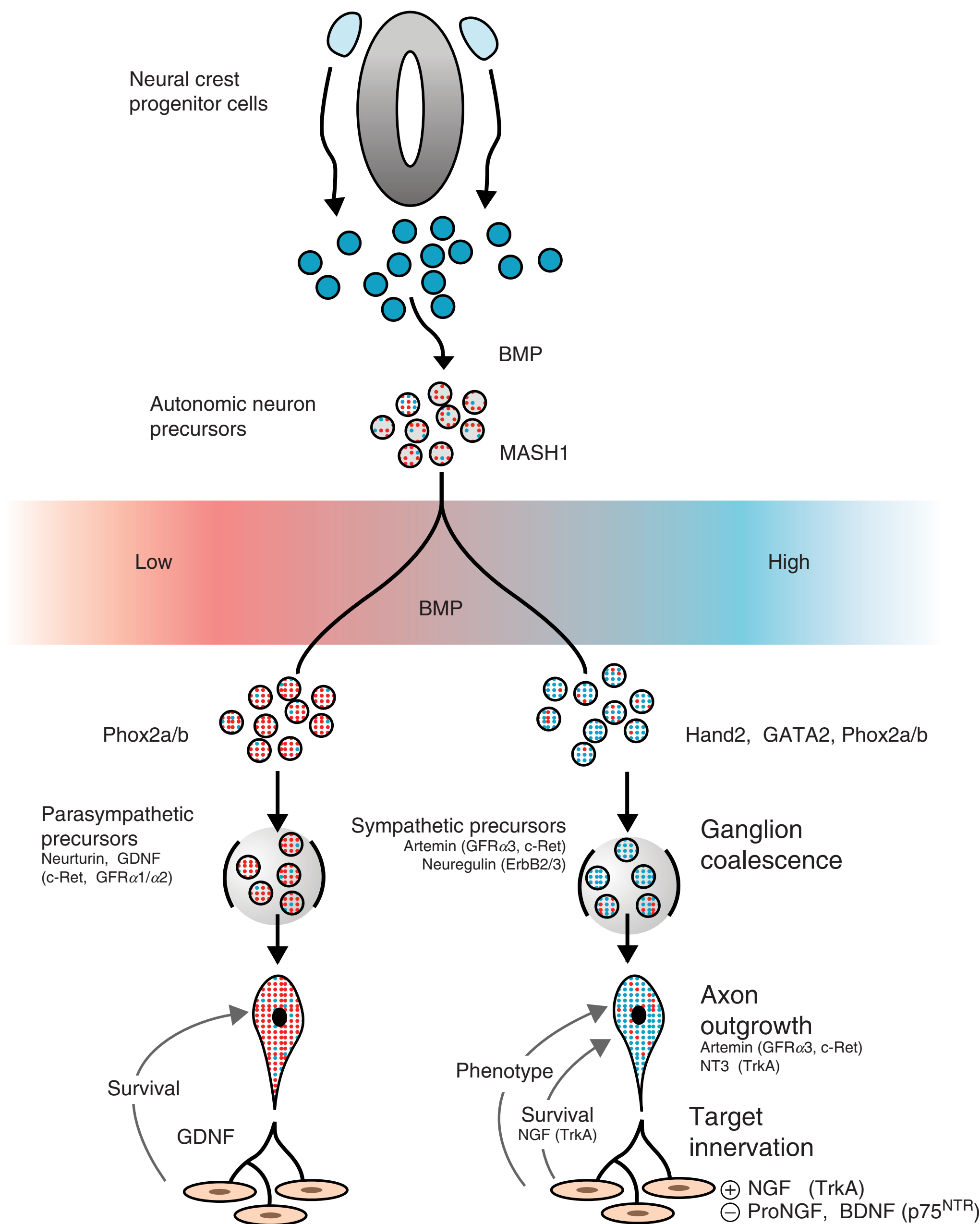

Figure 1 Schematic diagram indicating processes involved in the ontogeny of the autonomic nervous system, and the roles of some regulatory proteins. Neural crest gives rise to progenitor cells which differentiate into mammalian achaete–scute homolog-1 (MASH1)-expressing autonomic neuronal precursors under the influence of members of the bone morphogenetic protein (BMP) family. Neuronal identity appears to be determined within a BMP gradient, with sympathetic neurons differentiating within high concentrations and parasympathetic neurons differentiating within low concentrations. Parasympathetic neurons require expression of members of the Phox2 family of transcription factors, which are also required for sympathetic neurons, in addition to Hand2 and Gata2. Coalescence of precursors into ganglia requires artemin acting on the GFRα3/c-Ret receptor complex and neuregulin acting on ErbB receptors for sympathetic ganglia, and glial cell-derived neurotrophic factor (GDNF) and neurturin acting on GFRα1 or GFRα2 complexed with c-Ret. Sympathetic axon outgrowth requires both artemin and neurotrophin-3 (NT3), which acts on the TrkA receptor, whereas parasympathetic axon outgrowth may be dependent on GDNF or related proteins. Target-derived factors regulate target innervation: in sympathetic neurons, nerve growth factor (NGF) acting on TrkA can promote neuronal survival and increase innervation density, whereas proNGF and brain-derived neurotrophic factor (BDNF) acting selectively on the $p75^{NTR}$ diminish target innervation density. GDNF regulates innervation density of some parasympathetic targets. Target-derived factors can also influence neuronal neurochemical phenotype.

Table 1 Factors regulating autonomic nervous system development, and effects of mutations of regulatory genes

Factor	*Role in development*	*Effect of deletion*
MASH1, CASH1	Transcription factors specifying autonomic neuron identity	Autonomic neurons fail to form
Bone morphogenetic protein	Regulates formation of autonomic precursors, and differentiation of sympathetic and parasympathetic neurons	Sympathetic and parasympathetic neurons fail to form
Phox2a/b	Transcription factors required for catecholaminergic phenotype	Sympathetic and parasympathetic neurons fail to develop
Hand2, Gata2	Transcription factors specifying sympathetic fate	Absence of sympathetic neurons
Neuregulin, ErbB2/3	Promote sympathetic precursor migration	Inappropriate sympathetic trunk ganglion formation
Hepatocyte growth factor	Neuroblast survival and differentiation	Reduction in sympathetic ganglion neurons
Semaphorin 3A	Neural crest cell migration	Abnormal sympathetic ganglion formation
Neurotrophin-3	Sympathetic axon outgrowth	Impaired axon extension, fewer surviving sympathetic neurons due to inaccessibility to target-derived trophic factors; impaired cholinergic sympathetic phenotype
Glial cell-derived neurotrophic factor, c-Ret, GFRα1/2	Parasympathetic neuron survival, neuroblast migration; axon guidance	Reduced numbers of parasympathetic postganglionic neurons, impaired axon outgrowth
Nerve growth factor	Target-derived protein regulating sympathetic neuronal survival in early postnatal development, and target innervation density	Augmented cell death of sympathetic neurons; absent or diminished sympathetic innervation of some targets
Artermin, c-Ret, GFRα3	Sympathetic neuroblast migration; rostral sympathetic ganglion formation; sympathetic axon outgrowth	Misplaced superior cervical ganglion; impaired sympathetic axon outgrowth
Neurturin	Maintenance of parasympathetic target innervation	Reduced parasympathetic innervation

autonomic neuron progenitor cells differentiates from neural crest precursors under the influence of local expression of bone morphogenetic proteins (BMPs). Differentiation of both sympathetic and parasympathetic precursor cells is dependent upon their expression of the mouse or chick achaete–scute homolog transcription factors, MASH1 or CASH1, respectively, and animals lacking these proteins fail to form autonomic neurons. The transcription factors Phox2a and Phox2b are also involved in expression of catecholaminergic properties, which are characteristic of both sympathetic and parasympathetic neuronal precursors. Phox transcription factors are required for early survival of both sympathetic and parasympathetic autonomic neuron precursors. Sympathetic neuronal precursors are further dependent upon the Hand2 and Gata2 transcription factors.

Autonomic neuronal precursors migrate ventrally toward their ultimate destination, guided by local signaling proteins which, for truncal sympathetic neurons, include neuregulin and Sema3A, acting on the ErbB and neuropilin 1 receptors, respectively. Artermin, acting on the c-Ret and GFRα3 receptors, appears to play an important role in sympathetic ganglion coalescence and in providing chemotactic signals responsible for rostral migration of superior cervical ganglion precursor neurons. The factors regulating parasympathetic neuron migration are less understood, but evidence indicates that in some cranial parasympathetic ganglia, precursors are dependent initially upon glial cell-derived neurotrophic factor (GDNF) via the c-Ret and GFRα1 receptors for ganglion cell proliferation and migration, and subsequently on neurturin and the GFRα2 receptor for neuronal survival and ganglion development.

During normal development, different regions of the neural crest give rise to specific structures. In chick–quail chimeras, for example, rostral parasympathetic ganglion neurons derive from mesencephalic neural crest, whereas sympathetic ganglion neurons derive from the truncal neural crest extending below somite 4. However, premigratory precursor cells apparently are not committed to specific fates. Thus, neural crest cells from the vagal region that would normally give rise to enteric ganglion cells, when transplanted caudally, give rise to apparently normal sympathetic ganglion neurons and adrenomedullary cells. This suggests that neural crest derivatives at this stage remain multipotent and substantially plastic, and differentiation into mature neural crest derivatives is determined largely by peri- and postmigratory environments.

While most migrating neural crest cells become irrevocably committed during the process of migration and gangliogenesis, it is now clear that a small population of precursor cells remains undifferentiated

and may give rise to nascent neurons and glia throughout development and maturity. These presumptive stem cells may serve as a source of cells that can contribute to continued plasticity in the developing and adult organism.

Plasticity of Autonomic Neuroeffector Pathways

Axon Outgrowth

Following migration and coalescence of ganglion cells, connectivity between the central nervous system and peripheral targets must be established. Autonomic neurons elaborate axons in the prenatal and early postnatal periods. Preganglionic axons traverse the anterior region of the somitic mesoderm. Somitic mesoderm appears to play a critical role in preganglionic axon guidance, and removal or transpositioning of somites leads to major alterations in axon trajectory. Preganglionic axons project to the region of their intended ganglion, even if the ganglion has been removed. Once having entered the ganglion, preganglionic axons synapse with the postganglionic neurons; while some synapses are present in the prenatal period, the first postnatal week is characterized by dramatic synaptogenesis and establishment of a functional pathway, at least in the sympathetic inferior cervical ganglion of the rat. Ganglion neurons are initially polyinnervated by convergent inputs from multiple preganglionic neurons, and normal reductions through axon pruning taking place postnatally.

Autonomic postganglionic neurons elaborate axons shortly after exiting the cell cycle, and postganglionic axons first reach their intended targets in the late postnatal period in rodents; substantial increases in numbers of target projecting neurons occur through the first 2–3 postnatal weeks. In the case of sympathetic axonal outgrowth, this appears to be regulated predominantly by two locally produced proteins. Artemin, a member of the GDNF family of trophic factors, is produced by vascular smooth muscle cells in early development, and sympathetic axons at this developmental stage express the artemin receptors c-Ret and GFRα3. Neurotrophin 3 is also produced locally and activates sympathetic TrkA receptors, and is required for normal postganglionic axon outgrowth. The association of these growth factors with vascular smooth muscle is believed to account for the fact that sympathetic pathways associate closely with blood vessels as they course to their target destinations. Parasympathetic axon outgrowth is dependent upon GDNF and its receptors c-Ret and GFRα1. Disruptions of these growth factors or their receptors have serious consequences; without sufficient axon outgrowth, not only are targets hypoinnervated, but neurons are also in turn deprived of required target-derived survival factors and hence undergo abnormally extensive cell death, leading to fewer ganglion neurons. In some cases, diminished postganglionic axon outgrowth affects the mature neurochemical phenotype because of deprivation of target-derived proteins that influence neurotransmitter properties.

One feature of the postganglionic sympathetic system is a dramatic ability to reorganize in response to injury in the postnatal period. For example, innervation of the orbit normally derives exclusively from the ipsilateral superior cervical ganglion, and damage to this pathway in the adult results in sustained denervation and impairment, known clinically as Horner's syndrome. In this syndrome, the pupil is abnormally constricted (loss of excitatory sympathetic innervation to the pupil dilator muscle) and the upper eyelid fails to retract normally (ptosis, due to loss of excitatory sympathetic innervation to the superior tarsal smooth muscle which elevates the eyelid). However, in the neonatal rat, removal of one superior cervical ganglion results in sprouting of spared intracranial vascular sympathetic axons from the contralateral intact ganglion, which take atypical pathways to reach the orbital targets. Despite having significant phenotypic differences from normal resident innervation, such as expression of neuropeptide Y, as is typical of vascular sympathetic neurons but not normal resident tarsal muscle innervation, this aberrant pathway largely restores functional control of the target (**Figure 2**). The ability to establish contralateral orbital innervation is lost completely during the first postnatal month, largely through differentiation of smooth muscle-like myofibroblasts that define their trajectory to the orbit; this coincides with the time course reported for the loss of artemin expression during development in similar cell types, implicating a developmental reduction in artemin expression as a cause of the postnatal diminution in pathway plasticity.

Neuronal Survival: The Neurotrophic Hypothesis

Once the terminal axon reaches the target, a critical process takes place in which the relationship between target and neuron is established and refined, thus ensuring that the neuroeffector pathway is effective and appropriate. This process relies on target-derived proteins that can modify both neuronal survival and terminal axon growth.

The prototypical neurotrophin NGF has long been associated with sympathetic postganglionic axon sprouting *in vitro*, so it is not surprising that this protein would be considered to be the primary candidate for controlling sympathetic innervation. An abundance of

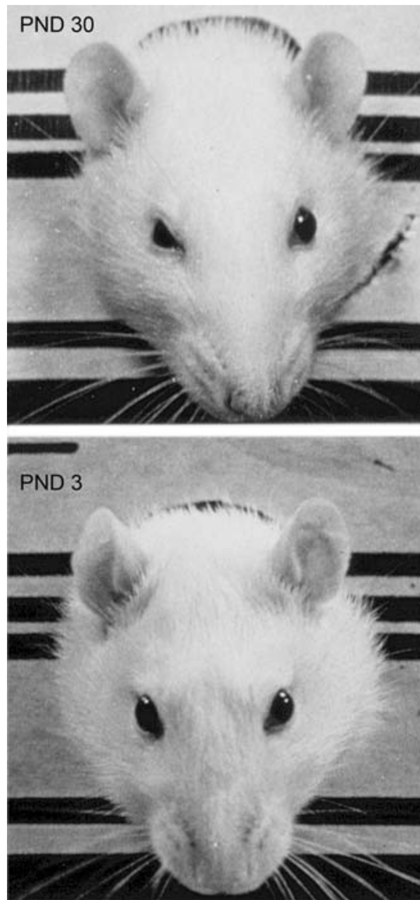

Figure 2 Plasticity in postganglionic pathway formation in the rat. The right superior cervical ganglion was removed from the rat in the top panel on postnatal day (PND) 30, whereas the same ganglion was removed from the rat in the bottom panel on PND 3. The photographs are of the awake rats at 3 months of age. Following PND 30 ganglionectomy, contraction of the superior tarsal muscle in the right eyelid is impaired, leading to ptosis and reduced palpebral fissure width. In the rat receiving ganglionectomy on PND 3, there is minimal evidence of impaired contraction; recovery is due to the formation of an atypical pathway deriving from intact contralateral neurons that normally project to cranial blood vessels to the denervated orbital targets.

information supports the idea that NGF (and many other trophic proteins in different contexts) is required for neuronal survival. Sympathetic neurons are produced in overabundance during development, and a relatively large proportion (in some cases >50%) undergoes developmental cell death in the perinatal period, leading to the ganglion cell numbers ultimately observed in the adult. Those neurons that do survive are believed to be the ones that have obtained access to adequate amounts of a target-derived survival protein (trophic factor, from the Greek term *trophos*, meaning 'to nourish'). However, trophic proteins are believed to be produced in limited quantities. In those sympathetic axons that reach the target first and have access to the greatest amounts of NGF, which binds to the NGF-selective receptor TrkA and is transported retrogradely to the cell body as receptor–ligand complex, programmed cell death (apoptosis) is prevented. Axons that cannot compete for adequate neurotrophic factor because they are misguided or arrive after target innervation has already achieved optimal density ultimately are eliminated through programmed cell death. The principle that an oversupply of developing neurons competes for target-limited trophic factor, thus matching neuronal numbers to target needs, represents the essence of the 'neurotrophic hypothesis.' While less is known about the trophic requirements of parasympathetic neurons, members of the GDNF family of ligands appear to serve similar roles. Modulating the amount of trophic factor available can induce considerable plasticity in the numbers of postganglionic neurons; addition of exogenous NGF or upregulation of its synthesis in peripheral tissues, such as epidermis, results in increased numbers of surviving sympathetic ganglionic neurons. Similarly, depletion of endogenous NGF during development, inactivation of the NGF gene, or null mutations of the TrkA gene encoding the NGF receptor all result in marked reductions in numbers of sympathetic ganglion neurons. A comparable situation has been shown to be the case for cranial parasympathetic neurons, the survival of which is reliant first upon GDNF and subsequently upon neurturin, acting, respectively, on GFRα1 and GFRα2 complexed with the coreceptor c-Ret.

Regulation of Target Innervation

The establishment of effective neuroeffector transmission is dependent upon the presence of appropriate numbers of terminal autonomic axons within the target tissue. Visceral targets show a wide range in the numbers of sympathetic and parasympathetic fibers present per unit tissue mass (innervation density). In light of NGF's well-documented role, not only in inducing sympathetic neuronal survival but also in eliciting robust outgrowth *in vitro*, it is not surprising that this neurotrophin would be a logical candidate for regulating sympathetic innervation density in peripheral targets. Indeed, early studies of several different targets revealed a positive correlation between levels of NGF mRNA or protein and the relative densities of sympathetic innervation. In keeping with this idea, mice with null mutations for NGF or TrkA fail to develop normal target innervation. Conversely, conditions favoring increased target levels of NGF can lead to increased target innervation. For example, spontaneously hypertensive rats display abnormally elevated levels of NGF in the mesenteric vasculature, and these vessels are also hyperinnervated by

sympathetic axons. Further, in keeping with the central tenet of the neurotrophic hypothesis, that neurons compete for limited amounts of trophic factors, neonatal destruction by capsaicin of sensory nociceptor nerves (which express TrkA and compete for target NGF) results in sympathetic axon hyperinnervation; this presumably occurs because reduced competition makes more NGF available to sympathetic axons. Accordingly, alterations in target NGF levels can result in significant plasticity in target innervation density.

While there is little doubt that NGF plays an important role in sympathetic target innervation, recent lines of investigation show that the relationship is far more complex. As indicated earlier, NGF is required for neuronal survival; therefore, loss of innervation in the absence of NGF or TrkA could be due solely to cell death rather than to an absence of local growth-promoting properties. Indeed, using mouse mutants lacking genes for both NGF and the apoptotic factor Bax (thus circumventing cell death that would normally occur in the absence of NGF), Ginty and colleagues found that the dependency on NGF for development of target innervation was highly variable, with innervation of some targets being drastically reduced (e.g., salivary glands, heart) while others were unaffected (trachea). This supports the idea that growth-promoting factors other than NGF contribute to target sympathetic innervation.

A second complicating factor is that, in addition to propulsive effects of proteins such as NGF and GDNF, autonomic target innervation appears to be controlled by proteins that are repulsive in nature. For example, sympathetic neurons are responsive to semaphorins and can be repulsed from targets expressing high levels of these proteins. Moreover, neurotrophins show surprising complexity. In addition to the TrkA receptor mediating sympathetic neuron survival and axon outgrowth, the pan-neurotrophin receptor $p75^{NTR}$ can facilitate ligand binding to TrkA, but can also mediate repulsive cues. Brain-derived neurotrophic factor (BDNF), a neurotrophin closely related to NGF, shows little affinity for TrkA but binds avidly to $p75^{NTR}$. When $p75^{NTR}$ is selectively activated, this inhibits sympathetic axonal outgrowth and contributes to sympathetic axon pruning (and in some cases can cause cell death). Importantly, in addition to BDNF, the precursor form of NGF, proNGF, shows relatively little affinity for TrkA but strongly binds $p75^{NTR}$, and, like BDNF, can be repulsive and pro-apoptotic to sympathetic neurons. Thus the extent to which NGF regulates target innervation density will depend upon which form predominates.

The balance between TrkA and $p75^{NTR}$ activation has important implications for developmental plasticity of sympathetic innervation. Some targets, such as those of the female reproductive tract, undergo considerable innervation plasticity in the postnatal period. For example, uterine innervation changes through puberty and beyond, under the influence of the gonadal steroid hormone estrogen; in fact, changes in estrogen lead to reductions in myometrial sympathetic innervation at the estrus stage of each estrous cycle. Available evidence shows that estrogen increases uterine expression of BDNF, and that fluctuations in this hormone underlie physiological axon pruning that takes place during puberty and throughout the reproductive cycle.

Neurochemical Plasticity

Neural crest progenitor neurons give rise to a richly diversified range of peripheral autonomic neurons. Fully differentiated parasympathetic neurons typically express a cholinergic phenotype, which includes choline acetyltransferase and vesicular acetylcholine transporter (VAChT), a nitrergic phenotype (neuronal nitric oxide synthase), and the neuropeptide vasoactive intestinal polypeptide (VIP). Most mature sympathetic neurons are noradrenergic, expressing the proteins tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), vesicular monoamine transporter-2 (VMAT2), and the norepinephrine transporter; a significant proportion of noradrenergic sympathetic nerves, mainly those innervating blood vessels, also contain neuropeptide Y. About 5% of mature sympathetic neurons, which project to eccrine sweat glands and periosteum, are cholinergic. Neural crest progenitor cells also give rise to autonomic ganglionic small intensely fluorescent (SIF) cells, which are dopaminergic (express TH but not DBH), and adrenomedullary chromaffin cells (synthesize epinephrine and contain phenylethanolamine *N*-methyltransferase (PNMT), in addition to TH and DBH).

The differentiation of parasympathetic and sympathetic neurons appears to be regulated by way of a BMP gradient; progenitors most proximate to BMP-producing tissues (e.g., dorsal aorta) acquire a noradrenergic phenotype, while those exposed to lower concentrations become cholinergic parasympathetic neurons. Differentiation of both types of neurons requires MASH1 and Phox2a/b transcription factors, and noradrenergic neurons additionally require the Hand2 and Gata2 transcription factors. Differentiation of neural crest progenitors into adrenomedullary chromaffin cells is, in part, under control of the local environment; corticosteroid derived from the surrounding adrenal cortex is required for PNMT expression. Local factors have also been implicated in phenotype acquisition and maintenance in SIF cells,

which are located proximate to blood vessels and may require endothelial factors for differentiation.

The common origins of both sympathetic and parasympathetic neurons underlie sustained similarities throughout development and presage responses observed with certain perturbations. Experimental observations in neural crest-derived precursor cells reveal that the biochemical machinery necessary for acetylcholine synthesis is present well before commitment to final cell fate. Similarly, catecholaminergic properties have also been described as a feature of autonomic neuron progenitors prior to expression of other pan-neuronal properties.

Transmitter Phenotype 'Switching'

While genetic programming and instructive signals during precursor migration and gangliogenesis appear to play a major role in determining autonomic neuronal phenotype, target-derived proteins are also important in determining or refining mature neuronal phenotype. The best studied example of this is the transition from an adrenergic to a cholinergic phenotype in some sympathetic neurons. Early studies showed that neonatal sympathetic neurons cultured under certain conditions (e.g., in culture medium conditioned by cardiac myocytes) lose their noradrenergic phenotype, and this is replaced over time by cholinergic properties. In an extensive series of experiments, Story Landis and colleagues extended these findings *in vivo* by showing that sweat glands, which are innervated by cholinergic sympathetic axons in the adult, are actually innervated by axons with a catecholaminergic phenotype at postnatal day 4; however, these catecholaminergic properties (dense core vesicles, catecholamine histofluorescence, and TH immunoreactivity) are downregulated over the next 10 days and are replaced by cholinergic properties (VIP immunoreactivity and cholinesterase activity). An impressive body of evidence has been accrued to support the idea that these neurons undergo a 'phenotype shift' from catecholaminergic to cholinergic. Evidence in favor of this hypothesis includes several findings: (1) destruction of catecholaminergic fibers by neonatal administration of selective neurotoxins 6-hydroxydopamine, guanethidine, or antisera to NGF prior to the shift prevents sweat gland cholinergic innervation, even though cholinergic nerves should be unaffected by these treatments, (2) transplantation of the sweat glands to ectopic sites where sympathetic nerves normally do not show a cholinergic phenotype (trunk hairy skin, ocular anterior chamber co-cultures with the superior cervical ganglion) induces cholinergic properties, (3) replacement of sweat glands with a target that normally is not innervated by cholinergic sympathetic axons (salivary gland) prevents cholinergic properties from appearing in the resident innervation, and (4) mouse mutants that fail to develop sweat glands also fail to develop cholinergic fibers to the footpad. Further studies have shown that the shift is facilitated by an interactive loop with the target, in which noradrenergic transmission accelerates acquisition of cholinergic properties, and that a similar phenotypic shift occurs to sympathetic innervation to the periosteum; periosteal cells induce a VIP and VAChT phenotype in resident innervation and also when transplanted to ectopic sites. The factors responsible for this shift have not been definitively identified, but members of the gp130 cytokine family, which includes leukemia inhibitory factor and cardiotropin, promote cholinergic properties in cultured neurons and are strong candidates.

Recent studies imply that the establishment of cholinergic traits in sympathetic neurons is more complex. Using antibodies with high sensitivity to VAChT, it appears that cholinergic properties are demonstrable in subpopulations of sympathetic neurons prior to target innervation, and that sweat gland-innervating fibers can express VAChT at an age supposedly preceding the phenotype shift. Thus, the extent to which cholinergic properties are conferred *de novo* by the target or are instead an intrinsic property of sympathetic neurons undergoing normal development *in vivo* remains uncertain. Nonetheless, it is clear that some targets produce factors that are capable of inducing neurochemical phenotype plasticity in developing sympathetic neurons.

Another factor that can influence neurochemical phenotype is injury. Normally, VIP-immunoreactive neurons are only rarely encountered in the superior cervical ganglion. Following axotomy, however, VIP is dramatically upregulated *in situ* or under culture conditions. This implies that the factors normally responsible for providing a stable neurochemical phenotype can be affected by injury.

See also: Autonomic Nervous System Development; Neural Crest.

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Enteric Nervous System Development

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Discovery of the Independent Function of the Enteric Nervous System

The discovery of the enteric nervous system (ENS) dates to the period of the American Civil War, when the presence of ganglia within the external muscle layers of the bowel (Auerbach's or myenteric plexus) and within the submucosa (Meissner's and Schabadasch's or submucosal plexuses) was described. Before the end of the nineteenth century, Bayliss and Starling had established that the 'local neural mechanism' (the ENS) mediates propulsive motility, which they called "the law of the intestine," independent of input from the central nervous system (CNS). While World War I raged, Trendelenburg emphatically confirmed this conclusion by eliciting "the law of the intestine," now renamed the peristaltic reflex, *in vitro*. Despite the clarity of this evidence, and the absence of its refutation, many biologists continue to believe that the ENS is an array of parasympathetic relay ganglia used by vagal and sacral nerves to control the gut. That they should do so is surprising because in his original definition of the autonomic nervous system, JN Langley clearly differentiated between the enteric and parasympathetic nervous systems. The parasympathetic and sympathetic systems were defined, respectively, by their craniosacral and thoracicolumbar outflows from the CNS, while the ENS, which can operate independently of CNS control, is a separate division that contains many neurons that lack a CNS innervation. The misapprehension of the independence of the ENS tends to trivialize its importance, decrease ENS research, and focus explanations of functional gastrointestinal disorders exclusively on the CNS.

Organization of the ENS

The ENS is comprised of myenteric and submucosal plexuses, which interconnect and innervate effectors, smooth muscle, blood vessels, and glands (**Figure 1**). Extrinsic motor and sensory nerves of vagal and spinal origin link the ENS and CNS. The number of enteric neurons ($>10^8$ in the human small intestine), which approximates that of the spinal cord, dwarfs the number of motor axons ($\sim10^3$) in the vagus nerves. This disparity critically influenced Langley's view of the enteric innervation but does not mean that the CNS is unable to influence the ENS. It can, and does, with effects that often become all too disturbingly apparent when anxiety strikes. In fact, investigators who selectively visualize the efferent vagal innervation have marveled at its apparent extent. Vagal axons, however, are only a small proportion of the vast numbers of fibers in the ENS. When all axons are demonstrated, fibers can be distinguished individually only by electron microscopy. Intrinsic reflexes, their integration within the ENS, and their coordination with myogenic activity (muscle and interstitial cells of Cajal (ICCs)), not the CNS, are what determine the basic motile and secretory behaviors of the gut. ICCs are electrically coupled to smooth muscle and act as pacemakers and intermediaries, which transmit signals from nerve to smooth muscle.

The sensory component of the extrinsic enteric innervation is very much larger than the motor; about 90% of vagal fibers are afferent. Pain and discomfort are conveyed to the CNS primarily by spinal nerves, while information that the vagi convey to the brain does not necessarily reach consciousness. Vagal sensory fibers are responsible for nausea and, when highly stimulated, also convey pain, but mostly they are involved in 'homeostasis,' the ramifications of which are not completely understood but may be beneficial. Vagal stimulation is utilized to treat depression and epilepsy, and it improves learning and memory in humans and animals. Vagal motor fibers have been postulated to act on enteric command neurons, allowing the CNS to exert a powerful overall effect, despite the intrinsic control of motility and secretion. The CNS thus operates in a role analogous to that of a chief operating officer, who directs overall operations but does not micromanage.

The anatomy, physiology, and neurochemistry of the ENS are complex. The histological organization of the ENS, for example, resembles that of the CNS and lacks the connective tissue sheaths that normally surround peripheral nerves. Because the ENS thus lacks internal collagen, neurons are supported by enteric glia, which are rich in glial fibrillary acidic protein and resemble CNS astrocytes. Enteric neurons, moreover, are phenotypically diverse. Every class of neurotransmitter and neuromodulator that has been described in the CNS is also represented in the ENS. Enteric interneurons, furthermore, are arranged in microcircuits, which are not yet understood.

ENS Control of Motile and Secretory Behaviors

The basic pattern of the peristaltic reflex has been ascertained, but motility is more than just peristaltic reflexes. The gut sometimes propels its content and sometimes mixes them. The ENS contains a library of

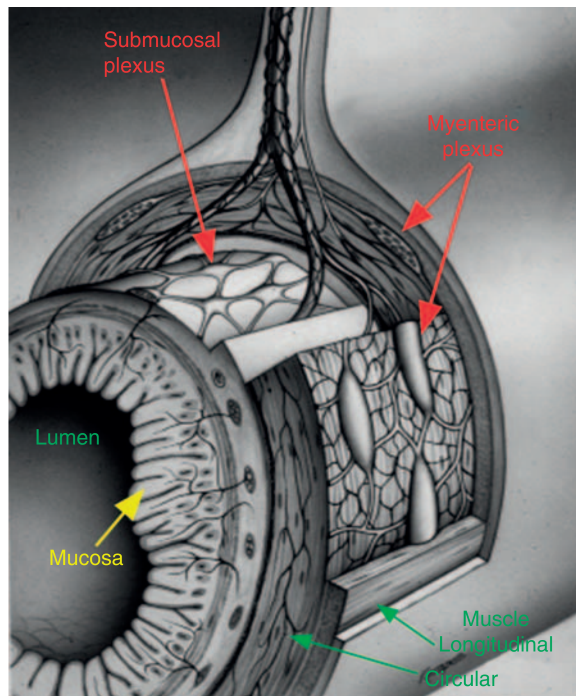

Figure 1 A drawing showing the organization of enteric innervation. Extrinsic nerves are depicted at the top of the figure reaching the bowel as perivascular (sympathetic) and paravascular (sensory, parasympathetic) bundles in the mesentery. The submucosal and myenteric plexuses are indicated. The myenteric plexus lies between the circular and longitudinal layers of the muscularis externa. The submucosal plexus can be seen in the cutaway view and in profile in the cross-sectional view of the gut. Nerve fibers enter the mucosa but are limited to the connective tissue of the lamina propria, and none enter the lumen.

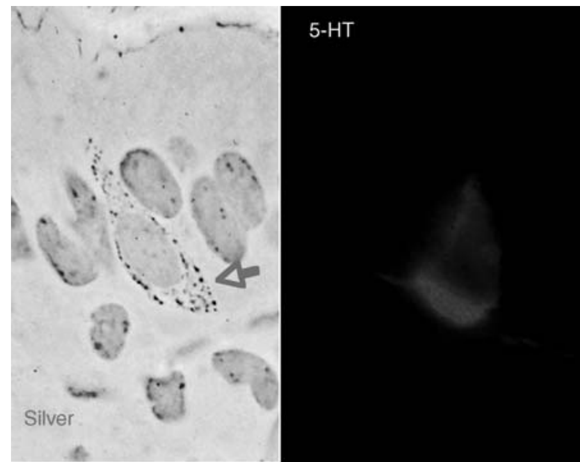

Figure 2 Enterochromaffin (EC) cells, which function as sensory transducers, can be demonstrated with silver (left) or immunocytochemically with antibodies to 5-HT (right). Silver stains depend on the ability of 5-HT to act as an agent that reduces silver salts to metallic silver, which precipitates to reveal the storage granules. Note that these granules (silver) and 5-HT are concentrated in the basolateral cytoplasm. This orientation reflects the basolateral secretion of 5-HT.

motor programs, from which it selects appropriate patterns. This library includes postprandial mixing to facilitate digestion and absorption, aborally directed power propulsion, orally directed power propulsion (associated with vomiting), and physiological ileus (in which motility stops). There is also an interdigestive, housecleaning program consisting of migrating motor complexes that sweep down the bowel. Choosing programs requires that the content of the lumen of the bowel be closely monitored.

No nerves actually enter the lumen (**Figure 1**); therefore, the ENS must be apprised of luminal conditions transepithelially. Enteroendocrine cells in the mucosal epithelium function as sensory transducers. The best known of these is the enterochromaffin (EC) cell, which contains serotonin, or 5-hydroxytryptamine (5-HT; **Figure 2**), which is secreted in response to increases in intraluminal pressure or, in the duodenum, applications of acid or glucose to their apical microvillous border. Also, 5-HT stimulates the mucosal processes of intrinsic primary afferent neurons (IPANs), subsets of which exist in the submucosal and myenteric plexuses. These cells do not project to the CNS but activate the intrinsic microcircuits that mediate peristaltic and secretory reflexes. IPANs thus allow the ENS to function independent of CNS input. Common motor neurons of the ENS relax or contract intestinal muscle, probably through the intermediation of ICCs. Excitatory motor neurons are cholinergic and also secrete a neurokinin; inhibitory motor neurons contain nitric oxide synthase but may also utilize adenosine 5′-triphosphate or vasoactive intestinal peptide (VIP) to exert relaxant effects. Additional enteric neurons are secretomotor and cause goblet cells to secrete mucous and crypt epithelial cells to secrete chloride and thus water, and they also regulate gastrointestinal peptide secretion by enteroendocrine cells. Secretomotor neurons are either cholinergic or VIP-secreting, without overlap. The IPANs of both plexuses are cholinergic and may also co-store peptides and a calcium binding protein, calbindin. In fact, about 70% of all enteric neurons are cholinergic; however, most of these neurons co-store additional peptides and/or small molecule neurotransmitters, such as 5-HT. Neurons of the ENS can be identified by a chemical code made up of the transmitter and other molecules that they co-express.

Serotonin Is Important in the Normal and Abnormal Behavior of the Bowel

Not only is 5-HT produced and secreted by EC cells to initiate reflexes, but it is also a neurotransmitter of long descending myenteric interneurons. Although

its CNS activity (seemingly related to everything that makes life worthwhile) is what made 5-HT famous, its concentration in the CNS pales beside that in the gut. The enteric epithelium, moreover, continually turns over. New cells are generated from stem cells in the necks of gastric glands and intestinal crypts, mature as they rise to the gastric surface and villus tips, and eventually die and slough into the lumen. The epithelial cells are thus constantly in motion. Because axons are not adapted to track moving targets, the axons of IPANs cannot make traditional synapses on EC cells but terminate as processes in the lamina propria of the mucosa. This type of paracrine junction is acceptable because the bowel is a gross and imprecise organ. Specificity of signaling is achieved, not by the close apposition of interacting elements, but by the ability of receptors to selectively respond to 5-HT. EC cells overcome the large and variable distance to the nerves they stimulate by secreting massive quantities of 5-HT. In fact, 95% of the body's 5-HT is located in the gut, and the EC cells represent a much larger store of it than do the neurons of the myenteric plexus. The overflow of 5-HT from EC cell secretion reaches the intestinal lumen and stocks platelets with 5-HT. Platelets do not synthesize 5-HT but take it up as they circulate through the bowel. The rate-limiting enzyme in 5-HT's biosynthesis is tryptophan hydroxylase (TpH). There are two TpH isoforms, TpH1 and TpH2. EC cells contain TpH1, and neurons of the ENS and the CNS contain TpH2.

The large quantities of constitutively secreted 5-HT, and its overflow, necessitate an efficient mechanism in the bowel for 5-HT inactivation. Inactivation is accomplished by 5-HT reuptake; this involves a plasmalemmal serotonin transporter (SERT), which is expressed in the gut by enterocytes and serotonergic neurons. Enteric SERT is identical to CNS SERT. Tricyclic antidepressants, serotonin selective reuptake inhibitors (SSRIs), and cocaine, which are in widespread use, and abuse, all inhibit SERT. These compounds potentiate 5-HT, but their enteric effects are limited by the presence of backup transporters, which include organic cation transporters (OCTs) and the dopamine transporter (DAT). The OCTs and DAT have a lower affinity than SERT does for 5-HT, but they have a high capacity, and they prevent catastrophe from occurring when SERT is knocked out. They do not, however, completely protect the bowel from SERT inhibition. Tricyclic antidepressants and SSRIs exert gastrointestinal 'side effects,' which are really direct effects. These include nausea, diarrhea, and perhaps as a result of the desensitization of 5-HT receptors, constipation. Diarrhea and constipation alternate in knockout mice, which lack SERT; these mice also lose water excessively in stool.

Major advances in the treatment of patients undergoing chemotherapy for cancer and in those suffering from irritable bowel syndrome (IBS) have highlighted the importance of enteric 5-HT. EC cells not only trigger intrinsic reflexes by secreting 5-HT and stimulating IPANs, but their 5-HT also activates extrinsic sensory nerves to initiate nausea and discomfort. Different subtypes of 5-HT receptor are utilized to activate, respectively, intrinsic and extrinsic nerve fibers. Submucosal IPANs, but are stimulated by 5-HT_{1P} receptors, which may be heteromultimers of other G-protein-coupled receptors. IPANs (as well as cholinergic motor neurons to smooth muscle) also express 5-HT_4 receptors at their terminals. The 5-HT_4 receptors enhance acetylcholine and calcitonin gene related peptide secretion and thus strengthen synaptic transmission in excitatory (prokinetic) pathways. Further, 5-HT activates extrinsic sensory nerves by stimulating 5-HT_3 receptors. Myenteric IPANs, which appear to drive the giant, sometimes painful contractions associated with the aboral power propulsion pattern of activity, are also stimulated by 5-HT_3 receptors.

The dichotomy of 5-HT receptor subtypes involved in intrinsic and extrinsic reflex stimulation makes therapy possible. Some 5-HT_3 antagonists, including ondansetron and granisetron, provide relief from the nausea that accompanies cancer chemotherapy without at the same time paralyzing the bowel by interfering with 5-HT-driven peristaltic and secretory reflexes, and some 5-HT_3 antagonists, particularly alosetron, also alleviate the discomfort associated with IBS. This is a major advance because the pain and discomfort of IBS are far more troubling to patients than the altered bowel habits that are another hallmark of that disease. Blockade of the giant contractions of power propulsion by 5-HT_3 antagonism may also contribute to the relief of pain and discomfort in IBS but may be counterproductive. Not only do 5-HT_3 antagonists block activation of the IPANs that initiate power propulsion; they also inhibit serotonergic fast excitatory neurotransmission within the ENS. Thus, 5-HT_3 antagonists are constipating and useful only to treat diarrhea-predominant IBS. It is possible that interference with the emergency aboral power propulsive pattern of activity contributes to the rare complication of ischemic colitis that has been linked to the use of 5-HT_3 antagonists in treating IBS.

In contrast to the slowing of motility associated with 5-HT_3 antagonists, the strengthening of neurotransmission in prokinetic pathways by 5-HT_4 agonists, such as tegaserod, is useful in the treatment of chronic constipation and constipation-predominant IBS. These compounds do not initiate reflexes and do not activate pain pathways but instead rely on

natural stimuli to get a refractory bowel moving again, and 5-HT_4 agonism strengthens propulsive motility. Therefore, 5-HT_4 agonists are effective when taken for their approved use.

The ENS Originates from Vagal, Truncal, and Sacral Regions of the Neural Crest

The complexity and unique nature of the ENS suggests that factors that drive its development will be different from those that govern development of the remainder of the autonomic nervous system. Meaningful research on ENS development dates from the demonstration by Yntema and Hammond that deletions of what they called the 'anterior neural crest' caused deficiencies to occur in enteric ganglia of chick embryos. Subsequently, Le Douarin deleted the crest from various regions of chick embryos, but she also replaced it with grafts of crest from quail embryos, creating interspecies chimeras. The grafted quail cells migrated seemingly normally in their new hosts. Quail cells express an identifying pattern of nucleolar-associated heterochromatin that enables them to be reproducibly identified in fields of chick cells (**Figure 3**). Quail cells can also be recognized immunocytochemically, as has been done in more recent studies, by using quail-selective antibodies. As a result, cells of neural crest origin can be identified in the target organs of chimeric embryos, which in combination with suitable grafts allows the sites of origin of crest-derived émigrés to be determined. The axial levels of origin of the crest-derived enteric neurons and glia have now definitively been mapped in avians through the study of interspecies chimeras. For some time, it was simply presumed that the origin of the mammalian ENS was more or less analogous to that of avians. More recently, vital dyes and genetic mapping techniques have been used to verify the correctness of that assumption. The ENS is derived from the postotic (vagal and rostral truncal axial levels equivalent to somites 1–7) and sacral (axial caudal to somite 28) regions of the crest (**Figure 4**).

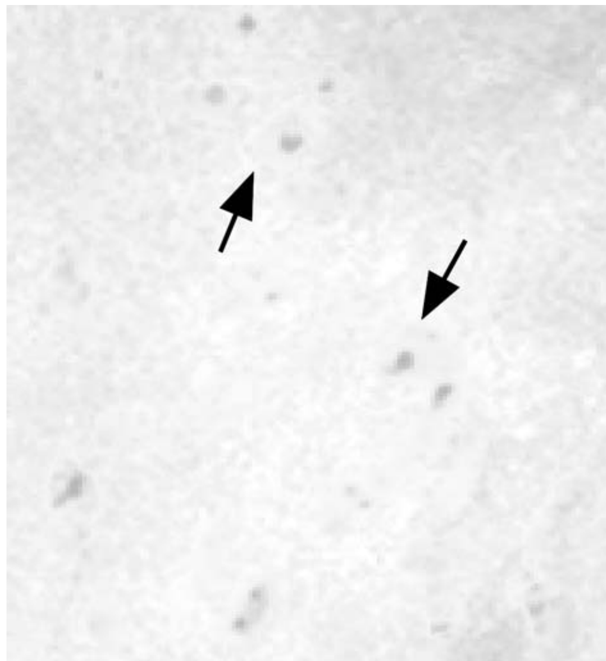

Figure 3 Quail cells (arrows) from a graft of neural crest are demonstrated in the bowel of a chick embryo to which they have migrated. The nucleolar-associated heterochromatin that characterizes the quail cell nucleus has been demonstrated by staining DNA with the Feulgen reaction. Quail cells and thus cells of donor neural crest origin can be distinguished from the chick cells of the host.

Enteric Neurons and Glia Develop from a Multipotent Precursor Population

Along with knowledge of the sites of origin of crest-derived cells came the counterintuitive discovery that differences between enteric neurons in the neural crest origins of their predecessors do not explain the phenotypic diversity of the ENS. The developmental potential of various regions of the crest, at least with respect to the ENS, does not appear

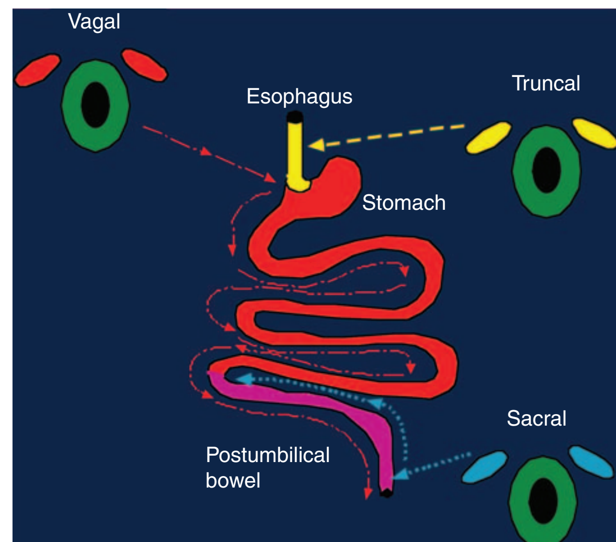

Figure 4 Cells that migrate from the vagal, rostral truncal, and sacral levels of the neural crest colonize the bowel. Vagal crest-derived cells (red) colonize the entire bowel, truncal crest-derived cells (yellow) colonize only the esophagus and immediately adjacent stomach, and sacral crest-derived cells (blue) colonize the postumbilical gut, which thus contains cells of both vagal and sacral origin (purple).

to be preset. For example, crest which, if left unperturbed, would form the adrenal medulla will instead migrate to the gut and give rise to an ENS when it is transplanted to the vagal region of a recipient embryo. This interchangeability of developmental potential of populations of crest cells has led to the concepts that there are defined pathways in embryos along which crest-derived cells migrate and that populations of crest cells are pluripotent; nevertheless, despite the pluripotentiality of the population, individual premigratory crest cells may not be pluripotent. Some crest-derived cells may already be specified before they migrate. For example, crest-derived cells that form sensory neurons migrate away from the neuraxis before those that give rise to melanocytes. The evident pluripotency of populations of crest-derived cells may be due in part, therefore, to their heterogeneity. Even if individual stem cells are not able to give rise to all the derivatives of the crest, a population may contain a sufficient number of determined precursors to allow all these derivatives to develop. According to this idea, if cells migrate to the skin, those precursors that are capable of forming melanocytes will develop, but if the same population finds itself in the bowel, those precursors that are capable of developing as enteric neurons and glia will now differentiate. Actually, cells committed to be melanocytes (which leave the neuraxis late) do not normally migrate to the bowel; they preferentially migrate along the dorsolateral pathway that leads them to the skin. The small population of melanogenic crest-derived cells that do migrate ventrally are filtered out of the population that colonizes the bowel as it passes through the caudal branchial arches so that the gut is nonpigmented. On the other hand, melanocytes develop in the gut when melanogenic precursor cells are experimentally planted there; the gut is also pigmented in chicks with the Silkie Fowl mutation, in which migratory controls fail. The crest-derived precursors of the ENS are thus not normally pluripotent, in the sense of being able to give rise to every type of cell that can be generated by the neural crest. Crest-derived cells that have completed their migration to the bowel, for example, cannot give rise to melanocytes or mesectoderm. Examinations of clones of enteric crest-derived cells, however, have established that they are multipotent, in the sense that they can give rise to glia or any of the many types of neuron that are found in the ENS. In any case, the unique properties of the ENS are determined, not by the site of origin of committed precursors in the neural crest, but by epigenetic factors, especially microenvironmental cues these cells encounter within the gut or while migrating to it.

Development of the ENS Is a Progression of Interacting Cell Autologous and Nonautologous Events

The developmental potential of enteric crest-derived cells decreases progressively as these cells age; moreover, crest-derived cells cannot respond to growth factors or molecules of the extracellular matrix unless they first express the relevant receptors. Cell autologous and nonautologous events thus combine and interact to define differentiation of the ENS. Developmental progress in the formation of enteric neurons and glia is marked by a series of transcription factors that must be expressed, receptors that must be displayed on plasma membranes, and growth factors that must be encountered. The multipotent precursors of the ENS, which are capable of giving rise to both neurons and glia, obligatorily express the transcription factors Pax3 and Sox10, which drive expression of Phox2b and the receptor tyrosine kinase, Ret. Knockout of Sox10 or Phox2b prevents the formation of any enteric neurons or glia, while knockout of Ret blocks the formation of enteric neurons below the level of the esophagus and immediately adjacent stomach. To activate Ret, ligands must first bind to a member of the glial cell-derived neurotrophic factor (GDNF) family receptor alpha (GFRα) molecules. Two Ret ligands function in ENS development to stimulate Ret, GDNF, which binds to GFRα1, and nurturin, which binds to GFRα2. Of these, GDNF is required first and by almost all neural precursors. Nurturin appears to be necessary for the formation of restricted later-arising populations of neurons found mainly in the submucosal plexus. GDNF promotes proliferation of crest-derived precursors, which are extensive in fetal bowel, and is also chemoattractive for crest-derived cells. GDNF is produced by non-crest-derived elements of the enteric mesenchyme and is thought to assist in preventing crest-derived precursors from migrating out to the gut during its colonization. Recent evidence suggests that the basic helix–loop–helix transcription factor, Hand2, is also essential in allowing enteric crest-derived cells to complete the series of steps that take them from precursors to neurons. The gut is colonized, neuronal differentiation commences in the absence of Hand2, but when Hand2 is deleted, the precursors express early neuronal markers but then freeze without terminally differentiating. Perhaps because they begin neuronal differentiation, progenitors do not shift by default toward the glial lineage, nor do the unfinished precursors die. Thus far, animals lacking Hand2 have not survived long enough to determine the fates of the neuronal precursor cells that cannot complete differentiation.

Hirschsprung's Disease Is the Most Obvious Developmental Disorder of the ENS

Defects in the genes required early in ENS differentiation are associated in humans with Hirschsprung's disease, or congenital megacolon. Hirschsprung's disease occurs when a variable length (long segment or short segment) of terminal bowel is aganglionic. The affected region of the gut is not denervated, but when the ganglia, which mediate the complex behaviors of the bowel, are lacking, the gut is as effectively obstructed as if it had been tied off with a suture. The gut dilates proximal to the aganglionic segment. Extrinsic nerves and descending nerve fibers from the proximal ganglionated portion of the gut form coarse trunks in the aganglionic region. *RET*, which is mutated in 3–35% of cases in which a gene has been identified, and the endothelin B receptor (*EDNRB*), which is mutated in 5–15% of cases, are the most common genes found to be mutated in association with Hirschsprung's disease. Others include genes encoding the ligands GDNF, GFRα1, and nurturin for RET and EDN3 (endothelin3) for EDNRB, as well as the transcription factors SOX10, PAX3, PHOX2B, and SIF1.

Hirschsprung's disease is the most visible and highly investigated enteric neuromuscular birth defect. Hirschsprung's disease is not uncommon, occurring in about one per 5000 births, except among Mennonites, where the incidence is one per 500 births because of inbreeding. Because both the development of glia and all types of enteric neurons fail, albeit segmentally, it is not surprising that expression of each of the genes linked to Hirschsprung's disease is required early in ontogeny. If left untreated, Hirschsprung's disease is lethal, but surgical removal of the aganglionic bowel prevents death. Surgery is thus life-saving, but it may not leave patients with a fully functional, inconvenience-free gut. Defects in motility leading to constipation and/or soiling may remain, even after the aganglionic tissue has flawlessly been removed, suggesting that the remaining ostensibly 'normal' ENS is really abnormal. The presence of ganglia thus does not guarantee that the ENS is normal.

Subtle ENS Defects May Arise as a Result of Mutations in Genes Required Late in ENS Development

Because enteric behavior is complex, the presence or absence of enteric neurons, which has historically been used by pathologists to assess ENS function, is inadequate. Intestinal neuronal dysplasia, for example, is a Hirschsprung's-like condition, associated with abnormal ganglia rather than aganglionosis, in affected regions. Intestinal neuronal dysplasia has been somewhat controversial, and some have doubted its existence, but it has been documented by competent pathologists and is mimicked in mice lacking Hox 11L1 and in rats that with a heterozygous endothelin B deficiency, suggesting that intestinal neuronal dysplasia is a real condition. Chronic idiopathic intestinal pseudo-obstruction (CIIP) severe enough to require bowel transplantation can even occur despite the presence of ganglia in the nonfunctioning gut. The occurrence of functional abnormalities of gastrointestinal motility, despite the presence of enteric ganglia, supports a hypothesis that there is a spectrum of functional disorders, from CIIP to IBS, that are the result of defects in genes that are not required early in the development of the ENS but which are needed to complete the development of particular subsets of neurons or to regulate synaptogenesis. Gene products required late in ENS ontogeny include the neurotrophin NT3 and its high-affinity receptor, TrkC. During the period when GDNF is required to drive proliferation of enteric crest-derived cells, NT3 does nothing to them. Crest-derived cells do not acquire TrkC or become NT3-responsive until after these cells have colonized the bowel. NT3, furthermore, is not needed for the development of all enteric neurons but only for subsets. One such NT3-dependent subset is the submucosal IPAN, which initiates peristaltic and secretory reflexes. After the deletion of NT3 or TrkC, therefore, the ENS appears to be normal and ganglia are present; however, the bowel will not function normally because it lacks a critical subset of its neurons.

Late events are important in ENS development. Early crest-derived precursors have been visualized as they migrate through the murine bowel. These cells, of vagal origin, migrate proximodistally in long chains of seemingly attached cells in the outer gut mesenchyme. The driving force for migration appears to be the proliferation of precursors at the leading edge of the colonizing population; however, the evident contact of leaders with stragglers raises the possibility that population pressure from stragglers contributes to the forward movement of émigrés. The location of the migrating cells in the outer gut mesenchyme is appropriate for the formation of the myenteric plexus. Sacral crest-derived cells delay their arrival in the postumbilical bowel until the arrival of their vagal counterparts but then ascend in the bowel in a countercurrent fashion. The submucosal plexus forms after the myenteric. The formation of the submucosal plexus results from a secondary perpendicular migration of crest-derived cells from the outer gut mesenchyme toward the mucosa. This secondary migration occurs because

netrins, which are secreted by the endoderm, attract crest-derived cells. Netrins act on the netrin receptor called 'deleted in colorectal cancer' (DCC), which is expressed by migrating crest-derived cells. Netrins are also secreted by developing pancreatic acinar cells and attract DCC-expressing crest-derived cells from the bowel, which migrate out of the gut and into the developing pancreatic buds. These crest-derived cells ultimately give rise to intrapancreatic ganglia. It is interesting that the enteropancreatic migration of crest-derived cells is followed by the extension of enteropancreatic nerve fibers from neurons in gastric and duodenal myenteric ganglia. These nerves ultimately connect the bowel to the pancreas, where they terminate in ganglia.

Gastrointestinal dysmotility disorders are neither well understood nor rare. These conditions are believed to be congenital when they occur in children, but that belief has not clarified their pathogenesis or provided a means of treatment. Although similar gastrointestinal dysmotilities also occur in adults, the possibility that adult disorders might, like their pediatric counterparts, be congenital has not been tested. Much of the research on ENS development has focused on the pathogenesis of Hirschsprung's disease. This focus is understandable because the aganglionosis of Hirschsprung's disease is so obvious; moreover, the current treatment of Hirschsprung's disease, while lifesaving, leaves much to be desired. Still, the paradigm that the presence of ganglia signifies a normally functioning ENS has proven to be false. It is thus possible that future research on the development of the ENS will be not only intellectually satisfying but fruitful in pointing the way toward understanding the pathogenesis of gastrointestinal conditions that are now mysterious, unpreventable, and poorly treated.

See also: Autonomic and Enteric Nervous System: Apoptosis and Trophic Support During Development; Enteric Nervous System: Neurotrophic Factors.

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Sexual Differentiation of the Brain

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Men and women differ not only in their anatomy and reproductive functions but also in cognitive and affective behaviors. On an average, men are generally better at tasks involving the mental rotation of objects, whereas females are superior at memory tasks in which they are asked to recall objects in a spatial array. There are also sex differences in the prevalence of mental disorders, with males more commonly diagnosed with schizophrenia, autism, and attention deficit hyperactivity disorder. Females, on the other hand, are more likely to suffer from disorders of depression and anxiety. During the past several decades, scientists have uncovered sexual dimorphisms in the central nervous system (CNS) of mammals that may contribute to the etiology of these sex differences. Studies on rodents suggest that many sexual dimorphisms in the CNS result from exposure to hormones at approximately the time of birth, lending credence to the idea that the masculinizing or feminizing seed is planted early in life. However, some areas of the brain remain vulnerable to hormones after this critical period and can incur morphological changes during adolescence and in adulthood. This article discusses instances of each in the mammalian CNS, the mechanisms that underlie these sexual dimorphisms, and examples of sexual differentiation of the human brain and behavior.

The sex of a mammal is determined at the time of conception when sperm from the male penetrates the egg and either contributes an X or a Y chromosome to the genetic makeup. If the sperm donates a Y chromosome, the offspring will be a male, and if it donates an X chromosome, the offspring will be a female. The sex-determining region Y (SRY) gene on the Y chromosome of the male is responsible for the formation of the testes, and in the absence of this gene, ovaries form. In males, it is the formation of the testes and the subsequent secretion of testicular hormones that masculinize the body. One of these hormones, anti-Müllerian hormone (AMH), suppresses the development of the female reproductive organs (e.g., the fallopian tubes and uterus), whereas another, the steroidal androgen testosterone (T), induces the formation of the male reproductive system (including the seminal vesicles and prostate) and external genitalia. In females, T and AMH are nearly absent, so male genitalia and internal sex organs fail to form and sex organs develop a feminine phenotype. Gonadal hormones can only influence genital phenotype during specific developmental periods; T in adulthood does not result in further development of male genitalia.

Testicular androgens such as T are primarily responsible for masculinization of the brain. In 1959, Phoenix and colleagues discovered that prenatal administration of T to female guinea pigs resulted in a defeminization of female sexual behavior. As adults, these early T-treated females (when administered estrogen and progesterone (E + P) to stimulate female sexual behavior) displayed little or no lordosis, the female rodent posture of sexual receptivity. However, these same females responded to testosterone in T, showing increased pelvic thrusting (typical of male mounting behavior). Similarly, adult males that were castrated immediately after birth showed increased lordosis after E + P administration as adults and decreased pelvic thrusting after T treatment. This outcome suggested that perinatal androgens are necessary for masculinization (promoting male-typical behaviors such as pelvic thrusting) and defeminization (interfering with female-typical behaviors such as lordosis) in male rodents. Females treated with T only in adulthood show little or no difference in sexual behavior, implying that rodents need 'androgen priming' in early development in order for adult testosterone to 'activate' behavior. This series of experiments laid the foundation for the organizational hypothesis, which postulates that behavioral sex differences result from (1) exposure to different hormones that act in early development to organize the neural machinery underlying behaviors and (2) exposure to different sex hormones in adulthood that activate the previously organized neural machinery. This conceptual framework has been amply confirmed in studies of nonhuman mammals.

Sexual Differentiation of the CNS

Exposure to steroid hormones at approximately the time of birth can also result in permanent morphological changes in the CNS, which may explain the lasting effects of early hormone exposure on adult behavior. In rodents, there are structural sex differences in several areas of the brain, one of the most robust of which is the sexually dimorphic nucleus of the preoptic area (SDN-POA). The volume of the SDN-POA is several times greater in males than in females, a difference which results from T exposure at approximately the time of birth. Castration of

newborn male rats decreases the size of the SDN-POA in adulthood, whereas T treatment of female rats just before and after birth increases adult SDN-POA volume. In the SDN-POA, masculinization results not from the direct activation of androgen receptors (ARs) by T or its metabolite dihydrotestosterone, as it does in the genitalia. Rather, T is converted to estrogens such as estradiol by the enzyme aromatase within the brain, and this brain-produced estrogen activates estrogen receptors (ERs) to masculinize the SDN-POA. The effect of activating ERs is to keep neurons in the SDN-POA from dying during the course of neuronal development. In turn, this rescuing of neurons from death by estradiol results in a greater SDN-POA volume in males than in females (**Figures 1(a)** and **1(b)**).

In another sexually dimorphic brain region, the anteroventral periventricular nucleus (AVPV), which is involved in regulating ovulatory cycles by influencing secretion of the luteinizing hormone, regional volume is greater in female rats and mice than in males. This sex difference is also due to the binding of aromatized metabolites of T to ERs. However, in the AVPV, ER activation promotes cell death, contributing to a larger female nucleus.

The spinal nucleus of the bulbocavernosus (SNB) provides another example of a sexually differentiated structure in the CNS in which gonadal hormones play a decisive role. The SNB is a sexually dimorphic group of motor neurons located in the lower lumbar spinal cord which innervates muscles involved in male copulatory behavior: the bulbocavernosus and

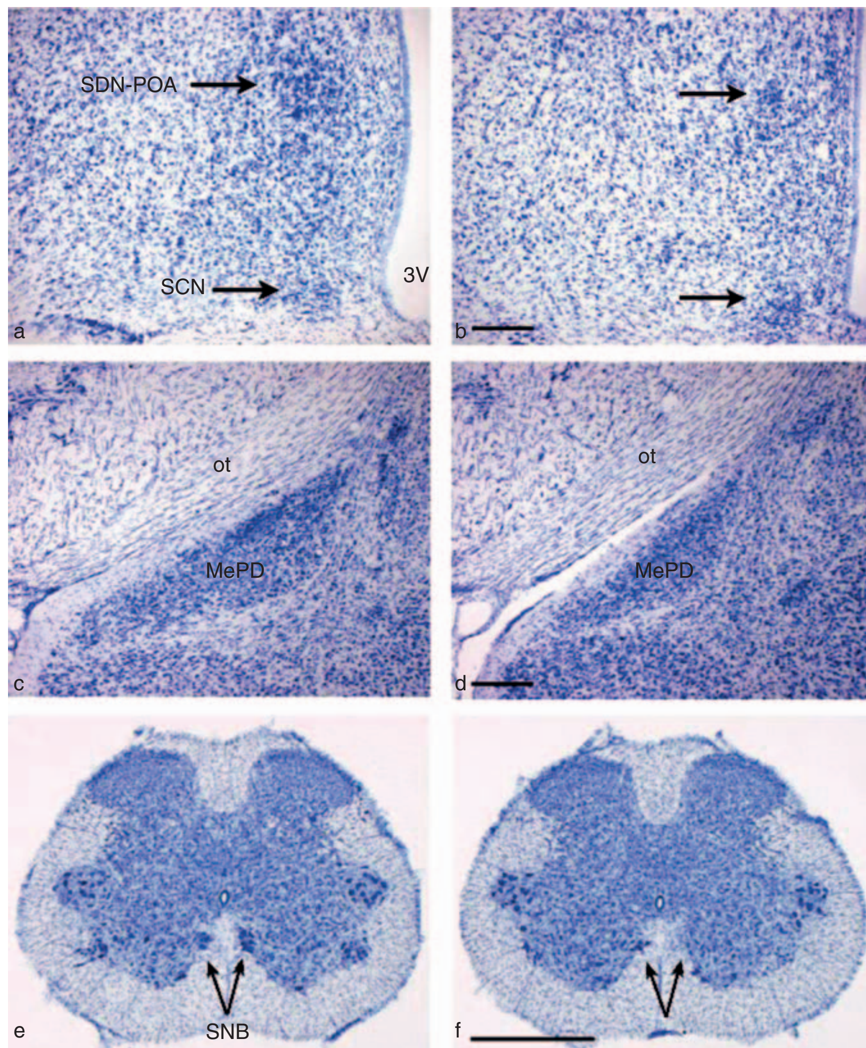

Figure 1 The sexually dimorphic nucleus of the preoptic area (SDN-POA) and the posterodorsal portion of the medial amygdala (MePD) are larger in males (left) than in females (right). There are also more motor neurons in the spinal nucleus of the bulbocavernosus (SNB) of males (left) than of females (right). SCN, suprachiasmatic nucleus.

levator ani perineal muscles. SNB motor neurons and the muscles they innervate are present at approximately the time of birth in both sexes, but they die in females postnatally while surviving in males. The SNB system is spared from death by early exposure to T in males (**Figures 1(e)** and **1(f)**). Neonatal castration of males causes the SNB system to die, whereas T treatment of females spares the system. However, unlike the SDN-POA, SNB cells are spared from apoptosis (cell death) by the action of androgens upon ARs and not ERs. Genetic male rats with a defective AR fail to develop a masculine SNB. It is also known that androgens do not act directly on SNB motor neurons to spare them from death. Rather, androgens first act in the periphery to spare the muscles that are innervated by the SNB, and this action secondarily spares SNB motor neurons from death.

Sexual Differentiation during Adolescence

The maturation of the reproductive system during adolescence is marked by a period of elevated gonadal hormones. It is likely that these gonadal hormones also play a role in molding both the male and the female adolescent brain, and emerging evidence in rodents suggests that pubertal hormones indeed organize brain morphology and behavior. For example, some sex differences in the brain develop during the course of adolescent development, as in the locus coeruleus (LC) and AVPV, where volume is greater in females, and in the primary visual cortex, where volume is greater in male rats. Both the LC and the AVPV enlarge in females during adolescence, although the mechanisms that underlie these increases in volume are not known (neurogenesis, increased migration, or cell differentiation?). There is evidence that sex differences in the primary visual cortex are the result of ovarian hormones that induce cell death in females during adolescence, suggesting that female gonadal hormones can also drive brain organization. Ovariectomy of female rats prior to puberty results in an increased number of neurons in the primary visual cortex compared to females with intact ovaries. The visual cortex of ovariectomized females becomes similar to males in both neuron number and volume.

Changes in synaptic organization also occur during adolescence, a process that has been best studied in the medial amygdala (MeA). The MeA is a brain region that receives input from olfactory and pheromonal systems by way of the olfactory bulbs, and it has been implicated in male sexual behavior. The MeA is approximately 150% larger in adult male rats than in females. A significant amount of remodeling, involving the pruning of dendrites as well as changes in dendritic spine density in the MeA, accompanies adolescent development in hamsters and may serve to organize aspects of cognition and behavior.

Studies of the Syrian hamster further indicate that adolescence is a second critical period for behavioral development. T during puberty is necessary for the full masculinization of male hamster sexual behavior since prepubertal castration decreases mounts and intromissions when the hamster is exposed to a receptive female in adulthood. Similarly, pubertal hormones appear to masculinize male agonistic behavior since castration prior to puberty decreases aggressive attacks and increases submissive behavior compared to animals that received T during puberty.

Sexual Differentiation in Adulthood

In certain areas of the CNS, sexual differentiation occurs in adulthood and is similarly dependent on gonadal hormones. In rats, portions of the MeA remain sensitive to circulating androgens into adulthood because castration of adult males causes some areas within the MeA to shrink to a size typical of females, whereas T treatment of adult females causes the same area to grow to a male-typical volume. The size of the posterodorsal medial amygdala (MePD; **Figures 1(c)** and **1(d)**, which receives olfactory and pheromonal information and is important for some aspects of male sexual behavior, appears to be solely dependent on adult hormones. MePD volume is 1.5 times greater in male compared to female rats and mice, but this sex difference can be abolished by castration of males or administration of T to females. Structural plasticity in the MePD is mediated in adulthood through activation of both ARs and ERs, and the overall amygdala also appears to grow at puberty in boys, but not girls, suggesting that it is sensitive to androgens in humans as well as in rodents.

Another brain sexual dimorphism, arginine vasopressin (AVP) fiber innervation of the septal area in rodents, depends on T in both early development and adulthood for full masculinization. Septal AVP innervation is greater in males and plays a role in pair bonding, parental, and aggressive behavior. Castration of adult males decreases septal vasopressin innervation, although AVP depletion following neonatal castration is more extensive than the reduction following adult castration. The sex difference in septal vasopressin in adulthood is primarily dependent on aromatized metabolites of T that act on ERs, although the AR also contributes to some degree to the perinatal organization of this system.

The SNB system, like septal AVP innervation, is influenced by hormones in both development

and adulthood. Castration of adult males causes widespread regression throughout the system: The muscles, the motor neurons, and their synapses all regress after castration in adulthood. These morphological changes are accompanied by a reduction in spinal reflexes of the penis that are necessary for male sexual behavior. Interestingly, although both SNB somas and dendrites shrink after castration, each appear to be regulated by distinct mechanisms. In rats, androgens act directly through ARs in SNB motor neurons to regulate soma size while acting indirectly, through AR in the target muscles, to regulate the length of SNB dendrites.

Hormonal Site of Action and Downstream Regulation

Although the particular hormones and receptors involved in sexual differentiation of brain and spinal cord structures are becoming clear, little is known about the location and cell types that these hormones act upon and the downstream genes regulated to masculinize the CNS. In the SDN-POA, T acts to prevent apoptosis; however, it is not known whether it is acting directly on neurons in the SDN-POA or in some other area that indirectly spares these neurons. In the SNB system, motor neuron survival results, secondarily, from the sparing of target musculature that provides SNB motor neurons with a signal that keeps them alive. Thus, it is possible that sexual differentiation of the SDN-POA and AVPV results from T acting on distant target cells. However, although it is known that T acts on the target musculature to spare SNB motor neurons, it is not known what cell types in the muscle (muscle fibers, fibroblasts, or Schwann cells) are involved. Similarly, in the SDN-POA and the AVPV, it is not known which cell type (neurons, astroctyes, or oligodendrocytes) provides the site(s) of action for T. The only morphological sex difference in rodents for which the cellular target of T is known is in the regulation of SNB soma size. In female rats heterozygous for the testicular feminization mutation, a mutation that renders the AR protein nonfunctional, testosterone treatment in adulthood increased soma size only in SNB neurons with functional ARs. This suggests that T acts directly via the AR in SNB motor neurons to regulate soma size.

Researchers are also just beginning to uncover the genes acting downstream from T. Evidence suggests that T may modulate the Bcl2 family of genes that are involved in regulating apoptosis in the SNB, AVPV, and the central division of the medial preoptic nucleus (which comprises a significant portion of the SDN-POA). Deletion of a member of the Bcl2 family, the Bax gene, which signals downstream regulators of cell death, decreases sex differences in neuron number in both the SNB and the AVPV by decreasing the amount of cell death. Likewise, sex differences are reduced in the SNB and AVPV in transgenic mice that overexpress the anti-apoptotic Bcl2 gene. Interestingly, T promotes cell death in the AVPV while preventing it in SNB and SDN-POA. In both cases, T appears to affect expression of Bcl2 family genes in such a way that it can spare cells in some areas and condemn them in others.

Neurotrophic factors, as mediators of hormone effects, also appear to play a role in the sexual differentiation of the CNS. In the SNB system, T-dependent signaling through the ciliary neurotrophic factor receptor α (CNTFRα) is necessary for the development of a sex difference. CNTFRα is highly expressed in SNB motor neurons and administration of CNTF to female rats rescues SNB motor neurons from death. Similarly, male mice lacking CNTFRα fail to develop a masculine SNB system. Evidence from our laboratory suggests that another neurotrophic factor, brain-derived neurotrophic factor (BDNF), plays a role in the arborization of SNB dendrites in adulthood. Castration of adult rats, which results in decreased SNB dendritic length, is paralleled by a decrease in BDNF signal in SNB motor neurons and their dendrites, whereas BDNF signal is unaltered in androgen-insensitive motor neurons of the nearby retrodorsolateral nucleus.

In the POA, T induces the formation of prostaglandin E_2 (PGE_2), which is involved in dendritic spine development. The proliferation of dendritic spines in the POA appears to be important in the organization of male sexual behavior. Pharmacological blockade of PGE_2 in male newborns inhibits the formation of dendritic spines, whereas administration of PGE_2 to newborn females increases spine formation. Neither manipulation of PGE_2 has an effect on SDN-POA volume, suggesting that T may regulate the expression of different genes to promote dendritic spine formation in the POA, on the one hand, and increase volume of the SDN-POA on the other hand. For other areas of the CNS where sex differences have been located, little is known about the genes acting downstream from T, primarily because not enough is known about the target cells that respond directly to T or its metabolites.

Sexual Differentiation of the Human Brain and Behavior

The role of gonadal steroid hormones in the masculinization of the human brain and behavior is not as clear as in rodents, partially because human

behavior is very complex. Humans are so dramatically influenced by social cues that it is difficult to decipher whether a particular behavior is the result of T exposure or a byproduct of the milieu in which they were raised. Many sex differences in behavior may simply reflect the fact that boys have a penis and girls have a vagina, because from the moment of birth something as simple as genital development will radically shape the child's social environment.

Much of what we have learned about the role of hormones in the development of human behavior has come from studies of people who have been exposed to unique cascades of hormonal development. One such example is found in genetic males (XY) with complete androgen insensitivity syndrome (CAIS), a disorder characterized by a mutation in the AR gene. In people with CAIS, the testes develop and release T, but in the absence of functional AR, T fails to masculinize the periphery, resulting in a female phenotype. These individuals are generally raised as girls, and as adults they self-identify as women. They do not differ from genetic (XX) females in sexual orientation, are just as likely to be married, and as children were involved in female-typical play. Since people with CAIS have ample T and functional ER, this suggests either that, unlike rodents, the aromatization of T to estrogen and subsequent activation of ERs is not responsible for the masculinization of behavior (and thus AR may be necessary), or that the social context in which these individuals are raised and continue to live outweighs any influence of gonadal hormones.

Studies of women with congenital adrenal hyperplasia (CAH), who are exposed to T in fetal development because their adrenal glands overproduce androgens, suggest that androgens may play a role in masculinizing the female brain. Females with CAH are more likely to engage in male-typical activities and are less interested in female-typical activities throughout life. In addition, although most women with CAH are self-reported heterosexuals, they are more likely to be homosexual than are other women. Women with CAH also show male-typical spatial performance, suggesting that androgens play a role in the masculinization of behavior in humans. However, the genitalia of these women are also slightly masculinized, which could affect the way these women are perceived either by their parents or by themselves.

Another strategy that has been used to study the effects of early androgen exposure on human behavior is through the examination of somatic markers that are affected by prenatal androgen exposure. These somatic markers of androgen exposure, including finger length measurements (ratio between second and fourth digit lengths (2D:4D)), otoacoustic emissions, eyeblink patterns, and limb measurements, can be co-varied across measures of sexual orientation, cognitive abilities, and sex-biased affective and mood disorders. Using these somatic markers, links have been made between an increased exposure to prenatal T and homosexuality in women. A relationship between somatic markers of T in male homosexuality is less clear. Examination of finger length ratios in males suggests that levels of prenatal testosterone may be related to some affective and mood disorders. Boys with autism and attention deficit hyperactivity disorder, which are more common in males, tend to have hypermasculine finger length ratios (smaller 2D:4D; increased prenatal testosterone), whereas boys with anxiety disorders have more feminine ratios (larger 2D:4D; decreased prenatal testosterone).

Differences in hormone exposure may contribute to structural sex differences in the human CNS, including the corpus callosum, anterior commissure, third interstitial nucleus of the anterior thalamus (INAH3), bed nucleus of the stria terminalis, Onuf's nucleus, and suprachiasmatic nucleus (SCN). There is also a sex difference in overall brain weight favoring men, although women have a greater cortical surface area. These sexual dimorphisms in brain morphology may contribute to gender differences in behavior and the prevalence of some mental illnesses. Very little is known about how sex differences in the human brain may be related to differences in behavior, although the sizes of two hypothalamic nuclei have been correlated to sexual orientation in men. Inspired by the dramatic sex difference in the SDN-POA of rats, in 1991 Simon LeVay compared nuclei within the preoptic area of heterosexual men and women and discovered that one of these nuclei, INAH3, was larger in heterosexual men than in women, and that homosexual men had a feminine-sized nucleus. On the other hand, the vasopressinergic subnucleus of the SCN was found to be larger in volume and contained twice as many cells in homosexual men compared to heterosexual men. In this instance, the nucleus of homosexual men was not feminized. Together, these findings indicate that the brains of homosexual men may be feminized in some areas but not in others. However, it is important to note that it is unclear whether these morphological differences in the brains of homosexual men are the result of androgen exposure or arise as a result of differences in experience and behavior.

In some areas of the brain, sex differences in morphology may exist as a means to minimize sex differences in behavior. Differences in gonadal hormone levels between men and women could potentially contribute to maladaptive sex differences in behavior,

with alterations in brain structure compensating for these differences. An example of such a phenomenon has been suggested in the prairie vole, in which the male and female both contribute to the care of their offspring. In females, changes in hormones that accompany pregnancy and childbirth prime maternal behavior. Males, on the other hand, are not exposed to these hormones and their parental behavior appears to rely on sexually differentiated vasopressin innervation. This more extensive vasopressin innervation of the septal area in males may then compensate for the lack of maternal hormone exposure and make male prairie voles more effective fathers. It is also possible that hormones *per se* are not the sole contributor to the sexual differentiation of brain and behavior. Evidence suggests that in some songbirds and perhaps in mice, the expression of sex chromosome genes within cells may exert some influence on sexual differentiation of the brain and behavior independently of gonadal steroids. However, gonadal hormones are the primary instigators of sex differences in neural structure.

In many ways, hormone exposure during critical periods of development affects us, partially because these hormones have made us look like a male or a female, and also because these hormones have influenced the development of our brain and subsequently our behavior. Hormones may also continue to act throughout our lives to influence behavior; decreases in gonadal hormones with aging are often associated with depression, anxiety, debilitated memory, and general unhappiness. Lifelong plasticity in areas such as the MePD and the SNB demonstrates how these changes in behavior may also be accompanied by changes in structure. It is likely that there are many additional sex differences in the CNS waiting to be revealed. As we discover more of these dimorphisms and uncover how these sex differences emerge, we gain a better understanding about the influence of hormones and why, in many ways, men and women are really not the same.

See also: Sexual Differentiation of the Central Nervous System.

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Sexual Differentiation of the Central Nervous System

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Introduction

Despite a wide variety of behaviors that are shared between male and female members of vertebrate species, certain traits are strongly associated with a single sex. Such behaviors are said to exist in two forms and are therefore dimorphic. Behaviors that are characteristic of males are designated as masculine and those that are characteristic of females as feminine. Sexually dimorphic behaviors are widespread among vertebrate species because nearly all reproduce sexually. Accordingly, dimorphic phenotypic traits have developed to support these sex-specific behaviors and the physiological processes that accompany them. Thus, males and females display distinct copulatory behaviors and neuroendocrine physiological responses. The importance of these sex-specific functions to vertebrate species is reflected in their widespread occurrence and in the reliability of developmental mechanisms that have evolved to ensure their unambiguous display. Sexually dimorphic behavior extends beyond reproduction and maternal behavior, although such differences can be more subtle and at times less reproducible.

Chromosomal Sex and the Developing Gonad

Masculinization of the body begins with a genetically controlled process termed sex determination that leads to the sex-specific differentiation of the gonad. The genetic sex of mammals is specified by the presence of either paired X chromosomes (females) or single X and Y chromosomes (males). The paternally derived Y chromosome contains the Sry (sex-determining region of the Y) gene, which encodes a transcription factor that causes the undifferentiated gonads to develop into testes; the absence of Sry expression leads to development of ovaries. Hormones secreted by the testes act on various tissues to masculinize the body, and it is testosterone secreted by Leydig cells in the testes that is responsible for masculinization of the central nervous system (CNS). In males, levels of testosterone are elevated significantly at approximately the time of birth (perinatal) and pass through the blood to the CNS, where they have free access to developing neurons and glia. Although all of the evidence to date supports a defining role for testosterone in sexual differentiation of the brain, it would be surprising if genes located on the Y chromosome, and therefore only present in males, did not influence masculinization of the CNS in some manner. Genetic studies in mice suggest that chromosomal sex does appear to impact certain behaviors, but these effects are relatively subtle compared with the robust changes caused by sex steroid hormones.

Major Principles and Model Systems

The developing brain is considered to be bipotential with respect to sexual differentiation, and in the absence of testosterone it develops phenotypic traits that are characteristic of females. For example, the ability of males to mount receptive females and the ability of females to display lordosis are dependent on the perinatal hormone environment. Similarly, whether the brain can induce a surge in secretion of gonadotropins from the pituitary gland, and thereby cause ovulation, is also determined by perinatal exposure to testosterone. Sex steroids also regulate expression of reproductive behavior and associated neuroendocrine events throughout life in response to what are described as activational influences of steroid hormones. The more permanent, developmental actions of testosterone are viewed as having an organizational action on the CNS and tend to be restricted to discrete periods of development. Thus, treating a female rat with testosterone during the first few days of life will permanently masculinize copulatory behavior and eliminate ovulation, but applying a similar treatment in adulthood does not. However, in adulthood, sex steroids regulate sexually dimorphic behavior and these activational actions are required for full expression of behavior (**Figure 1**).

The ability of perinatal testosterone to permanently alter neural structure and function is primarily due its action on brain development. The neural circuits controlling sexually dimorphic functions are thought to be specified during a developmental critical period occurring at or near the time of birth. For example, injecting female rats with testosterone during the first week of life generally increases the size of the sexually dimorphic nuclei, but treatments later on do not. However, sex steroid hormones continue to affect the structure and function of the CNS throughout life, but to a more limited degree than during the perinatal period. Gonadal hormones can alter patterns of connectivity, change expression of neurotransmitters and their receptors, and influence electrophysiological properties of neurons in adults. These effects can be very rapid (seconds to minutes) or can occur over extended periods (days to weeks). Thus, it is the combined action of sex steroid

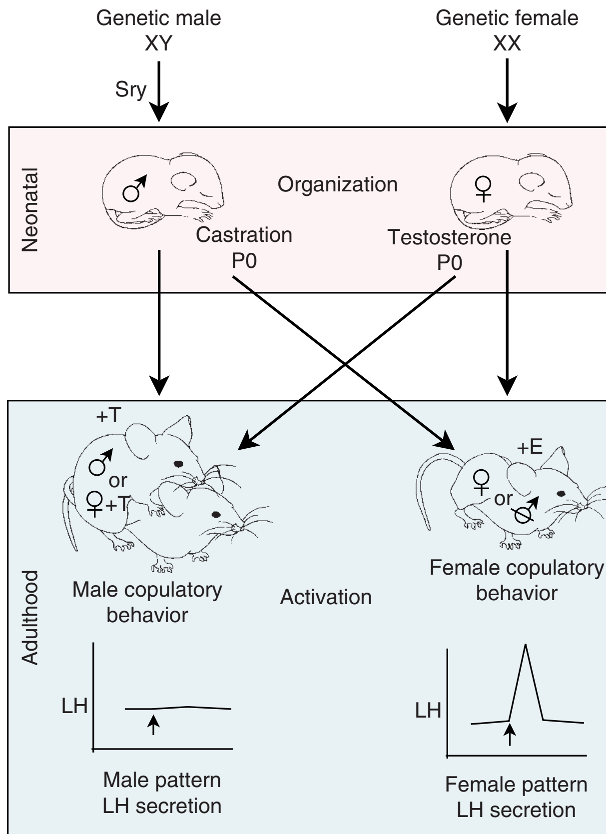

Figure 1 Sexual differentiation and hormonal activation. Sex determination is specified by the complement of sex chromosomes (X and Y). Expression of the Sry gene on the Y chromosome in males causes formation of the testes, which in rodents secrete testosterone at high levels during the neonatal period (the first few days of life). During this period, testosterone acts on the brain to 'organize' the structure of regions that mediate sexually dimorphic functions. During adulthood, sex steroid hormones such as testosterone (T) and estrogen (E) act on the brain to 'activate' expression of copulatory behavior, mounting in males and lordosis or arching of the back in females, as well as sex-specific patterns of luteinizing hormone (LH) secretion from the pituitary gland, which causes ovulation. Castration of male newborn mice, or treating females with testosterone, on the day of birth (P0) reverses expression of these sexually dimorphic functions.

hormones, acting during discrete periods of sensitivity during development, together with the more short-term activational actions of these hormones later in life, that determines display of sexually dimorphic behaviors and physiological responses. However, it is important to remember that levels of circulating hormones in adult animals are different in males and females, which may influence expression of phenotypic traits independent of the developmental actions of testosterone. Surprisingly, it is estrogen that effects many of the masculinizing developmental actions of testosterone in the brain through intracellular conversion of testosterone to estradiol (a form of estrogen) by the enzyme aromatase. Once formed in the cell, the estradiol binds to estrogen receptors, which function as ligand-activated transcription factors to control gene expression. By regulating expression of genes involved in multiple aspects of cell function and development, estrogen directs the formation of sexually dimorphic neural circuits that are presumably responsible for expression of sexually dimorphic behaviors and physiological responses.

Because of its prominent role in regulating reproductive function, the hypothalamus has been the focus of many studies on sexual dimorphism in the brain, and most sexually dimorphic nuclei have been found within the hypothalamus or in limbic regions that innervate it. Nuclei in the preoptic region of the hypothalamus are known to control copulatory behavior and ovulation, and generally these regions contain high densities of neurons that express receptors for testosterone and estrogen. In a variety of mammalian species, preoptic nuclei involved in these functions contain different numbers of neurons in males and females, as well as having sexually dimorphic patterns of connectivity. The medial preoptic nucleus of the hypothalamus (MPN) contains distinct components that are larger in males and contain more neurons. These cellular components were included within a region designated as the sexually dimorphic nucleus of the preoptic area (SDN-POA) by Roger Gorski and co-workers. This region is so dimorphic as to be identifiable in stained tissue sections without magnification. Treatment of females with either testosterone or estrogen increases the size of the SDN-POA to that characteristic of males, and removal of the gonads from newborn male rats reduces the size of the region. Most morphological sex differences follow this pattern of hormonally specified male dominance, but the pattern is reversed in another preoptic nucleus, the anteroventral periventricular nucleus (AVPV). The AVPV plays a critical role in the control of ovulation and is larger in females. Exposure to testosterone during perinatal life reduces the size of the AVPV and permanently alters numbers of distinct subpopulations of neurons in a cell-type-specific manner. For example, treatment of female rats with testosterone decreases the number of dopaminergic neurons in the AVPV but increases numbers of neurons that express the opioid peptide dynorphin in the same nucleus. In humans, the rostral hypothalamus contains several distinct cell groups, termed the interstitial nuclei of the anterior hypothalamus, and at least two of these nuclei have been reported to be sexually dimorphic. Although these findings are controversial, and present difficulties for functional interpretation, it seems clear that structural sex differences are not restricted to nonhuman species.

The MPN and AVPV receive inputs conveying a variety of sensory modalities, but the most direct are those than transmit olfactory signals. The medial nucleus of the amygdala and principal nucleus of the bed nuclei of the stria terminalis (BSTp) are sexually dimorphic nuclei (larger in males) that are key components of the pathway from the accessory olfactory bulb to the hypothalamus. Androgen receptors may play a more prominent role in regulating the development of these telencephalic nuclei than they do in the hypothalamus, and these regions appear to remain relatively sensitive to the effects of testosterone manipulations in adulthood. Breedlove and colleagues showed that castration of adult males decreased, and treatment of adult females increased, the size of the posterodorsal part of the medial amygdala. Concurrent changes in the hormone-treated animals in their responses to airborne cues from receptive females suggest that hormonal regulation of nuclear volume may underlie changes in the function of this sexually dimorphic olfactory pathway.

The most thoroughly studied sexually dimorphic motor pathway is the spinal nucleus of the bulbocavernosus (SNB), which innervates striated muscles of the perineum (**Figure 2**). The motor neurons of the SNB are present before birth in both sexes and innervate the muscles, but the muscles regress and the neurons die soon after birth in females unless they are exposed to testosterone. In contrast to the sexual differentiation of the preoptic region, which is largely mediated by estrogen receptors, the action of testosterone on the SNB relies solely on androgen receptors.

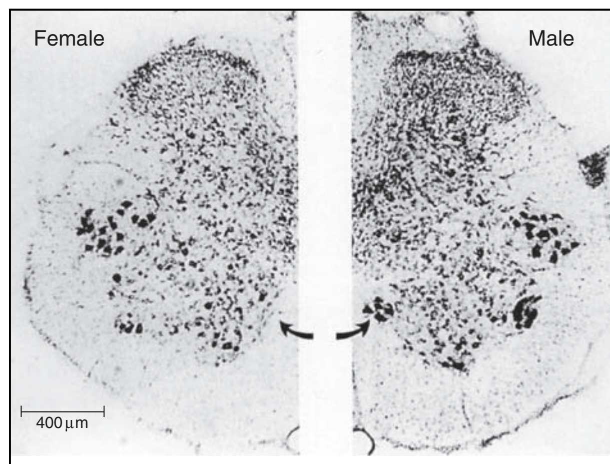

Figure 2 The spinal nucleus of the bulbocavernus (SNB). The SNB is found in the lumbar region of the spinal cord in rats. There are 3 times as many motor neurons in the SNB (arrows) of male rats as there are in females. Adapted from Breedlove SM, Jordan CL, and Kelley DB (2002) What neuromuscular systems tell us about hormones and behavior. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, and Rubin R (eds.) *Hormones, Brain and Behavior*, pp. 192–222. San Diego, CA: Academic Press.

However, testosterone does not act directly on the SNB to influence neuron number but, rather, acts on the perineal muscles and then secondarily affects the motor neurons of the SNB. Such target-dependent control of development is a common feature of neuromuscular systems. However, hormones can also act at multiple levels and at multiple times during development of neuromuscular systems. For example, in the frog vocal control neuromuscular system studied by Darcy Kelley and colleagues, both muscle cells and motor neurons express androgen receptors and appear to represent direct targets for hormone action on sexual differentiation of this system.

The avian song control system presents yet another pattern of steroid action. Male zebra finches sing more than females, and the system of nuclei responsible for song production is sexually dimorphic. Treatment of newly hatched female finches with estrogen masculinizes both the pattern of singing and the size of sexually dimorphic song system nuclei. Surprisingly, neither castration of young birds nor blockade of steroid receptors prevents sexual differentiation of these brain nuclei. *In vitro* studies of isolated brain slices provide a possible explanation: the active hormone (estrogen) is produced by forebrain neurons, which then directs sexual differentiation locally. However, even in identical culture conditions the slices derived from the brains of male birds produced more estrogen than those derived from females, suggesting that the male genotype may cause a higher level of hormone production in the male brain, independent of hormone levels produced by the gonad. Evidence from mammalian systems also supports a possible role for genetic signals in sexual differentiation of the CNS. Dispersed cultures of midbrain dopaminergic neurons derived from male and female mice placed *in vitro* on embryonic day 14, a time that precedes the normal increase in testosterone levels, nevertheless develop differently: the cultures derived from males end up with significantly more neurons. Moreover, similar embryonic cultures derived from mice lacking the Sry gene, and therefore lacking testicular testosterone secretion, have a greater number of dopaminergic neurons when derived from male embryos compared to those derived from females. The laboratory of Arthur Arnold used transgenic mice, engineered to display gonadal sex independent of chromosomal sex, to study correlations between Y chromosomal genes and sex differences in behavior and brain chemistry. Together, these findings suggest that multiple cell types may be masculinized by genetic signals. However, it should be noted that the overwhelming majority of sexually dimorphic traits evaluated in this transgenic paradigm support a dominant role for sex steroids in the control of sexual differentiation.

Neurobiology of Sexual Differentiation

Few studies have provided definitive proof that individual sexual dimorphisms are responsible for specific aspects of functional sex differences. In large part this is due to the fact that the study of sexual dimorphisms proceeded well ahead of our understanding of the functional neural systems that mediate sexually dimorphic behavior and physiology. However, an emerging perspective is that sexually dimorphic aspects of neural circuits conveying or decoding sensory information, or that control motor output systems, function differently in males and females because of structural differences in their organization. Alternatively, there may be differences in the way these pathways respond to changes in hormone levels acutely. Brain function is dependent on the numbers, organization, and patterns of connections that constitute functional neural systems, so it follows that developmental events that alter these parameters of neural circuits will impact their function. Numerous regions in the mammalian forebrain have been identified as sexually dimorphic, and their normal development is dependent on testosterone exposure during the perinatal period. For hypothalamic regions, conversion of testosterone to estradiol and activation of either or both estrogen receptors (ER-α and ER-β) are initial events in the process. These hormone receptors impact development through changes in gene expression that ultimately cause changes in cell number, morphology, neurochemistry, and connectivity among a wide variety of sexually dimorphic neural circuits.

Sex Steroids Regulate Programmed Cell Death

Assembly of neural circuits involves generation and differentiation of component populations of neurons, migration of neurons to resident sites, and establishment of functional connections with other parts of the nervous system. Hormonal regulation of neurogenesis does not appear to play a major role in specifying sexually dimorphic numbers of neurons in the CNS; however, estrogen appears to influence neurogenesis in mature animals. Nor does formation of sexually dimorphic nuclei appear to be influenced significantly by hormonal regulation of neuronal migration patterns; however, it is possible that experimental limitations are largely responsible for a lack of definitive information on migration. The cumulative evidence indicates that the major way in which sex steroid hormones alter neuronal number in sexually dimorphic regions of the CNS is by influencing cell death. Testosterone decreases cell death in the MPN, BSTp, and SNB, all of which have more neurons in males. In contrast, testosterone exposure increases cell death in the AVPV, where cell number is greater in females. In each of these regions, dying neurons display the morphological features of an apoptotic cell death mechanism, including DNA fragmentation. Moreover, cell loss in the AVPV appears to depend on activation of caspases, enzymes that mediate key aspects of the apoptotic cell death cascade. Although the evidence for cell death as a mechanism underlying sexual differentiation of neuronal number is most thoroughly documented for these nuclei, there is compelling support for cell death as a major determinant in several sexually dimorphic nuclei, in a variety of vertebrate model systems, indicating that hormonally induced control of cell number is a widespread neurobiological mechanism. Less clear is how sex steroid hormones bring about cell death. One possibility is that testosterone regulates expression of antiapoptotic genes such as members of the Bcl2 family, and overexpression of Bcl2 appears to function in a neuroprotective way. The laboratory of Nancy Forger showed that overexpression of Bcl2 in mice reduces the magnitude of sex differences in the SNB and AVPV, suggesting that testosterone regulates cell death in these nuclei through a Bcl2-dependent mechanism. Sex differences were eliminated in mice in which the proapoptotic gene Bax was deleted, demonstrating that Bax is required for sexual differentiation of these nuclei. However, it is a mistake to assume that all sexually dimorphic populations of neurons are regulated through differential control of such proapoptotic or antiapoptotic genes. For example, dopamine neurons in the AVPV are resistant to the developmental effects of manipulating Bcl2 expression or deletion of Bax, despite the overall effects on cell number in the AVPV, indicating that the reduction in dopaminergic neurons seen following exposure to testosterone is caused by a cell death program that is independent of Bcl2 family proteins. In addition to regulation of cell death, testosterone acting early in development may cause lasting changes in neurotransmitter phenotype. In the BST of the rat, perinatal steroids appear to specify a subset of galanin-containing neurons to co-express vasopressin, consistent with the notion that hormones acting perinatally may induce marked and lasting changes in gene expression that lead to sex-specific patterns of neuronal differentiation.

Sex Steroids Specify Patterns of Connectivity

Axon guidance The assembly of functional neural circuits requires neurons to establish polarity, undergo dendritic morphogenesis, and extend axons that traverse significant distances in the CNS guided to their

targets by a complex array of molecular guidance cues. Estrogen is capable of exerting a direct trophic action on neural tissue, especially if the neurons express estrogen receptors. For example, hypothalamic explants extend a profusion of neurites in response to estrogen treatment, suggesting that sex steroids can promote axonal growth. Two sexually dimorphic nuclei in the zebra finch song control system, designated the higher vocal center (HVC) and the robust nucleus of the archistriatum (RA), play important roles in the motor control of song production. A massive projection from the HVC to the RA is required for expression of song in adulthood, and this pathway forms in male but not female finches. Formation of this pathway can be induced in female finches by exposure to exogenous estrogen, which *in vivo* appears to be derived locally rather than from the gonad. In contrast, neurons in the preoptic region of the quail hypothalamus provide sexually dimorphic projections to the midbrain periaqueductal gray. These neurons contain aromatase and convert gonadally derived testosterone to estrogen during development. The homologous pathway in rats shows a similar sex difference, with more preoptic neurons innervating the periaqueductal gray in males than in females. The most dimorphic pathway in the mammalian forebrain appears to be a projection from the BSTp to the AVPV (**Figure 3**). The intensity of this limbic–hypothalamic pathway is at least an order of magnitude greater in males relative to the homologous pathway in females, and it appears to develop through a directed mechanism rather than forming in both sexes and then regressing in females. The molecular mechanisms directing development of the BSTp-to-AVPV pathway remain unknown, but it is clear that testosterone acts on the AVPV target cells to promote innervation by BSTp neurons. Because the BSTp is larger in males, it is not surprising that it provides a more robust projection to its targets in males. However, the density of projections from the BSTp to the AVPV in male rats is much greater than would be predicted simply on the basis of the greater number of neurons in the BSTp. Numbers of projection neurons are often influenced by the size of their targets, but the AVPV contains fewer neurons in males relative to females, which would argue for a weaker input in males if target size were controlling the number of afferents from the BSTp. Thus, exposure to sex steroids during the perinatal period decreases the number of cells in the AVPV while increasing the innervation of the remaining cells, resulting in a hormonally directed increase in the convergence of BSTp inputs onto AVPV neurons in males. Because the BSTp conveys olfactory information to the hypothalamus, the hormonally directed differentiation of the BSTp-to-AVPV pathway means that sensory information will impact certain hypothalamic circuits differently in males and females. Innervation of perineal muscles by the SNB is also regulated by target-derived factors. In this system, testosterone acts on the muscles to induce endogenous factors, which then act on SNB motor neurons to mediate

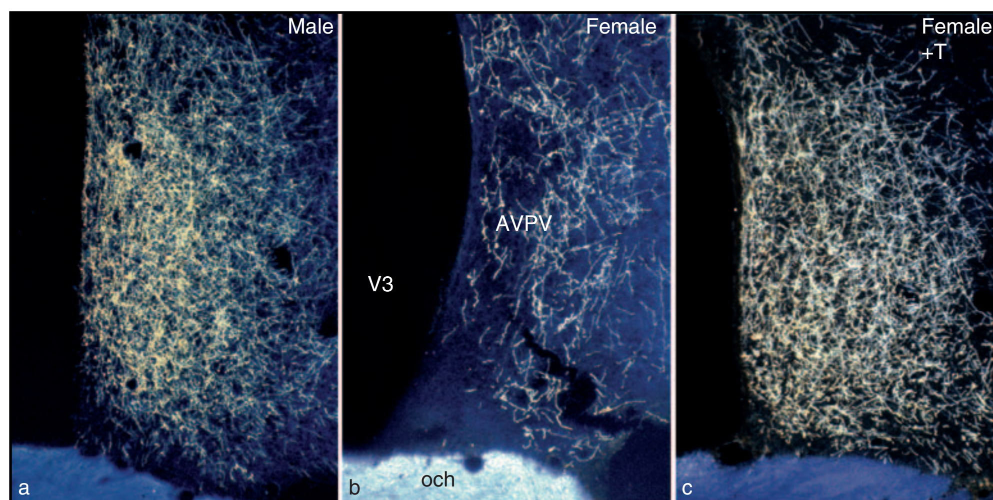

Figure 3 Sexually dimorphic limbic–hypothalamic pathway. The principal nucleus of the bed nuclei of the stria terminalis (BSTp) innervates the anteroventral periventricular nucleus (AVPV) of the hypothalamus. The axonal tracer PHAL was used to label neural projections from the BSTp to the AVPV in male (a), female (b), and testosterone-treated female (c) rats. Treatment of newborn females with testosterone induces innervation of the AVPV by BSTp neurons, thereby changing this pathway from the female to the male pattern. och, optic chiasm; V3, third ventricle. Adapted from Gu G, Cornea A, and Simerly RB (2003) Sexual differentiation of projections from the principal nucleus of the bed nuclei of the stria terminalis. *Journal of Comparative Neurology* 460: 542–562.

sexual differentiation of the SNB. One such factor has been identified as the ciliary neurotrophic factor, but other factors that influence growth and maintenance of neuromuscular junctions may also play a role in specifying innervation of perineal musculature by SNB neurons. The brain-derived neurotrophic factor (BDNF) is important for development of the avian song control nuclei, but in contrast to the SNB system, BDNF appears to act through both retrograde and nonretrograde signaling mechanisms.

Synaptogenesis Sex steroid hormones also appear to be involved in regulation of dendritic structure and synapse formation in sexually dimorphic regions. *In vitro* experiments document the ability of estrogen to promote dendritic length and branching in embryonic limbic and hypothalamic neurons. Estrogen specifies sexually dimorphic patterns of dendritic morphology in the hypothalamus of postnatal rats, and glial cells appear to play a determinant role in this process. Sexually dimorphic patterns of synapses on hypothalamic neurons have been identified that are determined by exposure to testosterone during perinatal life. In the arcuate nucleus of the hypothalamus, synapses on both dendrites and cell bodies of neurons are more abundant in females. In contrast, there is a different synaptic pattern in the ventromedial hypothalamic nucleus, in which the density of synapses is greater in males, indicating that there is considerable regional variation in sexually dimorphic patterns of synaptogenesis. Whether these different patterns of synaptology are due to differential innervation by regions with varying numbers of afferent neurons or are locally specified by postsynaptic mechanisms is unknown. The arcuate nucleus is innervated by the AVPV, which contains more neurons in females and provides more robust innervation of the arcuate nucleus. Similarly, several regions that have greater numbers of neurons in males innervate the ventromedial nucleus. However, inputs to the ventromedial nucleus from the BSTp are similar in males and females, even though the BSTp provides a sexually dimorphic projection to the AVPV, indicating that at least for the BSTp, hypothalamic targets play an important role in specifying patterns of innervation. Thus, in addition to regulating the numbers of neurons that reside in sexually dimorphic nuclei, perinatal testosterone specifies patterns of connectivity in multiple neural networks. The functional consequence of these sex differences is that the input–output relationships of neurons comprising key components of both sensory and motor pathways are different in males and females, which means that information is processed in unique ways, leading to expression of sex-specific behaviors.

The Role of Receptors for Estrogen and Testosterone

A key developmental factor in the regulation of sexual differentiation of the brain is expression of receptors for estrogen and testosterone by neurons in sexually dimorphic nuclei (**Figure 4**). All of the major sexually dimorphic nuclei in the mammalian brain contain high densities of neurons that express ER-α, AR, or ER-β during the perinatal period, when testosterone exerts its greatest effect on brain development. These receptor proteins function primarily as ligand-activated transcription factors that regulate cell development through regulation of gene expression. However, sex steroids can also influence the activity of neurons through what appear to be membrane-associated receptors. Because neural activity has a profound influence on neuronal differentiation and formation of connectivity, such membrane receptor-mediated effects of sex steroids may alter development of regions that do not express the nuclear steroid receptors in abundance. In the rodent, estrogen receptors are responsible for regulation of sexual differentiation of the hypothalamus, whereas the AR appears to mediate most of the sex-specific developmental events in telencephalic regions. This regional specification of receptor function may underlie the enhanced role of the AR in sexual differentiation of behavior in primate species, in which telencephalic regions play a more dominant role in controlling behavior.

The signaling events underlying the developmental actions of sex steroid hormones appear to interact at several levels with neurotrophin signal transduction pathways. For example, estrogen can activate the mitogen-activated protein kinase cascade in postnatal neurons as well as regulate the activity of transcription factors such as c-fos and the cAMP-regulated enhancer binding protein (CREB). These cellular signals in turn play an important role in coupling neurotrophin signaling to the nucleus. Work from the laboratory of Margaret McCarthy has directly implicated CREB in sexual differentiation of copulatory behavior. Few downstream targets of these hormonally activated signaling pathways have been identified, but similarities between some of the morphological effects of estrogen and molecules such as BDNF suggest there is a convergence of neurotrophin and sex steroid signaling on common pathways impacting development. These signaling pathways are commonly activated by a variety of environmental factors and are sensitive to the overall effects of experience. It is therefore intriguing to speculate that the observed interactions between developmental

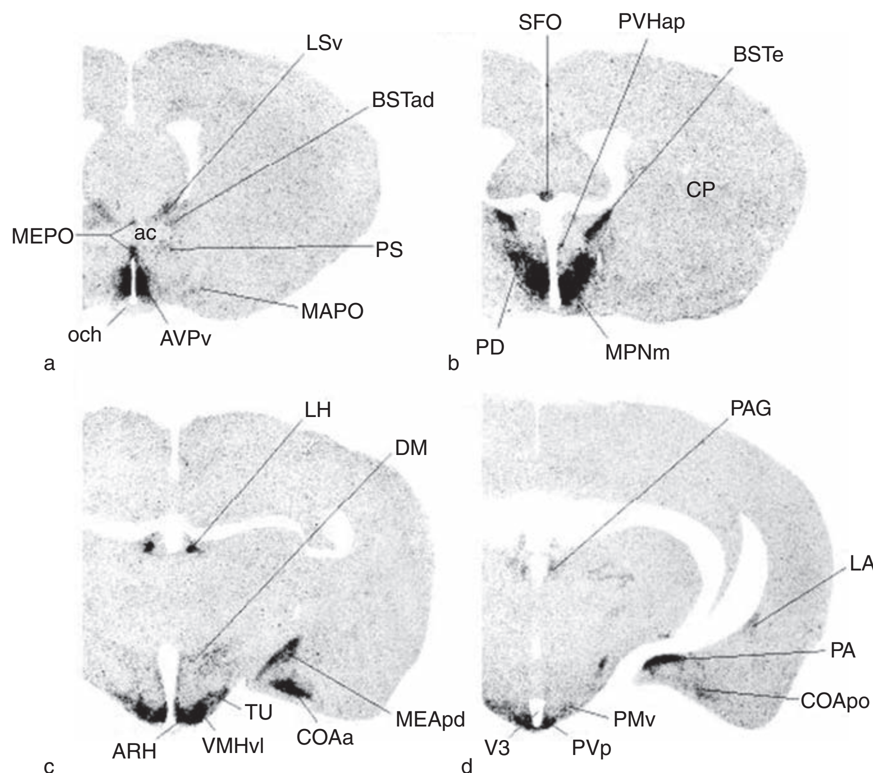

Figure 4 The distribution of forebrain neurons that express estrogen receptor (α form). Estrogen receptors are expressed in distinct regions of the forebrain in adulthood, as illustrated here by expression of ER-α mRNA, and during development. Sexually dimorphic regions of the forebrain tend to contain high densities of neurons that express ER-α. ac, anterior commissure; ARH, arcuate nucleus of the hypothalamus; AVPv, anteroventral periventricular nucleus; BSTad, anterodorsal nucleus of bed nuclei of the stria terminalis; BSTe, encapsulated (principal) nucleus of bed nuclei of the stria terminalis; COa, cortical nucleus of the amygdala (anterior part); COApo, cortical nucleus of the amygdala (posterior part); CP, caudoputamen; DM, dorsomedial hypothalamic nucleus; LA, lateral nucleus of the amygdala; LH, lateral habenula; LSv, lateral septal nucleus (ventral part); MAPO, magnocellular preoptic nucleus; MEPO, median preoptic nucleus; MeApd, medial nucleus of the amygdala (posterodorsal part); MPN, medial preoptic nucleus (m, medial part); PA, posterior nucleus of the amygdala; PAG, periaqueductal gray; PD, posterodorsal preoptic nucleus; PMv, ventral premammillary nucleus; PS, parastrial nucleus; PVHap, paraventricular nucleus of the hypothalamus (anterior parvicellular part); PVp, posterior periventricular nucleus; och, optic chiasm; SFO, subfornical organ; TU, tuberal nucleus; V3, third ventricle; VMHvl, ventromedial hypothalamic nucleus (ventrolateral part). Reproduced from Simerly RB (1995) Hormonal regulation of limbic and hypothalamic pathways. In: Micevych PE and Hammer RP Jr. (eds.) *Neurobiological Effects of Sex Steroid Hormones*, pp. 85–116. New York: Cambridge University Press.

events specified by hormones and the impact of external environmental influences on behavior may be linked through common intracellular signaling pathways.

See also: Sexual Differentiation of the Brain.

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AXON GUIDANCE AND SYNAPTOGENESIS

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Chemoaffinity Hypothesis: Development of Topographic Axonal Projections

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Introduction

A critical function of the vertebrate nervous system is to interpret the environment through the connections of various sensory organs. To accomplish this task, the incoming information must be organized in an efficient manner. Perhaps the most efficient organization is achieved through the use of topographic maps that are present throughout the organism to process sensory information. A topographic map is a projection from one set of neurons to another wherein the receiving set of cells reflects the neighbor relationships of the projecting set. In the nervous system of higher vertebrates, topographic maps are common and include sensory body maps, tonotopic maps, and maps of visual space. Furthermore, topographic maps persist in some form throughout the circuitry, from first-order to higher-order connections.

Axon guidance is critical to the formation of appropriate neural connections. However, once the primary growth cone reaches the tissue that it will innervate, the problem switches from finding the correct linear path to finding the correct point in a two- (or three-) dimensional space. This latter problem, known as target recognition, has long been postulated to be under the control of gradients of axon guidance molecules. The chemoaffinity hypothesis, formally proposed half a century ago by Roger Sperry, outlines a conceptual framework by which neural connections can develop a topographic map. The predominant model for studying map development has been the primary visual projection formed by the axons of retinal ganglion cells (RGCs) to their major midbrain target, the optic tectum (OT) of fish, amphibians, and chick, or the superior colliculus (SC) of mammals. The representation of the retina onto the OT or SC can be simplified to the mapping of two sets of orthogonally oriented axes: the temporal–nasal (TN) axis of the retina along the anterior–posterior (AP) axis of the OT or SC, and the dorsal–ventral (DV) axis of the retina along the lateral–medial (LM) axis of the OT or SC (corresponding to the ventral–dorsal OT axis in nonmammalian vertebrates).

Studies of amphibians and fish, as well as invertebrates, have provided much insight into mechanisms of topographic map development and will certainly continue to have substantial impact on the field. However, this article will focus on chicks and mice because of their similarities in map development, their closer relationship to human biology, and the greater availability of relevant genetic experimentation.

The Chemoaffinity Hypothesis

The chemoaffinity hypothesis, formally proposed by Roger Sperry in 1963, presaged the dawning of the era of molecular mechanisms of map development. Sperry expressed his hypothesis in the following passage:

> I still go back to my initial interpretation proposing an orderly cytochemical mapping in terms of two or more gradients of embryonic differentiation that spread across and through each other with their axes roughly perpendicular. These separate gradients successively superimposed on the retinal and tectal fields and surroundings would stamp each cell with its appropriate latitude and longitude expressed in a kind of chemical code with matching values between the retinal and tectal maps.

His critical suggestion was that these molecular tags were associated with the neural tissue (e.g., membrane bound) and might be distributed in complementary gradients that mark corresponding points in both the sensory (e.g., retina) and target structures (e.g., OT or SC). Although Sperry based this hypothesis on his studies of regeneration of the retinotectal projection in newts and frogs, it provided the fledgling field of map development with direction. The basic foundation of the chemoaffinity hypothesis has largely been borne out, but subsequent experimental studies and mathematical models have refined it substantially to add multiple gradients of attractants and repellents and to more accurately account for the sequential phases of complex behaviors exhibited by RGC axons during map development in the OT or SC of higher vertebrates.

Based on the chemoaffinity hypothesis, each point in the OT or SC would have a unique molecular address determined by the graded distribution of topographic guidance molecules along the two tectal axes, and similarly each RGC would have a unique profile of receptors for those molecules that would result in a position-dependent, differential response to them by RGC axons. Over several decades following Sperry's hypothesis, the specificity of the projections of RGC axons to tectal cells was investigated further by the tracing of axonal projections following experimental manipulations, first in the regenerating retinotectal system of fish and amphibians, and later in the developing retinotectal or retinocollicular

projections with the development of high-resolution axon tracing techniques that work effectively in chicks and mammals. This body of evidence supported the basic hypothesis that the establishment of topographic projections involves the positional marking of RGC axons and their target sites in the OT or SC.

Prior to the discovery of the first topographic guidance molecules, the ephrins, the most compelling evidence for their existence came from the work of Friedrich Bonhoeffer and colleagues, who developed and used elegant *in vitro* assays, most prominently the membrane stripe assay. These assays were the first demonstrations of the presence of membrane-associated molecules that meet the criteria for topographic guidance molecules, and they were instrumental in these molecules' eventual identification.

Discovery of Graded Molecular Guidance Cues

Many labs using numerous approaches have carried out searches for topographic guidance molecules. Several cell surface molecules, such as TRAP and TOP_{AP}, with graded or restricted patterns in the retina, OT, or SC consistent with a role in mapping, were identified prior to the mid-1990s, but functional studies have yet to show such a role. The first description of graded molecules that proved to have properties of topographic guidance molecules came only in the mid-1990s with the cloning of two related genes, ephrinA2 (originally called Eph Ligand Family-1, or ELF-1) and ephrinA5 (originally called repulsive axon guidance signal, or RAGS), both of which are ligands of the receptor tyrosine kinase, EphA3 (originally named MEK4), expressed in a graded pattern by RGCs.

Eph receptors and ephrin ligands were not only the first topographic guidance molecules to be discovered, but they also have been defined to have prominent roles in both AP and DV mapping, and many family members are involved. The Eph family is the largest known family of receptor tyrosine kinases, comprising 14 Ephs and eight ephrins in mouse and 15 Ephs and nine ephrins in chick. Signaling through Ephs and ephrins has been implicated in a wide variety of biological processes. Ephs and ephrins are separated into two subclasses based on homology, the EphA/ephrinAs and EphB/ephrinBs, within which receptor–ligand binding and activation are promiscuous; some minimal cross-talk occurs between subclasses. EphAs and EphBs, as well as ephrinBs, are transmembrane proteins; ephrinAs are glycosylphosphatidylinositol (GPI)-linked to the cell membrane.

Ephs and ephrins can signal bidirectionally into either the Eph- or ephrin-expressing axon, and in some instances, Eph and ephrin signaling can be bifunctional, resulting in opposing axonal responses (i.e., attraction or repulsion), depending on the context. As described below, EphAs and ephrinAs act bidirectionally in AP mapping, whereas EphBs and ephrinBs act both bidirectionally and bifunctionally in DV mapping. A few other receptor–ligand pairings have also been implicated in mapping; most notably, Wnt3 and its receptors Ryk and Frizzled have been implicated in DV mapping.

An interesting historical note is the cloning of repulsive guidance molecule (RGM). RGM is the molecular activity that Bonhoeffer and colleagues believed to be the repellent membrane-associated molecule enriched in posterior membranes defined using the membrane stripe assay, as well as a growth cone collapse assay. In fact, the search for RGM resulted in the discovery of RAGS (ephrinA5), a molecule with very similar properties (i.e., GPI linked, similar size, repellent for RGC axons, and expressed in a similar gradient in the OT or SC). However, RGM knockout mice do not exhibit mapping defects, although some findings using ectopic expression in chick retina of an RGM receptor, neogenin, have suggested a role for it in AP mapping.

Mechanisms of Map Development

Determining the process by which RGCs establish topographic connections is critical for defining the roles of graded guidance molecules in map development and creating conceptual or computational models of the process. Frogs, fish, chicks, and rodents have been the vertebrates predominantly studied as models for development of retinotopic maps (**Figure 1**). These species exhibit important differences in the development of the visual system and retinotopic maps, as well as substantial differences in the absolute size of the OT or SC; for example, the two-dimensional area of the chick OT is more than a thousand times greater than that in frog and fish during the initial events in map development. Although each species has unique features that can be exploited, they have substantial differences in mechanisms employed by RGC axons to target their correct termination zone (TZ) and therefore also in the actual roles of topographic guidance molecules in controlling these axon behaviors.

Multiple Phases in the Development of a Topographic Map

Development of retinotectal topography in chicks and rodents is a multistep process (**Figure 1(b)**). RGC axons enter their midbrain target at its anterior edge and extend posteriorly parallel to the AP axis of the OT or SC. In birds and mammals, essentially all RGC axons extend well posterior to the topographically appropriate location of their future TZ. Thus,

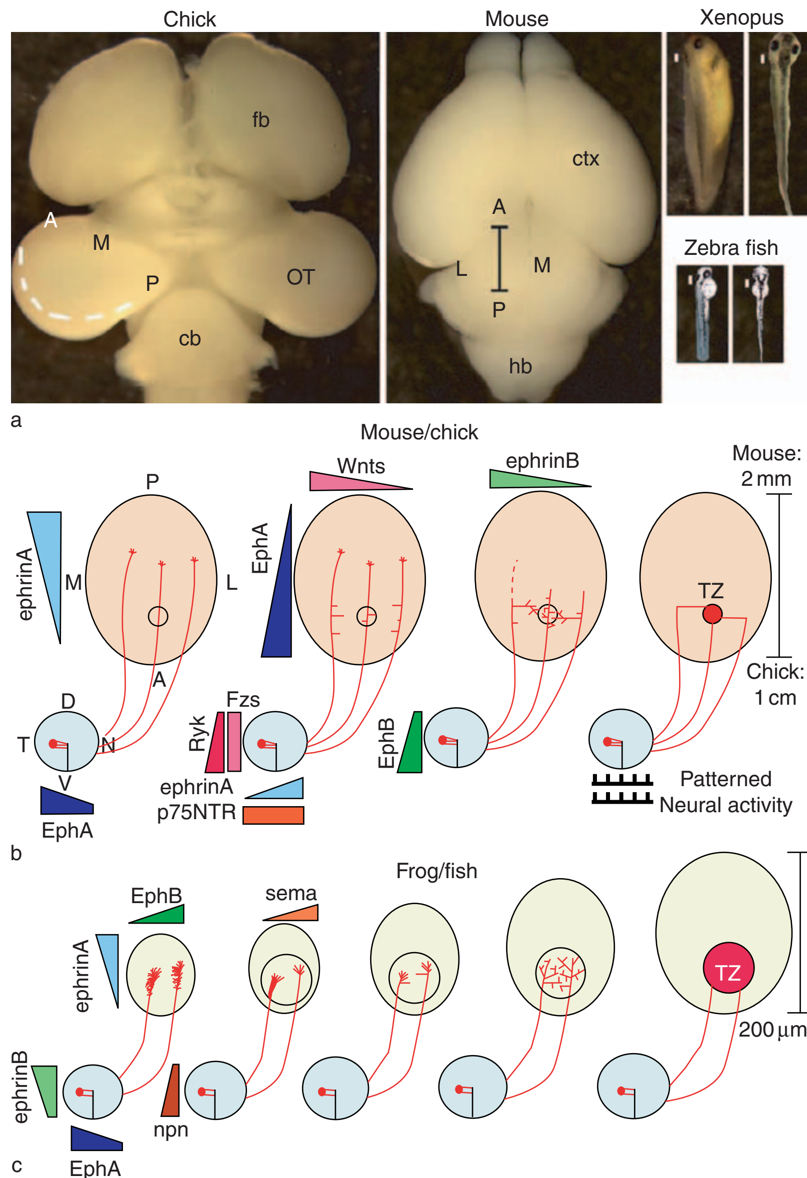

Figure 1 (a) Photographs, at critical ages during topographic map formation, of the primary model systems currently used for the examination of retinocollicular and retinotectal map development. All photographs are at the same scale. The mouse superior colliculus (SC), on the dorsal surface of the midbrain (bracket, 2 mm), is approximately as large as an entire developing zebra fish, and the chick optic tectum (OT) is approximately the size of an entire Xenopus tadpole at these ages. The Xenopus and zebra fish OT sizes are denoted by the small white bars adjacent to the brain. The mouse and zebra fish are used commonly for the potential genetic manipulations available in these organisms. Chick and Xenopus are especially useful for overexpression, mis-expression, and knockdown studies. Retinal ganglion cells from these organisms can be cultured and manipulated *in vitro*. (b, c) Mechanisms and molecules controlling retinotopic mapping in (b) mouse and chick and (c) frog and zebra fish. The names and distributions of molecules and activities demonstrated *in vivo* to control, in part, the mapping mechanisms used at each stage are listed. The gradients may represent the consensus distribution for a combination of related molecules (e.g., EphAs), which are not listed individually due to distinctions in the individual members expressed and the precise distributions between species. Molecules and activities other than those listed are likely to participate in topographic mapping. (b) In mouse and chick, retinal ganglion cell (RGC) axons enter the SC and OT, respectively, over a broad LM extent and significantly overshoot their future termination zone (TZ, circle). Interstitial branches form *de novo* from the axon shaft around the anterior–posterior (AP) position of the future TZ. Branches are directed along the lateral-medial (LM) axis of the OT or SC toward the position of the future TZ, where they arborize in a domain encompassing the forming TZ. The broad, loose array of arbors is refined to a dense TZ in the topographically appropriate location. (c) In frog and fish, the RGC growth cone extends to the TZ, where it forms a terminal arbor through a process of backbranching from the area immediately behind the growth cone. The tectum expands as terminal arborizations elaborate and refine into a mature TZ. cb, cerebellum; ctx, cortex; D, dorsal; fb, forebrain; hb, hindbrain; N, nasal; T, temporal; V, ventral. Modified from McLaughlin T, Hindges R, and O'Leary DDM (2003) Regulation of axial patterning of the retina and its topographic mapping in the brain. *Current Opinion in Neurobiology* 13: 57–69.

the growth cones of RGC axons in birds and mammals do not target their future TZ but instead extend a millimeter or more posterior to it. In addition, RGC axons from the same retinal location enter and grow across the OT or SC with an aberrantly broad distribution over its LM axis. Branches form *de novo* from the axon shaft hundreds of micrometers or even millimeters behind the growth cone. These interstitial branches form roughly perpendicular to the primary axon and preferentially extend along the LM axis toward their future TZ.

In summary, the topographically ordered connections of the mature projection are established by primary branches of RGC axons that form *de novo* interstitially along the length of RGC axons. These branches form along the shaft of the primary axon with a topographic bias for the correct position along the AP axis, which is the first indication of a topographic response. They subsequently extend along the LM axis to the appropriate site of the future TZ, where they go on to arborize.

AP Mapping

Historically, models of topographic mapping have been based on the action of molecular activities that promote axon growth. However, with the identification in the late 1980s by Bonhoeffer and colleagues of repellent activities concentrated in posterior chick OT that preferentially affect temporal RGC axons, it became clear that topographic mapping was controlled in a fashion different from that previously recognized. These findings eventually resulted in the incorporation of repellent activities into models of topographic mapping, but until recently these models focused on guiding RGC axons to their correct TZ. These models were based on a gradient-determined repulsion, in which an RGC axon would stop its growth when it reached a threshold level of repulsion found at the AP position of the future TZ. Although such models could explain retinotectal map development in amphibians and fish (**Figure 1(c)**), they can not account for aspects of map development along the AP axis in chick OT and rodent SC, including the initial AP overshoot of RGC axons and the subsequent topographic branching interstitially along the axon shaft.

Models for AP mapping in rodent SC and chick OT must explain the topographic bias in the formation of interstitial branches, which is arguably the key feature of AP map development. Models that in principle can account for topographic branching have been proposed recently, including models based on parallel gradients of a branch-repellent (or inhibitory) activity and a branch-promoting activity. However, the field has gravitated toward a model based on opposing gradients along the AP axis, each of which inhibits branching. This model is consistent with the gradients and actions of topographic guidance activities defined over the past few years and, it is important to note, can account for the phases exhibited by RGC axons during map development. As described below, the opposing gradients are based on opposing graded expression of EphAs and ephrinAs along both the TN retinal axis and the AP OT or SC axis, with one repellent gradient caused by EphA forward signaling and the other by ephrinA reverse signaling.

RGC axons respond differentially, in a manner relating to their origin along the TN retinal axis, to opposing gradients of repellent activities along the AP axis of the OT or SC. The low-to-high AP gradient of repellent activity has been shown by many complementary studies to be due to EphA forward signaling, controlled by a high-to-low TN gradient of EphA receptors on RGC axons and a low-to-high AP gradient of ephrinAs in the OT or SC (**Figure 2**). These studies include manipulating expression of EphA receptors by RGCs and of ephrinAs in the target. Recent experimental data have provided evidence that the opposing gradient of repellent activity is caused by ephrinA reverse signaling controlled by a low-to-high TN gradient of ephrinAs on RGC axons and a high-to-low AP gradient of EphAs expressed in the SC. The critical findings are that retinal axons are repelled by EphA7 in the "Bonhoeffer" stripe assay, a repulsion blocked by ephrinA5-fc that blocks EphA function, and that EphA7 knockout mice have retinocollicular mapping defects consistent with the loss of the high-to-low AP graded expression of EphA7 in the SC (EphA7 is not expressed by RGCs). Because ephrinAs are anchored to the cell membrane by a GPI linkage and lack an intracellular domain, to reverse signal they must associate with transmembrane proteins capable of activating intracellular signaling pathways. Recent studies have shown that the p75 neurotrophin receptor mediates the repellent effect of ephrinA reverse signaling on RGC axons on binding EphAs, and is required for appropriate development of the retinotopic map in the SC.

DV Mapping

Topographic specificity along the LM axis emerges through the bidirectional guidance of branches that form along RGC axons with an AP bias as described above. Branches that extend from RGC axons located lateral to their future TZ grow medially whereas branches that extend from RGC axons located medial to their future TZ grow laterally. Branches that reach

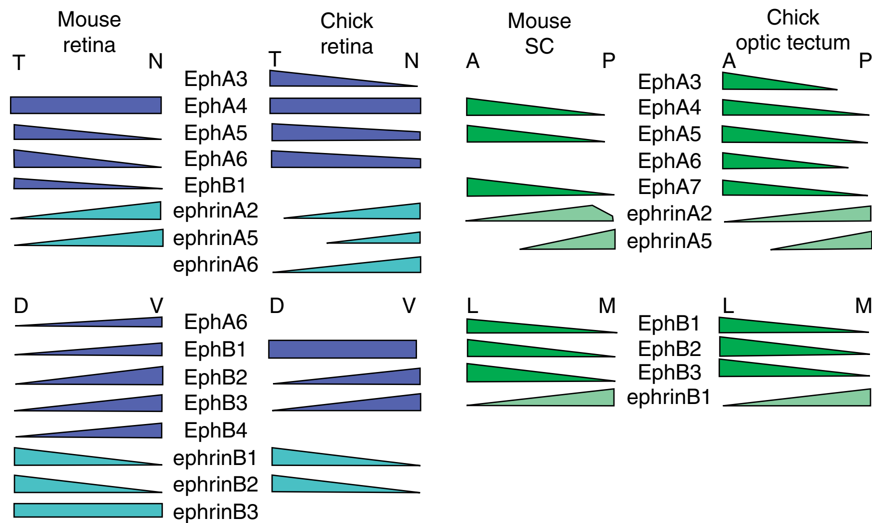

Figure 2 Distribution of Ephs and ephrins in the retina and optic tectum (OT) and superior colliculus (SC) of chick and mouse, respectively. The charts represent generalized patterns for individual Ephs and ephrins in the retina and OT or SC during critical stages in the development of the retinotopic map (E15–P7 in mouse and E6–E14 in chick). The charts are likely incomplete, and the distributions for several molecules are dynamic during development. The sizes and shapes of the gradients are generalized, and relative expression levels should not be inferred. A, anterior; D, dorsal; L, lateral; M, medial; N, nasal; P, posterior; T, temporal; V, ventral. Modified from McLaughlin T and O'Leary DDM (2005) Molecular gradients and development of retinotopic maps. *Annual Review of Neuroscience* 28: 327–355.

the area of the nascent TZ selectively form complex arbors. Therefore, not only is this guidance of interstitial branches a critical feature for DV retinotopic mapping, but the molecular mechanisms that control it must account for the bidirectional guidance along the LM axis of the OT or SC of branches of axons that have the same retinal origin and presumably express the same set of receptors at similar levels.

Studies of the molecular control of DV mapping have implicated EphB and ephrinBs, exhibiting both bidirectional signaling and bifunctional action, as well as Wnts and their receptors. In retina, EphB receptors are expressed by RGCs during map development in an overall low-to-high DV gradient, complemented by an overall high-to-low DV gradient of ephrinBs. In both chick OT and mouse SC, ephrinB1 is expressed in a low-to-high LM gradient, complemented by an overall high-to-low LM EphB gradient (**Figure 2**). Analyses of EphB2 and EphB3 mutant mice, with and without reverse signaling intact, show aberrant LM mapping due to defects in the guidance of interstitial branches and show that ephrinB1 acts as a branch attractant via EphB2/B3 forward signaling. Electroporation studies in chick corroborate these findings, and in addition show that high levels of ephrinB1 repel branches. Taken together, these studies show that in mice and chicks, ephrinB1 can act through EphB forward signaling as both an attractant and a repellent: a branch located lateral to its nascent TZ is attracted up the gradient of ephrinB1 toward it, whereas a branch located medial to its nascent TZ is repelled down the ephrinB1 gradient toward it. The primary axon is not influenced by ephrinB1, but instead the effects of ephrin-B1 are specific to interstitial branches and are context dependent: The location of the branch on the ephrinB1 gradient in relation to the location of its future TZ and its EphB level determine its response. In frogs, ephrinB reverse signaling has also been implicated in retinotopic mapping, but such a role has yet to be shown in mice and chicks (**Figure 1(c)**).

In principle, EphBs and ephrinB1 could account for DV mapping through their actions as bifunctional (i.e., attractant and repellent) and bidirectional (i.e., EphBs and ephrinBs both can act as receptors) guidance molecules. However, recent evidence suggests that Wnt signaling is also involved in DV mapping. Wnt3 is expressed in a high-to-low medial-lateral gradient in chick OT and mouse SC, and Wnt receptors, Ryk and Frizzled family members, are expressed in an overall low-to-high DV gradient by RGCs. Functional studies show that Ryk mediates RGC axon repulsion by higher levels of Wnt3 whereas Frizzled receptors mediate an attractant effect of lower levels of Wnt3 on dorsal RGC axons. Thus, the 'morphogen' Wnt3 appears to act as a topographic guidance molecule in the OT and to cooperate with EphB-ephrinB1 to regulate DV mapping. In zebra fish, Sema3D has also been implicated in DV retinotopic mapping by repelling ventral RGC axons from ventral OT.

Refinement of the Retinotopic Map

As described above, in mice and chicks, all arbors are formed by interstitial branches that preferentially arborize at, or in the vicinity of, the topographically appropriate location of the nascent TZ. In frogs, refinement of individual arbors involves a combination of directed branch dynamics; further, the continued but disparate growth of the retina and OT requires a continuous small-scale remodeling throughout life. These processes act to shape and refine developing arbors and are likely dependent on trkB/brain-derived neurotrophic factor (BDNF) interactions and neural activity. Compared to map remodeling in chicks or mice, refinement in fish and frogs is a precise shaping of arbors rather than major remodeling of a topographically diffuse projection that involves the elimination of substantial lengths of a primary axon and entire arbors that it might have formed along the eliminated segment.

In chicks and mice, the initial collection of arbors is loosely organized around the topographically appropriate position of the future TZ and requires a substantial degree of remodeling to develop the precise connections evident in the mature retinotopic map. Remodeling requires the removal of inappropriately located branches and elimination of overshooting portions of RGC axons. In mice, the remodeling of the retinocollicular projection occurs before the onset of vision but is coincident with a period of correlated spontaneous waves of activity that propagate across the retina. These waves are generated by networks of connections between RGCs and amacrine cells, a type of retinal interneuron, and correlate the activity of neighboring RGCs, thereby relating an RGC's position to its pattern of activity. These amacrine cell–RGC circuits are cholinergic and can be selectively disrupted by deletion of the $\beta 2$ subunit of the nicotinic cholinergic receptor. Analysis of $\beta 2$ knockout mice, which lack retinal waves but retain wild-type levels of RGC activity, show that correlated patterns of RGC activity are required for the large-scale remodeling of the retinocollicular projection into a refined map. Further, these studies demonstrate that a later resumption of correlated activity does not lead to proper map refinement, thereby revealing a brief early critical period for retinotopic map remodeling in mice.

Concluding Statements

From studies to date, it is clear that other receptor–ligand pairings are required to generate proper retinotopic maps. Several groups have carried out forward- and reverse-genetic screens to identify additional candidate genes involved in retinotopic mapping, or screens that could produce candidate genes as a by-product. For example, the large-scale Tubingen genetic screen in mutagenized zebra fish was designed in part to identify genes involved in RGC axon pathfinding and mapping. This near-saturation screen has resulted in the identification of about a hundred mutants, representing scores of genes; a subset of these mutants have defects in DV or AP mapping in the retinotectal projection. These mutants are being investigated further. In addition, microarray screens have identified many known and unknown genes expressed in gradients or restricted patterns along the TN or DV axes of the developing mouse retina, some of which will be found to be involved in retinotopic mapping.

It is interesting that Sperry's chemoaffinity hypothesis was, in part, a response to contemporaneous views that axonal projections could self-organize. Though it is clear that topographic guidance molecules are critical for mapping, the role that a system plays in its own development is an intriguing and understudied facet of mapping. It seems likely in many systems that the projecting axons themselves affect the development of the projection. For example, RGC axons may alter the expression levels and distributions of guidance molecules as they elaborate arbors in the target due in part to the presence of guidance molecules on RGC axons, including multiple ephrins and Ephs on RGC axons. Thus, guidance information itself is dynamic, and patterned activity has a role in mapping – therefore the progressive development of the topographic map may feed back on itself in multiple ways and influence its own development.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Topographic Maps: Molecular Mechanisms.

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Topographic Maps: Molecular Mechanisms

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Topographic maps are a widespread organizing feature of the central nervous system, especially in early sensory processing, whereby spatial relations between cells are preserved between the projecting set of neurons and the receiving set. That is, neighbors in the projecting set connect to neighbors in the receiving set. The effect is that neurons in higher circuits are arranged in a 'map' representing spatial or frequency information about the external world. As information moves toward higher processing centers, it remains organized topographically, so that, for example, neighboring columns of the visual cortex process information from neighboring areas of the visual world, and neighboring columns of the auditory cortex process sounds of similar frequencies.

Ongoing work has revealed two major themes in topographic map development. First, axon guidance cues and their receptors are expressed in gradients in the projecting and receiving layers, which guide projecting axons into a crude topographic map. Second, this crude map is then refined by activity-dependent remodeling of axonal projections, in which neural activity in the projecting layer leads to the elimination of axonal projections not well correlated with their neighbors. This article focuses mainly on the retinotectal projection because it is by far the best characterized of the topographic maps, but reference is also made to other systems, where the same general principles apply.

The Purpose of Topographic Maps

At first glance, it might be unclear why sensory processing is organized topographically. There is no homunculus 'viewing' a projection of the outside world inside the brain, and it would seem that it is the pattern of connections that matters for information processing, not whether neurons processing neighboring stimuli in the external world are neighbors themselves. However, the topographic organization of sensory processing does have important functional benefits. Much of sensory processing relies on comparing neighboring stimuli, most notably in center-surround receptive fields that allow the visual system to analyze edges and motion. The spatial proximity of neurons that respond to neighboring stimuli makes this kind of processing more efficient, since the transmission of action potentials is costly in both energy and time. In addition, some diffusible cues, like nitric oxide, rely on near-neighbor relations. Some circuits rely directly on axon length for processing; for example, the auditory system calculates interaural time difference using coincidence detectors with the delays inherent in action potential transmission to determine how long the sound from one ear must be delayed to match the timing of the sound from the other ear. In this case, the spatial arrangement of cell bodies relates directly to circuit function.

Perhaps more importantly, topographic mapping is efficient developmentally. An arbitrary spatial arrangement of neurons and their synapses, though theoretically possible, would require every connection to be specified individually, a daunting prospect in an organism with billions of neurons and trillions of synapses but only a few tens of thousands of genes. In contrast, topographic mapping allows axonal targets to be determined with only a few broad gradients and activity-dependent refinement of projections (see below). That is, the mechanism of topographic map formation may itself be the functional rationale behind topographic maps.

Historical Perspective

The topographic organization of sensory processing has been recognized since the nineteenth century, but the mechanism by which maps are established was debated until Roger Sperry's classic experiments in the 1940s and 1950s led to his proposal of the 'chemoaffinity hypothesis.' Using the regenerative capacity of amphibian nervous systems, Sperry severed frog optic nerves and allowed the retinal axons to reinnervate their target, the optic tectum (OT). Even if the optic nerve was artificially scrambled or if the eye was rotated 180°, axons always regrew to the correct targets according to the original topographic map. Strikingly, if the eye was rotated 180°, the frog behaved as if its visual world was upside down, as if the formerly 'top' part of the eye, now on the bottom, reconnected to the 'top' part of the retinotopic map in the tectum. These classical experiments argued for the 'chemoaffinity hypothesis,' that axonal connections are specified by complementary molecular identification tags on the axon and its target. To overcome the problem that there could not be enough molecules to mark each cell individually, Sperry proposed overlapping and orthogonal gradients to mark each cell with its 'latitude and longitude.' This combinatorial code would provide each cell with a unique molecular identity defined by expression levels of each tag. This model has been broadly validated with some

modifications (see below) in the last decade with the identification of these molecular tags, especially the Ephs and ephrins.

Axon Guidance Cues in Topographic Mapping

Ephs and Ephrins

The axon guidance cues most involved in topographic mapping are the ephrins and the Eph receptor tyrosine kinases. The Eph receptors (discovered in an erythropoietin-producing hepatocellular cell line) form the largest subfamily of receptor tyrosine kinases and are divided into two classes, EphA and EphB. Their ligands, the ephrins (Eph-receptor interacting proteins), are similarly divided into two classes, ephrinA and ephrinB; generally, EphAs bind ephrinAs while EphBs bind ephrinBs. EphrinAs are glycosylphosphatidylinositol (GPI)-linked to the membrane, while ephrinBs are transmembrane proteins. In addition to 'forward signaling' from ligand to receptor, ephrins are also capable of 'reverse signaling' from receptor to ligand, most clearly with ephrinBs through their intracellular domain, but potentially also with ephrinAs through an unknown mechanism. Ephrins and Ephs can be both attractive and repulsive guidance cues, but function only when membrane bound, suggesting that they guide axons through contact attraction or repulsion rather than long-range guidance.

Nasal–Temporal Mapping in the Retinotectal Projection

Retinal ganglion cell (RGC) axons project onto the OT in nonmammalian vertebrates (the 'retinotectal' projection), and onto the superior colliculus (SC) in mammals (the 'retinocollicular' projection). In both cases, the projection is topographic, such that nasal axons project posteriorly, and temporal axons project anteriorly (**Figure 1**). (For simplicity, this article refers to the retinotectal projection, with the understanding that similar principles apply in the retinocollicular projection.) Retinotectal topography can be observed by recording receptive fields of tectal neurons to see which part of the retina they respond to, and by anterograde and retrograde tracing, where a lipophilic dye is injected into the retina or tectum, to see which part of the other layer is connected by RGC axons.

Classic experiments by Bonhoeffer and colleagues in the 1980s using the 'stripe assay' showed that temporal axons were selectively repelled by membranes from the posterior tectum. Retinal axons were allowed to grow on alternating stripes of membranes from anterior and posterior tectum. Temporal axons grew only on anterior stripes, while nasal axons grew indiscriminately (**Figure 2**). The directed growth of temporal axons was due to repulsion from posterior stripes rather than attraction to anterior stripes, because heat inactivation of posterior membranes alone abolished the striped outgrowth pattern, while heat inactivation of anterior membranes had no effect. These findings confirmed the central principle of the chemoaffinity hypothesis, but with a surprising twist – instead of the prevailing idea of attraction between matching tags on axons and their targets, these findings suggested repulsion between axons and inappropriate targets.

Eventually, ephrinA2 and ephrinA5 were identified as the critical ligands in the tectum, and EphA5 and EphA6 (EphA3 in chick) as the receptors in RGCs. EphA receptors are expressed in a low nasal to high temporal gradient in RGCs, while EphrinAs are expressed in a low anterior to high posterior gradient in the tectum (**Figure 1(d)**). The same principle holds in another retinal target in mammals, the dorsal lateral geniculate nucleus (dLGN) in the thalamus, where ephrinA2 and ephrinA5 are expressed in a high ventral–lateral–anterior to low dorsal–medial–posterior gradient (see **Figure 4(b)**). Knocking out or mis-expressing ephrinA2/A5 disrupts retinotectal topography, though some retinotopy remains intact, suggesting that other molecules are involved (see below).

In the tectum, ephrinA expression is thought to be patterned by the transcription factor Engrailed-2, which, like the ephrinAs, is expressed in a low anterior to high posterior gradient. In the retina, the nasal–temporal axis is patterned by the transcription factors BF1 (expressed nasally) and BF2 (expressed temporally), and SOHo1 and GH6 (expressed nasally). SOHo1 and GH6 repress EphA3 expression in chick, suggesting a mechanism by which a low nasal to high temporal gradient of EphA expression is created.

RGCs also express ephrinAs themselves, in a high nasal to low temporal gradient (**Figure 1(d)**). EphrinA5 has been shown to silence EphA3 responsiveness through *cis*-interactions when the two are co-expressed (i.e., interactions on the same cell, as opposed to *trans*-interactions between ephrinA and EphA on different cells), suggesting that ephrinA expressed on nasal axons could silence what little EphA receptor they do have. Thus, the counter-gradient of ephrinA may enhance the gradient of EphA signaling in RGCs. Also, it has been suggested that ephrinA expressed by early-arriving retinal axons might sharpen the tectal ephrinA gradient, because nasal axonal arbors in the posterior tectum would express high ephrinA on top of tectal ephrinA, while temporal axonal arbors in the anterior tectum would express low ephrinA.

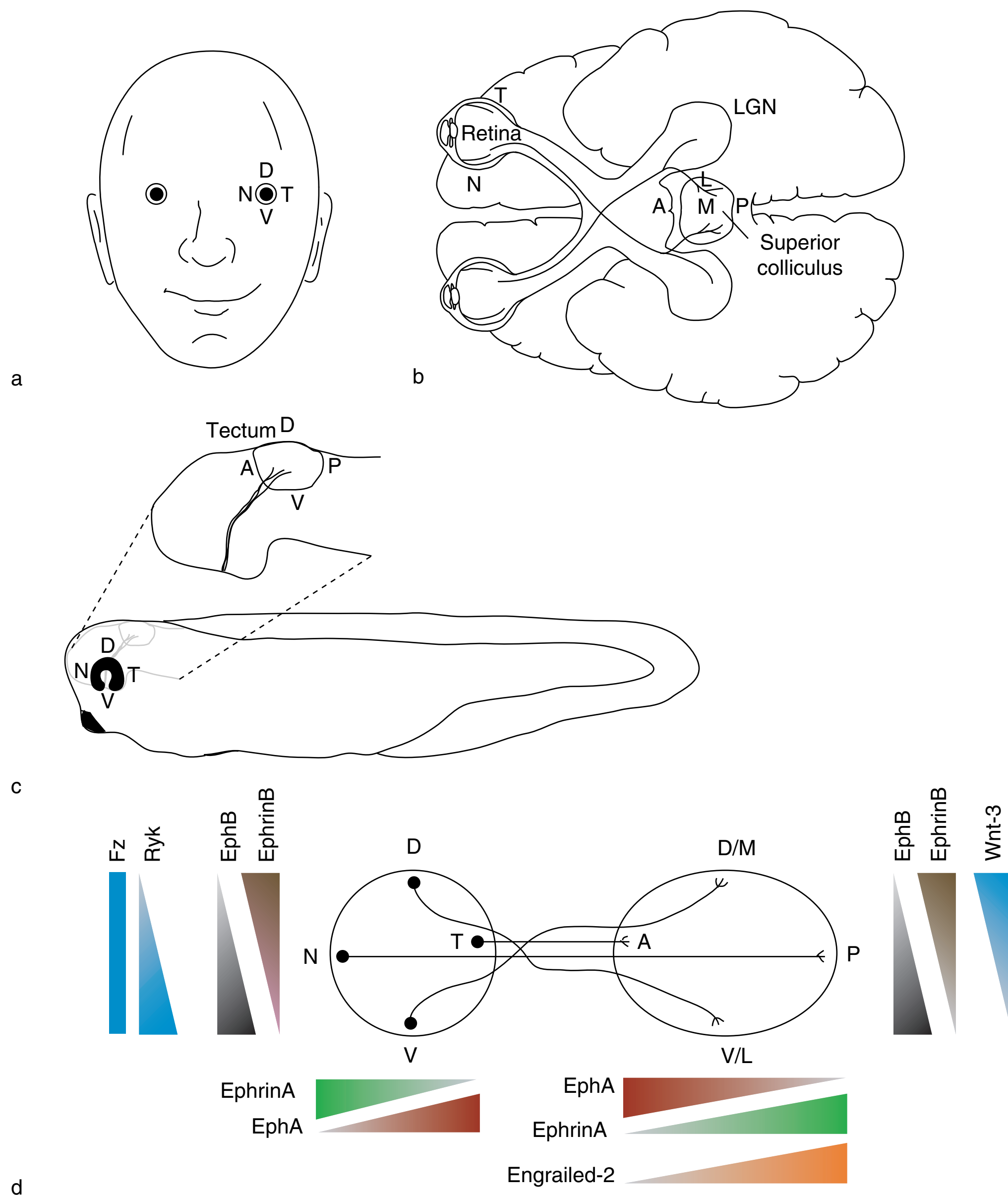

Figure 1 (a) Schematic of nasal–temporal and ventral–dorsal retinal axes. (b) The retinocollicular projection: ventral view of the human brain, showing the retina, lateral geniculate nucleus (LGN), and superior colliculus (SC). (c) The retinotectal projection: lateral view of a *Xenopus* embryo, showing the retina and tectum. (d) Schematic of retinotectal/retinocollicular topography and gradients of guidance cues and their receptors. Dorsal tectum in *Xenopus* corresponds to medial tectum in chick and medial SC in mouse; ventral tectum in *Xenopus* corresponds to lateral tectum/SC in chick and mouse.

Initially, tectal ephrinAs were thought to be purely repulsive. In this model, temporal axons expressing high levels of EphA receptor are repelled by the high levels of ephrinA in the posterior OT/SC and therefore remain in the anterior tectum, while nasal axons expressing low levels of EphA receptor are unaffected by ephrinA and continue to grow into the posterior OT/SC. Axon–axon competition and a hypothetical opposing gradient would explain why all axons did not simply crowd into the anterior tectum. (For a review of computational models of molecular gradients in retinotopic mapping, see 'Further reading.')

However, recent findings suggest that ephrinAs have concentration-dependent biphasic effects. EphrinA2 promotes *in vitro* retinal axon outgrowth at low concentrations and inhibits it at high concentrations, with the tipping point between positive and negative effects for a particular axon varying depending on its nasal–temporal position. Nasal axon growth is promoted by ephrinA2 up to a higher concentration than temporal

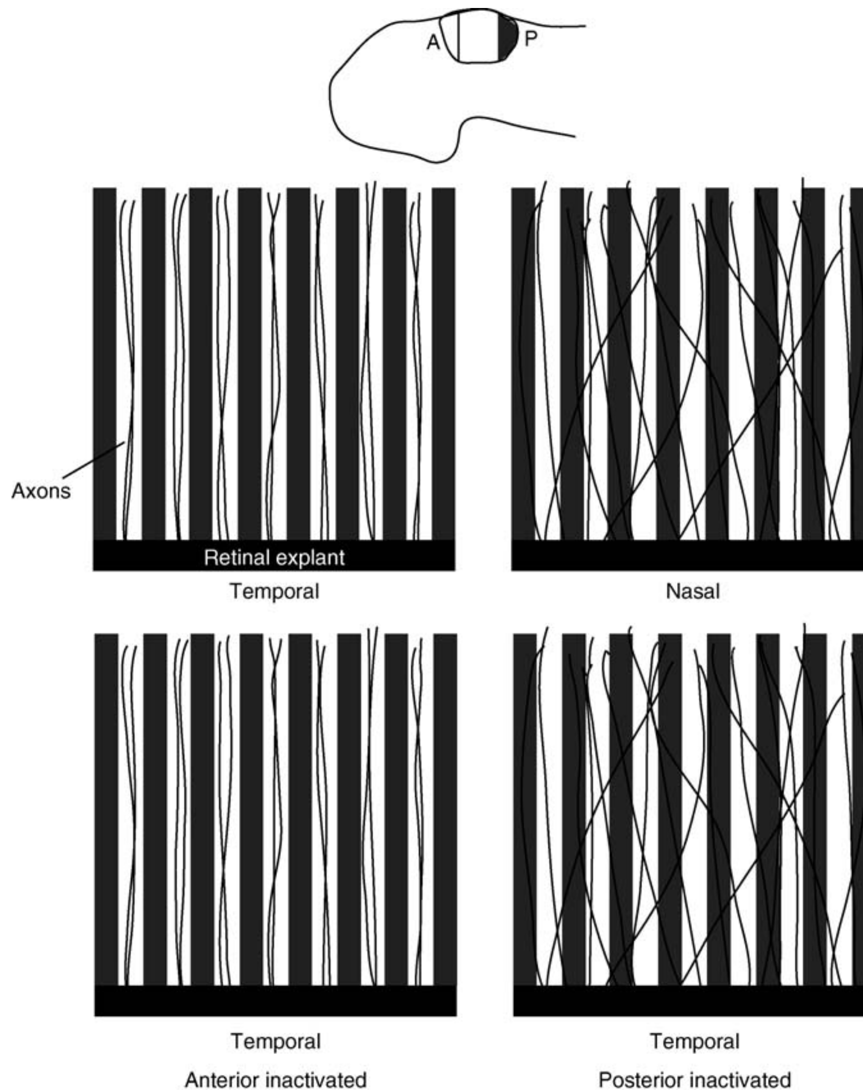

Figure 2 The stripe assay. Membranes from anterior (white) and posterior (green) tectum are laid down in alternating stripes, and retinal pieces are explanted on them so that axons will grow over the stripes. Temporal axons avoid posterior membrane stripes, whereas nasal axons grow indiscriminately. Temporal selectivity is abolished by heat inactivation of posterior membranes, but not anterior membranes.

axons. Each axon therefore terminates around the tipping point between a positive and negative response to ephrinAs. Another molecule mediating biphasic guidance is Engrailed-2. In addition to its role in transcriptional regulation of tectal ephrinA levels, Engrailed-2 attracts nasal axons but repels temporal axons. Unlike ephrins, Engrailed-2 passes directly into growth cones *in vitro* and may function by binding to intracellular signaling molecules rather than receptors; presumably, nasal and temporal growth cones differ in these intracellular molecules.

In frogs and fish, ephrin gradients act by controlling how far posterior the growing RGC axons extend: RGC axons grow 'up' an ephrinA gradient, and depending on how much EphA receptor they express, they stop at a certain zone, which becomes the target. In contrast, in birds and mammals, axons substantially overshoot the target (though still biased toward the target), and the critical step for topographic mapping is selective interstitial branching near the target zone (**Figure 3**). This difference may be due to species differences in size and developmental timing: frog and fish RGC axons innervate the tectum early, when it is still very small (150–200 μm), and both the retina and tectum continue to grow as the retinotopic map is refined. In contrast, the OT in chick and SC in mouse are relatively large (10 000 μm in chick, 2000 μm in mouse) when RGC axons arrive and do not grow substantially during map refinement. In addition to mediating retinal axon extension, EphAs and ephrinAs are thought to mediate selective interstitial

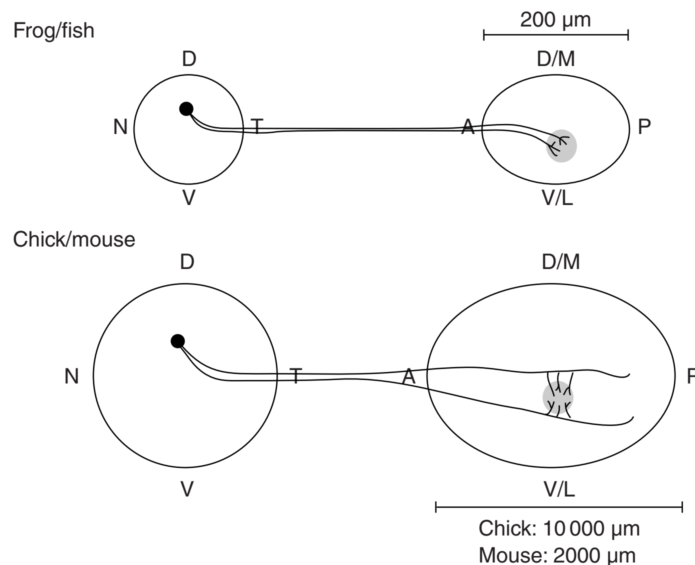

Figure 3 In frogs and fish, axons stop growing near the eventual target zone (gray circle) and arborize there. In contrast, in birds and mammals, axons considerably overshoot the target zone and selectively grow interstitial branches at the target zone. This difference may be due to the vastly different sizes of the tectum/SC between the two cases (bars).

branching by blocking branching posterior to the target zone. As with axon extension, branching anterior to the target may be blocked by axon–axon competition or by ephrinA biphasic action, in which branches need a certain threshold of ephrinA (attractive at low concentrations) to grow.

A straightforward nasal–temporal mapping only applies to organisms with mostly monocular vision, where each retina projects mainly to the contralateral OT. In organisms with binocular vision, such as humans, each retina projects to both sides of the brain. In the right retina, for example, the nasal half projects to the left side of the brain while the temporal half projects to the right side. Each retina therefore needs two topographic maps, and, indeed, the human embryonic retina displays a bidirectional high central to low nasal/temporal gradient of EphA receptors, while the dLGN displays a complementary gradient of ephrinAs (**Figure 4**). Remarkably, this finding was predicted by Sperry almost half a century ago.

Dorsal–Ventral Mapping in the Retinotectal Projection

Retinal axons also map topographically in the dorsal–ventral dimension. Dorsal RGCs map to the ventral tectum (lateral tectum in chick, SC in mouse), whereas ventral RGCs map to the dorsal tectum (medial tectum/SC). Until recently, dorsal–ventral mapping was poorly understood compared to anterior–posterior mapping, but in the last few years it has been shown that dorsal–ventral mapping is established by ephrinBs and EphBs, as well as Wnt-3 and its receptors Ryk and Frizzled.

EphrinBs are expressed in a high dorsal to low ventral gradient in both the retina and the tectum, while EphBs are expressed in high ventral to low dorsal gradients (**Figure 1(d)**). Like EphAs, retinal gradients of ephrinBs and EphBs are set up by transcription factors – Vax2 (expressed ventrally) and Tbx5 (expressed dorsally). Ventral Vax2 represses ephrinB expression, to create the high dorsal to low ventral ephrinB gradient, while dorsal Tbx5 represses EphB expression, to create the high ventral to low dorsal EphB gradient.

In frogs, reverse signaling by EphBs in the tectum onto ephrinBs in RGCs mediates dorsal–ventral mapping: ephrinB-expressing dorsal axons are attracted to EphB-expressing cells in the ventral tectum. Ectopic expression of ephrinB in ventral RGCs shifts their tectal projections ventrally, while expression of dominant-negative ephrinB in dorsal RGCs shifts their projections dorsally. Chick and mouse dorsal–ventral mapping appears to be achieved by forward signaling from tectal ephrinBs to RGC EphBs: interstitial branches of EphB-expressing ventral axons are attracted to ephrinB-expressing cells in the medial tectum/SC. Knocking out EphB2 and EphB3 in mice causes axonal branches to project too far laterally.

In chick and mouse, the counteracting force that pulls axons toward the lateral tectum/SC is created by a Wnt-3 gradient as well as biphasic action by

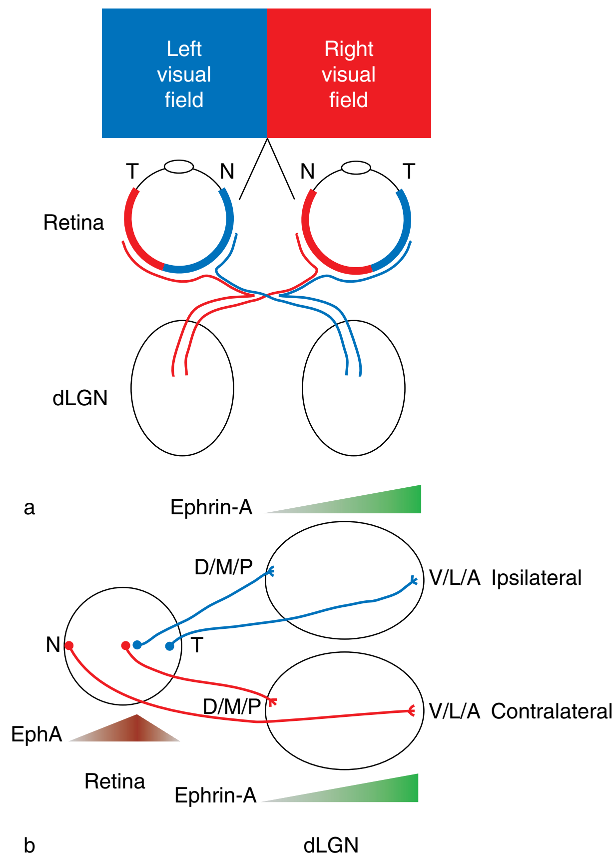

Figure 4 Schematic of retinotopic mapping in organisms with binocular vision. (a) Nasal axons cross at the optic chiasm to the contralateral side of the brain, while temporal axons project to the ipsilateral side, so that each side of the brain processes the contralateral half of the visual field. (b) Topographic mapping between the retina and the dLGN is achieved by a bidirectional high central to low nasal/temporal gradient of EphAs in the retina and a high ventral to low dorsal gradient of EphrinAs in the dLGN.

ephrinB. Wnt-3 is expressed in a high medial to low lateral gradient in the tectum. The receptor Ryk, which mediates repulsive responses to Wnt-3, is expressed in a high ventral to low dorsal gradient in the retina, while the receptor Frizzled, which mediates attractive responses to Wnt-3, is expressed evenly in the retina (**Figure 1(d)**). The Ryk-mediated repulsive response and Frizzled-mediated attractive responses both drive interstitial branches laterally: high-Ryk-expressing ventral axons are repelled by Wnt-3 while low-Ryk-expressing dorsal axons are attracted to low Wnt-3 levels (such as those in the lateral tectum), though still repelled by high Wnt-3 levels. This lateral-driving effect of Wnt3 counteracts the effect of ephrinB, which attracts axons medially. EphrinB itself may also have biphasic effects; in chick, high levels of ephrinB can be repulsive, indicating that axons from a given DV retinal position have an 'optimum' level of ephrinB that their interstitial branches grow toward.

Guidance Cue Gradients in Other Topographic Maps

Although the role of guidance cue gradients in topographic mapping has been most thoroughly studied in the retinotectal projection, similar principles apply in other sensory projections, including a role for ephrins and Ephs. In the thalamocortical projection, ephrinAs and EphAs play double roles (**Figure 5**). In early development, EphA receptors are expressed in a high anterior to low posterior gradient in the thalamus, and ephrinAs are expressed in a high posterior to low anterior gradient in the ventral telencephalon. Thalamic axons pass through the ventral telencephalon en route to the cortex, so this initial mapping step sorts them into cortical areas. For example, axons from the anterior thalamus end up in the anterior cortex, in the motor cortex, while axons from more posterior regions of the thalamus end up more posteriorly in the cortex, for example, in somatosensory or visual cortex. EphrinAs and EphAs are then reused later in development; both are expressed in high medial to low lateral gradients, with EphAs in the ventrobasal thalamus and ephrinAs in somatosensory cortex (S1). These complementary gradients arrange a somatotopic map in an analogous way to the retinotectal map: EphA-expressing medial thalamic axons are repelled away from the ephrinA-expressing medial S1 into the lateral S1. Thus, ephrinAs and EphAs are used twice in thalamocortical mapping: first at an intermediate target to mediate 'inter'-areal mapping and then within cortical areas to mediate 'intra'-areal mapping.

EphrinAs and EphAs also play a role in topographic mapping between the hippocampus and septum, and between motor axons and muscles. In both cases, the mechanism is similar to that described for AP retinotectal mapping: high-EphA-expressing axons are repelled from high-ephrinA areas of the target. In the auditory system, functional studies of topography have not yet been done, but there are gradients of EphA4 and ephrinB2 that could form the basis of a tonotopic map in the avian nucleus laminaris, the target of axons from the nucleus magnocellularis, which is in turn the target of cochlear ganglion cells. Interestingly, ephrins have a conserved role in topographic mapping beyond vertebrates: in *Drosophila*, the single Eph and ephrin homologs are required for topographic mapping in the visual system. The reuse of Ephs and ephrins across different projections suggests an evolutionarily efficient modular approach to topographic mapping that would

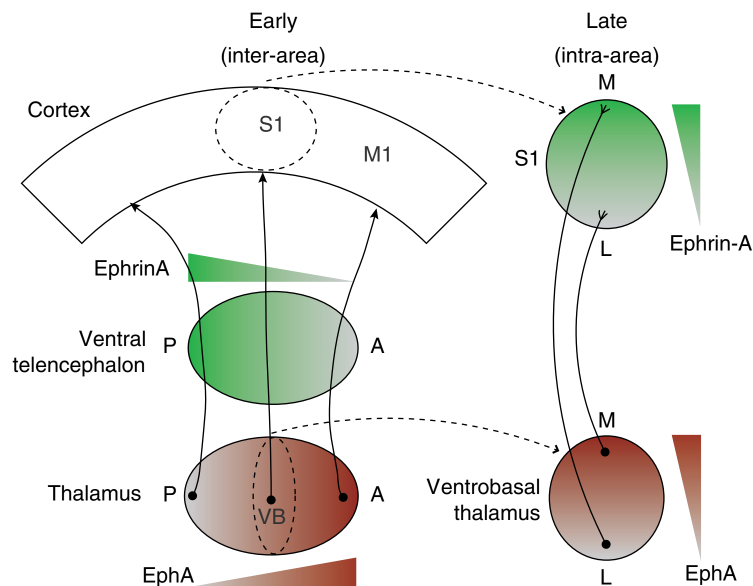

Figure 5 Topography in the thalamocortical projection uses EphA and ephrinA gradients twice. In early development, thalamic axons are sorted toward the correct cortical areas by an ephrinA gradient at an intermediate target, the ventral telencephalon. Later on, thalamic axons are sorted within each cortical area by a second ephrinA gradient.

allow newly evolved projections to be patterned by co-opting a standard package of Eph and ephrin gradients.

Activity-Dependent Refinement of Topographic Maps

Correlated RGC Firing Is Required for Topographic Map Refinement

Molecular gradients are insufficient to establish a refined topographic map, most likely because they act over too long a range to ensure precise mapping at the cellular level. Moreover, in frogs and fish, retinotectal topography shifts throughout life, as the retina grows radially at the periphery while the tectum grows linearly at the posterior edge. Retinotopic map refinement occurs through correlated activity in RGCs, where cells that are close together fire around the same time, while cells that are far apart do not. Correlated retinal firing occurs naturally in vision; neighboring RGCs have neighboring receptive fields, so they are likely to receive, and transmit, similar input. In amphibians and fish, where embryos develop externally, the retina becomes responsive to visual input around the time when axons arrive in the tectum, so normal visual input may suffice to provide the correlated firing patterns needed to refine retinotectal projections. However, in mammals, where embryonic retinas develop in the dark, correlated firing is provided by spontaneous 'retinal waves' of depolarization that move across the retina. These waves have been observed by multielectrode arrays as well as calcium imaging of depolarization. Because they occur every few minutes and the depolarization only lasts for a few seconds, cells that fire together temporally are very likely to be located together spatially.

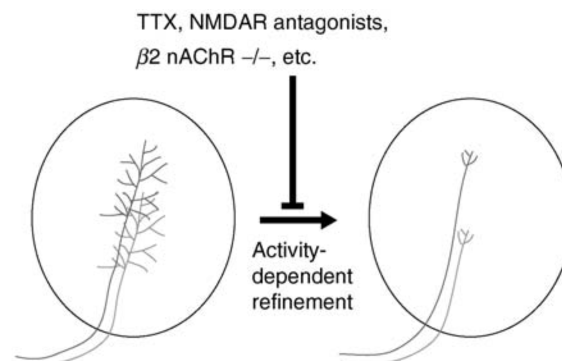

Figure 6 Activity-dependent mechanisms are required to refine initially broad axonal arbors to create precise retinotopy. Refinement can be blocked by interfering with correlated neuronal activity in RGCs, for example, with TTX, NMDAR antagonists, knockout of the β2 subunit of the nicotinic ACh receptor, and rearing under stroboscopic conditions.

Eliminating correlated retinal firing blocks the refinement of retinotectal mapping, as measured by the size of axonal arbors or receptive fields (**Figure 6**). Blockade of neuronal activity in the retina causes abnormally broad axonal arbors and receptive fields

in the tectum, even though the basic shape of the topographic map remains intact, suggesting that molecular gradients suffice for a crude topographic map, but refinement requires activity. This finding holds whether neuronal activity is abolished pharmacologically, by intraocular injection of tetrodotoxin (TTX), or by genetic knockout, for example of voltage-dependent sodium channels. Importantly, it is not electrical activity in RGCs *per se* that matters, but correlated firing patterns. The $\beta 2$ subunit of the nicotinic acetylcholine receptor (nAChR) is required for spontaneous waves of activity in the developing retina, but not for neuronal activity *per se*. Mice lacking $\beta 2$ nAChR exhibit random spontaneous retinal activity instead of retinal waves, and indeed develop abnormally large axonal arbors, just like those of TTX-injected animals. Correlated retinal firing can be inhibited in fish, which lack retinal waves, by rearing them under stroboscopic illumination, which artificially increases correlation of firing between RGCs that are far apart; this treatment, like the $\beta 2$ nAChR knockout, decreases retinotopic refinement.

Additional support for this model comes from artificial ocular dominance bands induced by transplanting extra eyes onto frog embryos, where normally only one eye innervates each tectum. Ectopic and native RGCs innervate the same tectum, and presumably express and encounter the same set of molecular gradients, yet the two projections gradually segregate into exclusive ocular dominance bands reminiscent of those discovered by Hubel and Wiesel in the cat visual cortex. Ectopic RGC innervation is presumably an extreme case of uncorrelated firing, as RGCs from two different eyes would be even more uncorrelated than RGCs far apart in the same eye. Ocular dominance bands are thus an extreme example of activity-dependent refinement of topography, as two sets of axons uncorrelated with each other segregate within a region defined by the coarse topography set up by molecular gradients.

Postsynaptic NMDA Receptors Induce Map Refinement by Eliminating Inappropriate Connections

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that are blocked by a magnesium ion unless the dendrite is already depolarized, meaning that NMDARs act as ‘coincidence detectors’ that only activate when more than one synapse fires nearly simultaneously onto the dendrite. In long-term potentiation, Ca^{2+} influx through NMDARs activated by coincident stimulation leads to signaling that strengthens the synapse, and it is thought that a similar mechanism operates in the stabilization of synapses that are ‘in sync’ with their neighbors and the withdrawal of axonal branches from synapses that are ‘out of sync.’ Indeed, blocking NMDARs in postsynaptic tectal cells prevents retinotopic refinement of the presynaptic retinal axons. In addition, calcium/calmodulin-dependent kinase II (CaMKII), which is activated by NMDARs, is required in postsynaptic tectal dendrites for pruning presynaptic RGC axonal arbors. Time-lapse imaging in dually innervated frog tecta shows that axonal branch elimination rates, but not addition rates, are higher in areas dominated by axons from the other eye. This bias is NMDAR dependent, suggesting that correlated firing acts through NMDARs to eliminate axonal branches not well correlated with their neighbors.

The nature of the retrograde signal activated by postsynaptic NMDARs to axonal branches, telling them whether to stabilize or retract, remains unclear, although a few candidate mechanisms exist. Nitric oxide (NO) has been suggested as a retrograde messenger since NO synthetase is expressed in tectal cells and NO collapses growth cones *in vitro*. However, blocking NO synthetase appears to slow down, but not ultimately prevent, retinotopic refinement in mouse and chick, while it has no effect on refinement in frogs and fish. Brain-derived neurotrophic factor (BDNF) is produced by tectal neurons where it is released by neuronal activity, and it promotes axonal branching and synapse formation, making it a good candidate for an activity-dependent retrograde messenger. However, the effects on axonal branch dynamics of blocking neuronal activity do not match those of blocking BDNF, suggesting that BDNF may not directly mediate the effects of correlated neuronal activity, but rather modulates them in a complex way that is not fully understood. Work in fish suggests a role for arachidonic acid, a cleavage product of diacylglycerol; both applying arachidonic acid globally and inhibiting its release by postsynaptic cells block retinotopic refinement. Cell adhesion molecules may also serve as activity-dependent retrograde messengers. Applying antibodies against neural cell adhesion molecule (NCAM) or glycoproteins to the tectum inhibits retinotopic map refinement. NCAM, N-cadherin, and glycoprotein L1 are inserted into the membrane in an activity-dependent fashion in other systems, suggesting that they may similarly be inserted by NMDAR activation to aid in axonal branch or synapse stabilization. These candidate mechanisms all seem to converge on interrelated presynaptic signaling pathways involving Ca^{2+}, phospholipase C (PLC), growth-associated protein 43 (GAP-43), Rho GTPases, and others, ultimately

influencing the actin cytoskeleton to cause stabilization or retraction of axonal branches.

Conclusions

Our understanding of the molecular mechanisms behind topographic mapping has increased greatly in the last decade. An emerging principle is that topographic mapping efficiently uses a relatively small number of genes to pattern connections among billions of neurons. Matching gradients of axon guidance cues in the target layer and their receptors in the projecting layer set up a coarse topographic map, while activity-dependent mechanisms refine this map to make it more precise by eliminating inappropriate connections. The conservation of the molecular principles of topographic mapping across phyla and across different neural projections suggests that these principles may be a basic method of nervous system organization that evolution has repeatedly turned to. Future work will surely shed further light onto the development of topographic maps.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Chemoaffinity Hypothesis: Development of Topographic Axonal Projections.

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Axonal Pathfinding: Theoretical and Computational Models

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Introduction

Building a brain requires the precise formation of connections between vast numbers of neurons, often separated by significant distances. Axon pathfinding is thus a crucial process in the development of a functioning nervous system. A better understanding of the principles and mechanisms underlying axon pathfinding has both clinical and broader practical importance. From a clinical perspective, our ability to treat or prevent some neurological defects will be improved by a better understanding of how axon guidance can fail. Furthermore, the regeneration of damaged nerves requires axons to reconnect to their appropriate targets; hence, understanding axon guidance will be necessary for the development of therapeutic techniques. In a broader context, our understanding of axon pathfinding ties into our understanding of the nervous system as a whole: what principles underlie its formation and function? can we harness those principles in order to improve our own engineering processes, such as in the construction of self-wiring computers?

Mathematical and computational models are very useful tools for understanding the constraints on nervous system development. Ultimately, such constraints are quantitative and set by the physics of the system; hence, they must be modeled mathematically to yield the best predictive power and generate strong hypotheses. Models may also prove valuable in the development of therapies. For instance, how can axons be made to grow toward a specific target area? What additional information is needed by axons which are failing to develop correctly? Sufficiently detailed theoretical models have the potential to guide experimental research in axon pathfinding through simulations done *in silico*.

Experimental Data

Guidance Cues

Axons grow along their correct trajectories by following a molecular map consisting of spatiotemporal patterns of guidance cue molecules. Four main families of molecules have been identified based on their guidance abilities – the netrins, the Slits, the semaphorins, and the ephrins – consisting of approximately 100 distinct molecules altogether (although several other classes of molecules also provide guidance information for axons, including the neurotrophins and some classical morphogens). The netrins were first identified as attractive guidance cues which direct contralaterally projecting neurons toward the midline. The transmembrane proteins DCC and Unc-5 have been shown to act as receptors for netrin-1. Both netrins and their receptors are highly conserved between species. Netrin-1 is known to have a bifunctional role, typically attracting growth cones expressing only the DCC receptor but repelling growth cones expressing both DCC and Unc-5. The Slits and their receptors, the roundabout family (the Robos), were first identified as repellents preventing contralaterally projecting neurons from recrossing the midline. Subsequently, they were also shown to stimulate axon outgrowth and branching. The semaphorins appear to act primarily as short-range cues which repel axons from particular regions or, by forming the walls of corridors, hem axons into a preferred path. However, they have also been reported to act as long-range chemoattractants. Semaphorins are classified by their structure into eight groups. They signal through multimeric receptor complexes, with the precise structure of a complex determining its specificity for a semaphorin subgroup. Semaphorin receptor molecules include the neuropilins, the plexins, and the cell adhesion molecule L1. The ephrins are substrate-bound molecules best known for their role in the formation of topographic maps in the central nervous system. For example, the graded expression of the Eph tyrosine kinases – the ephrin receptors – in the retina combined with graded expression of the ephrins in the tectum aid in the formation of an ordered topographic mapping between the two structures. Similar strategies appear to orchestrate the formation of other topographic maps. Although ephrin/Eph signaling induces axon repulsion in these examples, under other contexts the ephrins can also act to attract axons.

The Growth Cone

Growing axons are tipped by special sensorimotor structures known as growth cones. These probe their local environment and, depending on the signals they detect, direct axon outgrowth, turning, branching, and pruning. Growth cones exhibit complex morphology, as illustrated in **Figure 1**. They are conconceptually divided into three sections: an actin-rich peripheral region, a transitional region, and a central region containing organelles and microtubules. Fingerlike protuberances extending from the edge of the growth cone known as filopodia are supported by bundles of filamentous actin (F-actin). These appear to act as sensory devices, extending the effective

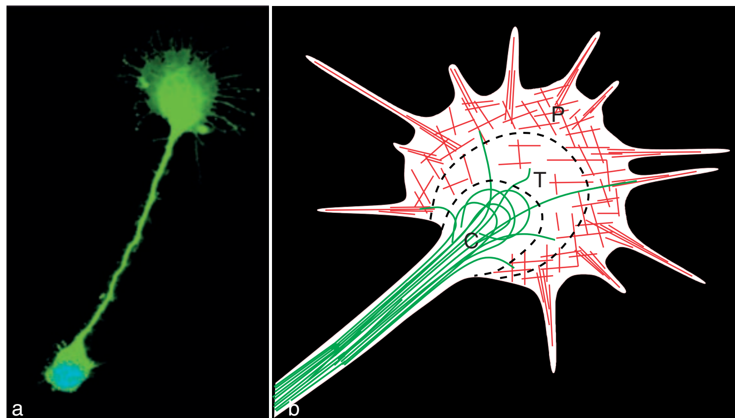

Figure 1 (a) Rat superior cervical ganglion neuron *in vitro*. The neuron was grown on a substrate of laminin for 48 h and then fixed and stained for β-tubulin. The cell body is at the bottom left, and the growth cone is at the top right. (b) Anatomy of the growth cone. This figure illustrates the division of the growth cone into three domains: transitional (T), peripheral (P), and central (C). The peripheral domain is rich in actin, which exhibits two forms of organization: loosely linked networks giving rise to lamellipodial structures and tight bundles supporting filopodia. The central domain contains organelles and microtubules which extend from the axon shaft into the growth cone. Rat SCG neuron image courtesy of Z Pujic.

sensing range of the growth cone. Structures based on more chaotic meshworks of F-actin are known as lamellipodia and have also been implicated in growth cone movement.

Growth cones undergo constant morphological change thought to be driven by remodeling of the actin network controlled by actin-binding proteins such as myosin, ARP2/3, and WASP. Growth cone motility appears to be driven by an 'actin treadmill.' Unpolymerized G-actin diffuses outward into the peripheral region and preferentially polymerizes near the cell membrane. The entire network of polymerized actin is drawn toward the central region by myosin and undergoes depolymerization in the transitional region. This constant cycle of polymerization, retrograde flow, and depolymerization is thought to generate traction when coupled to a permissive substrate through adhesion molecules.

Microtubules also play a significant, although unclear, role in growth cone motility and axon guidance. Interactions between microtubules and actin in the transitional region appear to have a strong influence on axon outgrowth and guidance.

Guidance cues influence growth cone behavior, and subsequent axon outgrowth, through cytoskeletal effectors activated or inhibited by cascades of intracellular second messengers triggered by receptor binding. Several molecules have been implicated as playing roles in this process, particularly calcium and the cyclic nucleotides cAMP and cGMP. These have been the focus of much attention, with the finding that, in some circumstances, changing the relative concentrations of these molecules within the growth cone can switch the effect of several guidance cues from attraction to repulsion or vice versa. Further intriguing findings have demonstrated that protein synthesis occurring locally within the growth cone is necessary for correct growth cone behavior.

Theoretical and Computational Models of Axon Pathfinding

Axon Extension and Branching

Experimental evidence suggests that tubulin molecules are synthesized only in the soma and then assembled into microtubules predominantly in the growth cone. This implies that axon outgrowth is limited by the rate at which microtubules can be transported to regions of active extension. A number of theoretical models have explored this idea, including various effects such as diffusive and active transport of tubulin monomers, competition between neurites for tubulin, viscoelastic stretching of axon segments, calcium-induced microtubule depolymerization, and varying intrinsic rates of tubulin polymerization and depolymerization within different growth cones. These models have been successively refined, ultimately incorporating compartment-based modeling with dynamic compartment allocation. Most strikingly, this modeling program has demonstrated that small variations in polymerization and depolymerization rates in the growth cones of different neurites can lead to sharp changes in elongation rate, including growth cone pausing and neurite retraction.

Growth cone behavior and axon extension are thought to be mediated by partially independent but related processes. This has led to modeling work

focused on characterizing the interaction between the two. The majority of this work has been phenomenological, using sophisticated mathematical machinery to better describe experimental data. Work in the mid-1980s showed that in some circumstances, axon elongation can be regarded as a one-dimensional random walk, with parameters varying with neuronal type, substrate, and chemical environment. In the mid-1990s, this was extended by performing correlation analysis which showed that axon outgrowth dynamics exhibit significant anticorrelation. Subsequent analysis showed significant correlation between the dynamics of microtubule polymerization in the central region and growth cone advance.

Growth Cone Morphology

The complex and dynamic nature of growth cone morphology further complicates our understanding of the contribution of growth cones to axon pathfinding. By statistically analyzing time-lapse images of growth cones undergoing dynamic changes in morphology, researchers have developed probabilistic rules specifying the likelihood of filopodial initiation and retraction, and also the spatial distribution of filopodia in terms of a limited number of parameters. In this model, growth cone morphology is described by the instantaneous length and angle of each filopodium, and the dynamics of the filopodia are characterized by the following parameters: the rate at which filopodia extend, the rate of retraction, the average rate at which new filopodia are initiated (modeled as a Poisson process), and the shape parameters for a gamma distribution that gives the time over which a filopodium extends before retracting. The model also specifies a simple conditionally random rule for where a filopodium initiates, and it assumes that filopodia extend radially from the center of the growth cone. Computer simulation then gives qualitatively realistic morphologies, which also satisfy quantitative constraints such as the correct average number of filopodia. By mapping the effects of external cues onto the parameters of the model, one can hope to gain some intuition as to how those cues might operate.

Other researchers have directly modeled the processes underlying actin dynamics in order to understand filopodial formation, stability, and behavior. This analysis indicates that the maximum length of a filopodium is determined by the number of bundled actin filaments in its core. For less than approximately 10 bundled actin filaments, the strain exerted on the bundle by the membrane is sufficient to cause buckling for even very short filopodia. As the number of included filaments increases, it becomes less likely that a filopodium will buckle; however, more G-actin is required for the structure to continue extending. Thus, when the number of filaments is too large, a filopodium is also unable to extend. The best trade-off between stability and G-actin depletion is achieved with approximately 30 actin filaments. The model predicts average filopodia lengths between 1 and 10 μm, which are in agreement with experimental data. Similar work has examined the mechanisms behind lamellipodial structures.

Axon Turning and Guidance

Axons are thought to be guided by external cues through two processes: gradient-based guidance, in which the growth cone attempts to climb or descend a concentration gradient (chemoattraction or chemorepulsion), and contact-mediated guidance, in which the growth cone interacts with small regions of highly concentrated guidance cues and modulates its behavior accordingly. For gradient-based guidance, the growth cone must detect a potentially shallow gradient in the presence of noise, whereas for contact-mediated guidance the growth cone is essentially involved in a search process.

Gradient guidance Single cell chemotaxis – the attraction or repulsion of organisms such as bacteria, leukocytes, or slime molds by chemical gradients – has received a large amount of theoretical attention. Much of this can be directly applied to the case of the growth cone. Of particular relevance is a seminal contribution by Berg and Purcell, who argued that gradient detection by any small sensing device is fundamentally limited by statistical fluctuations, both due to variations in local ligand concentration and due to the inherent stochasticity of receptor binding. Growth cones are believed to sense and respond to gradients by comparing receptor binding across their spatial extent: the side of a growth cone exposed to the highest concentration of ligand will, on average, also display the largest amount of receptor binding. If growth cones do use such a spatial-sensing strategy, then in order for a growth cone to detect and reliably respond to a chemical gradient, the noise due to fluctuations in receptor binding cannot be much larger than the difference in receptor binding across its spatial extent. By modeling the physics of receptor–ligand interaction, one can estimate the limitations growth cones face when responding to chemical gradients. If the root mean squared error in a concentration measurement is given by σ_C, then the error associated with taking the difference between two such measurements is $\sigma_{\Delta C} = \sqrt{2\sigma_C}$. This gives an order of magnitude lower bound on the difference in concentration, ΔC, that the growth cone can

detect: $\Delta C_{\min} \approx \sigma_{\Delta C}$. Using a simple model of receptor binding mechanics, it has been shown that for the timescale on which growth cone behavior usually occurs ($\sim$100 s), growth cones can detect gradients with a steepness of between 1% and 10%, depending on whether the guidance cue is diffusing freely or is substrate bound. However, over much longer periods (several days), experimental work has demonstrated that growth cones can respond to gradients of 0.2% or less across their spatial extent. Furthermore, at sufficiently high and low concentrations, almost all or almost no receptors are bound: this leads to further reductions in sensitivity. Experimental and theoretical work has confirmed that the highest sensitivity is achieved when approximately half of the receptors are bound, which occurs when the concentration is equal to the dissociation constant for binding.

Aside from these general constraints on gradient detection, models have also been developed which directly simulate the behavior of growth cones and axons in the presence of guidance cue gradients. Several models have focused on biochemical networks which are putatively responsible for growth cone motility and guidance. Such models are difficult to construct, partly due to the complexity of growth cone biochemistry and partly due to the lack of experimental data on important quantities such as reaction rate constants, concentrations, and interactions between molecular species. One model has focused on the Rho-GTPase signaling network, which is known to play an important role in actin-driven cell motility. Due to incomplete experimental data, the investigators took a qualitative approach, simulating the behavior of several plausible interaction networks and kinetic constants and using the results of these simulations to form hypotheses about the underlying mechanisms of growth cone motility. They found that the Rho-GTPase network undergoes a sharp transition in its dynamics when a threshold concentration of a particular signaling molecule is reached. The authors linked these two dynamic behaviors to different modes of growth cone motility, developing a model which could reproduce some experimentally observed phenomena.

Other models have placed less emphasis on specific biochemical mechanisms and have focused instead on the potential role of filopodia in axon guidance or on more general signal-processing strategies that a growth cone may implement, such as temporally or spatially averaging receptor inputs in order to reduce noise. Spatial averaging involves pooling the inputs from multiple receptors, whereas temporal averaging combines information from different time points. An interesting conclusion from this study is that spatial averaging provides the most benefit when the average is taken over approximately one-third of the growth cone's spatial extent. This optimum averaging range occurs because although spatial averaging reduces noise in a local concentration measurement, it also reduces the spatial resolution of the measurement. Because gradient detection requires concentration measurements to be made at multiple locations, the advantages gained in noise reduction are offset by the loss in resolution. Assuming growth cones use such a strategy, this has implications for the intracellular signaling network, suggesting that second messenger molecules implementing the spatial averaging process must diffuse at a rate much slower than expected for cytosolic compounds. One possibility is that spatial averaging is achieved through membrane-bound molecules.

In addition to detecting a chemical gradient, the growth cone must also amplify the possibly extremely shallow gradient in receptor binding in order to achieve a definite motile response. Understanding the mechanisms underlying this amplification has been a general focus for experimental and modeling work on microbial chemotaxis. In one influential model, amplification is achieved by coupling the external signal to a pattern formation system involving local activation and long-range inhibition. The system begins in a spatially symmetric, but unstable, steady state. Symmetry is broken by the external signal, which pushes the system into a stable, asymmetric state that reflects the direction in which the symmetry was broken. A difficulty with this approach is that the system then becomes stuck, unable to respond to new inputs such as a change in the external signal. Several additional mechanisms have been proposed to work around this, each postulating a second process which serves to reset the system to its original, unstable state. Further generalizations of this class of models suggest mechanisms for the formation of filopodia and generate testable predictions for the spatiotemporal distribution of such structures.

Contact-mediated guidance Axons are also guided by cues which are more tightly localized in space, referred to as short-range or contact-mediated cues. For example, filopodial contact with single cells expressing appropriate cues can entirely redirect an axon's trajectory. For this kind of guidance, noise is less of an issue because the signal is essentially binary: either the growth cone contacts the cue or it does not. In this situation, an appropriate theoretical framework is that of stochastic search. The question of how filopodial dynamics of the growth cone affects its ability to locate and respond to highly localized guidance cues has been addressed with the aid of the models describing growth cone morphology in terms of filopodial dynamics. The efficacy with which a growth cone is able to locate a guidance cue has been mapped against

the parameters defining the dynamics, suggesting some behaviors one might expect to observe depending on the geometry of the guidance cue distribution. This work suggests that filopodial dynamics are set, and possibly modulated, in order to increase a growth cone's ability to detect and respond to relevant cues. In a more abstract approach, growth cone movements were described by a combination of stochastic (e.g., deflection by random adhesion to the substrate) and deterministic (e.g., a tendency to move in the direction of past axon extension) motions. The growth cone was found to more effectively respond to short-range guidance signals when the two processes contributed equally. This prompted the authors to propose that growth cones modulate the relative influence of stochastic and deterministic movements depending on the importance of short-range cues at different stages in development – a suggestion consistent with experimental observations of growth cone behavior.

Axon–Axon Interactions

The models described so far consider the guidance of single neurons in isolation. However, the development of the nervous system involves the correct guidance of many axons simultaneously, and it is well established that axons use one another as additional sources of information during development. One of the earliest computer models of axon guidance attempted to explain the characteristic 'sheetlike' pattern of axon outgrowth observed in the formation of the ventral commissure of the spinal cord. Using a descriptive model of individual axon behavior, including several experimentally observed features of ventral commissural axon growth – a tendency for straight growth, for initial outgrowth to be directed ventrally, and for growing axons to extend preferentially over the surrounding matrix and not other axons – this work attempted to distinguish the most important features of individual axon behavior for the formation of axon sheets. From computer simulations, the authors concluded that initially polarized outgrowth, a suitably high density of neurons, preferential adhesivity for extension over the substrate rather than other axons, and a tendency for straight growth were sufficient to generate the observed patterns. Another model examined the possibility that growth cones secrete diffusible guidance cues in order to attract or repel one another to create or break up axon bundles.

Topographic Map Formation

A specific example of axon guidance that has been well studied theoretically is the formation of the topographic map between the retina and optic tectum/superior colliculus. In 1963, Roger Sperry first proposed that such maps could arise because gradients of molecular labels in the retina are matched to gradients of labels in the tectum. The subsequent discovery of gradients of Eph receptors and their ligands, the ephrins, in the retina and tectum confirmed this prediction. However, a large number of experiments investigating how such matching might work in detail have suggested that several other constraints are also important. Since the 1970s, numerous theoretical models of such map formation have been proposed in order to gain insight into this complexity. Some of the simplest propose sorting mechanisms, whereby an initial random map is refined by comparing the retinal origin of axons terminating at neighboring sites in the tectum. Others have hypothesized that tectal labels are at least partly induced or modified by transport of retinal labels into the tectum. Several models have highlighted the importance of competition in map formation, both between axons for tectal target space and between tectal targets for axons. Another important theme has been cooperative effects between axons, somewhat similar to the axon–axon interactions discussed previously. Increasing data on the precise role of Eph/ephrins in map formation have provided new challenges for such models, many of which are yet to be addressed.

Guidance Cue Patterning

A further area of active research aims to understand how guidance cue patterns are generated in the first place, and how effectively particular patterns can guide axons. The modeling of gradient systems in developing organisms has a long history, and gradients are thought to be a primary means for generating spatial ordering. In general, molecules expressed as gradients in order to provide spatial information are known as morphogens, and several classical morphogens have been shown to also guide axons.

A number of models have been proposed to explain how gradients of appropriate shape and stability could be set up. The simplest model assumes that the molecule of interest is diffusing away from a continuous source through a homogeneous medium. More complex models recognize the inhomogeneous nature of the medium, degradation of molecules, binding of molecules to cells, endocytosis, and active transport processes. A further complication which arises when attempting to generalize results from one experimental model to another is that of scaling: gradients form on a typical length scale, and different species have embryos of different sizes at developmental stages when axon wiring is forming. Hence, a system which works in one embryo may not work in another.

Additional constraints are placed on the formation of gradients useful for axon guidance. The ability of a

growth cone to detect and respond to a gradient varies with background concentration and gradient slope. The minimum gradient that a growth cone can detect over a particular background concentration specifies limitations on gradient-based guidance. It allows the construction of optimal gradients, in which the gradient slope is always equal to the minimal detectable gradient for the growth cone. Following this line of argument, coupled with estimates of parameters central to the model, it can be shown that the maximum distance over which growth cones can be guided by an optimal gradient is on the order of 1 cm.

Future Directions

This article provided an overview of the kinds of models which have been applied to axon guidance and how these have helped us to understand axon pathfinding. However, research of this kind is still at an early stage. Important questions remain to be answered and are the focus of active research. For instance, how sensitive can growth cones be to gradients of guidance cues? How close do they come to achieving fundamental sensitivity limits? What are the actual mechanisms they use to detect gradients? How do developing neurites integrate information from multiple guidance cues? What searching strategies do growth cones use to locate local guidance cues? What roles do axon branching and pruning play in axon guidance? How do microtubules and the F-actin cytoskeleton interact to support axon outgrowth and steering? How much of a role do axon–axon interactions play in the formation of the nervous system, and when are they important?

In addition to fresh modeling approaches, answering these questions will require significant experimental advances. A wealth of experimental data is available on axon guidance, but most studies have been aimed at identifying guidance cue molecules or intracellular molecules mediating or eliciting particular behaviors. Although such data are obviously crucial, in order to generate sufficiently constrained models, data of a more quantitative nature are needed. Recognizing this need, experimental techniques for producing well-controlled and flexible patterns of guidance cues have been developed. Ultimately, these techniques should allow us to develop better constrained models and, using them, obtain additional power to tease apart the mechanisms and principles underlying axon guidance.

Finally, the discovery that new neurons are constantly being born in adult brains opens up another area for exploration. These nascent neurons must somehow find their way to their appropriate niches and extend axons to make functional connections. Modeling axon guidance thus has a central role to play in understanding both the initial development and the normal functioning of the nervous system.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Axonal Regeneration: Role of Growth and Guidance Cues; Growth Cones.

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Axon Guidance: Guidance Cues and Guidepost Cells

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Guidepost cells, also called landmark cells, were originally used to refer to specific cells in the developing insect limb that help peripheral nerve cells find their central targets. As vividly depicted by their name, these cells provide local cues to guide pioneer neurons to navigate through an unfamiliar territory in embryonic tissues. In combination with other cellular mechanisms, such as selective adhesion, they ensure the proper development of stereotypic neural connections that underlie complex behavior.

Although they have been historically studied in insects, guidepost cells have been demonstrated in many organisms as well as in both peripheral and central nerve systems. In fact, they now take a broader meaning and more appropriately refer to the intermediate targets in the course of neural development. In addition to guiding axons, they provide local guidance cues to control many other processes during nerve development. This article reviews the historical studies in insect cells, their role in midline crossing, and their emerging function in synapse formation.

Historical Studies of Guidepost Cells in Grasshoppers

The concept of 'guidepost cells' was first suggested by Australian biologist Michael Bate in his seminal study of peripheral pioneer neurons in grasshopper *Locusta migratoria*. In insects, peripheral sensory neurons are produced by a small number of epidermal cells at the body surface. Most of these cells invade their central targets by hopping along the preexisting nerve fibers that are established by pioneer neurons early in development. So how do pioneer neurons connect to the central nervous system (CNS) in the first place? To understand this, Bate followed them in the developing antenna and limbs by both light and electron microscopy. He discovered that these neurons, also with cell bodies born at the edge of each appendage, have to extend their axons through the entire length of the antenna or the limb to reach the CNS. Interestingly, through the course of their journey, not all axons follow a linear path, the shortest route. Instead, some of them, especially those in the limb, take an indirect route and change growth directions several times before reaching the central target. Based on this observation, Bate concluded that the connection path is determined by the interaction of the pioneer axons and the extrinsic cues in the limb. He suggested that these cues are provided by a group of cells, 'the signpost,' that "occur consistently at intervals along the developing appendage and seem to provide a series of stepping stones between the tip of the limb and its base."

The use of 'guidepost cells' or 'landmark cells' was adopted in subsequent studies of peripheral pioneer neurons in grasshopper limb buds, in which the simple nerve connection and the easy access for experimental manipulation provided an attractive system to further test this idea. Using newly developed staining techniques, many more cells have been found that can potentially serve as guidepost functions in the insect limb. They have some characteristic features, which were later used as major criteria to define guidepost cells in other systems: (1) they are located along the route of pioneer axonal path; (2) they are contacted by the growth cone of pioneer axons; (3) they are separated from each other but within reach of growth cone filopodia; (4) they are distinct from neighboring cells because they can be labeled by specific antisera on the surface, and they do not line up to form a continuous path for pioneer growth cones to follow; and (5) they form a special connection with axons because dye can pass from pioneer neurons to these cells. These physical and morphological attributes lead to a 'guidepost hypothesis,' which suggests that the placement of a series of distinctive cells is used to guide pioneer neurons along their trajectory from a distance.

The hypothesis was later tested in a cell ablation experiment, in which the putative guidepost cells that can be recognized immunologically were selectively killed with high-intensity light. The removal of guidepost cells in the developing grasshopper limb buds caused the pioneer neurons to wander away from their normal axonal pathway, and often resulted in the formation of multiple branches. Therefore, cells in the embryonic tissues are present to guide pioneer neurons during early development.

Guidepost Cells and Guidance Cues in Axon Guidance

Following the initial studies in the grasshopper, guidepost cells have also been described in several other invertebrates, including leech, moth, and fruit fly. Although the use of the term has been limited to these simple systems, the cellular studies of axonal pathfinding in grasshoppers have provided two important concepts that have simplified our understanding of

how complex neural pathways are connected. First, they have revealed that the long and sometimes irregular axonal trajectories can be broken down into short segments, and at each segment axonal growth is regulated by local cues at choice points. These choice points contain a cluster of individual cells as the guidepost cells in insects or often a group of functionally specialized cells, which are referred to as intermediate targets in vertebrates. Second, they have shown that complex neural networks are established in two phases, by pioneer neurons that enter the axon-free environment in early development and by later born neurons that face a more complex environment filled with intertwined nerve fibers from early projecting neurons. Whereas the pioneer neurons are guided by local guidance cues, those later developing axons can follow preexisting nerve fibers by selective fasciculation, a cellular mechanism that has also been well studied in grasshoppers.

How do guidepost cells regulate the growth of axons? What are the guidance cues they provide to change the growth direction of developing axons? Ramon y Cajal proposed a century ago that diffusible chemicals in the embryonic environment could guide axonal growth over a long range, just like the chemotaxis of single motile cells. They do so by attracting growth cones, the motile sensors at the leading edge of a neuron that have exquisite sensitivity to detect chemical cues in the embryonic tissues. *In vitro* studies of cultured neurons initially demonstrated the existence of factors secreted by intermediate targets of axons. In the past 15 years, many molecules have been identified by genetic screening and biochemical purification to serve as guidance cues. They include several families of highly conserved extracellular molecules – Netrins, Semaphorins, Slits, and Ephrins – as well as classic neurotrophins and morphogens.

The biochemical and biophysical properties of guidance cues separate them into two classes, diffusible and contact mediated, although their distinction has become less clear in recent studies. Diffusible cues are secreted by cells from a long distance and can either attract axons toward the target or repel axons away from it. Contact-mediated cues are often associated with extracellular matrix or cell surface and regulate the adhesiveness of the growth environment, so they either provide a permissive surface on which axons grow or create an unfavorable region that axons tend to avoid. As shown by embryological, tissue culture, and genetic studies, these molecules are present in developing nervous system and work together to control the growth and guidance of axons during their long journey to their eventual synaptic partners. The following sections review recent studies of midline guidance in both invertebrates and vertebrates and use it as an example to demonstrate now a simple change in axon growth direction is regulated by guidance cues at a specific choice point.

Guidepost Cells at the Midline

The midline in the animal CNS is important for establishing neural pathways used for bilateral communication. In the insect nerve cord or the vertebrate spinal cord, a group of neurons called commissures extend their axons across the midline. Once crossed, the axons turn rostrally, join the longitudinal nerve tracks, and eventually synapse on the contralateral side of the brain.

Cells in the ventral midline have been found to serve as intermediate targets to guide the commissural axons to cross the midline. In flies, there are several midline glial cells that are important for controlling the cross because mutations affecting their formation greatly reduce the fidelity of the growth of commissures crossing the midline. In the vertebrate spinal cord, the main action occurs at the floor plate, a region that contains several layers of neuroepithelial cells with distinct morphological and immunological features at the ventral midline. They are derived from the initial neural fold, but their properties are induced by a molecule, Sonic hedgehog, secreted from the notochord that lies ventrally below them. In mouse mutants of Danforth's short tail or Gli2 (a zinc-finger transcription factor) in which the floor plate is missing, commissural axons grow abnormally when they reach the ventral midline. The same has been documented in zebra fish, in which mutations affecting floor plates also result in the misguidance of spinal commissural axons. Therefore, the ventral midline cells provide a unique system for understanding axon guidance at a choice point.

Attracting to the Midline

The initial characterization in grasshoppers demonstrated that a single filopodium of the sensory growth cone makes a direct contact with guidepost cells and leads the axon in the correct direction. The distance between each pair of guidepost stops is less than 100 μm, which might be sufficient for filopodia to search in space and find the guidepost cells by differential adhesion. However, for commissural neurons, especially those from vertebrates, their targets are hundreds of micrometers away. How do they know where to find the intermediate target?

The ventral midline cells appear to secrete diffusible molecules that attract commissural growth cones

to cross midline. The presence of diffusible factors was first demonstrated in the study of vertebrate commissural neurons in culture. When the floor plate from chick or rodent embryos was cultured adjacent to the dorsal spinal cord explant in three-dimensional collagen gels, commissural axons in the explant grew out toward the floor plate. Subsequent biochemical purification identified a family of secreted protein Netrins that can stimulate commissural axon outgrowth and attract them toward COS cell aggregates that produce the protein. Netrins are secreted by the floor plate cells and bind DCC, a cell surface receptor that is exclusively expressed on commissural neuron axons. Genetic analyses in mice, fruit flies, as well as *Caenorhabditis elegans* have established that this ligand and receptor pair is required for midline attraction because mutations in these two genes lead to the failure of commissural axons growing toward the midline. However, interestingly, in the mouse netrin-1 mutant spinal cord, some axons still reach the floor plate. This is due to the presence of another molecule, Sonic hedgehog, also secreted from the floor plate.

How do soluble factors attract axons over a long distance? Target-derived factors have been thought to form a diffusible gradient that guides the growth cone, a theory proposed by Ramon y Cajal after the discovery of growth cones. Recent studies of netrin expression in chicks demonstrated that proteins secreted in the spinal cord do form a dorsal–ventral gradient, with the highest amount concentrated at the floor plate. In addition, growth cones in culture can respond to netrin gradients and turn toward the source that provides the protein. Therefore, chemoattraction provides a simple mechanism to explain the guidance of embryonic axons toward their targets.

Repelling Away from the Midline

After commissural axons are attracted to the midline, most of them do not stay there but instead leave the midline and project to the contralateral side of the spinal cord. A remarkably conservative and reproducible feature is that they cross the midline only once. How is this achieved?

The guidepost cells that provide attraction at the ventral midline also secrete another family of extracellular molecule, Slits. These molecules bind and activate cell surface receptor Robos on the commissural axons and repel them away from the midline after crossing. This was first demonstrated in a genetic screen in flies, in which a mutation in Slit caused all the commissural neurons to collapse at the midline. Interestingly however, the initial study of the Robo receptor revealed a very different phenotype. With only Robo eliminated, commissural axons freely cross and recross the midline and wrap around it to give the roundabout phenotype that is very different from Slit. This is because there are multiple Robo isoforms in flies. When another receptor Robo2 is also deleted in flies, the same Slit phenotype is observed, suggesting that both Robo1 and Robo2 can signal commissural axons to leave the midline, whereas only Robo1 is needed to prevent recrossing. Therefore, slits provide a negative guidance cue to commissural axons and directly activate Robo receptors to drive them away from the midline, thus preventing axon from recrossing. The same molecular mechanism is also used in vertebrates. When all three Slit genes are deleted from the mouse spinal cord, a considerable amount of commissurals are stalled near the floor plate.

In addition to preventing commissural axons from recrossing, the Slit proteins secreted by the midline cells serve as a guide to determine how far each interneuron axon should extend and which fascicle to join. Three Robo 1 homologs are differentially expressed on axonal fascicles, with Robo only on the medial fascicle, Robo and Robo3 on the intermediate ones, and all three (Robo, Robo2, and Robo3) on the most lateral one. The amount of Robo expressed on their surface provides a combinatorial code to determine the position of longitudinal fascicles. When the receptor level is perturbed by overexpression or knockdown, the fascicle positions along the lateral axis either shift away or move closer to the midline accordingly.

Slits are expressed in the midline at the same time as the attractive factor netrins. How do the commissural axons avoid being repelled from the midline before crossing? In flies, this is accomplished by an intracellular protein Comm, which appears to interact with Robo receptors and keeps them away from the growth cone membrane before crossing. In post-crossing axons, Comm expression is downregulated and thereby surface Robos are increased to respond to the repulsive signal from slits. Interestingly, no Comm homolog has been found in vertebrates, but a third Robo-like receptor, Robo3/Rig1, which is expressed also only on the precrossing axons, appears to serve the same function. In the Robo3/Rig1 mouse mutant, commissural neurons reach the ventral side of the spinal cord but stay away from the floor plate, mimicking the defect initially found for the Comm mutation in flies.

Adhesion at the Midline

The initial study of pioneer neuron guidance in grasshoppers suggests that cell adhesion may play an important role in controlling growth direction by

guidepost cells. Studies in the past 20 years have identified many cell adhesion molecules that form homophilic or heterophilic dimers on the cell surface. In the vertebrate floor plate, several immunoglobulin superfamily cell adhesion molecules – axonin-I, NrCAM, and NgCAM – have been shown to be involved in proper control of the behavior of commissural axons at the floor plate. If their functions are perturbed by antibodies in chick embryos, the axons reach the floor plate but fail to cross the midline and turn to the ipsilateral side of the spinal cord.

Another cell surface molecule, Fasciclin II (FasII), provides a permissive substrate for the later born neurons to join the fascicle and extend along the longitudinal axis after crossing. In fly FasII mutants, these bundles do not form tightly, even though they can turn and move away from the midline, as repelled by slits.

Guidepost Function in Synapse Formation

The concept of guidepost cells has also been used in a different developmental context. During the development of neural circuits, axons are guided to different regions of the nervous system, where they contact their synaptic partners. Synaptic target selection and synapse formation are also critical steps to achieve the precise assembly of neuronal circuits. Several studies have shown that distinct populations of guidepost cells are important at the level of synaptogenesis. The following sections summarize the discoveries of these studies.

Caja-Retzius Cells and Certain GABAergic Interneurons as Guidepost in Hippocampus

In hippocampus, the cell bodies of pyramidal neurons are located in the basal region and send out dendrites toward the pial surface. Distinct subcellular domains of pyramidal dendrites are innervated by two populations of afferents. Distal portions of pyramidal dendrites receive input from the ipsilateral entorhinal afferents, whereas the proximal dendrites form synapses with the commissural fibers. This layer structure is established during development with the help of two populations of guidepost cells. Caja-Retzius cells and a set of GABAergic interneurons are early developing neurons found in the two afferent layers. They synapse with the entorhinal afferents and the commissural axons, respectively. These synapses are transient in nature since some of the Caja-Retzius cells and GABAergic interneurons die later. The disappearance of transient synapses is accompanied by synaptogenesis of afferents with the pyramidal dendrites as the synapses are transferred from the guidepost cells to mature synaptic targets.

The significance of the guidepost cells in the development of hippocampal circuit was demonstrated by cell lesion experiments. When the Caja-Retzius cells are ablated, the laminar innervation of the entorhinal axons is impaired. It is interesting to note that the ingrowth of entorhinal axons into the hippocampus precedes the extension of pyramidal dendrites. It is conceivable that the early existence of the Caja-Retzius cells is important to hold the presynaptic terminal in place before the true postsynaptic target arrives. Since in the absence of the Caja-Retzius cells laminar projection of the entorhinal axons is impaired, this suggests that early synaptogenesis might be important for the stabilization of axon arbors. Indeed, several *in vivo* time-lapse studies have shown that synapse formation in the CNS is very dynamic, with constant synapse formation and disassembly, and branch addition and retraction. The presence of synapses on axon branches increases the stability of the branches. Therefore, the laminar distribution of the guidepost cells provides a scaffold for the presynaptic terminals on the afferent fibers, which consequently stabilizes the axonal arborization and achieves laminar innervation.

Subplate Neurons as Guidepost in the Maturation of Visual Cortical Circuit

In the mature visual system of vertebrate animals, thalamic inputs directly innervate primary visual cortex layer 4 neurons. Functional organization of the visual cortex, such as ocular dominance columns and orientation columns, emerges through specific synaptic circuit formation. Interestingly, thalamic inputs first form synapses with another population of neurons, the subplate neurons, before connecting to the layer 4 neurons. At this early time, subplate axons innervate the layer 4 neurons and relay the information from thalamus to the cortex. Later, during the critical period of cortical activity-dependent plasticity, the subplate neurons gradually die through programmed cell death. In the meantime, adult circuit forms in which thalamic inputs directly synapse onto layer 4 neurons.

Ablation of subplate neurons results in the failure of segregation of the thalamic inputs into ocular dominance columns and the formation of orientation columns. This strongly suggests that subplate neurons are essential for synaptic remodeling and maturation of neural circuit. In support of this notion, one study showed that subplate ablation prevents the upregulation of $GABA_A$ receptor expression and perturb the maturation of inhibitory circuits in the layer 4 neurons. Collectively, subplate neurons act as a relay station at early stages of cortical development and are indispensable for patterning mature synaptic circuits.

Vulval Epithelial Cells as Guidepost in the Development of the Egg-Laying Synaptic Circuit

In *C. elegans*, egg-laying behavior is controlled by a pair of motor neuron HSNs. The cell bodies of HSNs are situated just posterior to the vulva, with its axon guided ventrally and then anteriorly. The HSN axons defasciculate dorsally from the ventral nerve cords near the vulval opening and innervate the vulval muscles by forming a cluster of neuromuscular junctions onto the muscle arms. The HSN axons also form synapses onto the VC motor neurons in this region, which in turn innervate muscles. Genetic and developmental analysis revealed that the surrounding vulval epithelial cells play an important guidepost role in the formation of this egg-laying neural circuit. In the absence of these epithelial cells, HSNs form ectopic synapses, which are located more anteriorly than normal. Molecularly, an immunoglobulin superfamily protein, SYG-2, was found to perform the guidepost function. SYG-2 is expressed transiently by the guidepost epithelial cells at early stages of HSN synapse formation. SYG-2 directly binds its receptor on HSN, another immunoglobulin superfamily protein, SYG-1, and clusters SYG-1 at the segment of HSN axon near the vulva. SYG-1 induces accumulation of synaptic vesicles/presynaptic active zone components and directs the location and target selection of HSN presynaptic specialization. In the absence of functional SYG-1 or SYG-2, HSN has a reduced number of synapses formed at its normal location. Instead, the majority of synapses are formed onto adjacent body wall muscles, inappropriate synaptic targets, at anterior ectopic locations. Developmentally, the axons of HSN reach the synaptic region prior to the outgrowth of the postsynaptic VC dendrites. Presynaptic terminals can be observed in this segment of the HSN axons before the VC dendrites reach the same region. The transient synapses of HSN are likely to form directly onto the guidepost cells before being 'handed over' to the late maturing postsynaptic targets. The guidepost vulval epithelial cells have several important functions in the development of the egg-laying organ. These cells attract the migrating sex myoblast, which gives rise to the vulval muscles. They also stimulate the branching of the VC motor neurons. Therefore, guidepost cells not only spatially and temporally control the maturation of the egg-laying neurons and muscles but also regulate the assembly of the neural circuits at the synapse formation level.

The three types of synaptic guidepost cells mentioned previously are probably examples of similar cell types that have not been discovered. Guidepost cells seem to be particularly important in synapse formation where there is temporal discrepancy between axonal and dendritic development. It is conceivable that guidepost cells stabilize axons by forming transient synapses, which disappear upon the arrival of true postsynaptic dendrites.

Conclusion

The function of guidepost cells in axon guidance is well established. They are frequently the sources of axon guidance molecules that attract or repel axon growth cones. The emerging roles of guidepost cells in synapse formation and neural circuit maturation reveal previously unknown complexity during synaptic circuit assembly.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Axonal Pathfinding: Netrins; Axonal Regeneration: Role of Growth and Guidance Cues; Growth Cones.

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Axon Guidance by Glia

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Evidence for Conducive Functions of Astroglia

Astroglial Cells Constitute Axonal Growth Pathways *In Vivo*

Astrocyte-derived basal laminae Astrocyte surfaces were originally recognized as a favorable substrate for axon outgrowth. Müller cells, for example, produce a specialized structure toward the inner surface of the retina, the glial endfeet. Retinal ganglion cell axons elongate along the endfeet-derived basal lamina that contains characteristic extracellular matrix (ECM) components such as laminin-1, collagen IV, heparan sulfate proteoglycan (HSPG), and nidogen. The response of axonal growth cones to laminin-1 is regulated by integrins, heterodimeric receptors of the ECM.

Astrocyte monolayers Astrocyte monolayers constitute an excellent growth substrate for axons *in vitro*. For example, type I protoplasmic astrocytes with extended surfaces prepared from embryonic or perinatal central nervous system (CNS) tissues are more efficient than those obtained from later postnatal stages. Primary astrocytes are more efficient soon after plating and tend to lose beneficial properties with time. Subtypes may exist that are less supportive for axon extension. The neurite growth-promoting properties are also regulated by the spatial arrangement of astrocytes.

Astrocytes in three dimensions It has been observed that astrocytes that still support axon growth when used as monolayers may have inhibitory properties when assembled in tissuelike structures. For example, dorsal root ganglion axons confronting astrocytes lodged in three dimensions in a cellulose acetate tube will grow readily into tubes filled with embryonic but not with aged or matured astrocytes. The model in this regard mimics the dorsal root entry zone, a reputed stop area for centripetal axons in the adult. In this context, the astrocyte–meningeal cell interface may construct a growth barrier.

Multiple Control Mechanisms Regulate the Astrocyte-Dependent Establishment of Cortical Connections via the Corpus Callosum

The Corpus Callosum and the Glial Sling

Anatomy of the corpus callosum and the blueprint hypothesis The corpus callosum connects the left with the right hemisphere in the developing nervous system. In the mouse, it consists of approximately 3 million myelinated fibers that link corresponding regions of the cortices. More that 50 human genetic aberrations have been described that lead to some form of dysgenesis of the corpus callosum. Two populations of midline glia, the indusium griseum and the glial wedge, are necessary for the adequate guidance of axons in this process. The emerging glial structures construct a transient bridge of astrocytes that connects the left with the right hemisphere of the developing telencephalon. This glial bridge, also called the glial sling, supports the reciprocal growth of cortical axons, and the experimental interruption of the sling leads to the formation of acallosal mice. In this situation, the cortical connecting axons role up on either side of the cerebral midline and form the bundles of Probst, longitudinal fascicles of misdirected axons. Growth promotion of cortical axons can be restored by the implantation of nitrocellulose filters that are covered with embryonic astrocytes-derived membranes. The blueprint hypothesis of axon growth states that channels walled by astrocyte surfaces might provide a mechanical growth and guidance substrate for growth cones. Molecular specializations of both growth cone and astrocyte surfaces are presumably implicated in the regulation of these interactions.

Molecular bases of neuron–glia interactions The mechanistic concept of axon guidance has been modified and geared toward an interpretation which emphasizes molecular signals in the growth environment, the readout by specific growth cone-based receptors, and integration of these influences by signal transduction cascades which eventually modulate growth cone movements (**Table 1**). Thus, a conduit function of astroglia based on the chemorepellent slit-2 has been proposed as an additional guidance principle in the corpus callosum. The molecular analysis of the signaling system involved in corpus callosum formation has progressed and additional gene families have been identified. Thus, the wnt5 protein is expressed by cells of the glial wedge and plays a role in guidance of connecting axons on the contralateral side of the developing corpus callosum formation. Wnt5a-dependent guidance in this context is mediated by the protein tyrosine kinase Ryk that is required in the axon.

Cell Adhesion Molecules Play a Pivotal Role in Neural Cell Interactions

General Definition of Cell Adhesion Molecules

Cell adhesion molecules (CAMs) were first identified during the study of sponge aggregation and

Table 1 Families of adhesion molecules involved in axon guidance

Adhesion molecules	*Features*	*Functions*
Ig superfamily (IgSf)	At least one immunoglobulin (Ig-like) domain, often in combination with fibronectin type III domains	Ca-independent adhesion, either in the homophilic or in the heterophilic mode
L1CAM, NCAM	First IgSf members found in the nervous system	Axon growth and guidance, synapse formation, and plasticity; human L1CAM mutated in the human MASA syndrome that is associated with mental retardation
TAG-1	GPI-linked member of the IgSf	Axon fasciculation
Cadherin superfamily	Characterized by at least one cadherin domain	Primarily homophilic, Ca^{2+}-dependent adhesion; roles in sorting out of cells, synapse formation
N-cadherin	Classical cadherin	Neuron–neuron and neuron–astrocyte adhesion; elimination leads to premature death and cardiovascular malformations
Laminin-1	Founding member of the laminin gene family that comprises more than ten members; laminins are heteromultimers formed by α, β, and γ chains; several genes for each subunit have been distinguished	Excellent axon growth promoting substrate and, for this reason, obligatory cell substrate constituent in many cell culture protocols; as a component of basal lamina, laminin-1 promotes axon growth in the peripheral nervous system; the outgrowth promotion is read out via integrins
Integrins	Large family of heterodimeric receptors formed by α and β subunits; many genes for either type of subunit have been described	Integrins are receptors of extracellular matrix constituents; a subgroup interacts with the sequence RGD in target molecules; more than 20 Itg receptors have been identified
Netrin-1, -2	Homologies to the γ_1 subunit of laminin-1	Netrins are released by midline glia and build up a chemotactic gradient that attracts axons from commissural neurons

subsequently discovered in mammals. One distinguishes Ca^{2+}-dependent and Ca^{2+}-independent adhesion mechanisms that are served by separate gene families, the immunoglobulin (Ig) and the cadherin gene superfamilies. CAMs interact either in the homophilic mode with identical members of a gene family or in the heterophilic mode with distinct partners that may or may not belong to the same gene family.

The Cadherins and Ca^{2+}-Dependent Adhesion

N-cadherin was the first classical cadherin discovered in the CNS and it mediates neuron–neuron and neuron–astrocyte interactions. Classical cadherins contain five cadherin repeat motifs, calcium binding sites, and a transmembrane domain that result in an overall molecular mass of approximately 100 kDa. Subgroups of cadherin-related neuronal receptors and of protocadherins as characterized by the cadherin domain have been discovered, many of which are expressed in the CNS. There, expression patterns correlate with neuroanatomical networks, and current concepts propose a functional role in wiring and synaptogenesis within these systems. This interpretation is consistent with the fact that classical cadherins function in a homophilic, calcium-dependent manner. The constitutive cadherin domain motif has also been found in so-called unconventional cadherin-like molecules that play a role in epithelial cell junction formation or in *Drosophila* tumors (e.g., the gene *fat*). The cytoplasmic domain of classical cadherins interacts with specialized linker proteins called catenins. These are required for the functional activity of cadherins, and the component β-catenin is involved in signal transduction to the cell nucleus and is an important mediator of the wnt signaling pathway, which links cadherins to differentiation processes and to cancer pathology.

Immunoglobulin Superfamily

The Ig superfamily constitutes the other dominating family of CAMs in the nervous system. Its characteristic feature is the Ig domain that consists of 90–100 amino acids arranged in seven antiparallel β-pleated sheets that fold into a globular structure. In many molecules, the Ig loop is combined with one or several fibronectin type III (FNIII) domains, followed by a transmembrane domain. Some IgCAMs are connected to the membrane by a glycosylphosphatidylinositol (GPI) anchor that confers augmented mobility within the membrane plane. GPI-linked proteins are thought to be associated with transmembrane glycoproteins that convey signal transduction processes – for example, the neurexin family member Caspr/paranodin in the case of the IgCAM contactin. On the mechanistic level, Ig superfamily members mediate calcium-independent adhesion processes that

may be either homophilic or heterophilic. Several neuronal adhesion molecules with pronounced expression on axonal surfaces, such as L1CAM or TAG-1/axonin-1 CAM, have been grouped as AxCAMs, highlighting their prominent role in axon fasciculation.

IgCAM Interactions Launch Signal Transduction

Functional participation of IgCAMs in neurite outgrowth requires the activation of downstream signaling mechanisms, including the variation of intracellular calcium in the growth cone. The transduction mechanisms elicited by IgCAMs converge with those launched via N-cadherin and necessitate the basic fibroblast growth factor (bFGF) receptor that *cis* interacts with these CAMs within the membrane. Selected isoforms of NCAM are expressed by astrocytes *in vitro* and mediate neuron–astrocyte adhesion. With regard to heterophilic interactions, the small isoform of the receptor protein tyrosine phosphatase (RPTP)-β/ζ is expressed by astrocytes, a transmembrane protein that interacts with the neuronal adhesion molecule contactin, and other CAMs of the Ig superfamily. These examples illustrate the functional involvement of IgCAMs in conducive neuron–astrocyte interactions. Mutations of the human L1CAM lead to mental retardation, hypotrophy of the corticospinal tract, and hydrocephalus, emphasizing the importance of the Ig superfamily for development of CNS structures.

Integrins as Extracellular Matrix Receptors

The integrins constitute the third prominent gene family of membrane-based CAMs. These consist of α and β subunits that assemble to heterodimers and mediate the interactions of neural cells with ECM components. In some cases, interactions between integrins and Ig superfamily members have been reported, and the activation of integrins involves signal transduction pathways with small GTP-binding proteins such as *ras*.

Evidence for Segregating Functions of Glia

Gene Families That Mediate Axon Growth Inhibition Counterbalance Adhesion Molecule Gene Families

Growth cone collapse The investigation of neural cell interactions resulted in the identification of the Ig and cadherin superfamilies and of growth- and motility-promoting ECM constituents. However, in the 1980s it became clear that in addition to growth promotion, growth inhibitory molecules also contribute to the regulation of cell migration and growth cone movement (**Table 2**). The phenomenon of growth cone collapse had been described in the context of the interaction of sympathetic with retinal neurites, or when growth cones were confronted with myelin fractions or membrane preparations obtained from inhibitory territories. These interactions invariably resulted in collapse and retraction of the growth cone that remained paralyzed for 30–60 min *in vitro* prior to resuming growth and exploratory behavior. It was quickly realized that this inhibitory effect might equally affect guidance and inhibition of regeneration.

Eph kinases and ephrins The systematic investigation of the visual projection led to the identification of RAG (retinal axon guidance molecule), a GPI-linked protein with a gradient-like distribution in the tectum that proved homologous to a gene family subsequently renamed ephrins. One distinguishes the GPI-linked ephrins A, which interact with complementary EphA-type tyrosine kinases, from the transmembrane ephrins B, which recognize the EphB-type tyrosine kinases. Both groups contain a large number of both ephrin- and Eph-type kinase genes, and a certain degree of freedom in the mutual combinations has been noted. A remarkable trait of the Eph kinases and the corresponding ligands is that the molecules are expressed in reciprocal gradients, which is similar to the concept that Sperry developed in his chemoaffinity hypothesis that posited a chemical cell surface expressed code of positional information. Manipulation of expression levels of Eph genes resulted in a graded repositioning of axonal projections in the visual system. These studies provided impressive support both for the Sperry hypothesis and for the notion that the Eph–ephrin signaling system is an important contributor to the code. Whether the members of the pairs are expressed in neuronal and glial lineages, respectively, remains to be established.

Semaphorins and neuropilins Another gene family mediating growth cone collapse is the semaphorins, which comprise an increasing number of members and have been described in *Drosophila*, chicken, mouse, and human. A constitutive structural feature of these proteins is the sema domain of 500 amino acids that is shared by all family members. Semaphorin-dependent growth cone collapse is mediated by the plexin receptors, which can be categorized into subtypes A–D. In some cases, the plexins interact in the membrane in *cis* with the neuropilins NP1 or NP2 that act as coreceptors in these cases. The semaphorin IIIa dimer, for example, interacts with

Table 2 Gene families involved in repulsion and inhibition

Inhibitory molecules	*Features*	*Functions*
Semaphorins	Characterized by the semaphorin domain; seven structural classes reported in vertebrates and invertebrates	Sema3 molecules inhibit axon growth from several classes of neurons.
Plexins and neuropilins	Transmembrane semaphorin receptors of the growth cone	Detect and mediate Sema-dependent axon growth and guidance.
Ephrins	Family of genes that is divided into the GPI-linked ephrin A and the transmembrane located ephrin B molecules	Ephrins are the long sought-after ligands of the Eph kinases, originally described as orphan receptors.
Eph kinases	Tyrosine kinases located in the membranes of growth cones; one distinguishes Eph-A and Eph-B kinases; Eph kinases activated by interaction with complementary ephrin-A or -B ligands	In the CNS, activation of Eph kinases by ephrins in certain (but not all) combinations leads to growth cone collapse. Eph kinases and ephrins construct complementary gradients in the nervous system that encode positional information, for example, in the visual system.
Nogo proteins	Nogo proteins A–C have been described; they belong to the inhibitory components of myelin that are involved in the prevention of regeneration (in addition to MAG and OMgp)	Nogo proteins activate the Nogo receptor NgR that interacts with the low-affinity NGF receptor p75. Activation of these receptors results in growth cone collapse.
Chondroitinsulfate proteoglycans (CSPGs)	Composed of a core glycoprotein and at least one chondroitin sulfate carbohydrate chain; CSPGs include the lectican family, NG2, and phosphacan	CSPGs are found enriched in glial boundaries during development and in glial scars of the lesioned CNS. They are associated with axon growth inhibition. Axon growth stimulatory chondroitin sulfates have also been described.
Tenascin-C and tenascin-R	Composed of egf-type repeats and fibronectin-type 3 modules and represent the best-characterized glycoproteins of the neural extracellular matrix; tenascins assemble to multimers and are distributed in discrete patterns	Tenascins are inhibitory in certain situations and are capable of forming boundaries *in vivo* and *in vitro*. On the other hand, stimulation of axonal growth has also been reported. These molecules are thus best suited to mediate the ambivalent influences of astrocytes on axon growth and guidance.

plexin A1 and neuropilin 1 to launch growth cone collapse using a signal transduction pathway that involves small GTP-binding proteins, such as Rho and Rac1. The contributions of these inhibitory molecules in the context of neuron interactions with astrocytes remain to be worked out in detail.

Nogo and myelin-based inhibitors By comparison, more is known about the myelin sheath-derived inducers of growth cone collapse that are believed to underlie myelin-dependent inhibition of regeneration. These include the Nogo glycoproteins, with Nogo-A as the principal myelin-based inhibitor of growth, a member of the larger Reticulin gene family. Nogo-A contains a region called Nogo-66, a looplike structure which induces growth cone collapse by interacting with the complementary Nogo receptor NgR. NgR is GPI anchored to the growth cone membrane and part of a receptor complex that also contains the low-affinity NGF receptor p75. Interestingly, two other myelin components inhibitory to axon growth have been detected – the Ig superfamily member MAG (myelin-associated glycoprotein) and OMGP (oligodendrocyte–myelin glycoprotein). Both are also able to activate the Nogo receptor complex, which suggests a common downstream pathway of myelin-dependent inhibition. This pathway involves the downstream activation of RhoA family-type proteins that play a major role in growth cone collapse (**Table 3**).

Glial Boundaries between Developmental Compartments of the Central Nervous System

Rhombomeres of the hindbrain The brain stem is subdivided into rhombomeres, and the paraxial mesoderm is substructured into somites. The rhombomeres in the hindbrain emerge beginning with the six somite (6s) stage and can be identified as a series of eight swellings along the neuraxis which are separated by grooves at stage 16s. The axons of motor neurons located in pairs of rhombomeres are destined to innervate specific branchial arches and leave the even but not the odd-numbered rhombomeres at defined exit points. The motor nuclei of the cranial nerves and their motor tracts are confined to subsets of rhombomeres along the rostrocaudal axis. Cells of neighboring compartments do not mingle, and intercellular communication by gap junctions is limited to cells within a given compartment. The apparent segmentation of the hindbrain correlates with

Table 3 Gene families involved in choice decisions at the midline

Floor plate-based signals	*Growth cone-based receptors*	*Functions and comments*
Netrin-1, -2 (laminin gene SF; unc-6 in the nematode)	Dcc ('deleted in colorectal cancer', IgSF)	Netrin attracts commissural neurons toward the floor plate. The gene products unc-5 and unc-6 were found to control circumferential axon growth in the nematode.
Sonic hedgehog (Shh)	Patched and smoothened	Shh is a morphogen with fate-instructing functions in early development (e.g., induces the floor plate). It serves as chemoattractant at this stage.
Slit-1, -2, -3 (extracellular matrix components)	Robo-1, -2, -3 (IgSF members)	Slit builds a chemorepellent gradient to drive axons away from the floor plate after crossing. When the robo receptor is mutated, the axons circle incessantly back and forth across the midline ('roundabout' mutation in *Drosophila*). There are several *slit* and *robo* genes in mammals.
Sema3a	Neuropilin and plexin-A, -B, and -C receptors	Acts as chemorepellent that prevents return to the floor plate after crossing.
Ephrin B2	EphB kinases	The Eph–ephrin connection mediates membrane-based reciprocal avoidance behaviors in certain combinations.
NrCAM (IgSF member)	Tag-1/axonin-1 (IgSF member)	The NrCAM–Tag-1 interactions are required to enable the growth cone to cross the midline.

distinct expression patterns of transcription factors of the homeobox gene family that code positional identities in *Drosophila* and vertebrates. The limits of expression of these genes in the mouse spinal cord progress in a caudorostral direction and are strictly coherent with boundaries of rhombomeric pairs in ascending order. Also, other genes are expressed in register with rhombomere boundaries, such as various members of the Eph tyrosine kinase and complementary ephrin ligand gene families and zinc finger transcriptional activators such as *Krox-20*. The Hox code can be altered by treatment with retinoic acid, which caudalizes the spinal cord, including cranial nerve nuclei. These findings concur to qualify the rhombomeres as true compartments with homeotic identity.

The prosomeric model of the prosencephalon Analogous patterns of transcription factors at more rostral positions of the neuraxis have prompted the view that compartments may also exist in diencephalic or telencephalic structures. A detailed analysis of the distribution of numerous transcription factors, such as Otx2, Emx1, Emx2, and Gbx2, in the developing rostral CNS has founded the view that one compartment yields the mesencephalon and six prosomeres preconfigure the diencephalon and the telencephalon with its cortical hemispheres. A more detailed assessment of compartments of the CNS is beyond the scope of this article and is available elsewhere. Interestingly, early axonal pathways often follow the boundaries between transcription factor expression territories. This is also true for interrhombomeric boundaries, where the extension of axonal fiber pathways has been documented.

Specialized cells in the compartment interface Closer inspection of the cellular populations in boundary regions revealed a reduced interkinetic nuclear migration of neuroepithelial cells and an increased intercellular space. IgCAMs such as NCAM that are expressed in rhombomeres may mediate intercellular adhesion in this context. The boundary cells exhibit an unusual fan-shaped array and abut with their endfeet on both the pial and the ventricular surfaces. In zebra fish, a so-called glial curtain has been proposed to separate individual rhombomeres. It has been envisaged that the specialized boundary cells construct a privileged pathway for outgrowing axons. An instructive influence of glial cordones on axon pathways is supported by transplantation experiments in which fiber tracts follow ectopic boundaries. The mechanism and molecular bases of hypothesized boundary functions for axon guidance are unknown, and differential adhesion or local inhibition concepts are being discussed.

Midline Glia as a Signaling Center for Growth Cone Guidance Decisions

Genetic control of the midline in *Drosophila* In *Drosophila*, the midline of the ladderlike nervous system involves the so-called midline glia, which is controlled by a battery of genes. Upstream, *single-minded* regulates the fate determination of midline cells in general. Glial cells emerge under the regulatory influence of *spitz*, a homolog of transforming growth factor-α, and the transcription factor *pointed*, which intervenes in the expression of *gcm* (glial cell missing), an additional gene required for the generation of glial cells in *Drosophila*. Homologs of *gcm*

have been described, but they seem to play a different role in vertebrates. Elimination of the midline glia prevents the separation of commissural fibers and eliminates the longitudinal connections of the characteristic ladderlike nervous system. The commissural fibers of the system begin to extend between three pairs of midline glia cells and the pair of MP1 neurons. Separation of the commissural fiber systems involves migration of the midline glia in the correct direction. A genetic screen has led to the identification of a number of additional genes which control this migratory behavior, one of which, called *klötzchen*, seems to implicate the spectrin cytoskeleton of midline glia. In all cases, deficits in midline glia migration lead to errors in the separation of the connecting commissural fiber systems.

The midline glia in the optic nerve chiasm A comparable class of boundaries associated with glial cell types has been described in the midline of the developing nervous systems of vertebrates. Thus, glial cells separate the left and right axonal projection systems at the optic chiasm. Advancing axons are attracted by netrin, interact with this glial population, and are directed either to the ipsilateral or to the contralateral cortex, as required in the context of binocular vision. Several candidate molecules have been considered to be involved in the directional growth cone choice. Among these are the glycoprotein L1, the hyaluronate receptor CD44, and chondroitin sulfate proteoglycan(s), as visualized by the expression of chondroitin sulfate epitopes in boundary glia. Chondroitin sulfate proteoglycans (CSPGs) are thought to indicate inhibitory territories in the developing nervous system that are also secured by ephrins.

The floor plate of the vertebrate neural tube Analogous situations are also observed in the floor and roof plates of the developing spinal cord (**Figure 1**). In the latter case, the dorsal midline cells express keratan sulfate and chondroitin sulfate epitopes linked to proteoglycan core proteins. Thereby, the CSPGs outline a boundary region that is not traversed by the commissural axons. When chondroitin/keratan sulfate-expressing proteoglycans are exposed as patterned substrates alternating with the glycoprotein laminin-1, various cultured neurons and their processes avoid the proteoglycan-rich regions and grow out on the laminin-1 substrates.

In the ventral half of the spinal cord, commissural axons of the dorsal horn are attracted toward the floor plate that releases the chemoattractant netrin-1. Netrin-1 binds to the growth cone-based receptor deleted in colorectal cancer (*dcc*) of the Ig superfamily. This mechanism is highly conserved because it has already evolved in the nematode *Caenorhabditis elegans*, in which unc-5 guides circumferential axons via the unc-6 receptor – homologs of netrin-1 and dcc, respectively. Sonic hedgehog (*Shh*) represents a second chemoattractant that acts in a similar direction. When the axons reach the midline, the commissural fibers interact with the floor plate via an adhesive mechanism that involves the Ig superfamily members NrCAM as expressed in the midline and TAG-1/axonin-1 as heterophilic ligand in the growth cone membrane. The GPI-linked axonin-1/TAG-1 protein is downregulated after the crossing has occurred and L1CAM appears on the longitudinally oriented fibers. Concerted interactions of these Ig superfamily members thus seem to be required to regulate the crossing step. Subsequently, the axons lose responsiveness to the attractant netrin-1 and develop

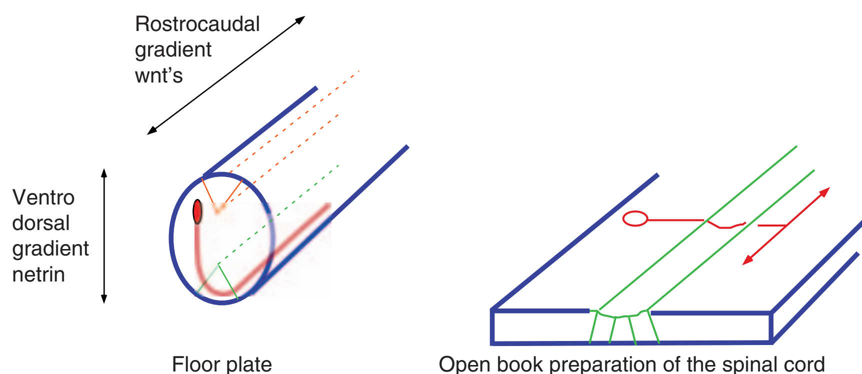

Figure 1 Axon guidance at the midline of the developing spinal cord. The figure shows the developing spinal cord of the rat at approximately embryonic day 13. At this stage, axons emanating from the commissural neurons migrate toward the ventral midline. There, axons contact the floor plate, midline glia (outlined in green) that defines an axon growth choice point. The growth cone migration and its behavior at the midline can be monitored *in vitro* with the so-called 'open book' preparation (right). Many genes are involved in growth cone guidance at the midline, some of which are listed in **Table 3**.

a sensitivity to the chemorepulsive signaling protein slit. This glycoprotein is released by midline glia and induces growth cone repulsion mediated by the Ig superfamily receptor roundabout (*robo*). *Robo* as well as *slit* were originally discovered in *Drosophila*, in which axons fail to stay on the ipsilateral side after crossing the midline, leading to the characteristic picture of incessantly circling axons. Several *slit* and *robo* genes have since been identified in mammals. A further inhibitory system is provided by semaphorins of the sema3 class and the complementary plexin and neuropilin receptor complexes NP1 and NP2, which mediate growth cone collapse. In addition, evidence has been presented that Eph kinase–ephrin interactions are also involved in the midline choice decision of the growing corticospinal projection. Thus, ephrin B3 is a constituent of the midline and prevents recrossing of these axons when they enter the gray matter after having migrated to the contralateral side of the spinal cord. Subsequently, the wnt proteins are involved in stimulating neurite outgrowth and regulating anterior–posterior guidance of commissural axons. These proteins utilize at least three signal transduction pathways and are implicated in many aspects of axon growth and interneuronal wiring (**Table 3**).

The Ambivalent Properties of Astrocytes Are Reflected by Extracellular Matrix Components That Embody Both Axon Inhibitory and Stimulatory Properties

Glycoproteins of the Extracellular Matrix

The pericellular space is organized by a superstructure of interacting glycoproteins and proteoglycans of the ECM. Astrocytes *in vitro* produce many of the ECM glycoproteins originally described in other tissues, namely fibronectin, laminin-1, vitronectin, thrombospondin, and tenascin-C. Laminin-1 is a functional component of astroglial endfeet in limiting membranes, for example, in the developing retina, and forms an excellent growth substrate for axon extension of many neuronal cell types. The chemodiffusible chemoattractants netrin-1 and netrin-2, which guide outgrowing commissural axons toward the floor plate of the midline in the spinal cord, are structurally related genes. Fibronectin has been found in association with blood vessels, a structure in which astrocytes contribute to the formation of the blood–brain barrier which isolates the CNS from the bloodstream.

The Tenascin Gene Family

Tenascin-C is transiently expressed by immature astrocytes in the developing CNS in which its distribution follows functional neuroanatomical subdivisions. For example, in the barrel field it delineates the emerging barrel field structure in layer IV. The glycoproteins of the tenascin gene family are characterized by structural motifs common to tenascin-C (Tnc), tenascin-R (Tnr), tenascin-X (Tnx), tenascin-Y (Tny), and tenascin-W (Tnw). A cysteine-rich N-terminus is followed by a series of egf-type repeats, fibronectin type III modules, and, finally, homologies to fibrinogen-β and -γ at the C-terminus. Different from this organization, a tenascin-like pair-rule gene in *Drosophila* contains the characteristic egf-type repeats but is devoid of other structural elements. The egf-type repeats of tenascins display a characteristic arrangement of cysteines that has also been found in the ECM protein reelin and is distinct from the one in the egf-type repeat modules of Notch or the laminin genes. The N-terminus links monomers in Tnr to trimers and in Tnc to hexamers under nonreducing conditions. As viewed with the electron microscope, the hexamer appears as a typical six-armed structure that has been designated hexabrachion and seems to be conserved during evolution. Two isoforms which are distinguished by one FNIII motif have been described in Tnr, a gene which is expressed in oligodendrocytes at later stages of development.

Multiple Isoforms of Tnc

Tnc possesses an alternative splice site between the fifth and the sixth FNIII module, where as many as six and nine additional FNIII repeats can be inserted in mouse and human Tnc, respectively. Up to 30 alternatively spliced variants in this region of Tnc have been revealed, approximately 50% of the theoretically possible 64 isoforms assuming a binary combinatorial code. In the human, theoretically 512 or 2^9 splice variants are conceivable on the ground of 9 potential alternatively spliced modules. Therefore, Tnc seems suited to specify pericellular microenvironments or to distinguish glial sublineages. Tnc is associated with numerous pathological conditions, including glial tumors.

Tnc Is a Multimodular and Multifunctional ECM Component

The characteristic hallmark of Tnc is its antiadhesive property for many cell types. In situations that expose a Tnc-rich environment alternating with laminin-1, the glycoprotein deflects growth cones and neuronal cell bodies, consistent with its boundary-like distribution in several CNS territories. On the other hand, homogeneous substrates containing Tnc promote neurite outgrowth of most neuronal cell types studied to date. Neurite outgrowth promotion could be mapped to the distal splice site that surrounds

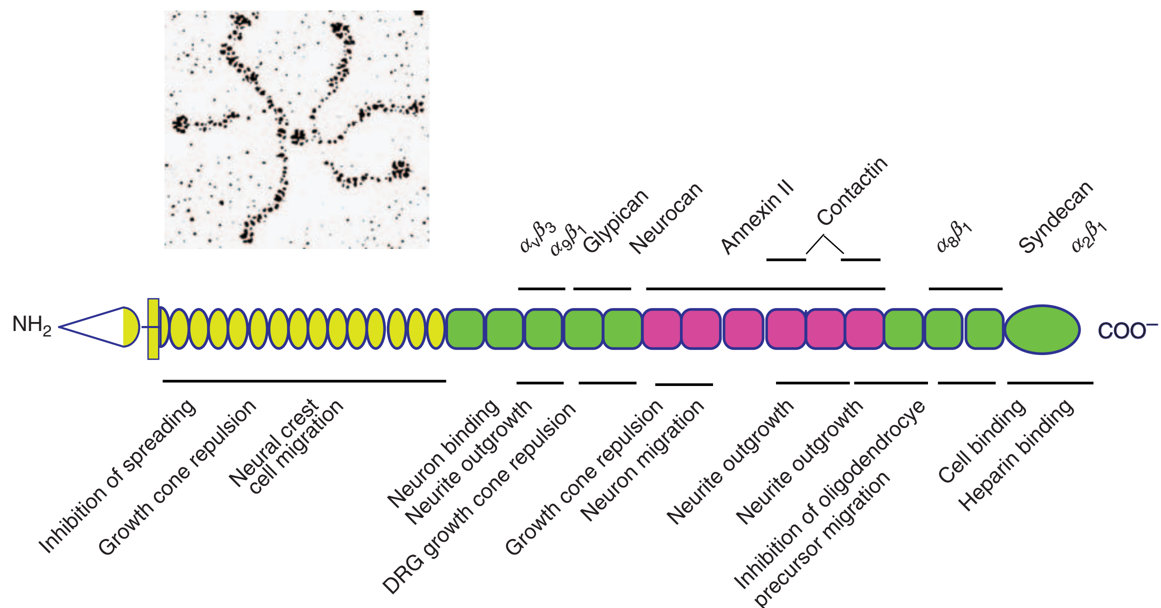

Figure 2 Structure–function relationship of tenascin-C. The glycoprotein of the extracellular matrix (ECM) tenascin-C is characterized by a modular structure with fibronectin type III domains (boxes), some of which are alternatively spliced (pink boxes). On the N-terminal end, the sequence comprises egf-type repeats (yellow elipses). Many functions have been ascribed to tenascin-C that are mediated by distinct receptors and/or ligands. The corresponding binding sites are underlined and complementary receptors are indicated. $\alpha_n\beta_m$ pairs of letters indicate specific integrin heterodimers.

cassette D. A site interfering with the motility of oligodendrocyte precursors was located to the FNIII module pair TNfn78, whereas the antiadhesive qualities could be mapped to other domains (**Figure 2**). Several Tnc receptors have been described, including the Ig superfamily member contactin, which mediates Tnc-dependent stimulation of neurite outgrowth, and the integrins $\alpha_v\beta_3$, $\alpha_7\beta_1$, $\alpha_8\beta_1$, and $\alpha_9\beta_1$. In the ECM, Tnc interacts with the proteoglycans phosphacan and neurocan. A number of studies on the Tnc −/− mutants suggest altered responses to stress, behavioral modifications, and deficits in the stem cell compartments of several organs, including the brain, during development, in the adult, and in response to lesion. These observations and the strong association of Tnc with human pathology in CNS cancer, hippocampal sclerosis, and various types of lesion warrant further studies of this versatile multifunctional glycoprotein. The antiadhesive glycoproteins of the CNS also comprise the thrombospondins, which have been found to mediate an astrocyte-derived signal for synaptic maturation.

Proteoglycans of the Extracellular Matrix

Key features of proteoglycans and heparan sulfate proteoglycans Proteoglycans represent the second class of ECM components expressed in the CNS. These components are defined as glycoproteins that carry at least one covalently linked glycosaminoglycan chain. One can distinguish HSPGs, which are mainly membrane bound, from CSPGs and keratan sulfate proteoglycans (KSPGs) of the nervous system, which are preferentially recovered from saline detergent-free extracts.

Two important HSPG subfamilies are the membrane-based syndecans and the GPI-linked glypicans. Central roles of the HSPGs may reside in the support of signaling processes in the context of tissue development. Thus, bFGF binds to a specific motif in heparan sulfate carbohydrate chains that expose the factor to its (the FGF receptor is a membrane bound tyrosine kinase) receptor. Similar mechanisms have been proposed for wnt signaling that plays important roles in axon growth and synapse formation.

CSPGs and KSPGs of the central nervous system With regard to CSPGs, the members of the lectican family – brevican, neurocan, versican, and aggrecan – have been identified in the CNS. These CSPGs possess a binding site for hyaluronic acid, a lectin-type sequence, and further distinct structural motifs. Versican is expressed by mature oligodendrocytes, whereas neurocan and aggrecan have been detected in neurons. The core glycoproteins may carry the HNK-1 epitope, a carbohydrate structure also expressed by neural recognition molecules, or other N-linked carbohydrates, for example, of the Lewis X-type, which are

recognized by specific monoclonal antibodies. Further CSPGs that have been described in the CNS with the help of specific monoclonal antibodies include CAT 301 and NG2, a marker of oligodendrocytes. CAT 301 upregulation during early embryonic development of the spinal cord depends on activation of the NMDA receptor, suggesting a role in plasticity. The possible roles of CSPGs in synaptic reorganization have been highlighted by the finding that injection of chondroitinase ABC, which degrades the particular glycosaminoglycans of CSPGs, restores plasticity of the adult visual cortex.

Inhibition of axon outgrowth by CSPGs On the functional level, many studies support the view that CSPGs inhibit axon outgrowth in an otherwise supportive environment. Thus, DRG axons extend profusely on a laminin-1-coated substrate but strictly avoid territories that have been replenished with CSPG preparations. Comparable findings have been obtained with defined CSPGs such as neurocan and versican and a variety of cell types, including neural crest, which led to the conclusion that CSPGs play a central role in axon growth inhibition and guidance. These observations motivated the analysis of CSPGs in the context of regeneration inhibition. Numerous studies concur that CSPGs are strongly upregulated by reactive astrocytes in a broad spectrum of lesion paradigms. Therefore, these components are considered an important inhibitory compartment that plays a pivotal role in the lack of regeneration of the CNS. This interpretation has gained support by the observation that injection of chondroitinase ABC into the lesioned spinal cord enhances plasticity and reactive sprouting and hence entails some improvement of the afferent sensory function. Several possibilities are conceivable to explain the mechanistic aspects of the inhibitory properties of CSPGs, and it is plausible that these components bind growth cone collapse, inducing molecules such as semaphorins to particular structural sequences in the glycosaminoglycan chains. On the other hand, CSPGs have been found associated with axon growth and regeneration in the peripheral nerve, which emphasizes that overall matrix composition and the lineage and age of the neurons involved need to be considered.

Phosphacan and Receptor Tyrosine Phosphatases of the Central Nervous System

The CSPG phosphacan/DSD-1-PG DSD-1-PG/phosphacan is one of the more abundant soluble CSPGs in postnatal mouse brain and is homologous to the secreted proteoglycan phosphacan from rat tissues. The GAGs of phosphacan are composed of chondroitin sulfate (CS)-A and CS-C motifs, a keratan sulfate chain, and the DSD-1-epitope. This unique structure was discovered with the MAb 473HD, requires the sulfation of the carbohydrate backbone, and contains CS-D dimers and dermatansulfate. The DSD-1-epitope displays neurite outgrowth promoting properties, which possibly involves its capacity to bind pleitrophin. Phosphacan is a splice variant and corresponds to the complete extracellular region of the largest isoform of the transmembrane receptor protein tyrosine phosphatase-β (RPTP-ζ/β). RPTP-ζ/β proteins occur as large or short receptors which possess a transmembrane domain and two cytoplasmic tyrosine phosphatase modules. The additional phosphacan short isoform (PSI) that corresponds to the N-terminal sequence has been described in the mouse, and several isoform variants have been found in *Xenopus*.

Receptor protein tyrosine phosphatases in neuron–glia interactions The different isoforms of RPTP-ζ/β are developmentally regulated, and astrocytes from various parts of the CNS express the short RPTP-ζ/β receptor. RPTP-ζ/β_{long} is expressed in the ventricular zone of the developing and the subventricular zone of the adult CNS and also by oligodendrocyte precursors. Neuronal expression has also been observed which may partially be due to PSI that is strongly expressed by cortical neurons. The spatiotemporal expression patterns of RPTP-ζ/β isoforms during development, maintenance, and pathology of the CNS have been correlated with cell–cell signaling, cellular proliferation, migration, differentiation, axon outgrowth, synaptogenesis, synaptic function, and tissue regeneration. Based on the prominent glial expression of phosphacan and RPTP-ζ/β receptors, the proteins have been considered as possible mediators of neuron–glia interactions. In the adult CNS, phosphacan occurs in the perineuronal nets of parvalbumin-expressing neurons, surrounding axon terminals and glial endfeet but not the synaptic clefts. It has been hypothesized that CSPGs associate with hyaluronic acid in these structures to build a neural ECM comparable to that in connective tissue. CSPGs in the perineuronal ECM may constitute an important element in limiting synaptic plasticity. On the functional plane, phosphacan interacts with the Ig superfamily members contactin/F3/F11, axonin-1/TAG-1, NrCAM, and NgCAM and hence might intervene in both homophilic and heterophilic interactions. Therefore, it is plausible to assume that IgSF constituents represent neuronal ligands of RPTP-ζ/β receptors expressed in glial membranes and serve as molecular mediators at the interface between these two cellular lineages. Interestingly, both RPTP-ζ/β

and the IgCAMs are linked to signal transduction pathways and hence will act back on the expressing cells in the context of reciprocal signaling mechanisms. In particular, the phosphotyrosine–phosphatase modules of RPTP-ζ/β may antagonize tyrosine kinase-based activities. ECM ligands of phosphacan include tenascin-C and tenascin-R. The integration into ECM superstructures might explain to some extent why the elimination of the phosphacan gene does not yield serious developmental deficits in mice.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axonal Pathfinding: Extracellular Matrix Role; Axonal Regeneration: Role of Growth and Guidance Cues.

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Corticospinal Development

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Introduction

The corticospinal (CS) system is the principal motor system for controlling skilled movements. In humans, this system is so important that damage to it during development or later in life invariably produces significant motor impairment. The primary motor cortex and adjoining premotor and somatic sensory cortices are the principal origins of the CS system. Neurons in these cortical areas project to the spinal cord through the CS tract. This is the last motor pathway to develop, growing into the spinal cord during the late prenatal and early postnatal periods. Even with this early start, development of the CS system is protracted, in humans especially, in whom it takes 10–15 years to gain some of its mature characteristics. It is remarkable that most CS system development occurs while the individual is acquiring motor skills during early postnatal life.

Like other neural systems, development of the CS system depends on a complex interplay between factors intrinsic to the developing central nervous system (CNS) – including the CS neurons themselves, the regions through which CS axons grow to reach their spinal gray matter targets, and the spinal gray matter – and on neural activity and behavioral experience. This article first considers milestones of CS system development in several species, including humans, and then examines, in a bottom-up sequence, the critical steps in development of the CS system's role in skilled movement control.

Phases and Time Course of CS System Development

While there are a bewildering number of steps leading to development of this motor system, four major steps can be identified, and each is governed by different mechanisms. First, precursor cells differentiate into CS neurons; this occurs prenatally. Second, CS axons grow through the brain and into the spinal cord to form the CS tract. Once CS axons are in the cord, there is outgrowth from the tract into the gray matter. This is the pathfinding stage and leads to the initial selection of postsynaptic targets. Pathfinding occurs primarily prenatally in humans, monkeys, and cats but primarily postnatally in rodents. Both the commitment to become a CS neuron and axon outgrowth and guidance to postsynaptic target neurons are determined by cell-specific and regional-intrinsic factors. Through genetic approaches, many key molecules have been identified recently. The third step is to establish connectional specificity at the circuit level, which involves refinement of the gray matter terminations. This typically includes both elimination of axon branches and growth of new branches to nearby targets. For spinal circuits, physiological studies in humans indicate that this happens during the first 2 years, while anatomical studies in animals show that refinement occurs within the first postnatal weeks and months, depending on the species. The rapid phase of myelination of CS tract axons occurs during this period. Fourth, the cortical motor representation forms, and the CS system's role in limb control is expressed. During this period, many other neural systems are developing and contributing to movement control. An individual's motor experience and other activity-dependent processes during the third and fourth stages have important long-term developmental functions.

CS Neuron Differentiation

CS neurons are layer-5 pyramidal neurons in a variety of cortical motor and somatic sensory areas. Early in development, CS neurons are distributed from the frontal to the occipital poles but later are restricted primarily to the posterior frontal and anterior parietal cortices. The reduction in the number of CS neurons is principally due to the elimination of spinal axon branches, not widespread cell death. The homeodomain transcription factor Otx1 is required for axon branch elimination. Mice in which the gene for Otx1 has been deleted fail to develop the limited distribution of CS neurons restricted to the somatic sensory and motor cortices.

Significant progress has been made recently in determining factors important for differentiation of CS neurons from precursor cells. It has been proposed that the combinatorial expression of many genes delineates the population of CS neurons. Experiments show that some of these genes are needed for development of the CS tract. For example, in mice without the forebrain embryonic zinc fingerlike transcription factor (Fezl), cortical neurons fail to grow an axon into the caudal brain stem and spinal cord (**Figure 1**). Moreover, the loss of Fezl results in the loss of other molecular markers of layer 5 and 6 neurons, including another transcription factor required for CS tract formation, Ctip2. This suggests that Fezl acts upstream to regulate neuronal differentiation.

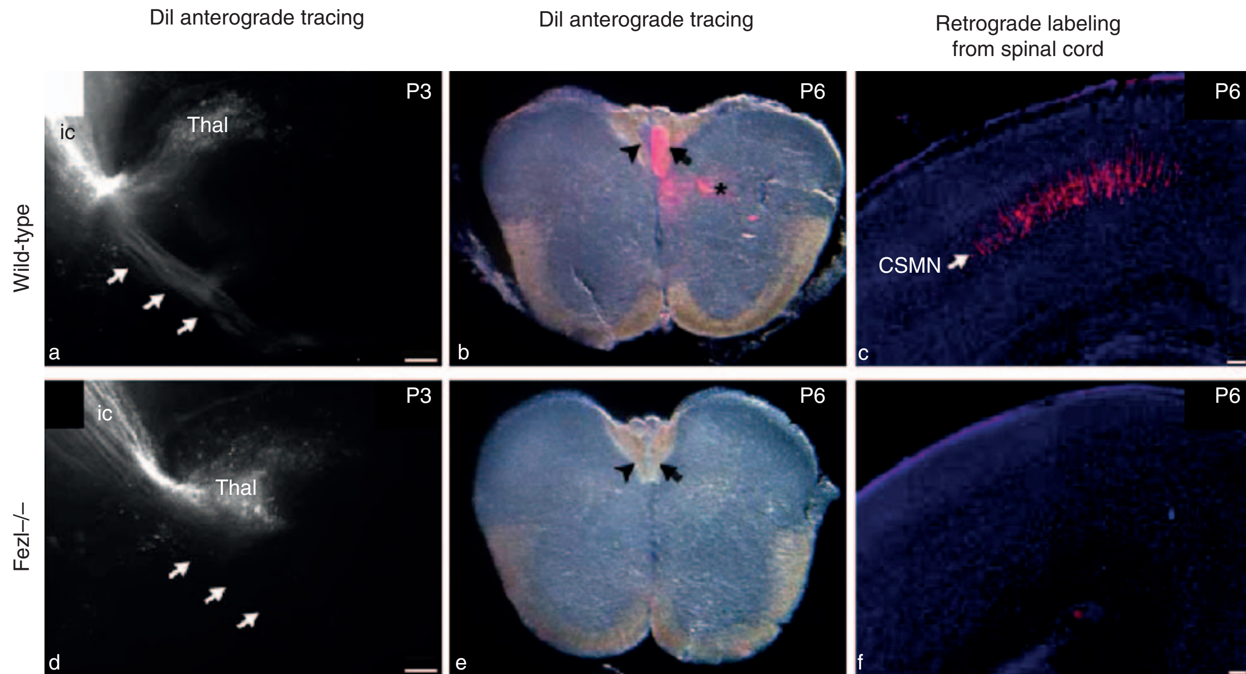

Figure 1 Forebrain embryonic zinc fingerlike transcription factor (Fezl) is important for specification of CS neurons. Images along the top row are from a wild-type mouse and along the bottom row, from a Fezl −/− mouse. (a, d) Anterograde labeling (bright white) of cortical efferent axons. Note that the wild-type shows projections to the brain stem and spinal cord (arrows), but the knockout mouse does not; both show projections to the thalamus (thal). (b, e) Anterograde labeling (red) of CS axons in the spinal cord of the wild-type but not the knockout mouse. (c, f) Retrograde labeling (red) of CS neurons in the wild-type mouse only. Scale bars = 100 μm. DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyamine perchlorate; Ic, internal capsule; CSMN, corticospinal motor neuron; P3, postnatal day 3; P6, postnatal day 6. From Molyneaux BJ, Arlotta P, Hirata T, Hibi M, and Macklis JD (2005) Fezl is required for the birth and specification of corticospinal motor neurons. *Neuron* 47: 817–831.

Development of the Projection to the Brain Stem and Spinal Cord

Pathfinding from Cortex and Subcortical White Matter Tracts

Traversing the CNS to reach its targets is a complex problem for any projection neuron, but particularly for a CS neuron because its axon has the longest distance to travel. Pathfinding is organized by complex tissue molecular cues that are detected by the primary growth cone of the axon. Studies suggest that cortical pyramidal cells simplify the task of reaching their final targets, for example, spinal cord neurons, by achieving a sequence of intermediate targets, such as first projecting into the internal capsule. Many chemorepellant and chemoattractant molecules have been identified as necessary for keeping CS neuron axons ‘on tract,’ else the axons stray into aberrant locations.

Decussation and Midline Crossing

Functionally, the CS system exerts its effects on the contralateral spinal cord and contralateral muscle through a descending projection that is primarily crossed. Most axons decussate in the medullary pyramid. In the rodent, the axons course from the ventral to dorsal surfaces as they cross from one side to the other, forming an X shape (**Figures 2**(**a**) and **2**(**b**)). Three sets of molecules and receptor families have been identified that play key roles in the crossing of CS axons: (1) the L1 cell adhesion molecule (L1-CAM), which is a member of the immunoglobulin superfamily of cell adhesion molecules; (2) the Eph A4 receptor and Ephrin B3, which is a protein that repels axon growth and is the membrane bound ligand for EphA4; and (3) members of the roundabout (Robo) family of transmembrane proteins and Slit, another protein with axon growth-repulsive properties.

L1-CAM is expressed along the CS tract; CD24, which is a ligand for L1-CAM, is localized at the pyramidal decussation. L1 knockout mice display profound errors in CS axon decussation in the medulla and guidance into the appropriate spinal white matter regions (**Figure 2**(**b**)). Human L1 mutations produce hypoplasia of the CS tract and spasticity, as well as a variety of other brain structural and functional impairments. Patients with L1 mutations show elevations in transcranial magnetic stimulation (TMS)

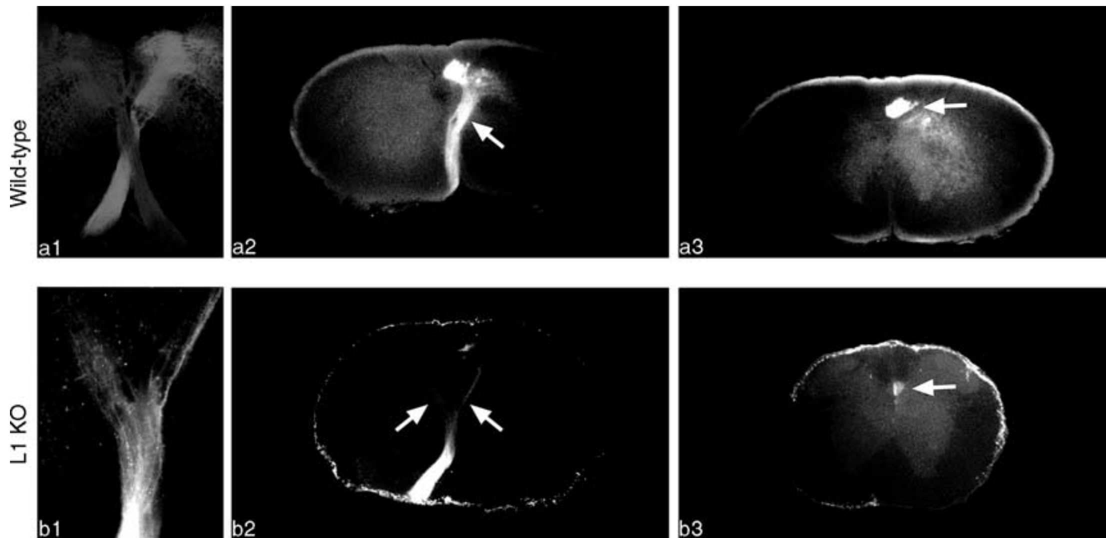

Figure 2 The pyramidal tract decussation and the role of L1-CAM in forming the contralateral descending CS projection. (a1) Image from a normal mouse showing the pyramidal decussation. Axons from the two sides are labeled red and green. (Image courtesy of Drs. Kyoko Itoh and Vance Lemmon.) (a2) Normal decussation in a wild-type mouse (arrow; similar to the red axons in (a1)). (a3) Descending axons in the CS tract (arrow). (b) The decussating axons from a L1-CAM mutant. (b1), (b2) Aberrant decussation from an L1-CAM mutant ((b1) is a high-magnification view). (b3) There are fewer contralateral descending axons (arrow) and no ipsilateral descending axons. Arrows in (a2) and (b2) mark decussating axons; arrows in (a3) and (b3) mark the presence of the dorsal corticospinal tract in the dorsal column. Magnification ×23 (a2, a3, b2, b3), ×44 (b1). From Itoh K, Cheng L, Kamei Y, et al. (2004) Brain development in mice lacking L1-L1 homophilic adhesion. *Journal of Cell Biology* 165: 145–154.

thresholds and increased latencies for evoking motor responses. These CS functional defects correlate with impairments in performance on several tests of hand/digit dexterity.

CS axons in EphA4 knockout mice decussate in the pyramid and descend into the appropriate white matter regions of the cord, but they terminate bilaterally in the spinal gray matter (**Figure 3**). This is because ephrin B3 is a midline growth barrier. Normally, it is present extensively along the spinal midline (**Figure 3**), thereby limiting recrossing. In the medulla, ephrin B3 is located only dorsal to the pyramidal decussation and at this level does not block CS axon crossing. Two Robo proteins, Robo1 and 2, are receptors for Slit. Robo is expressed on CS axons. CS axons may be prevented from aberrant recrossing through interactions between these Robo proteins on developing axons and Slit at the floor plate of the hindbrain and spinal cord. A third member of this family, Robo3, is functionally different from the other two members because it is required for decussation. In patients with a rare genetic disorder, horizontal gaze palsy with progressive scoliosis, mutation in the Robo3 gene prevents decussation of the CS tract. While most CS axons decussate in the medulla, approximately 10% do not and project to the ipsilateral cord. Moreover, once in the spinal cord, many CS axons – whether or not they decussate in the medulla – cross the midline before terminating on spinal neurons. The logic of the pattern of decussation of CS axons is not yet known.

Collateral Branching into the Gray Matter

Once CS axons reach the caudal brain stem, a small contingent pioneer the path into the spinal cord, followed later by waves of axons that further populate the CS tract. Long-distance growth of the primary descending CS axon is followed by formation of side, or collateral, branches that extend into the surrounding gray matter after a variable delay period. Spinal gray matter innervation is mediated by target-specific chemotropic factors that induce branching. In tissue explant experiments, for example, neurites from a portion of the prospective forelimb area of the sensorimotor cortex grow toward a cervical spinal explant but not to a lumbar explant.

Development of Connectional Specificity between CS Axon Terminals and SC Neurons

Topographic Refinement of CS Terminations in the Spinal Gray Matter

When CS axons initially grow into the spinal gray matter, their termination pattern is not what it is in maturity. The most common pattern is that CS axons have a widespread spinal termination pattern found early in development that is subsequently refined, or ‘pruned,’ to a more restricted distribution in maturity (**Figure 4(a)**). Terminations that are normally present

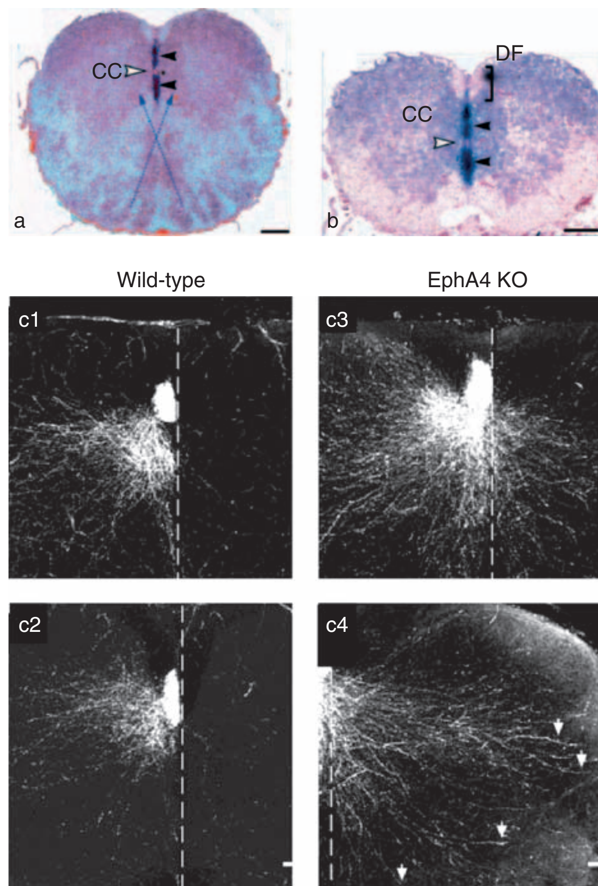

Figure 3 Ephrin B3 and its receptor EphA4 prevent recrossing of CS axons. (a, b) Ephrin B3 messenger RNA expression (dark blue staining; arrow heads) in the caudal medulla of a normal mouse, at the level of the pyramidal decussation ((a), dashed arrows) and in the spinal cord (b). (c1)–(c4) Region of the central canal. (c) Effects of EphA4 knockout on the development of the laterality of CS axon terminations in a 7-day-old mouse. In the normal mouse, terminations remain predominantly contralateral to the CS neurons of origin (c1) and (c2), whereas in the knockout mouse (c3) and (c4) there are abundant ipsilateral terminations. The arrows in (c4) point to aberrant terminations in the ventral and lateral gray matter. The distribution of terminal axons is also more extensive in the knockout mouse, suggesting a role for ephrin-Eph receptor interactions in determining regional distributions as well as laterality. Scale bars = 200 μm. (a, b) From Kullander K, Croll SD, Zimmer M, et al. (2001) Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. *Genes & Development* 15: 877–888. (c) From Coonan JR, Greferath U, Messenger J, et al. (2001) Development and reorganization of corticospinal projections in EphA4 deficient mice. *Journal of Comparative Neurology* 436: 248–262.

early in development and are eliminated later on are often termed 'transient terminations'. The rhesus monkey shows a pattern of progressive increase in CS axon terminal growth during early postnatal development rather than a broader distribution of terminations that is subsequently refined. **Figure 4(b)** shows that terminations within the central region of the gray matter become progressively denser (more red), especially within the lateral motor nucleus (yellow arrow). Irrespective of the particular pattern, the refined distribution of connections ultimately is achieved late in development: by 6–7 weeks in the cat, 8 months in the monkey, and after several years in the human. An important question for future research is why the CS system in some animals prunes back exuberant connections while others use a delayed outgrowth strategy.

CS terminations early in development are able to excite their spinal targets. This has been shown in the cat and rat by electrically stimulating CS axons in the CS tract and recording postsynaptic field potentials (i.e., focal synaptic potentials) in the spinal gray matter. In immature cats, for example, stimulation of the CS system evokes postsynaptic responses throughout the dorsoventral extent of the gray matter (**Figure 5(a)**). This broad distribution is similar to the one of CS terminations. By contrast, in mature animals, when CS axon terminals are restricted to the middle region of the cord, responses are largely limited to the same middle layers (**Figure 5(b)**). The refinement process restricts the effects of CS activation to particular spinal motor circuits. *In vitro* studies in rats show that synapse elimination is *N*-methyl-D-aspartate receptor-dependent. Although the anatomy and physiology of this process have been studied, the functional logic of the final termination patterns of CS axons has yet to be elucidated.

During postnatal development, local CS axon branch growth and presynaptic bouton formation are abundant (**Figure 6**; see also **Figure 4(b)** and increasing termination density). When axon branch elimination is present (**Figure 4(a)**), local growth occurs concurrently and complements the refinement process. Local axonal branch growth leads to a remarkable increase in the strength of CS evoked responses during early development (**Figure 5**; note increase from 30 to 700 μV range). As CS axon terminals mature, they also are better able to recruit spinal motor circuits into action (**Figure 7**). This has been tested in the cat by stimulating CS axons in the pyramid and recording motor responses in peripheral motor nerves. Immature CS connections require stronger electrical stimulation of CS axons, and a greater number of electrical stimuli, to evoke a motor response (**Figure 7(a)**) compared with more mature animals (**Figure 7(b)**). While comparable spinal recordings in the developing monkey have not been done, TMS has been shown to be effective in evoking motor responses only after about 3 months of age. This correlates with the progressive late growth of CS axon terminals and is consistent with the finding in the cat (**Figure 7**) that CS connections with spinal circuits become more effective in evoking motor responses later in development. In the monkey,

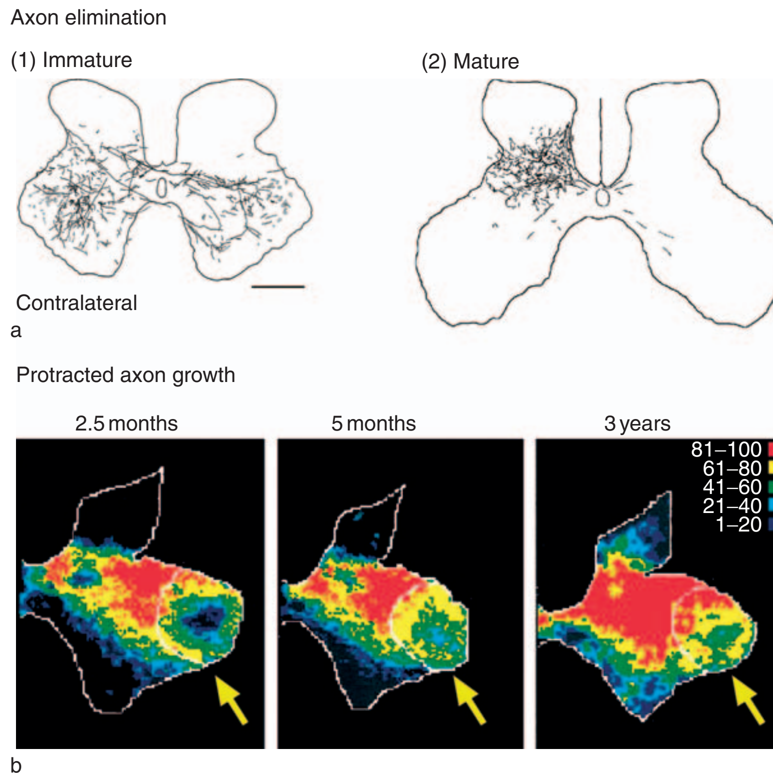

Figure 4 The distribution of CS axon terminations is refined postnatally. (a)(1) Extensive dorsoventral and bilateral CS terminations in a 5-week-old cat. (a)(2) An adult cat, where terminations are predominantly contralateral and restricted dorsoventrally. Scale bar: 1 mm. (b) Increase in CS axon terminal density in monkeys at three different ages. These sections were taken from the rostral portion of the first thoracic spinal cord segment. Color codes the density of terminations, on a scale from 1 to a maximum of 100. The arrow marks the region of the lateral motor nucleus, where motor neurons innervating distal arm muscles are located. Note that at 2.5 months, very little of the nucleus is densely labeled (red), and there are areas without any label, whereas at 3 years, the entire nucleus is labeled, and labeling in the outer rim is dense. (a1) From Li Q and Martin J (2000) Postnatal development of differential projections from the caudal and rostral motor cortex subregions. *Experimental Brain Research* 2000: 134–198. (b) From Armand J, Olivier E, Edgley SA, and Lemon RN (1997) Postnatal development of corticospinal projections from motor cortex to the cervical enlargement in the macaque monkey. *Journal of Neuroscience* 17: 251–266.

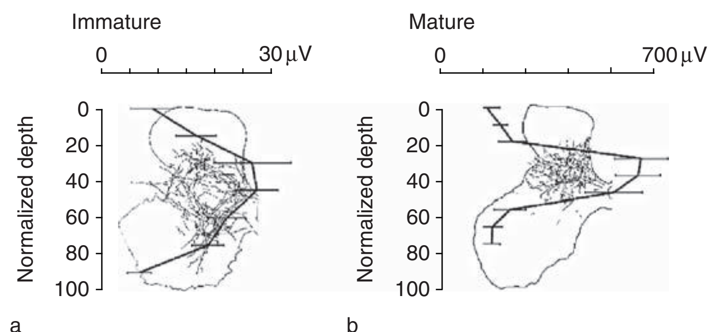

Figure 5 CS synaptic refinement. The amplitude of the monosynaptic CS evoked response is plotted at different depths (normalized; dorsal horn surface is zero and the ventral horn ventral surface, 100). Each graph overlies a schematic view of the distribution of terminations at that age. (a) The immature cat (5-week-old) shows small postsynaptic responses from the dorsal to the ventral surface. (b) By contrast, responses in the mature cat are restricted to the middle region, where the densest terminations are located. From Meng Z and Martin JH (2003) Postnatal development of corticospinal synaptic actions. *Journal of Neurophysiology* 90: 683–692.

TMS studies have also shown that there is an exponential increase in CV and decrease in TMS threshold within the first 1.5 years and a slower increase in CV and decrease in threshold later in development. Stronger connections between the motor cortex and spinal cord motor circuits are critical for the CS system's control over motor behavior.

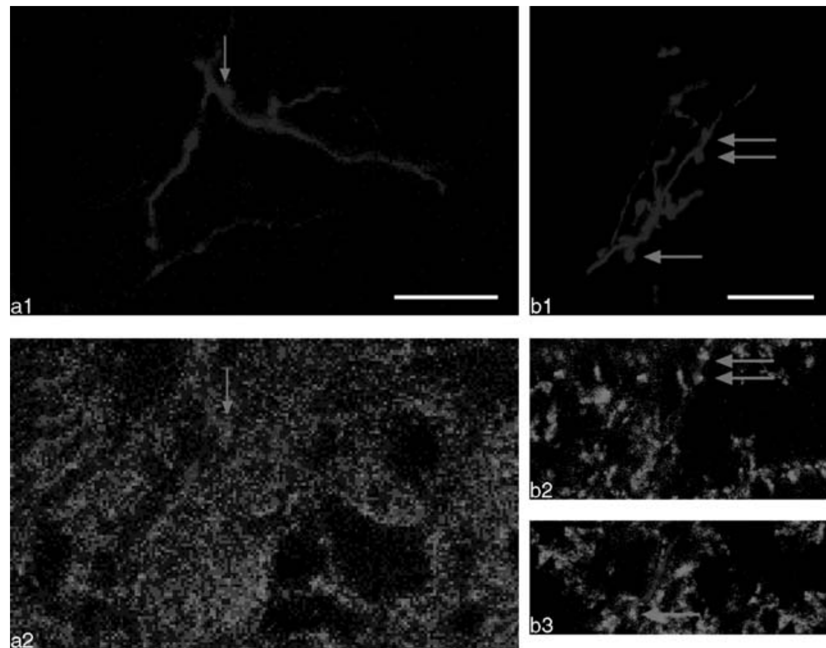

Figure 6 Confocal images from (a) an immature cat (4-week-old) and (b) a mature cat ((a1), (a2), (b2), and (b3): 1 μm optical slices; (b1): 7 μm projection image). The top two images show CS axons labeled with the anterograde tracer BDA (green). The lower row of images shows staining for the synaptic vesicle protein synaptophysin (red), together with axon staining (green). Double labeled boutons are yellow and highlighted with the yellow arrow. (a1) CS axon terminal with sparse branches. (b1) Highly branched mature axon terminal. Scale bar = 25 μm (a1, a2), 50 μm (b1–b3). From Meng Z, Li Q, and Martin JH (2004) The transition from development to motor control function in the corticospinal system. *Journal of Neuroscience* 24: 605–614.

Electrophysiological studies in humans using TMS also point to an early period of transient ipsilateral and ventral CS terminations, as in the cat. TMS up to about 1 year evokes bilateral motor responses (**Figure 8(a)**), consistent with bilateral terminations. The amplitude of the ipsilateral response becomes reduced relative to the contralateral response during the first year (**Figure 8(a)**). This is similar to the early postnatal elimination of ipsilateral CS branches in the cat. The TMS threshold for evoking muscle contraction is lower in preterm and very young infants, only to become higher during the first 3 postnatal months. This could be due to the elimination of some ventral CS axon terminal branches, thereby reducing synaptic effects. Between about 1 and 15 years, TMS threshold systematically decreases (**Figure 8(b)**). This threshold reduction is due to several factors, including increased CS axon terminal branching in the spinal cord, stronger connections in the spinal gray matter, and increased myelination of CS axons, which results in more-synchronous activation of spinal motor circuits.

Activity-Dependent Refinement of CS Axon Terminal Connections

The basis for the CS system's motor control functions depends on a refined pattern of connectivity between zones in the motor cortex that integrate motor control signals from various sources and spinal motor circuits. What drives postnatal refinement of CS axon terminations in the spinal gray matter? Recent studies show an important role for the level and pattern of CS neuronal activity in shaping development of the regional distribution of their spinal terminations, consistent with activity-dependent synaptic competition. Activity of the CS system can be selectively reduced by intracortical infusion of the γ-aminobutyric acid agonist muscimol and augmented by electrical stimulation of CS axons. Unilateral activity reduction results in an aberrant pattern of contralateral spinal terminations. The axons populate more dorsal laminae (**Figure 9(b)**; yellow). This defect reflects a failure of silenced axons to maintain connections established earlier during development as well as a failure to add new branches and presynaptic sites (**Figures 9(c)** and **9(d)**). Animals with this aberrant pattern of CS terminations have significant arm control impairments (see section on motor skill development).

The contralateral active CS system not only develops the normal contralateral projection but also maintains significant ipsilateral terminations. Thus, the reduction in termination space of the silenced side is balanced on that side by maintenance of ipsilateral

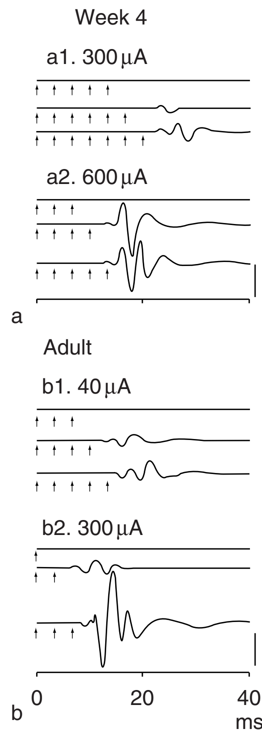

Figure 7 Ability of immature cat CS tract to recruit threshold motor responses is much less than in the adult. Each trace is an average recording from the median nerve contralateral to pyramidal tract electrical stimulation. The arrows indicate the time of occurrence of pyramidal stimuli. Note that more pulses and higher currents are needed to evoke responses in the 4-week-old animal than in the adult cat. Calibrations: (a) 500 μV; (b) 300 μV. From Meng Z, Li Q, and Martin JH (2004) The transition from development to motor control function in the corticospinal system. *Journal of Neuroscience* 24: 605–614.

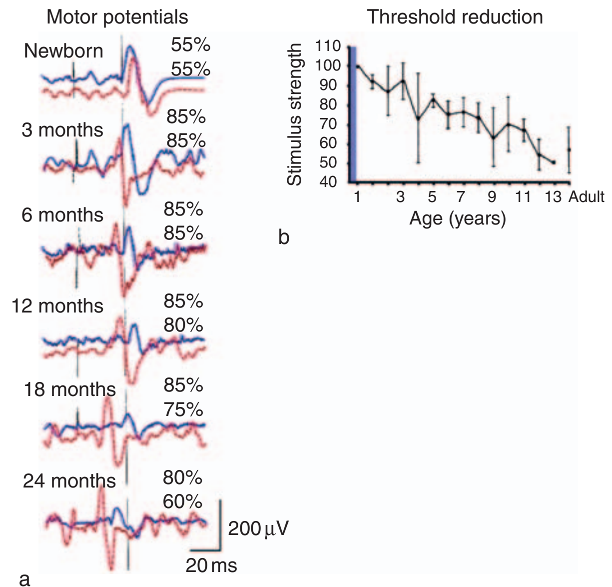

Figure 8 Transcranial magnetic stimulation (TMS) is used to activate the human CS system noninvasively. (a) Contralateral (red) and ipsilateral (blue) motor responses evoked by TMS over the motor cortex; 100% is maximal stimulus current. The amplitude of the ipsilateral response (blue) decreases with age. (b) The current for TMS to evoke a threshold motor response decreases from about the first to the 14th year. The *y*-scale of the graph plots current, as mean maximal current ±SD. Prior to 1 year of age, responses (blue) can be evoked at a lower threshold than at 1 year. (a) Modified from Eyre JA, Taylor JP, Villagra F, Smith M, and Miller S (2001) Evidence of activity-dependent withdrawal of corticospinal projections during human development. *Neurology* 57: 1543–1554. (b) From Nezu A, Kimura S, Uehara S, Kobayashi T, Tanaka M, and Saito K (1997) Magnetic stimulation of motor cortex in children: Maturity of corticospinal pathway and problem of clinical application. *Brain & Development* 19: 176–180.

terminations of the active system, which are the terminations that are normally eliminated. These topographic changes persist into maturity. The presence of ipsilateral terminations of the active side developing in parallel with the silenced CS terminations implies competition between the two sides during early development. Consistent with this idea is the observation that bilateral inactivation of the motor cortices produces a relatively normal pattern. This suggests that the changes in the laterality of CS terminals occurring after activity blockade are due to activity-dependent competition between developing CS terminals. The overall density of terminations after bilateral inactivation, however, is less than in controls, suggesting that the silenced CS terminations are less effective in competing with other spinal neural systems in securing terminations.

Another way to examine the role of activity of the two sides in shaping the mature pattern of terminations is to augment activity unilaterally. Electrical stimulation of CS axons in the medullary pyramid for several weeks during early postnatal life results in maintenance of substantial ipsilateral (and contralateral) terminations at 8 weeks (**Figure 10(a)**). Normally, there are predominantly contralateral terminations at this age. Projections from the nonstimulated side were displaced dorsally and laterally as a consequence of this CS system stimulation (**Figure 10(c)**). Both the maintenance of ipsilateral terminations and the displacement of nonstimulated axons are consistent with the activity-dependent competition model: The stimulated axons are more competitive at securing spinal synaptic space at the expense of the nonstimulated axons.

In hemiplegic cerebral palsy, the pattern of functional CS connectivity tested with TMS is similar to the anatomical distribution of CS axons in cats after unilateral CS system inactivation. TMS of the less impaired side evokes bilateral responses (**Figure 11**). TMS of the impaired side fails to evoke significant responses (not shown in figure). The aberrant ipsilateral effects are observed only after CS damage early in development, possibly before ipsilateral CS terminations are eliminated; in adults, stroke that produces hemiparesis does

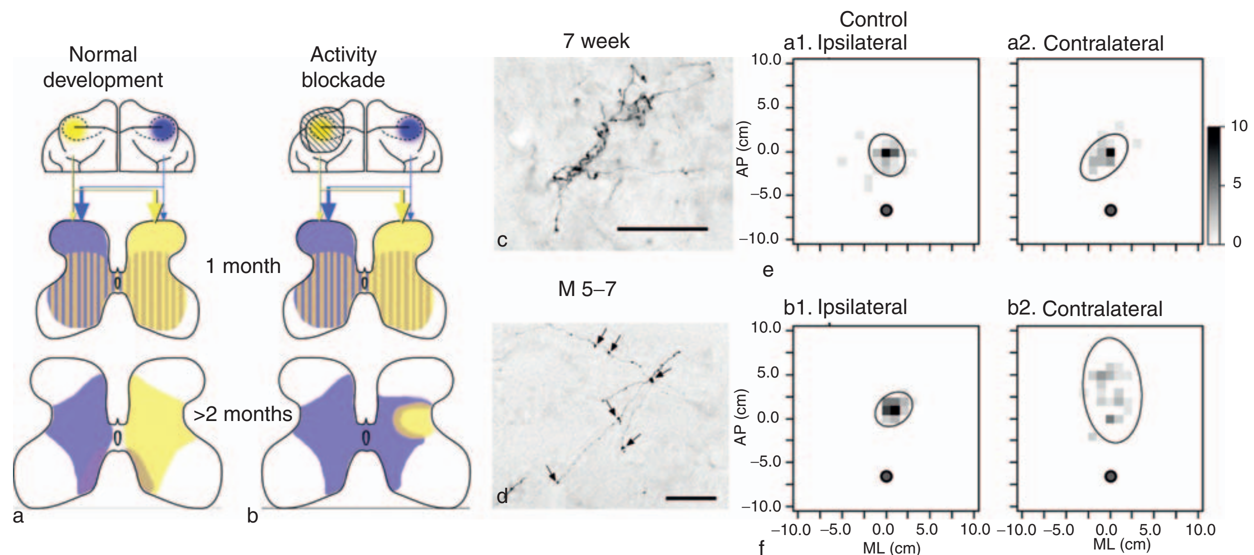

Figure 9 (a) Normally there are overlapping CS terminations in the spinal cord at 1 month in the cat. These terminations are refined into predominantly contralateral terminations after 2 months. (b) After motor cortex inactivation between weeks 5 and 7, the silenced axons (yellow) failed to populate as much of the contralateral gray matter. In contrast, the active side (blue) has a bilateral termination pattern. The ipsilateral axons are largely maintained transient terminations. (c) Morphology of a normal CS axon terminal at 7 weeks, and (d) morphology after inactivation. Arrows in (d) point to presynaptic boutons. Presynaptic boutons were too many to label in (c). Scale bars: 100 μm. (e, f) Results of behavioral experiments in which animals were trained to reach and grasp a food reward located at the origin (0,0). The start of the reach is indicated by the filled circle. (e) Data from an animal that received unilateral saline infusion into the primary motor cortex. (f) Animal that received unilateral muscimol infusion to block activity. Each square indicates the location of the end point of a reach. The more end points at that spot, the darker the square. The ellipses enclose the end points of 95% of the reaches. The area and the location of the ellipses are measures of accuracy. Reaching accuracy for the control animal is normal for both limbs. By contrast, accuracy for the contralateral limb in the animal in which the motor cortex was inactivated was significantly worse than for the ipsilateral side, which was normal. The end points for the contralateral side were both overshot (i.e., farther from the origin) and more dispersed. AP, anteroposterior; M5–7, muscimol inactivation between postnatal weeks 5–7; ML, mediolateral. (a–d) From Friel K and Martin JH (2005) Role of sensory–motor cortex activity in postnatal development of corticospinal axon terminals in the cat. *Journal of Comparative Neurology* 485: 43–56. (e, f) From Martin JH, Hacking A, and Donarummo L (2000) Impairments in prehension produced by early postnatal sensorimotor cortex activity blockade. *Journal of Neurophysiology* 83: 895–906.

not augment the ipsilateral response. These findings are consistent with the hypothesis that during early development, the impaired side is rendered much less competitive in securing and maintaining spinal synaptic space than the normal or less impaired side is.

Use-Dependent Development of the CS Tract

Motor experience comprises both directed control of muscle by motor centers in the brain and spinal cord and sensory information received by the nervous system as a consequence of movement. To determine the role of motor experience on CS development, limb use can be prevented or curtailed specifically during key periods when the CS system is developing. Preventing limb use during early development has a profound effect on CS axon terminal development. One way to prevent limb use during development is to block contraction of selected forelimb muscles using botulinum toxin A. Like motor cortex activity blockade, preventing limb use prevents the growth of CS axon terminals and presynaptic sites (**Figures 12(a)** and **12(b)**). When limb use is regained, axon branching and presynaptic site density remain significantly reduced compared with controls, which indicates that CS axon terminals do not recoup lost connections (**Figures 12(c)** and **12(d)**); moreover, the regional distribution of CS axon terminations remains aberrant (**Figures 12(e)** and **12(f)**). These findings are consistent with the synaptic competition model, similar to what is observed after CS neural activity blockade. However, there is an important distinction: Preventing motor experience changes the patterns of neural activity in the developing motor and somatic sensory system but does not eliminate activity, as in the blockade experiments. This suggests that the developing CS system uses forms of temporally specific activity-dependent plasticity, such as long-term potentiation and long-term depression, to shape connections and function.

Development of CS Control of Skilled Motor Behavior

What is the relationship between development of the CS system and development of skilled limb control?

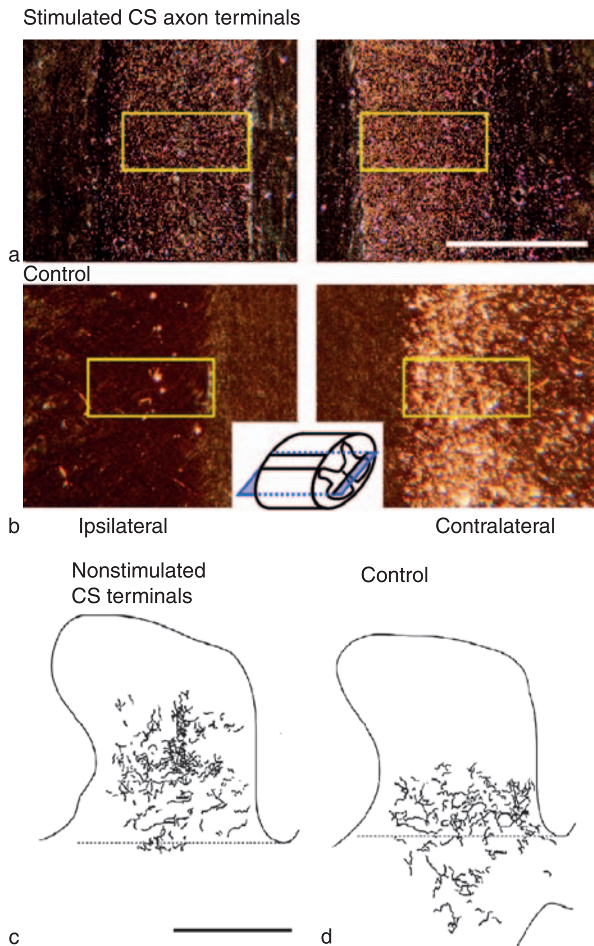

Figure 10 (a) CS tract electrical stimulation during early development promotes maintenance of ipsilateral terminations (left). Axons were labeled with wheat germ agglutinin conjugated with horseradish peroxidase, which appears as bright bronze particles. Inset shows plane of section in (a) and (b). Distribution of CS axon terminals from (c) the nonstimulated side (i.e., ipsilateral to the side of stimulation) and (d) a control stimulation (reticular formation). The intra-axonal distribution of tracer used in parts (c) and (d) is shown as the solid black lines. The dotted lines mark the ventral boundary of lamina 6. Note that stimulation displaced developing CS terminals dorsally in (c). Scale bar = 1 mm (a–d). From Salimi I and Martin JH (2004) Rescuing transient corticospinal terminations and promoting growth with corticospinal stimulation in kittens. *Journal of Neuroscience* 24: 4952–4961.

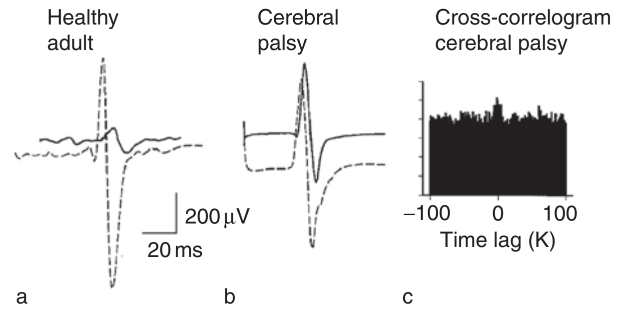

Figure 11 Ipsilateral (solid line) and contralateral (dotted) motor responses (flexor digitorum indicis) evoked by TMS in (a) a control adult and in (b) a patient with spastic hemiplegia acquired as a consequence of a stroke during infancy (i.e., cerebral palsy). Note that there is an abnormally large-amplitude ipsilateral response in the patient. (c) Correlogram for simultaneous electromyographic recordings from the two sides. The *y*-scale of the graph plots probably of occurrence $\times 10^{-2}$, from 0 to 15, and the *x*-scale plots time lag in milliseconds (K). The peak at zero indicates likely activation of electromyogram on both sides by a common signal. This suggests bilateral CS axon terminations. From Eyre JA, Taylor JP, Villagra F, Smith M, and Miller S (2001) Evidence of activity-dependent withdrawal of corticospinal projections during human development. *Neurology* 57: 1543–1554.

In many species there is clear evidence of co-development of skills, the spinal terminations of the CS tract, and the cortical motor representation. Monkeys begin to develop relatively independent finger movements during the period when CS axon terminals become dense within the intermediate zone and when CS terminals establish synapses within the motor nuclei. Humans develop relatively independent finger movements after about 1–2 years, as the thresholds for evoking motor responses begin to decline. However, as the CS system matures, other motor systems develop, as do sensory and cognitive systems. All these changes are likely to contribute to the expanding motor repertoire. Thus, because many CNS systems are developing during these early periods, establishing causal links between CS system developmental milestones and development of skilled control is difficult. Another way to obtain insight into how the CS system contributes to developing motor skills is to perturb normal CS tract development by changing CS activity levels or motor experience and determine the effect of these manipulations on skilled limb control.

Early postnatal blockade of motor cortex activity, which alters the termination pattern of CS tract axons, has a profound effect on visually guided skilled limb control. After the blockade is removed, there is a permanent aiming error in reaching, in which the end point of the movement overshoots the target (**Figure 9**(e)). During visually guided locomotion, there is also a movement end point defect, in which the foot is placed too far forward on the substrate. The common hypermetric defect (i.e., overreach and overstep) suggests a common underlying mechanism, such as a deficit in recruiting a muscle synergy for stabilizing limb position at the end of the movement. There also is a grasping impairment. After animals' motor experience is prevented and they are then allowed to regain limb use, their aim of reaching movements is normal, but their digit coordination during grasping is impaired (**Figure 12**(g)), similar to the grasping defect after postnatal inactivation.

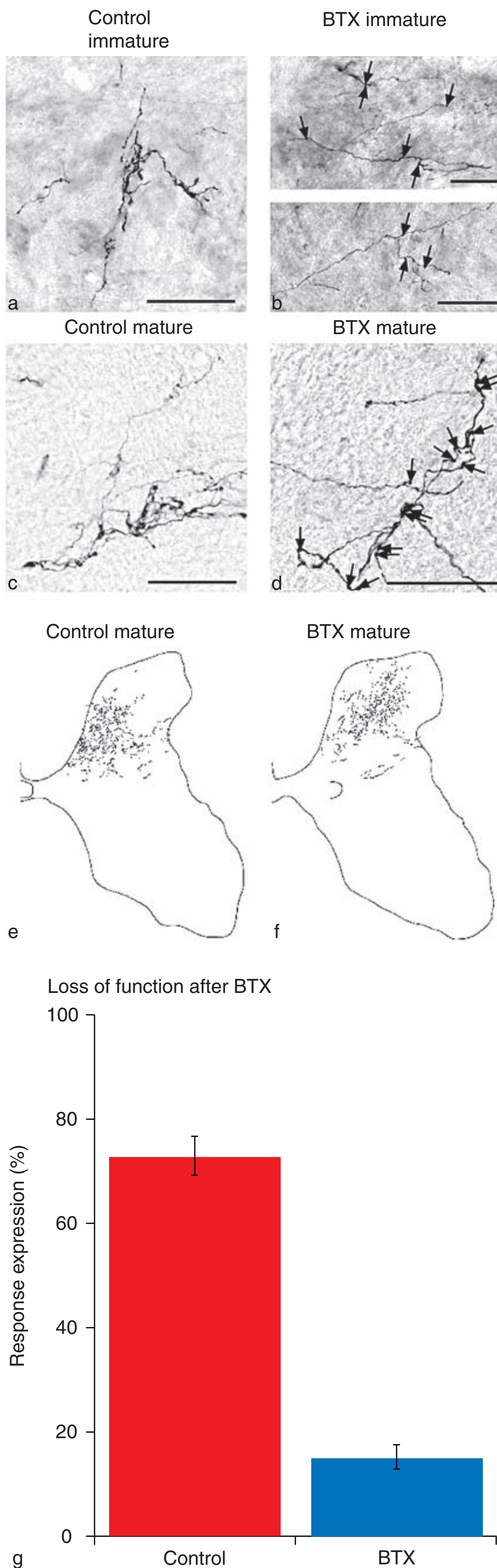

Figure 12 Effects of preventing limb use in a cat between weeks 3 and 7. Morphological changes after preventing limb use persist into maturity. (a, c) Control micrographs of normal corticospinal axon terminals at 8 weeks and in maturity, respectively. (b) Micrograph of terminal at 2 months, which is immediately after the period of disuse. (d) Micrograph of terminal in a mature animal in which BTX was injected into forelimb muscles between weeks 4 and 7. Arrows in (b) and (d) mark axon varicosites (boutons). Calibrations in (a)–(d): 100 μm. (e) Regional distribution of contralateral CS terminals in a mature control and (f) in a mature animal in which BTX was injected into forelimb muscles between weeks 4 and 7. Calibration for (e) and (f) corresponds to the length of the bar in (c), but representing 500 μm. (g) Once the effects of BTX wear off, there is a permanent coordination impairment. The occurrence of coordinated digit flexion and forearm supination is plotted (mean ± SD) for the control side (red) and the side in which BTX was injected (blue). BTX, botulinum toxin, From Martin JH, Choy M, Pullman S, and Meng Z (2004) Corticospinal development depends on experience. *Journal of Neuroscience* 24: 2122–2132.

Development of the Cortical Motor Map

The CS tract carries the output from the primary motor cortex, as well as adjoining regions. In maturity, several characteristics of the motor representation in the primary motor cortex are thought to be critical for the normal expression of skilled movements, including a requisite size of the representation and the need for particular joints to be represented. In animals, the motor map can be assessed using microstimulation, whereby electrical stimulation through a microelectrode is used to excite a small population of cortical neurons. In development, the motor representation is first detected at 2 months in the cat; prior to this, motor cortex microstimulation does not evoke motor responses. The absence of a motor map before week 8 could be due to several factors, including weak intracortical synapses and weak CS synapses on spinal motor circuits (see **Figure 7**).

During the following month, there is an increase in the percentage of sites from which microstimulation evokes a motor response and a concomitant decrease in the current threshold (**Figure 13**). The threshold reduction suggests more-efficient activation of spinal motor control circuits, reflecting synaptic strengthening at both the cortical and the spinal levels (see **Figure 6**). The joints and muscles represented increases, from only proximal forelimb movements and muscles initially to both proximal and distal. In addition, as animals grow older, effects at multiple joints are produced at a higher percentage of sites. These multijoint sites are thought to play a role in encoding interjoint motor synergies. The role of experience and CS system activity in shaping motor map development is being studied in the cat: Training reduces the threshold for evoking responses and

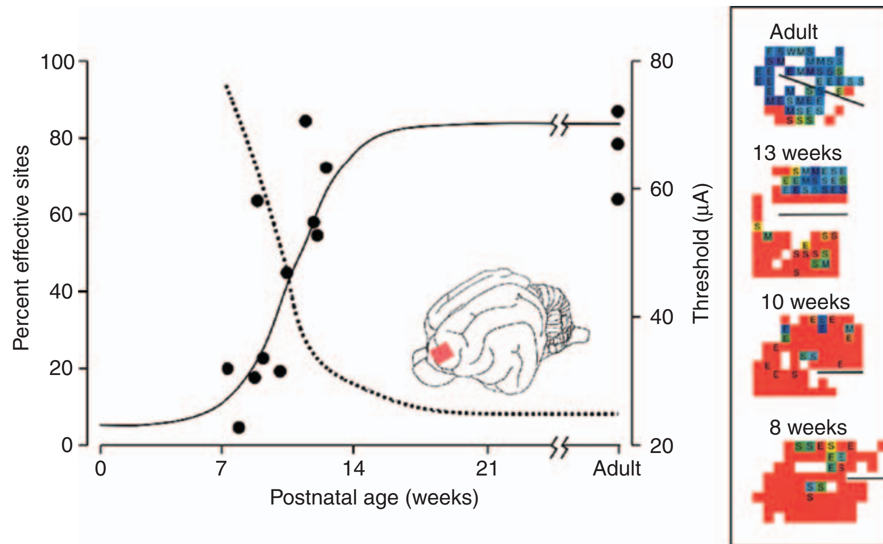

Figure 13 Development of the cortical motor map. The graph plots the percentage of sites from which microstimulation evoked a motor response at the various ages tested (solid line and dots; left scale) and the mean threshold for evoking the responses (dashed line; right scale). Each dot shows percentage data from a separate animal. Maps of the forelimb area at four selected ages are shown on the right. Color represents the amount of current needed to evoke a response. This threshold reduction can be seen on maps of the forelimb area of the cat motor cortex as a change from a preponderance of high-threshold (red) to low-threshold sites (blue). Red corresponds to a stimulation current of 100 μA, indigo to 1 μA. The general location of this area is indicated as the red box on the inset brain drawing. For the various maps, the lines indicate the cruciate sulcus, which roughly divides the rostral and caudal forelimb areas. E, elbow; M, multijoint responses; S, shoulder; W, wrist. From Chakrabarty S and Martin JH (2000) Postnatal development of the motor representation in primary motor cortex. *Journal of Neurophysiology* 84: 2582–2594.

increases the representation of coordinated muscle synergies, whereas preventing motor experience augments thresholds and reduces muscle synergy representation. These representational changes return to control levels several months after normal experience returns, reflecting plasticity that persists throughout the animal's life.

Conclusion and Implications for Rehabilitation

The CS system develops over a protracted pre- and postnatal period before achieving the mature distribution of spinal terminations. Activity-dependent synaptic competition between developing CS axon terminals, and possibly with other spinal synapses, shapes the formation of a complex set of stable connections between the motor cortex and particular spinal motor circuits. Both the level of CS neural activity and the pattern of activity, as reflected in the patterns of limb use, are critical to this system's development. The outcome is incorporation of the CS system into the developing motor systems and the expression of this system's contributions to skilled motor behavior.

Activity- and use-dependent development of the CS system assures that neural events and experience, as connections are being made, play an important role in forming the circuits for controlling movements that an individual makes throughout life. However, activity and use dependence also creates a vulnerability to deviations from an optimal functional state of the motor systems. It has been suggested that the vulnerability produced by activity-dependent competition between the developing CS tracts on the two sides of the brain could lead to a progressive worsening of the effects of a perinatal CS system lesion.

Can activity-dependent competition be harnessed to promote CS system function after early brain damage? Lessons can be learned from visual system development. It is well known that periods of monocular visual deprivation produced by cataracts or strabismus can lead to a worsening of sight as a child grows up. After correction of the ocular defect, vision is promoted in the impaired eye by periods of monocular deprivation of the normal-functioning eye. Visual acuity in the unimpaired eye is maintained at a normal level by permitting daily controlled visual experience. Work in the developing cat CS system points directly to overcoming functional impairment by rebalancing CS terminations later in development. There also is evidence in humans that rehabilitation strategies can be used to rebalance function in hemiplegic patients. A motor behavioral analog of monocular deprivation

is constraint-induced therapy, in which children with spastic hemiplegia have their unimpaired (or less impaired) arm physically restrained. Constraint-induced therapy forces them to use their affected arm exclusively during daily activities, which can lead to improvement in motor function. Convergence of animal research and human clinical studies leads to the optimistic view that some forms of developmental motor disorders might be significantly improved by harnessing activity-dependent processes shaping development of the CS system.

See also: Anterior-Posterior Spinal Cord Patterning of the Motor Pool.

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Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems

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Introduction

The functions and signaling mechanisms of many important axon guidance molecules may be described individually, but here we attempt to take a more systematic view to describe how various chemotropic guidance cues and adhesion molecules work together to help build precise connections in sensory systems, from the periphery to the brain. We focus on the cell surface/secreted molecules that regulate important pathfinding events in the vertebrates, especially in the mammalian sensory systems. Among the sensory systems, the pathfinding mechanisms are best understood in the visual and the olfactory systems, and to a lesser extent in the somatosensory system. The auditory and the gustatory systems, however, have not been very well characterized.

Building the Visual System

In vertebrate animals, visual stimuli are transmitted by retinal ganglion cells (RGCs) from the retina to subcortical relay stations and eventually to the primary visual cortex. The underlying pathway is formed during development by axons navigating through complex environments, involving decisions at multiple steps, in a remarkably stereotypical and precise manner.

Pathfinding and Topographic Mapping by RGCs

RGC axons project from the retina to the superior colliculus (SC) in mammals, or to the optic tectum (OT) in amphibians and avians, before reaching the lateral geniculate nucleus (LGN) of the thalamus. Significant progress has been made in revealing how RGC axons are guided to make several important choices: where to exit the retina, where to form the optic nerve, to cross or not to cross at the optic chiasm, and how to map the visual field topographically onto the SC and the LGN.

Finding and exiting the optic disk The first step in building the visual pathway is for the RGC axons to exit the retina and project into the optic nerve. RGC axons travel in radial routes inwardly toward the fovea of the retina, where the optic disk is located. Studies in zebra fish have revealed that RGCs express the chemokine receptor CXCR4, while the optic disk expresses the ligand stromal cell-derived factor-1 (SDF-1), and that SDF-1 attracts RGCs toward the optic disk (**Figure 1(a)**). In addition, cell adhesion molecules and bone morphogenetic protein (BMP) receptor type I, chondroitin sulfate proteoglycan (CSPG), and Sonic hedgehog (SHH) molecules have also been implicated in directing this process. Once the RGC axons reach the optic disk, they require netrin-1 to project into the optic nerve head (**Figure 1(b)**). In netrin-1 and DCC (deleted in colorectal cancer) mutant mouse embryos, many RGC axons fail to exit the eye and instead they project aberrantly into other regions of the retina, resulting in the hypoplasia of the optic nerve.

Forming the optic nerve and growing toward the optic chiasm After the RGC axons exit the eye, their growth along the optic nerve is guided in part by Slit proteins (Slit1 and Slit2). Slit1 and Slit2 are repellent molecules for RGC axons and are expressed in overlapping and complementary domains surrounding the optic nerve and adjacent to the optic chiasm (**Figures 1(b)** and **1(c)**). In this way, Slit1 and Slit2 help establish repulsive barriers for a narrow corridor that channels retinal axons toward the optic chiasm. In the absence of both Slit1 and Slit2, retinal axons are defasciculated and project ectopically into the preoptic area. Slits also help to prevent RGC axons from growing back toward the other eye after they reach the chiasm. In zebra fish, mutations in the Slit receptor gene *Robo2* lead to random projections of RGC axons and complete disruption of optic nerve formation. The responses and sensitivities of RGC axons to Slit proteins appear to be regulated by heparan sulfotransferases.

Crossing or not crossing at the optic chiasm At the optic chiasm, RGC axons have to decide whether to stay on the ipsilateral side or to cross the midline to the contralateral side. The percentage of RGC axons crossing at the optic chiasm differs among different species. Fish and birds have no binocular vision and all of their axons cross the midline. In higher mammals, RGCs located in the nasal retina project their axons to the contralateral side, while axons from RGCs of the temporal retina stay ipsilaterally. In mammalian species with less binocular vision, such as mice, only a small subpopulation of RGCs in the ventrotemporal (VT) region of the retina will project their axons ipsilaterally. Studies in *Xenopus* and mice

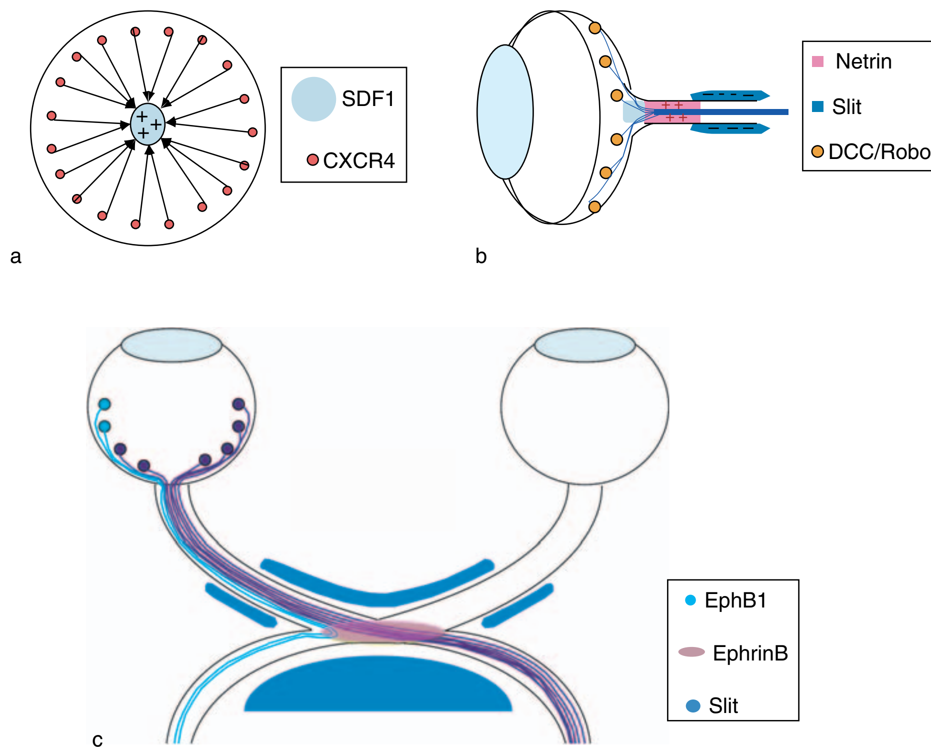

Figure 1 Molecules guide retinal ganglion cells at the optic disk, optic nerve, and optic chiasm. (a) Expressed stromal cell-derived factor-1 (SDF-1) attracts the chemokine (CXCR4)-expressing retinal ganglion cells toward the optic disk. (b) Netrin-1 attracts retinal ganglion cells into the optic nerve. Slit proteins prevent retinal ganglion cell axons from straying away. (c) Slits channel retinal ganglion cell axons toward the optic chiasm. EphrinB prevents EphB1-expressing retinal ganglion cells from crossing the optic chiasm.

have demonstrated that the repulsive ligand ephrinB is expressed at the optic chiasm. EphrinB prevents the crossing of ipsilaterally projecting RGC axons which express the receptor EphB1 (**Figure 1(c)**). In EphB1 null mice, ipsilateral projections are dramatically reduced and almost all RGC axons cross the midline. The expression of EphB1 in these noncrossing RGCs is likely to be regulated by the zinc-finger transcription factor Zic2 and the Lim-homeodomain factor Islet2.

Topographic mapping in the optic tectum/superior colliculus Leaving the optic chiasm, RGC axons project to several subcortical regions. The most prominent midbrain target is the optic tectum of fish, amphibians, and avians, or the SC of mammals. In the OT/SC, RGC axons form an ordered map of the visual field, the positions on the retina being topographically mapped onto the OT/SC along two orthogonally oriented axes: the temporal–nasal (T–N) axis of the retina maps along the anterior–posterior (A–P) axis of the OT/SC (**Figure 2**, upper panel), whereas the dorsal–ventral (D–V) axis of the retina maps along the lateral–medial (L–M) axis of the OT/SC (**Figure 2**, lower panel). The T–N to A–P mapping is primarily governed by the repulsive guidance of the EphA–ephrinA interactions. The EphA expressions in RGCs have a high-to-low gradient along the T–N axis, whereas the ephrinA expressions in the OT/SC show a low-to-high gradient along the A–P axis. These two countergradients together control either the sites of RGC axon termination in fish and amphibians, or the locations of interstitial collateral sprouting in avians and mammals (**Figure 2**, upper panel). The D–V to L–M mapping is achieved by functions of two sets of guidance cues, ephrinB and Wnt. EphrinB is expressed in a high-to-low gradient along the M–L axis in the OT/SC, while EphB is expressed in a low-to-high gradient along the V–D axis in the retina. High-level EphB signaling results in repulsion, while low-level signaling leads to attraction. EphB–ephrinB signaling results in a net attraction of all RGC axons to the medial part of the OT/SC. This is balanced by Wnt protein signaling. Wnt3 causes repulsion of RGC axons through the Ryk receptor, but attraction is through the Frizzled (Fzl) receptor. Wnt3 is expressed in a high-to-low gradient along the M–L axis in the OT/SC. Ryk is expressed in a low-to-high gradient along the V–D axis in the retina, while Fzl expression is equal along the V–D axis. The net result is that Wnt3 causes RGC axons to project toward the lateral part of the OT/SC. The combination of ephrinB and Wnt signaling is required for topographic mapping of RGC axons from the retina to the OT/SC (**Figure 2**, lower panel). This projection can also be refined by

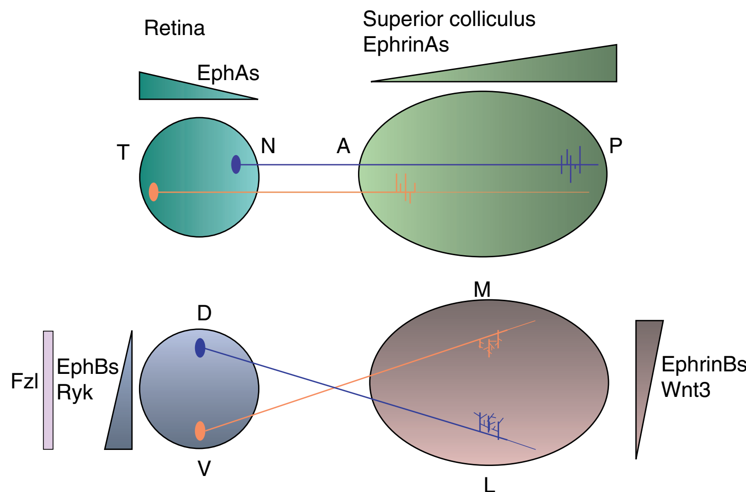

Figure 2 Topographic mapping of retinal ganglion cell projection in the superior colliculus, showing the temporal–nasal (T, N), the anterior–posterior (A, P), the dorsal–ventral (D, V), and the lateral–medial (L, M) axes. Schematic representations of graded receptors (EphA, EphB, Fzl, Ryk) and ligands (Wnt3 protein, ephrinA, ephrinB) that guide retinocollicular topographic mapping.

spontaneous correlated neural activity during a brief critical period.

Topographic mapping and segregation into eye-specific layers in LGN The lateral geniculate nucleus of the thalamus is the other main target of RGCs and is the relay station for visual input to the cortex. RGC axons again form ordered projections that map the visual field onto the LGN. Molecular mechanisms similar to those that govern the retinotopic map in the OT/SC also regulate the retinogeniculate projections. Gradients of ephrins in the LGN and countergradients of Ephs in RGCs regulate the topographic mapping in the LGN. Moreover, in mammals, input from the two eyes segregates into stereotyped eye-specific layers in the LGN. The precise pattern of segregation requires the function of EphrinAs, whereas the segregation of the eye input *per se* depends on neural activity.

From LGN to the Visual Cortex

The next connection in the visual pathway in mammals is for LGN neurons to relay the visual input that they receive from RGCs to the visual cortex. Compared with the pathfinding of RGCs, our understanding of the molecular mechanisms that guide the LGN neurons projections is quite limited. Here we summarize the current findings in this field, focusing on the guidance cues that are supported by *in vivo* evidence from mutant mice.

Getting to the cortex via thalamocortical projections LGN neurons follow precise pathways to relay information from the thalamus to the visual cortex. Their axons first project ventrally through the ventral thalamus. As they approach the dorsal border of the hypothalamus, they make a sharp turn and extend dorsolaterally through a narrow corridor, called the internal capsule (IC), into the ventral telencephalon. All thalamocortical axons (TCAs) likewise follow this path. Slit1 and Slit2 present in the hypothalamus prevent the TCAs from entering into the hypothalamus and also push TCAs away from the midline and toward the IC. Projecting into the IC requires recently identified 'corridor cells,' which express the membrane-bound form of neuregulin (Nrg1-CRD) that helps to attract and/or permit the growth of thalamic axons into the IC (**Figure 3**). Further extension of these axons to the cortex depends on the secreted form of neuregulin, containing an immunoglobulin-like (Ig) domain (Nrg1-Ig), which is expressed in the pallium and acts as a long-range chemoattractant (**Figure 3**). TCAs express the receptor Erb4 for both isoforms of neuregulin. Thalamocortical projection is severely disrupted in either the neuregulin or the Erb4 knockout mice in manners consistent with the roles of corridor cells and neuregulin in attracting TCAs. In addition, netrin-1 restricts the width of the internal capsule, and the semaphorin Sema6A is involved in the guidance of some TCAs.

Innervating and mapping in the visual cortex The molecular guidance cues that direct either the LGN axons toward the visual cortex or the subsequent axon branch projections into layer IV of the cortex are unknown at present. In terms of cellular events, axons initially accumulate and wait below the developing visual cortex in a zone called the subplate, which contains the first postmitotic neurons of the cerebral cortex, the subplate neurons. Subplate

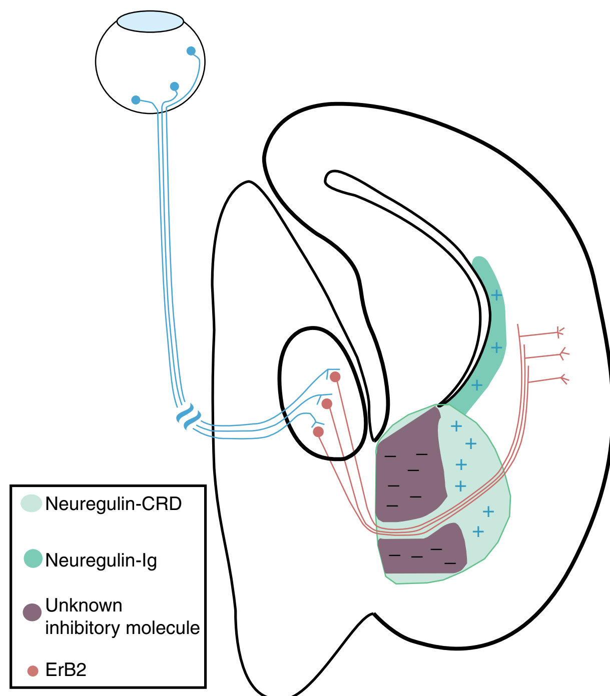

Figure 3 Thalamocortical axon projections are guided in part by neuregulin isoforms. Thalamocortical axons which express ErB2, the receptor for neuregulin, project through a narrow corridor toward cortex. The corridor is made attractive by the presence of a membrane-bound form of neuregulin (Nrg-CRD). In addition, a secreted form of neuregulin containing an immunoglobulin-like (Ig) domain (Nrg-Ig) acts as a long-range attractant for these axons.

neurons have been shown to play important roles in controlling where LGN axons wait and where they project, but the identities of the guidance molecules expressed by subplate neurons are unknown. LGN axons also form topographic maps of the visual field in the visual cortex. Here again, interactions between gradients of EphA/ephrinA direct the formation of the map, but they are not the only factors. Moreover, activity-dependent processes are likely to play a major role in shaping the visual connectivity in the cortex.

Building the Olfactory System

Odorants are detected by olfactory sensory neurons (OSNs) residing in the nose of mammals. OSNs project their axons to the olfactory bulb (OB), the first relay station in the olfactory pathway, and form a sensory map that encodes the quality of odorous chemicals. Mitral (and tufted) cells in the olfactory bulb project to multiple olfactory centers via the lateral olfactory tract (LOT). In the following sections, we review receptors and cues that have been shown to play important roles in guiding the OSN projection and the formation of LOT.

Formation of an Olfactory Sensory Map in the Olfactory Bulb

In the nasal epithelium of mammals, each OSN expresses only one odorant receptor (OR) from a family of about 1000 genes that all encode seven-transmembrane proteins. All neurons expressing the same OR, although randomly distributed in a broad circumferential zone along the dorsoventral axis in the epithelium, project convergently to a pair of glomeruli, each located at a stereotyped position on one side of the olfactory bulb. In this way, the identity of a chemical group (or groups) that is recognized by a given OR is mapped onto two anatomically conserved locations in the bulb (**Figure 4**). How do neurons expressing a specific OR find their target with such precision?

ORs play important roles in OSN targeting. Since the choice to express a given OR is tightly linked to the choice of the axonal projection site in the olfactory bulb, a model in which ORs dictate OSN axon targeting was proposed and supported by several lines of evidence. mRNAs of ORs are found in the OSN axons. OR proteins are concentrated not only in dendrites (to detect odorants) but also on axon termini in the glomeruli. Swapping either the entire coding sequence or the DNA sequence for a few amino acids of one OR with those of another resulted in altered OSN axonal convergence points in the bulb.

How might ORs influence where OSNs form the glomeruli? Two recent studies have shed a light on this puzzle. The first study strongly suggests that ORs signal through G-proteins to regulate intracellular cyclic adenosine monophosphate (cAMP) levels and that different cAMP concentrations determine the differential expressions of axon guidance molecules along the A–P axis. All OSNs express the olfactory-specific G-protein, G_{olf}, throughout their life span. In addition, they express the generic α subunit of G-proteins, G_s, at younger stages. ORs are coupled to these stimulatory G-proteins through a conserved DRY (Asp-Arg-Tyr) motif in their cytoplasmic loop after the third transmembrane domain. Mutating this G-protein binding site on a given OR, I7, caused the mutant ($I7_{DRY}$) OSNs to project diffusely in a broad domain in the olfactory bulb and failure to form glomeruli. This lack of axonal convergence can be partially reverted by expressing either a constitutively active G_s or a constitutively active cAMP response element-binding protein (CREB) in $I7_{DRY}$ neurons. Interestingly, co-expressing a dominant-negative protein kinase A (PKA) with the wild-type I7 results in these neurons projecting to a drastically anteriorly shifted position. In contrast, putting either a constitutively active G_s or a constitutively active PKA into

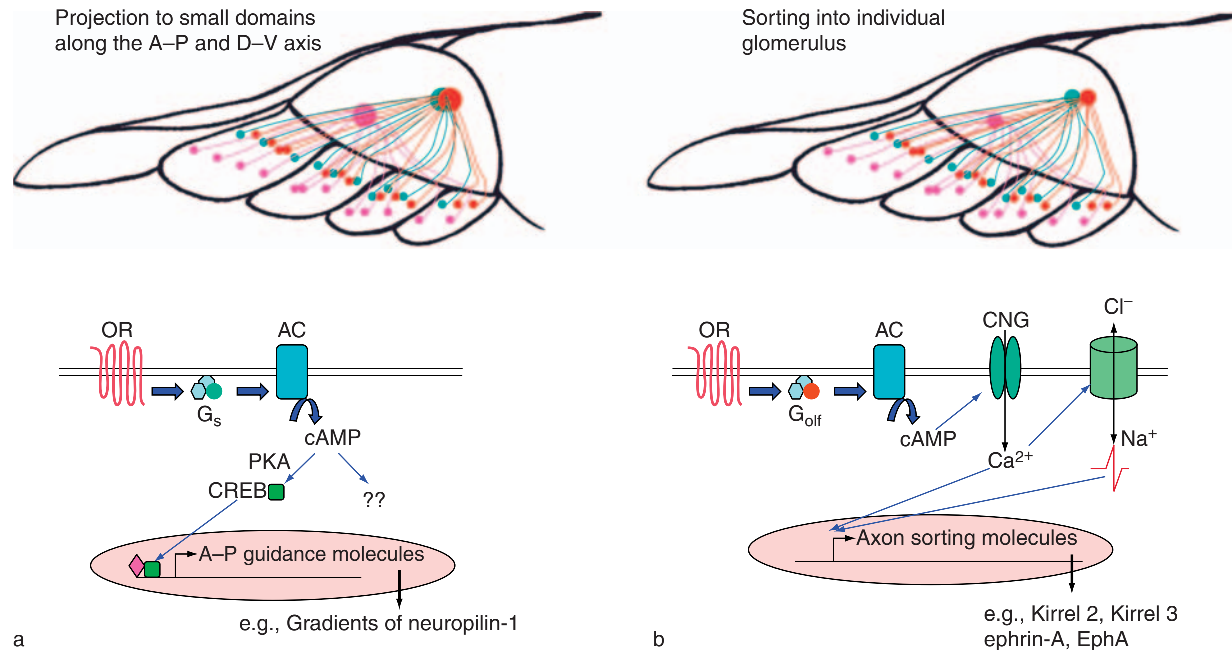

Figure 4 Formation of the olfactory sensory map is guided by odorant receptors (ORs). (a) ORs activate G-protein (G_s)signaling/cAMP signaling to guide the projection of olfactory sensory neurons along the anterior–posterior (A–P) axis and dorsal–ventral (D–V) axis to a small domain in the olfactory bulb. (b) Odorant receptors activate olfactory-specific G-protein (G_{olf}) signaling to induce calcium entry and neuronal depolarizations. These neuronal activities in turn regulate the expression of axon sorting molecules that help sort olfactory sensory axons into distinct glomeruli. AC, adenylate cyclase; CNG, cyclic nucleotide gated channel; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; PKA, protein kinase A.

I7-OSNs, causes them to converge at a much more posterior location in the olfactory bulb. These results led the authors to propose a model in which ORs instruct OSN targeting indirectly via OR-derived cAMP signals (most likely by activating G_s) and that different amounts of cAMP regulate the expression of A–P axon guidance molecules, probably through activating CREB (**Figure 4(a)**). Indeed, the authors have found that expression of the neuropilin-1 receptor positively correlates with the cAMP level, and that neuropilin-1 is expressed in a low-to-high A–P gradient in the glomeruli layer of the olfactory bulb. However, this model also implies that each OR can determine a unique level of cAMP, a hypothesis that remains to be tested. Moreover, since cAMP has been shown to have profound effects on growth cone turning responses, it is conceivable that OR-derived cAMP also control OSN growth cones navigation locally.

Another study used transgenic mice in which the majority of OSNs express one particular OR. This has led to the discovery that ORs also control the expression of the homophilic adhesive molecules (such as the Ig domain containing Kirrel2/Kirrel3 proteins) and the repulsive molecules (such as ephrinA5/EphA5) in an OR-specific and activity-dependent manner (**Figure 4(b)**). Different ORs, through OR-evoked activity, determine the ‘on’ or ‘off’ expression of certain cell adhesion or repulsion molecules, such that different classes of OSNs have distinct combinatorial adhesive/repulsive codes. Once OSNs project to a narrow domain in the olfactory bulb, the adhesive/repulsive molecules then control the sorting of olfactory axons expressing different ORs into different glomeruli within that domain, by both homophilic and mutual repulsive interactions. At the present, it is not known how ORs control the expression of these sorting molecules through OR-dependent neuronal activity. Neural activity may be spontaneous and random, as spontaneous activity is known to be required for the formation of the olfactory map. Spontaneous and odor-evoked activities are also required for the maintenance of the olfactory sensory map.

From the Olfactory Bulb to Higher Olfactory Centers

Mitral and tufted (M/T) cells are the projection neurons in the olfactory bulb; they send axons through the lateral olfactory tract onto several structures of the olfactory cortex. M/T axons are known to wait in the LOT (for about 2 days in the mouse) before sending collateral branches to the olfactory cortex. M/T cells with dendrites that receive input from one particular glomerulus project collaterals to multiple cortical areas in a highly distributed and complex, but not random, manner. Because the organizational

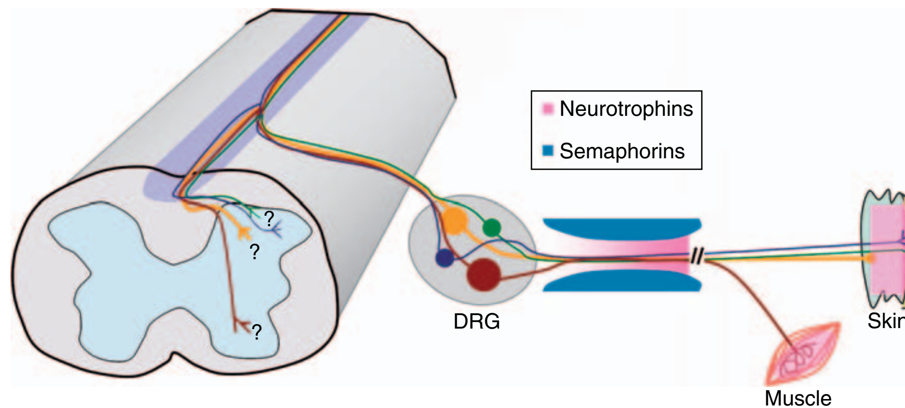

Figure 5 Peripheral projections of somatosensory neurons are guided in part by neurotrophins and semaphorins. Schematic representation of axonal projections of dorsal root ganglion (DRG) neurons. The molecules that guide the central axons into different layers of the spinal cord are unknown and are depicted as '?' in the drawing. Two classes of molecules, neurotrophins (including nerve growth factors, brain-derived neurotrophic factor, neurotrophins 3, 4, and 5, and glial-derived neuronal factors) and semaphorins, direct the peripheral axon outgrowth of sensory neurons by acting as chemoattractants and surround-repellents, respectively. Neurotrophins also promote the innervation, axon arborization, and sensory ending formation of DRG neurons in the target organs such as skin and muscle.

principle of the M/T projections in olfactory cortices is unclear, studies of this pathfinding process have largely focused on the formation of the LOT.

M/T cell projection to and along the LOT requires both short-range and long-range guidance cues. The short-ranged cues are believed to be at the surface of specialized cells called LOT cells. These cues help guide the M/T axons into the LOT and promote their elongation, although their molecular identities are unknown. M/T axonal projections in the LOT are also guided by long-range chemorepulsive molecules expressed in the septum, such that the axons avoid innervating septum regions *in vivo*. Slit1 and Slit2 together account for the repulsive ligands expressed in septum that repel M/T axons expressing Robo receptors. In Slit1 and Slit2 double-mutant embryos, M/T projection is completely disorganized, with many axons entering the septum region.

Building the Somatosensory System

The vertebrate somatic sensory system transmits to the brain information about physical stimuli that the body experiences. The stimuli can be painful, thermal, mechanical, or proprioceptive. Each of these modalities is detected by distinct types of primary sensory neurons and is processed by different central pathways. Although it is generally true that the body surface is topographically mapped onto subcortical processing centers and ultimately to the somatosensory cortex, surprisingly little is known about the molecular mechanisms that control the establishment of the somatosensory map and the neural circuit for each distinct modality.

The cell bodies of somatosensory neurons reside in a series of ganglia located outside the spinal cord (dorsal root ganglia) or the hindbrain (trigeminal ganglia), with the exception of the facial proprioceptive neurons, the cell bodies of which reside in the trigeminal mesencephalic nuclei inside the brain stem. Each sensory neuron grows two major branches stemming from a unipolar axon: a peripheral axon that innervates a specific body target, such as skin, viscera, or muscles, and a central axon that projects into the spinal cord or brain stem and forms specific synapses (**Figure 5**). Therefore, each neuron has two pathfinding tasks, one to the periphery and the other to the central nervous system (CNS), and the two processes must be coordinated such that the body is faithfully mapped onto the brain. The molecular mechanisms that guide the central axons of sensory neurons are largely unknown at present, but significant progress has been made in understanding the guidance of peripheral somatic sensory axons.

Peripheral Growth and Projection of Somatic Sensory Neurons Are Guided by Neurotrophins and Semaphorins

The growth of peripheral sensory axons follows stereotyped trajectories; this process is partly guided by neurotrophins (NTs) and semaphorins (**Figure 5**). NTs (including nerve growth factor, brain-derived neurotrophic factor, NT3, NT4, NT5, and glial-derived neuronal factor) are expressed and secreted by peripheral target tissues. They exert many biological effects on sensory neurons, acting not only as survival and differentiation factors, but also as chemoattractive factors for sensory axons. Both *in vitro* and

in vivo experiments have demonstrated that the chemotropic effects of NTs (for promoting axon elongation and target innervation) can be separated from their chemotrophic (survival) effects. For example, nerve growth factor-soaked beads, when embedded *in vivo* in embryos, can attract sensory axons growing toward them. The peripheral axons grow in fascicles, forming nerve bundles. This fasciculation is regulated by class III secreted semaphorins, which are expressed in a pattern surrounding the axon pathways, and sensory neurons express receptors that recognize these repellents (i.e., neuropilins 1 and 2 and plexins A3 and A4). Mice deficient in any of these receptors or semaphorin3A have severely defasciculated sensory axons. Besides NTs and semaphorins, little is known about the peripheral target selection and innervation process of sensory axons.

Summary

In summary, we have briefly reviewed the axon guidance molecules that work in concert to orchestrate the pathfinding processes that lead to the formation of visual, olfactory, and somatosensory systems. A general scheme is emerging, in that sensory neurons form topographic maps in their projections to the brain, such that sensory information is transformed into distinct spatial activities for the brain to process. These maps are guided first roughly by molecular gradients, and then by refinement with neuronal activity-dependent axon sorting. En route to their targets, neurons are guided to project through stereotyped pathways. These pathways are laid down by a combination of attractive and repulsive cues that usually result in a narrow channel for axons to navigate.

See also: Axon Guidance by Glia; Axon Guidance: Guidance Cues and Guidepost Cells; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Axonal Regeneration: Role of Growth and Guidance Cues; Olfactory Neuron Patterning and Specification; Optic Nerve Optic Chiasm and Optic Tracts; Optic Tectum: Development and Plasticity; Retinal Development: An Overview; Retinal Development: Cell Type Specification.

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Growth Cones

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Introduction

Neurons communicate with one another through synaptic connections on their dendritic and axonal processes. These processes can be highly elaborate, and axons in particular can grow long distances along highly precise routes to acquire their appropriate targets. At the tip of each growing process is a specialized motile structure called the growth cone. The growth cone contains the cellular machinery that drives process extension. This extension is not random but is guided by signals in the surrounding cellular and extracellular environment. These signals are called guidance cues. Guidance cues are recognized by receptors whose activation controls the direction of growth cone advance through a complex network of signaling pathways that is still being elucidated.

Growth Cone Structure

A growth cone's shape varies greatly depending on its rate of advance, the physical properties of the substratum on which it is growing, and its responses to guidance cues in the outside environment (**Figure 1**). A growth cone extending on a flat, featureless, and adhesive surface *in vitro* has a characteristic shape (**Figure 2**). At the tip of a growing axon is a fan-shaped structure which can be divided into three regions: the central, transitional, and peripheral domains. The central domain is closest to the axon and set back from the leading edge. It is rich in microtubules that splay out from the axon as they enter the growth cone. The peripheral domain is at the leading edge of the growth cone. It is composed of thin lamellar sheets and long thin protrusions called filopodia. The predominant cytoskeletal component of lamellae and filopodia is fibrillar actin. Actin is tightly bundled into a rodlike arrangement that fills each filopodium. Actin is organized into a highly cross-linked and branched network with the growing ends pointed predominantly toward the growth cone's leading edge in lamellipodia. The transition zone, as its name implies, is the region between the microtubule-rich central domain and the actin-rich peripheral domain.

Growth Cone Motility

Axonal extension is driven by the motile force generated by the cytoskeletal machinery within the growth cone. Growth cone motility is thought to be the result of force that is generated by a cycle of actin polymerization at its leading edge, myosin-driven retrograde flow of actin polymers through the peripheral domain, depolymerization of actin as it approaches the transition zone, diffusion of the released actin monomers back to the leading edge, and their repolymerization there (**Figure 3**). The resulting actin cycle generates a tank-tread-like flow of polymerized actin from the front to the back of the growth cone. When the actin-based tank tread gets a grip on the substratum, it can advance the leading edge and draw the growth cone forward. Coupling of the cytoskeleton to the substratum is accomplished by specialized receptors on the surface of the growth cone that recognize and bind to specific molecules in the outside environment. These receptors are indirectly linked to the actin cytoskeleton within the growth cone by cross-linking proteins. Receptors that provide this motility-enhancing function include integrins that recognize extracellular matrix components like laminin, and cadherins or immunoglobulin (Ig) superfamily members that recognize homophilic or heterophilic cadherins or Ig superfamily members expressed on adjacent cell surfaces.

Growth Cone Steering

Time-lapse observations of growth cones *in vitro* and *in vivo* demonstrate that both filopodia and lamellipodia are highly motile structures. Individual lamellae or filopodia appear to advance stochastically, at times and directions that are difficult to predict. Those that do not establish a secure contact with the substratum are soon withdrawn and resorbed, whereas those that establish a secure contact are more likely to become stabilized and persist. Growth cones facing a sharp boundary between a more permissive and a less permissive substratum tend to consolidate advances on the more permissive area while withdrawing more often from the nonpermissive area. In this way, differential adhesion experienced by spatially distinct lamellar and filopodial processes can steer growth cones onto a more permissive area of the substratum.

The growth cone is a highly polarized structure and advances only at its leading edge. Its ability to make sharp turns is limited by this extreme polarization, and it is relatively difficult to induce a turn, by any means, that is greater than about 90°. Growth cones are sensitive to the topology of the surface on which they extend. They tend to orient in the same direction as a grooved or filamentous substratum on which they are growing. A growth cone generates tension on the axon as it advances, and this tension reorients and stimulates additional growth in the

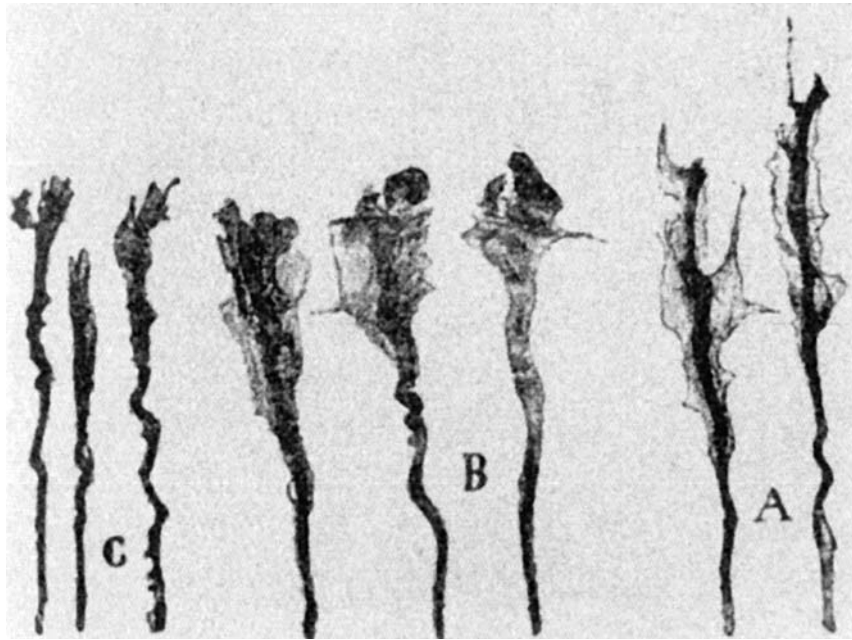

Figure 1 Growth cones were originally described by the famous neuroanatomist Ramón Y Cajal more than a century ago. This is a drawing he made to show the many different shapes they assume (grouped as A, B, and C by Ramón Y Cajal). Some of the variability comes from the stochastic manner in which individual filopodial and lamellar processes advance and withdraw, some is the result of growing in very different environments, and some is due to inherent differences in the overall motile state of the growth cone. From Ramón Y Cajal S (1890) Sur l'origine et les ramifications des fibres nerveuses de la moelle embryonaire. *Anatomischer Anzeiger* 5: 609–613. In Ramón Y Cajal S (ed.) (1995) *Histology of the Nervous System* (Swanson N and Swanson LW, trans.). New York: Oxford University Press. Originally published 1909.

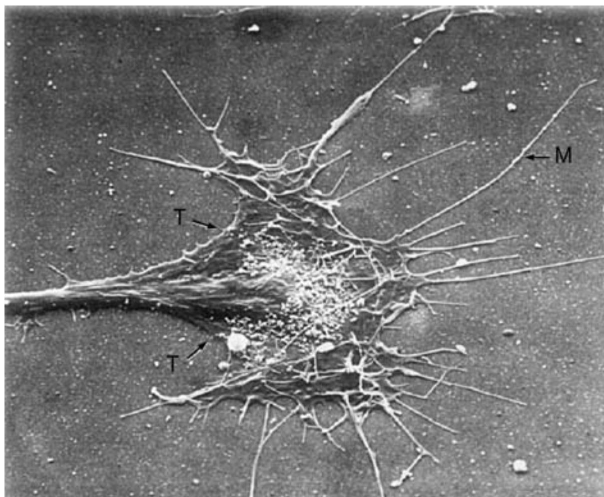

Figure 2 A scanning electron micrograph of a cultured growth cone. Note the thin lamellae and long, thin filopodia (M) projecting radially away from the leading edge and sides of the growth cone. In contrast, few filopodia originate from the traiting edges (T) of the growth cone. This growth cone is approximately 20 μm across. From Wessells NK and Nuttall RP (1978) Normal branching, induced branching, and steering of cultured parasympathetic motor neurons. *Experimental Cell Research* 115(1): 111–122.

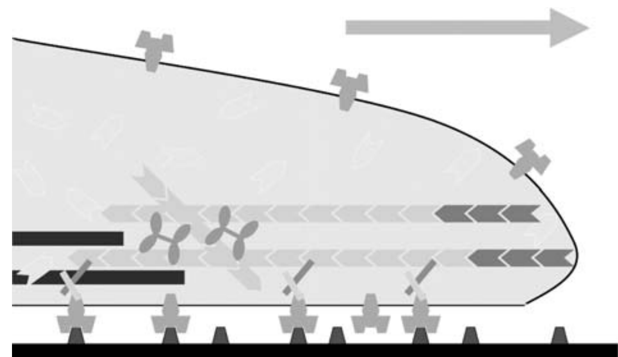

Figure 3 A schematic of the motile machinery at the leading edge of a growth cone that is advancing towards the right. Microtubles (olive) are confined to the proximal portion of the growth cone while fibrilar actin (rose) reaches into the distal most leading edge where it extends by polymerization (red). The resulting actin fibrils are contracted and moved rearwards by myosins (blue). A continuous cycle of actin polymerization at the leading edge, retrograde flow, and depolymerization at the rear is established. This cycle drives the growth cone forward over a substratum (black) decorated with specific permissive molecules (green) that are recognized by complementary cell surface receptors (light blue) which are linked to fibrilar actin (purples).

same orientation. This is likely to represent a reinforcing mechanism by which growth cones are steered toward more-permissive substrata as processes better stabilized against the substratum are likely to generate more tensile force than poorly stabilized processes. This differential tensile force would be expected to stimulate further additional advance toward a point of contact.

The consolidation of adhesive gains or responses to tensile force can be related to changes in the

organization of the growth cone cytoskeleton. Microtubules and fibrillar actin are in a kind of dynamic equilibrium that maintains their relatively high respective concentrations in the central and peripheral domains of the growth cone. The retrograde flow of actin in the peripheral domain tends to carry or push microtubules away from the leading edge. Some individual microtubules polymerize more rapidly than the retrograde flow of actin for brief periods and advance into the peripheral domain, but they cannot sustain high rates of polymerization long enough to remain there, and they get pushed or carried back to the central domain. In this way, a small subset of microtubules constantly probe the more peripheral regions of the growth cone. When filopodia or lamellae make contact with a highly permissive substratum, for example a preferred cell surface, the retrograde flow of actin is slowed by strong coupling to the substratum. Microtubules are then able to advance toward the site of contact (**Figure 4**). This invasion of microtubules stabilizes the advanced process and appears to bias further extension in the same direction. Treatments that prevent microtubules from polymerizing quickly make growth cones largely insensitive to external guidance cues. This suggests that stabilization of advancing microtubules as they probe the peripheral domain may help lock in actin-based advances at the leading edge.

A second method by which growth cones can be steered is by modulating the propensity of the leading edge to advance. Any localized change that promotes actin polymerization at the leading edge should tend to advance that region of a growth cone, while conversely, localized signals that inhibit actin polymerization or accelerate retrograde transport should induce retreat. Repellent guidance cues like semaphorin 3A can shut down actin polymerization at the leading edge, halting forward advance. When applied locally, semaphorin 3A discourages the advance of nearby lamellae more effectively than those at a distance. Lamellae that are less subject to the repellent signal advance more successfully than those that are close to the repellent stimulus. The growth cone consequently turns away from the localized source of repellent. Many attractants can be expected to work in a converse manner, locally promoting actin polymerization and the local advance of the leading edge of the growth cone.

Guidance Cues and Their Receptors

Growth cone advance in the developing nervous system is not random but highly stereotyped and organized. Axons generally follow reproducible routes to their appropriate targets, indicating that they are guided there by information in their surrounding environment. To be useful, this information must be distributed in a reproducible and spatially heterogeneous pattern. In consideration of what is known about growth cone motility and steering, this information is

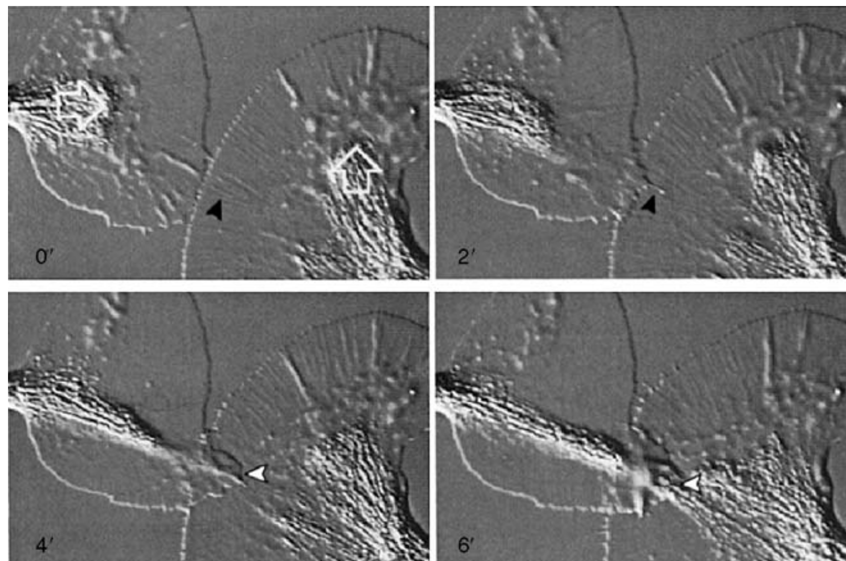

Figure 4 Microtubules within a growth cone are reoriented by contact with another cell surface. A sequence of differential interference contrast photographs of cultured snail neurons begins at 0′ with the initial contact between two growth cones. At 2′, the actin-rich leading edge has advanced markedly at the point of contact. Microtubules in the central domain already appear to be turning toward the point of contact. By 4′, microtubules in the left-most growth cone have advanced dramatically toward the portion of the leading edge that is growing rapidly onto the other cell surface. Black arrowheads mark a filopodium from the left-most growth cone extending on the second growth cone, white arrowheads mark a growing lamellum extending from the left-most growth cone onto the other growth cone. From Lin CH and Forscher P (1993) Cytoskeletal remodeling during growth cone-target interactions. *Journal of Cell Biology* 121(6): 1369–1383.

likely to fall into at least three general categories. The first is physical features that make advance easier in one direction than another. For example, axons are sometimes canalized into a specific route by dense tissues on either side of their path. The second is permissive molecules whose distribution can guide axons by making some pathways better for outgrowth than others. This would in principle be a powerful method of determining where nerve tracts form; however, permissive molecules have rarely been found to be expressed in a prepattern that correlates well with axonal outgrowth. Permissive molecules are often more broadly distributed than the trajectories of forming axonal tracts. Finally, signaling molecules can steer growth cones by locally altering cytoskeletal dynamics. Thus far, it is these signaling cues that seem to play the most important role in guiding axons *in vivo*.

A wide range of disparate signaling molecules have been shown to help guide axons *in vivo*. Some of the first to be characterized include members of the semaphorin, netrin, ephrin, and slit families. A large proportion of these family members can act as guidance molecules, although it would be wrong to think of them as being dedicated to this function alone since many contribute to other developmental and signaling events. In addition to these well-recognized guidance families, signaling molecules that were originally identified as morphogens have been strongly implicated as guidance cues, including members of the Wnt, BMP, and hedgehog families. Additional guidance cues have been identified in the cadherin and Ig superfamilies, among others.

For the most part, each guidance cue has a well-defined receptor or receptor complex which must be expressed by a growth cone for it to be sensitive to that particular cue. For example, each semaphorin is recognized by specific members of the plexin family, ephrins by the members of the eph family of transmembrane tyrosine kinases, and slits and netrins by select members of the Ig superfamily. Most often a traditional ligand–receptor pairing triggers a signaling event within the growth cone that alters its direction, but in some cases, homophilic interactions underlie important guidance events, for example between Down syndrome cell adhesion molecule Ig superfamily members in invertebrates. It is interesting that many of the ligands that act as guidance molecules have some of the same general characteristics as receptors, and in some instances it can be shown that 'reverse signaling' occurs where the presumptive ligand acts as a receptor and the presumptive receptor acts as its ligand. Since a growth cone's response to a particular cue in its environment depends on its ability to recognize the cue, some guidance decisions depend critically on the expression of accessory proteins that control receptor expression. For example, whether an axon chooses to cross the midline of the fly central nervous system can be determined by the expression of a protein called commissureless which in turn controls the axonal expression of a receptor for midline repellents.

Signaling molecules that guide growth cones can be further subdivided into those that serve as attractants or repellents. Attractive interactions can be detected in several different ways. *In vitro*, attractants can sometimes promote axonal outgrowth. A surer test for attractant activity is to demonstrate that a localized source of signal turns or attracts axons (**Figure 5**). Similarly, *in vivo*, ectopic expression of an attractant will orient sensitive axons towards higher levels of expression, while knockdowns of an attractant are expected to reduce axonal trajectories towards the normal source of signal. Repellents are easily detected *in vitro* by their ability to quickly paralyze growth cone motility. A localized source of soluble or surface bound repellent can redirect growth cones away from a localized source of signal. *In vivo*, misexpression of a repellent will exclude axons from an area they would normally enter, or when knocked down, encourage sensitive axons to invade an area they would normally avoid. Interestingly, the same guidance cue can act as either an attractant or a repellent. This is observed in the case

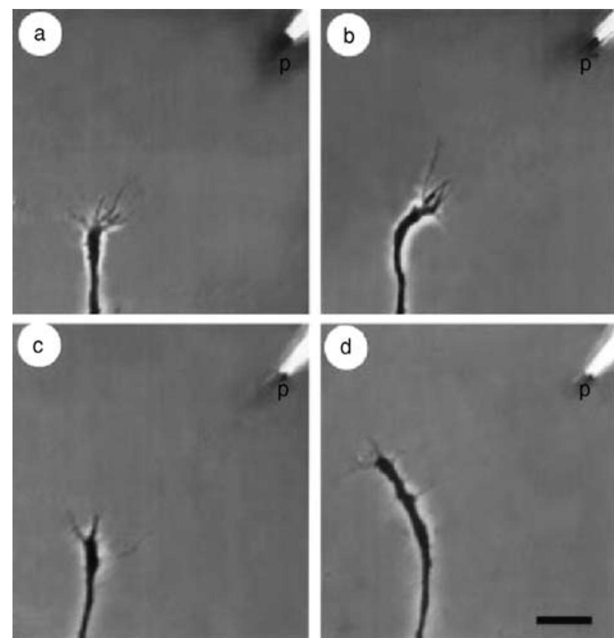

Figure 5 The conversion of an attractant into a repellent induced by a cyclic adenosine monophosphate (cAMP) antagonist (a, b). A cultured frog growth cone is induced to turn toward a source of brain-derived neurotrophic factor (BDNF) ejected from a pipette (p). (c, d) After treatment with the cAMP antagonist Rp-cAMPs, growth cones turn away from a source of BDNF. Scale bar = 20 μm (d). From Song HJ, Ming GL, and Poo MM (1997) cAMP-induced switching in turning direction of nerve growth cones. *Nature* 388(6639): 275–279.

of netrin-1. Attraction to netrin-1 is mediated by the expression of the DCC receptor while repulsion is mediated by expression of the unc-5 receptor.

Guidance cues may act either on contact or at a modest distance. Many guidance cues are transmembrane proteins or are coupled directly to lipids on the cell surface. Unless cleaved by a protease, these cues cannot diffuse away from the cell that produces them and are recognized only by growth cones that touch them. Other guidance cues are secreted and can act at a distance. Thus far it appears that this distance is relatively small, perhaps only a few hundred micrometers or so. This relatively limited range may be partially determined by the required steepness of the concentration gradient required to orient growth cones. An additional limiting factor is that some secreted guidance cues are charged and diffuse poorly through the extracellular matrix. Thus, most growth cone guidance decisions are largely local in nature and are determined by the expression of guidance cues on immediately adjacent surfaces or nearby tissues.

Receptor Signaling

Since many of the receptors for guidance cues discovered over the past decade are novel, the signaling pathways through which they control growth cone motility and orientation are only now being determined. Presumably, their signaling must converge on common pathways that control actin polymerization, retrograde flow, and microtubule dynamics. Among the key downstream signaling intermediaries are the Rho family of small guanosine triphosphatases, RhoA, Rac, and CDC42, which are well known to affect actin polymerization and organization in nonneuronal cells. Activation of many of the known guidance receptors stimulates or inhibits the activity of one or more Rho family members. These in turn modulate the activity of a complex circuit of downstream effector proteins which reorganize the cytoskeleton. A second signaling intermediary of some importance is the divalent cation calcium. Its overall intracellular concentration has been shown to affect growth cone motility, while localized intracellular perturbations in calcium levels can induce localized changes in the cytoskeleton and steering of the growth cone. The activation of some but not all guidance receptors induces significant changes in intracellular calcium that are important in the growth cone's response. A third key signaling component appears to be cyclic nucleotides such as cyclic adenosine monophosphate (cAMP). Altering their internal concentrations can alter how growth cones respond to a given guidance cue. In the most dramatic examples, altering cAMP levels can convert an attractant into a repellent or a repellent into an attractant (**Figure 5**). From these observations it is evident that the internal state of the growth cone affects how it responds to outside guidance cues.

The growth cone has been a source of fascination ever since it was first described by the famous neuroanatomist Ramón Y Cajal more than a century ago. During the intervening years, great progress has been made in elucidating the basic mechanism through which growth cones advance and identifying many of the guidance cues that steer growth cones. Even so, much remains to be discovered. The signaling pathways through which guidance receptors control the cytoskeleton are only partly known, and a fuller understanding of this signaling circuitry will be essential in understanding how growth cones integrate and respond to multiple competing guidance cues. From a developmental perspective, much of the information about where and when guidance cues affect axon trajectories is fragmentary and incomplete. A more systematic, systems-based examination of all the guidance cues involved in determining any given axonal trajectory will be required to fully explain how growth cones are guided to their correct targets.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Axonal Pathfinding: Extracellular Matrix Role; Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh); Axonal Pathfinding: Netrins.

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Optic Nerve, Optic Chiasm, and Optic Tracts

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Introduction

The projection neurons of the vertebrate retina are the ganglion cells, which extend axons across the inner surface of the retina in the optic fiber layer and exit the eye via the optic nerve head. These optic axons subsequently course along the optic nerve to the base of the brain at the optic chiasm, where, with optic axons from the opposite nerve, they intermingle and sort to form the optic tracts. The final destination of these axons is the target visual nuclei in the brain (**Figure 1**). A small proportion of optic axons course in an accessory optic tract posterior to the main optic tract (and innervate a distinct group of accessory optic nuclei), and there exist a small number of centrifugal fibers innervating the retina as well as commissural fibers of the supraoptic tracts. The contributions of the accessory optic, centrifugal, and supraoptic axons vary depending on the species and developmental age (there is also a retino-retinal projection that is entirely eliminated during development), and are not be considered further in this article.

Organization of the Optic Pathway

The retinofugal pathway establishes synaptic connections in both the diencephalon and the mesencephalon, where axons or collateral branches leave the pathway and innervate target visual nuclei. Different optic axon classes, arising from morphologically and physiologically distinct retinal ganglion cell classes and often discriminable by their size, depart the pathway at different locations to innervate distinct targets: for example, the retinal projection to the hypothalamus departs the pathway at the optic chiasm, while the projection to the midbrain extends through a continuation of the optic tract to the superior colliculus (optic tectum). This optic innervation forms a 'retinotopic map' in some of these target nuclei, by which the array of ganglion cells across the retina is faithfully replicated by the order of their synaptic connections within the target. Retinotopic precision in primates is greatest in the projection to the dorsal lateral geniculate nucleus, the thalamic relay nucleus that projects upon the primary visual cortex.

In mammals, many optic axons from the temporal retina do not cross the midline of the optic chiasm but rather turn to project into the optic tract ipsilateral to their origin. The area of the temporal retina giving rise to this nondecussating, or uncrossed, projection varies across species, dependent on the extent of binocular overlap within the visual field and constrained by the position of the eyes in the head. This 'partial decussation' at the optic chiasm enables the binocularly congruent parts of the two retinas to project directly to the same side of the brain, where processing of the two retinal images in the visual cortex mediates stereoscopic depth perception. Mammals with forward-facing eyes tend to have greater binocular overlap though a narrower field of vision relative to those with laterally placed eyes (**Figures 2(a)** and **2(b)**). The balance between these parameters in any species reflects the behavioral ecology of the organism. For example, rodents and rabbits have laterally positioned eyes, enabling a visual field adapted to viewing a broad expanse while monitoring for predators from above and behind (**Figure 3**). Additionally, in such species, the proportion of cells in the temporal retina giving rise to uncrossed optic axons is as low as 10%. This proportion rises in species with forward-facing eyes, such that virtually every ganglion cell in the temporal retina of primates projects ipsilaterally. The resulting narrower field of vision in primates, on account of having eyes in the front of the head, is partially offset by the ability to turn the head in many directions. The greater proportion of retinal ganglion cells with uncrossed optic axons in the temporal retina in primates, coupled with their smaller receptive fields, renders the population of binocularly driven cortical neurons sensitive to slight retinal disparities, thereby providing the substrate for fine stereoscopic depth perception.

The partial decussation of optic fibers at the chiasm is a feature unique to mammals. In most nonmammalian vertebrates, such as the favorite experimental models of fish and chick, the eyes are positioned laterally, maximizing the breadth of the visual field and minimizing or eliminating regions of binocular overlap. This arrangement of the eyes with respect to the brain results in an optic chiasm with no decussating fibers and a completely crossed retinal projection. Some nonmammalian species with frontally placed eyes, such as owls and frogs, make use of other neural pathways that cross the midline at locations central to the retinofugal targets in order to achieve binocularity.

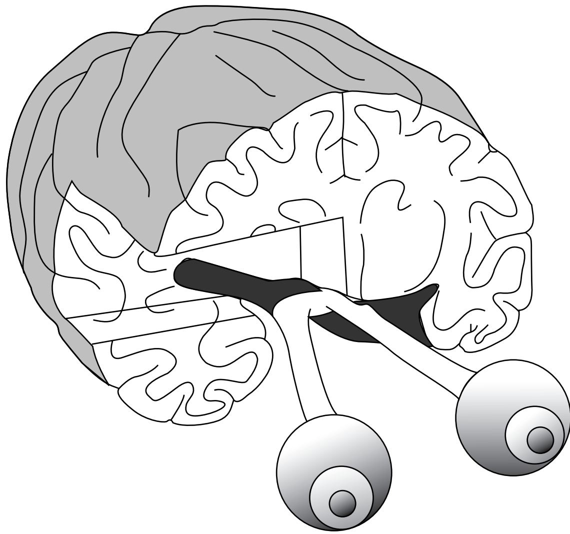

Figure 1 The optic nerves, chiasm, and tracts are defined by the course of the optic axons as they project from eye to brain. The optic pathway becomes fused with the base of the brain in the region of the optic chiasm (dark stipple, posterior chiasm and tract). The optic nerve (light stipple, anterior chiasm and nerve) comprises the second cranial nerve. Unlike the other cranial nerves, the optic nerve is a part of the central nervous system (as is the retina), being a developmental outgrowth of the ventral diencephalon. Modified from Hoyt WF and Lewis O (1963) The primate chiasm. *Archives in Ophthalmology* 69: 69–85.

Fiber Organization in the Optic Nerve, Chiasm, and Tract

Traditional accounts of the primary visual pathway assumed that optic axons coursed in a retinotopically ordered fashion along the length of the optic nerve, and that axons arising from binocularly conjugate retinal loci were brought together in the optic chiasm to yield a fiber order within the optic tract that accurately anticipated the binocular and retinotopic features of the visual targets. Clinical observations following focal damage to the optic pathway were, however, often incompatible with those traditional accounts: for example, selective impairments of distinct visual functions, affecting binocularly disparate retinal loci, were often a consequence of partial damage to the optic tract, rather than precise, binocularly conjugate, blind fields ('scotomas'), as should be expected from standard textbook accounts of the visual pathways. Subsequently, neuroanatomical investigations in mammals revealed that optic axons are only very coarsely organized into a retinotopic fiber order at the optic nerve head, and that this order deteriorates along the course of the nerve. In particular, the fibers arising from the nasal and temporal half-retinas are intermingled extensively in many mammalian species,

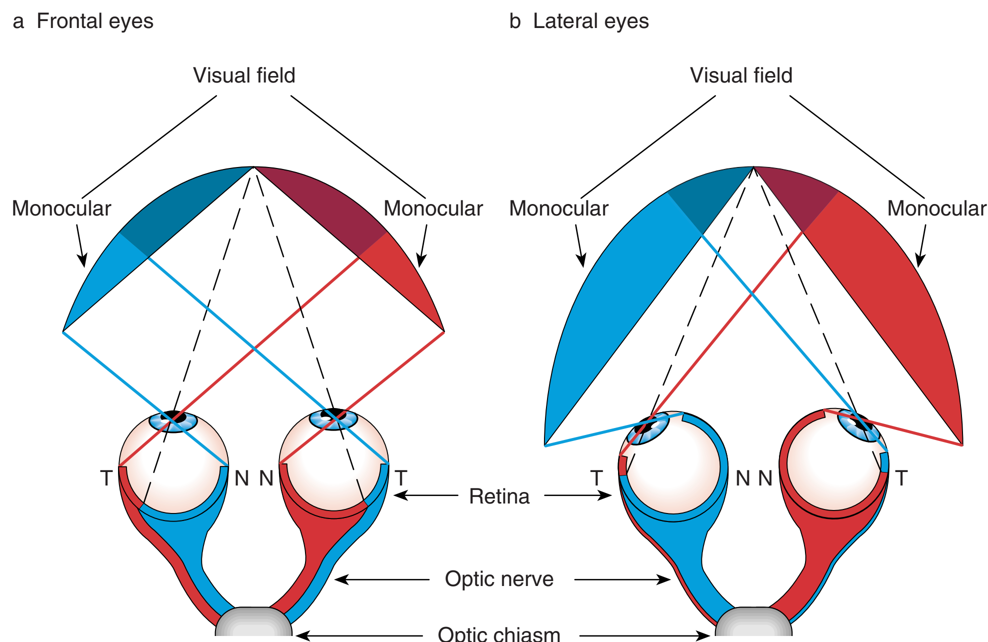

Figure 2 The visual field of mammalian species with frontally facing eyes (a) displays more binocular overlap but a narrower overall field of vision compared to mammals with laterally placed eyes (b) who tend to have a smaller binocular overlap but a wider overall field of vision. Red represents information from the right visual field and blue represents information from the left visual field. Note that both eyes transmit visual information from both visual hemifields; in animals with frontal eyes, this information comes more equally from both eyes, while in mammals with lateral eyes, one eye carries most of the information from a single hemifield. N, nasal; T, temporal. Modified from Jeffery G and Erskine L (2005) Variations in the architecture and development of the vertebrate optic chiasm. *Progress in Retinal and Eye Research* 24: 721–753.

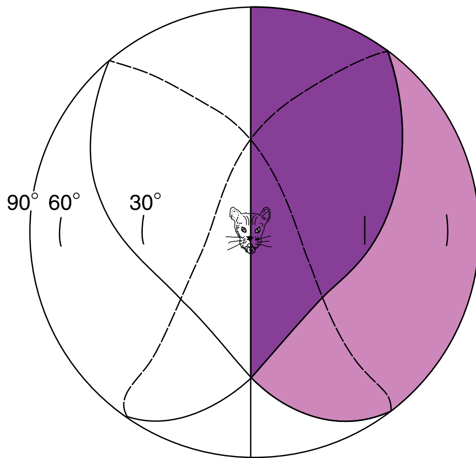

Figure 3 One visual hemifield is shown for the rat, with the binocular portion (dark purple) viewed by the right eye's temporal retina (situated ventrotemporally as a crescent upon the retina) and the left eye's corresponding nasal retina (situated between that eye's temporal retina and the optic nerve head). The remaining portion of the left eye's retina nasal to the optic nerve head views the peripheral (monocular) visual field (light purple). Despite the relatively lateral position of the eyes in the head, the rat's binocular visual field at its widest extent approaches 100°s of visual angle. Modified from Hughes A (1979) A schematic eye for the rat. *Vision Research* 19: 569–588, and from Reese BE (1988) 'Hidden lamination' in the dorsal lateral geniculate nucleus: The functional organization of this thalamic region in the rat. *Brain Research Reviews* 13: 119–137.

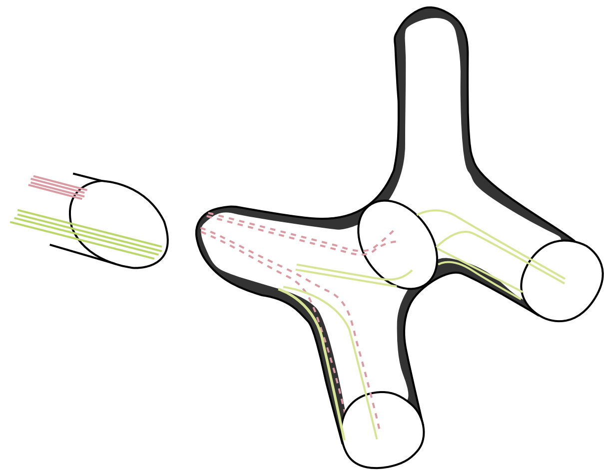

Figure 4 Optic axons become segregated by functional class (two of which are indicated here in different colors) as they pass through the chiasmatic region. This is evidenced in all adult mammalian species as a segregation of axons according to their size within the optic tract. Modified from Reese BE and Ho K-Y (1988) Axon diameter distributions across the monkey's optic nerve. *Neuroscience* 27: 205–214.

so that fiber position in the nerve cannot be a basis for the partial decussation at the optic chiasm. Further, an entirely different organizing principle is found central to the optic chiasm: while functionally distinct optic axons are intermingled within the optic nerve, they are partially segregated within the tract (**Figure 4**). In addition, those distinct axon classes regain some degree of retinotopic fiber order upon entering the tract. Rather than being a passive conduit for bestowing a retinal map upon the visual targets by maintaining precise neighbor relationships, the optic pathway displays conspicuous fiber rearrangements along its length. These observations on the mature visual pathway indicate that optic axons must change their ordering as they navigate a course from eye to brain during development.

Development of the Optic Pathway

Axonal Outgrowth and Optic Nerve Formation

The embryonic retina develops as an out-pouching of the diencephalon, the optic vesicle. This vesicle gradually invaginates to produce the optic cup, the inner surface of which becomes the germinal neuroepithelium producing all of the neurons of the mature retina; the outer surface becomes the nonneuronal pigmented epithelium. The neck of the optic vesicle, the optic stalk, differentiates into the optic nerve as the first postmitotic cells of the retinal neuroepithelium (the ganglion cells) extend axons, which grow among the cells of the stalk within its ventral wall. Those stalk cells, in turn, differentiate into the glia of the developing optic nerve, yielding a mature nerve composed of fascicles of axons and interfascicular astrocytes and oligodendrocytes. Electron microscopic and dye-labeling studies have confirmed that growing optic axons frequently change position between the fascicles, so that neighbor relationships are not maintained along the developing nerve. By the time the growing optic axons arrive at the base of the brain, those from different quadrants of the retina are conspicuously intermingled in the developing rodent and carnivore optic nerve.

Decussation Decisions at the Optic Chiasm

The microenvironment of the developing optic pathway changes dramatically at the base of the brain: axons no longer course in fascicles amid glial cell bodies, but rather course subpially among radial glial processes which extend from somata at the ventricular surface. As the axons from the two nerves meet, those from the nasal retina of each eye cross the midline; those from the temporal retina, by contrast, adopt an uncrossed course. Distinct populations of glial processes and neurons inhabit this 'decision region' prior to and during axonal invasion, and within this region the growth dynamics of newly arriving optic axons change, evidenced by the changing morphology and advance rate of their growth

cones. *In vitro* assays implicate a membrane-bound molecule to which only the temporal retinal growth cones are sensitive, acting as a contact-mediated inhibitory substrate, probably via a receptor–ligand interaction. One such ligand–receptor pair mediating divergence at the optic chiasm has recently been identified as belonging to the Eph/ephrin family, which is known to mediate a number of neuronal and glial interactions in the developing nervous system.

Curiously, a portion of optic axons from the temporal retina of hypopigmented eyes are misdirected at the chiasmatic midline, crossing the midline to project aberrantly into the opposite optic tract, suggesting a reduced sensitivity to the midline signal. Such a reduced sensitivity of axons from temporal retina may also occur during later development in normally pigmented nonprimate mammals, since the later-generated cohorts of retinal ganglion cell in these species extend axons that all decussate, irrespective of retinal locus. The molecular signature of ganglion cells from temporal retina during early development is different from that of their decussating neighbors forming at later developmental stages and contributes to the ability of those later-extending temporal fibers to cross the midline. The transient expression of Zic2, a zinc finger transcription factor, in retinal ganglion cells giving rise to uncrossed axons is necessary to prevent these fibers from crossing the midline at the optic chiasm. Thus, like other neuronal populations in the developing brain and spinal cord, retinal axons are molecularly specified to respond to signals at midline choice points such as the optic chiasm.

With respect to development of the optic chiasm, a unique but experimentally informative species is the frog, which initially has an entirely crossed visual projection. Beginning at metamorphosis, as the eyes shift from the side of the head to the front, the transforming tadpole develops a small ipsilateral component arising from the temporal retinal periphery. This projection is thyroxine dependent and corresponds with the appearance of a thyroxine-responsive stem cell population in the peripheral retina.

Chronotopic Fiber Reordering

The axons of progressively later-generated cohorts of retinal ganglion cells (destined to differentiate into distinct functional classes) grow amid the older optic axons down the nerve, but as they enter the optic chiasm and tract, they become segregated according to their time of arrival, creating a 'chronotopic' map. This is evidenced in electron micrographs by the accumulation of growth cones in the subpial parts of the optic tract, and by the selective labeling of older axons in the deeper parts of the tract. The fiber reordering has been related to the changing glial environment mentioned earlier, and to an associated changing molecular milieu in which proteoglycans create an unfavorable environment for newly arriving axons. This chronotopic reordering of optic axons ultimately yields a mature optic tract in which functionally distinct optic axons, having differentiated into their discriminable size classes, are segregated (**Figure 4**).

Optic Tract Formation: Retinotopic Reordering and Positioning

As the optic axons enter the tract, they regain a degree of retinotopic order that is not present in the prechiasmatic optic nerve: axons from dorsal and ventral retina are intermingled within the nerve, yet they become segregated across the width of the optic tract, establishing a coarse mapping of this retinal axis. The cause of this retinotopic reordering is unclear, but since the change is relatively sudden, it is likely to be triggered by some changing feature of the immature diencephalon. As this new fiber order, created afresh in the pathway at the base of the brain, presages the orderly termination of axonal arbors in the target nuclei, a role in the formation of retinotopic maps within the targets is suggested.

Independent of signals that reorder the optic axons relative to one another, other cellular and molecular features of the perichiasmatic region may be critical for determining the locus of pathway formation. For example, embryonic neurons in the chiasmatic region may provide a template for the future optic pathway. Ablation of these neurons prevents pathway formation, leaving optic axons stalled within the prechiasmatic nerves. Mutations producing a completely uncrossed optic pathway (shown in both humans and in dogs, in which the optic nerves fail to fuse) may reflect an anomalous specification of these cells or their surface properties that normally defines the characteristic crossing of the retinal axons. Other local features at the surface of the diencephalic neuroepithelium may provide a template for the developing optic tract, defined by both growth-promoting and unfavorable substrates for axonal elongation. These appear to be independent of other fiber tracts in the vicinity (at least in amphibians), since manipulations that allow optic axons to invade a less mature diencephalon, prior to the formation of these other pathways, do not prevent the formation of the optic tract.

Target Innervation

As optic axons invade their targets, their growth rate slows down and the morphology of their growth cones increases in complexity as they begin to form terminal arborizations. The interactions leading

to target recognition, axonal invasion, and synapse formation are still relatively undefined, but are likely to be different in midbrain and thalamus, suggested by *in vitro* studies, transplantation studies, and studies of their normal and abnormal patterns of innervation. Innervation of the lateral geniculate nucleus occurs via collateral sprouting after optic axons have extended within the optic tract beyond the level of the target, suggestive of a precise target-recognition mechanism. Yet, deprived of normal visual targets, optic axons will elaborate axonal arbors and form retinotopically organized synaptic connections in nearby nonretinal targets in the thalamus, provided that those nuclei have been suitably deafferented. Innervation of the optic tectum, by contrast, may be mediated by a longer range diffusible attraction, since transplantation studies have shown that this target can guide optic axons to it via novel (nonoptic) pathways. As occurs at the optic chiasm and in other regions of the central nervous system, receptor–ligand interactions are important for setting up the initial retinotopic map in target regions of the diencephalon. Molecules of the Eph/ephrin family, along with transcription factors, extracellular matrix proteins, neurotrophic factors, and cell death-regulating factors, including gradients of guidance molecules in the target regions responding to corresponding gradients in the retina, all contribute to the process of topographic mapping.

The foregoing developmental considerations are of interest not only because they begin to clarify the mechanisms governing axonal navigation and pathway formation: they also make comprehensible the mature organization of the optic nerve, chiasm, and tract. This in turn provides a fuller understanding of the disorders of vision that result following focal damage to the pathway.

Genetic Mutations in Pigment-Related Genes Reduce Binocular Vision by Reallocating Retinal Fibers at the Optic Chiasm

As mentioned previously, in mammals with reduced pigmentation, such as found in albino species, a portion of the uncrossed retinal projection fails to be inhibited by the midline at the optic chiasm and incorrectly crosses into the contralateral optic tract with the normally decussating fibers (**Figure 5**). Decades of study have revealed the precise wiring defects that result from the aberrant crossed projection within the lateral geniculate nucleus, as well as the downstream consequences within the primary visual cortex. These studies have determined that the fiber abnormality in hypopigmentation mutants arises from a lack of melanin in the eye rather than being due to any abnormality within the optic nerve or chiasm. Indeed, a study of mouse lines having mutations in genes that decrease pigment specifically in either the retinal pigment epithelium or in the outer choroidal layer of the eye have confirmed that only those with reduced or absent pigment in the retinal pigment epithelium display a smaller uncrossed retinal projection indicative of a fiber misrouting at the optic chiasm.

A number of inherited human diseases affect both pigmentation and vision, on account of absent or abnormally produced melanin in the retinal pigment epithelium. The visual system defects in these individuals include hypersensitivity to light, nystagmus, reduced visual acuity, and reduced binocular vision and depth perception, the last because of the retinal axon misrouting defect, thus leading to severe visual impairment in affected individuals. Similar abnormalities result from mutations in a variety of different

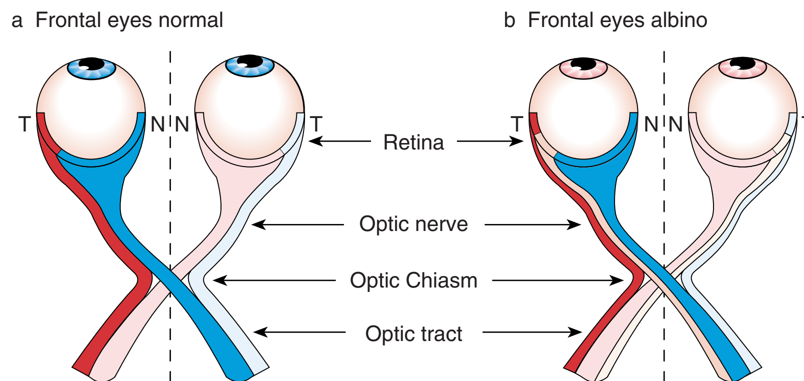

Figure 5 As in **Figure 2**, information from the right visual field is represented in red while that from the left visual field is depicted in blue. An albino individual from a mammalian species with frontal eyes (b) has reduced binocular vision due to the reallocation of fibers and a misrouting of some normally uncrossed fibers at the optic chiasm (represented by lighter coloring of fibers in the optic nerve, chiasm, and tract). N, nasal; T, temporal.

genes, the common feature of each phenotype being reduced or abnormal melanin in the retinal pigment epithelium. These genetic disorders include the tyrosinase-negative albino, in which no melanin is formed in the skin, hair, or eyes. The albino patient represents the most severe form of oculocutaneous albinism. Mutations in other genes of the melanin synthesis and melanosome biogenesis pathways likewise result in the visual disturbances found in albinism. Hermansky–Pudlak syndrome (HPS) results from mutations in a cluster of genes involved in various aspects of melanosome biogenesis. Ocular albinism (OA1) is yet another melanosome biogenesis disorder that affects vision. While treatment for these disorders is not yet possible, we do have mouse models of each genetic mutation and further research is under way to determine the causes and potential cures.

See also: Retinal Development: An Overview.

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Activity in Visual Development

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Introduction

The development of neuronal circuits involves initial coarse wiring under the guidance of molecular cues and the refinement of connections through mechanisms that are governed by patterned spontaneous activity and sensory experience. This fine-tuning of neuronal circuits is evident in the development of sensory maps in the brain. Sensory maps are organized layouts of neurons in which cells that prefer specific stimulus features are found in close physical proximity. The term map can apply either to the central representation of the sensory periphery, as in the case of retinotopic or other topographic maps, or to the orderly representation of higher order stimulus features such as orientation or interaural time difference.

Neural activity can be either permissive or instructive for circuit formation. When serving a permissive role, the presence of activity acts as a switch to regulate other downstream signaling events. In this case, neural activity essentially converts neurons from one state to another. An example of this is the ability to delay the onset of the critical period for plasticity by dark-rearing animals. Even brief visual experience can activate critical period plasticity in such animals. In contrast, an instructive role for activity is where the specific levels or patterns of neuronal firing carry information that allows different neurons to be distinguished from one another exclusively on the basis of these activity patterns. It is often difficult to prove unambiguously that activity plays an instructive role in a system because the simplest experiments involving blocking activity cannot distinguish between instructive and permissive roles. Nonetheless, there is compelling evidence from experiments in which activity patterns but not levels are altered, such as the ocular dominance shift in strabismic animals or the requirement for retinal waves of activity in the refinement of the retinocollicular map, that activity can also play an instructive role.

Activity in the developing visual system is not limited to that driven by visual experience. Throughout the visual system, in the thalamus, colliculus, and cortex, visual experience appears to be important in the maintenance of functional maps, but early development in these structures actually precedes vision. In this latter case, internally generated patterns of spontaneous activity play a key role in circuit refinement.

Sources of Activity in the Developing Visual System

In mammals, retinal ganglion cell (RGC) axons reach their targets before mammals' eyes open, and even before they have functional photoreceptors. In these early stages of development, in the absence of light responses, retinas are spontaneously active. These early activity patterns are called 'retinal waves' because they propagate across the ganglion cell layer, correlating the firing of tens to hundreds of RGCs. The synaptic circuits that mediate retinal waves are transient, with retinal waves disappearing as light responses are first developing. In contrast, in lower vertebrates, such as turtles, frogs, and chicks, there is an extended time during which waves and visual responses overlap. The synaptic mechanisms underlying retinal waves are described elsewhere in this encyclopedia.

An interesting feature of retinal waves is that they coincide with the period of development when visual responses are first detected in the retina. In mice, light-evoked responses have been detected as early as P10, which is 3 or 4 days before eye opening. In ferrets, which are born at approximately the same developmental stage as mice but have an elongated developmental period lasting 4 weeks until eye opening, light-driven responses are detectable in the dorsal lateral geniculate nucleus of the thalamus (dLGN) and visual cortex as much as 14 days before eye opening. Several experiments in mice, rats, and ferrets indicate that both spontaneous and light-evoked activity are detected in visual cortex before eye opening, indicating that both may influence developmental events. Visual deprivation by dark rearing, even when the eyelids are closed, alters the refinement of circuits within the retina and dLGN. Similarly, dark rearing and/or pharmacological manipulations of spontaneous activity have distinct influences on the development of RGCs in turtle retina, which have an extended period of light-evoked activity and retinal waves.

Once the eyes open, vision improves quickly, as determined by several measures. There is an immediate and steady increase in acuity and contrast sensitivity and a more gradual increase in spectral sensitivity. Neurons tuned to several features of the visual scene can be detected at eye opening, including ocular dominance and orientation.

Another source of neural activity that may be critically important for development is the activity patterns that are intrinsic to local circuits. Even in the absence of sensory input, there is a tremendous amount of spontaneous activity, some of which can be highly patterned. For example, spindle waves, which are fast oscillations in the cortical field potential generated by thalamocortical circuits, persist when the eyes are removed.

Retinotopic Maps in Tectum/Superior Colliculus

The two primary targets of RGCs in the brain are the superior colliculus (SC) and the LGN of the thalamus. In these targets, RGCs establish an arrangement of connections in target fields, termed a retinotopic map, that reflects the spatial arrangement of the RGCs in the retina, and eye-specific maps with inputs from the two retinas layering in neighboring but nonoverlapping regions.

The precise retinotopic and eye-specific targeting of RGCs axons observed in the adult emerges from initially diffuse and overlapping projections, prior to visual experience. There is a clear role for both neural activity and molecular factors, such as the ephrins and their corresponding receptors, for the establishment of these maps, although the relative importance of the two throughout the process of axon targeting and refinement is the subject of ongoing research. This article reviews the evidence that activity plays a role in the establishment of retinotopic maps.

The degree of retinotopic mapping can be assayed by different techniques. Most studies have relied on small focal injections of anterograde tracers (e.g., DiI) into the retina to visualize the axonal arbors in the SC/tectum, which is referred to as the termination zone. In addition, retinotopic maps have also been assayed by the spatial distribution of RGCs that are labeled by focal injections of retrograde tracers into the SC. Third, physiological measures in *in vitro* slices containing the optic tract and SC can assay the number of functional retinal inputs onto individual SC neurons. Last, *in vivo* physiological measurements of receptive sizes of SC/tectum neurons reveal the physiological consequences of topographic refinement.

The first preparations used for establishing a role for activity in retinotopic map formation were frogs and fish. These species have two advantages. First, topographic refinement occurs throughout life. The retina is constantly adding new cells at its periphery, whereas the tectum grows from the caudal end. Consequently, the retinal projections must constantly shift in order to maintain a retinotopic map. Second, in these species, RGC axons regenerate after injury, and hence maps can be studied while reforming in this more adult stage. Blockade of activity during either development or regeneration does not affect the course topography of projections but does profoundly affect the development of fine topography – the projections that mediate the fine point-to-point connectivity between RGCs and tectal neurons. These classic experiments led to the generation of a major dogma in developmental neuroscience that molecular cues mediate the development of course maps, whereas activity is important for the establishment of fine topography.

There has been growing evidence for activity also playing a role in the refinement of maps in mammalian systems. One fundamental difference between refinement in mammals and in frogs and fish is the location of axon branches that undergo refinement. In frogs and fish, refinement is mediated by small-scale changes in higher order axon branching emerging from the tip of the RGC axon. In contrast, during the development of retinocollicular maps in mammals (and similar to retinotectal maps in chicks), RGC axons overshoot their targets in the A–P axis. Branching in the appropriate retinotopic location occurs along the RGC axonal shaft, at sites anterior to the growth cone. Then, in what appears to be a distinct process, the overshooting axon and, in some cases, entire axonal branches are eliminated.

Pharmacological blockade in mammals leads to small, although significant, effects on the final level of retinotopy. To demonstrate a role for correlated retinal activity, mice that lack β_2-containing nicotinic acetylcholine receptors (nAChRs), which exhibit a pattern of retinal activity in which RGCs spike in a seemingly random pattern with little correlation between the spike trains of neighboring RGCs, have been examined. β_2-nAChR−/− mice exhibit less retinotopic refinement than wild-type mice. The absence of retinal waves in β_2-nAChR−/− mice is correlated with the irregular refinement of retinotopic maps despite the presence of approximately normal levels of activity in individual RGCs. Similar results were obtained with intraocular injections of nAChR antagonists, indicating that disruption of retinal waves can prevent the retinotopic refinement of retinocollicular projections.

Eye-Specific Maps in the Lateral Geniculate Nucleus

RGC axons project to the dLGN of the thalamus terminating in regions that are organized topographically and are segregated into eye-specific layers (i.e.,

projections from one eye end in regions spatially distinct from those of the other eye). When RGC projections from the eyes first grow into the dLGN, they are partially intermixed. The eye-specific layers then emerge gradually as the termination fields of the eyes segregate into regions containing either ipsilateral or contralateral retinal projections. This process is known to be activity dependent since intracranial infusion of TTX, a blocker of voltage-activated sodium channels, prevents segregation. Moreover, experiments have revealed that the activity driving this segregation comes from the retina since prolonged desynchronization of spontaneous retinal activity by parmacological disruption of nAChR activation in the eye also prevents layer formation. Blocking spontaneous activity in a single eye also significantly alters the distribution of RGC axons, indicating that competition from the two eyes is critical for the formation of eye-specific layers.

A wide array of transgenic mice and pharmacological manipulations have been used to gain insights into the mechanisms that mediate the formation of eye-specific layers. A few studies have taken advantage of the fact that the cellular basis of retinal waves switches from one mediated by nAChR to one mediated by ionotropic glutamate receptors to transiently block retinal waves. If nAChR-mediated retinal waves are eliminated during the initial period of refinement, either by pharmacological manipulation or by using β_2-nAChr−/− mice, eye-specific layers fail to form. However, even in the absence of the initial establishment of layers, RGC axons segregate into local eye-specific regions, with ipsilaterally regions segregated into small islands within the contralateral region. Thus, axons segregate without forming distinct eye-specific layers, indicating that eye-specific segregation and layer formation are separable processes that may occur through different mechanisms.

Retinal activity is essential not only for the establishment but also for the maintenance of eye-specific layers. In ferrets, intraocular injections of APB block glutamate-mediated waves after layers have been established and it has been shown to result in desegregation. In no-b-wave (*nob*) mice, RGCs fire in very frequent synchronous bursts that desegregate after layers have been established.

Whether retinal waves provide an instructive or permissive signal for driving eye-specific segregation is controversial. β_2-nAChR−/− mice do not form eye-specific layers, but pharmacological and genetic manipulations that significantly disrupt nearest neighbor correlations by increasing the uncorrelated firing of RGCs do not prevent layers from forming. The resolution of this controversy may rely on gaining insights into what aspect of the highly correlated activity is critical or driving refinement. Information required for activity-dependent segregation might be encoded in the slow periodic firing generated in individual neurons by waves. These periodic bursts of action potentials lead to substantial increases of intracellular calcium concentration in the participating neurons. There is growing evidence that periodic changes in intracellular calcium occurring on the order of minutes can profoundly influence a variety of intracellular processes. Thus, the periodicity of circuit activation may be tuned to the periodicity of intracellular signaling required to ensure the normal maturation of neurons in the retina or the segregation of retinal inputs in the dLGN.

Similarly, important information might be encoded in the spatial pattern of the activity. Synchronous activation of cells contains no distinct spatial information regarding the relative positions of cells involved in each event. However, the propagating activity seen in the retina synchronizes the activity of subsets of cells, thereby encoding their relative positions. A single retinal wave synchronizes firing of cells along a wavefront with a particular orientation on the retina, generating an activity pattern that might be used to establish orientation selectivity in visual cortical neurons. Activity patterns averaged over a large number of waves would lose orientation information but would maintain highly correlated firing among neighboring neurons, thus providing information that might be used to establish topographic projections.

The resolution of the question of whether the retinal waves are instructive or permissive for map refinement will rely on better targeted disruptions of spontaneous retinal activity based on a deeper understanding of the cellular mechanisms underlying plasticity.

Ocular Dominance Column Formation and Plasticity

The segregation of retinal inputs from each eye into eye-specific layers in the dLGN sets the stage for further segregation of eye-specific inputs in the thalamocortical projection to primary visual cortex. Transneuronal labeling studies in which radioactive amino acid or other anterograde neuronal traces that can jump synapses such as wheat germ agglutinin–horseradish peroxidase (WGA-HRP) are injected into one eye reveal a high degree of segregation of thalamocortical afferents into ocular dominance columns (ODCs) in layer 4 of the visual cortex of carnivores and certain primates, including humans.

Note, however, that eye-specific segregation is not evident in the visual cortices of all mammalian species: mice, rats, and even highly visual animals such as squirrels lack ODCs. Regardless of whether a species has segregated ODCs in the binocular zone of its visual cortex, binocular responsiveness (along with orientation and direction selectivity) of neurons is an important emergent property of the cortex not found at earlier levels of the visual system in normal adult animals.

This binocularity has proven to be a remarkably useful tool for studying cortical developmental plasticity. The pioneering work of Hubel and Wiesel revealed that deprivation of visual information through one eye by eyelid suture or image defocusing, known as monocular deprivation (MD), results in a dramatic shift of the responsiveness of cortical neurons to favor the nondeprived eye. These changes in the response properties of individual neurons are accompanied in most cases by a corresponding loss of visual acuity through the deprived eye, or amblyopia. The ocular dominance (OD) shift in response to MD is particularly powerful during a limited critical period in development, although evidence suggests that some degree of shift is possible even in adults. During the critical period, MD for as little as 1 day leads initially to a reduction of responsiveness to the deprived eye followed by an enhancement of the response driven by the nondeprived eye.

These changes are stronger and more rapid in extragranular (outside layer 4) layers of visual cortex, suggesting that plasticity of local cortical circuitry may guide the process. Nonetheless, the MD shift does propagate to cells in layer 4 and ultimately back to the dLGN. With less than 1 week of MD in the cat, the axonal arbors of thalamocortical neurons representing the deprived eye shrink, whereas those representing the nondeprived eye expand. Bulk transneuronal labeling from the eyes, as well as physiological assays, also reveals a gross shrinkage of deprived eye ODCs and a corresponding expansion of nondeprived eye columns. This propagation of OD plasticity back to successively earlier stages of the visual processing stream suggests the existence of retrograde messengers. This is further supported by numerous experiments in which blockade of spiking activity or of synaptic transmission through *N*-methyl-D-aspartate (NMDA) receptors in cortical neurons results in a disruption of the OD plasticity of thalamic afferents.

In addition to MD, misalignment of the eyes, or strabismus, during the critical period can also lead to a shift in the distribution of OD responses of cortical neurons, including the selective loss of binocular responses accompanied by a sharpening of ODC borders. This observation, together with the fact that comparable periods of binocular deprivation do not result in significant loss of visual responsiveness, argues that the equilibrium of inputs representing the two eyes is the result of a developmental competitive process.

This raises the question of whether a competitive process such as ocular dominance plasticity could be responsible for the initial segregation of ODCs. This question remains unresolved. Computer simulations, as well as the finding that RGC axons spontaneously segregate in an activity-dependent manner into ODC-like stripes in the optic tectum of fish and amphibia, indicate that the information contained in the spontaneous firing of retinal ganglion cells should be sufficient in principle to drive segregation into ODCs without the need for a molecular scaffold. On the other hand, ODCs are evident soon after thalamocortical innervation prior to the onset of the critical period for MD effects, and they do not appear to be disrupted by monocular enucleation at this early time. The presence of spontaneous activity within the already segregated dLGN at this stage, however, does not permit activity-dependent segregation of thalamocortical inputs to be ruled out at this point.

Molecular Mechanisms of Plasticity

It has become increasingly clear that developmental plasticity in the visual system is not mediated by a single mechanism. For example, in the dLGN of the ferret, segregation of eye-specific layers does not appear to require NMDA receptor (NMDAR) activation, whereas the segregation of inputs from on-center and off-center RGCs into sublaminae in the dLGN is prevented by application of NMDAR antagonists. In the visual cortex, NMDARs appear to play a key role in ocular dominance plasticity because pharmacological blockade or genetic knock-down of cortical NMDARs prevents the shift of OD in response to MD. The fact that current through NMDARs in response to presynaptic glutamate release is blocked by Mg^{2+} ions except when relieved by concurrent depolarization of the postsynaptic neuron allows NMDARs to serve as molecular detectors of correlated pre- and postsynaptic firing. Consistent with the role of NMDARs in the induction of synaptic plasticity, mutant mice deficient in the alpha isoform of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) or the serine/threonine phosphatase calcineurin, required for NMDAR-mediated long-term potentiation and long-term depression, respectively, lack normal OD plasticity.

Long-lasting neuronal plasticity generally requires protein synthesis. This is also true for OD plasticity

because application of cyclohexamide to visual cortex (but not LGN) to inhibit protein synthesis prevents the OD shift. The identities of the gene products required for OD plasticity have not been revealed. However, at least two key regulators of gene transcription, the extracellular signal-related kinase (ERK) and cyclic adenosine monophosphate response element-binding (CREB) transcription factors, which have both been implicated in long-term synaptic plasticity, are required to produce an OD shift. These protein synthesis-dependent pathways may be important for long-lasting structural plasticity, such as axonal arbor and dendritic spine remodeling in visual cortex.

Structural plasticity involves both the assembly of new connections and the dismantling of existing connections. Existing connections may be stabilized by interactions with the extracellular matrix and through cell–cell adhesion and signaling. Consistent with this model, activity of the serine protease tissue plasminogen activator has been shown to facilitate the OD shift during the critical period, leading to dendritic spine remodeling. After the critical period, a large degree of plasticity can be restored under conditions that reduce signaling by outgrowth inhibitory molecules such as chondroitin sulfate proteoglycans or the myelin inhibitor receptor NogoR.

These myriad molecular signaling cascades all nonetheless share a requirement for discriminable differences between the patterned neural activity in the two eyes. The ability of cortical neurons to detect these differences appears to rely critically on the balance between excitation and inhibition in the circuit. Evidence points to the developmental maturation of inhibitory circuitry, GABAergic basket cells in particular, as a key event for initiating the critical period for OD plasticity. The critical period in mice is opened precociously by augmenting the immature endogenous inhibitory circuitry with administration of the $GABA_A$ receptor partial agonist diazepam. The excitatory–inhibitory balance may be regulated in part by the activity-regulated expression of brain-derived neurotrophic factor (BDNF). Such activity-dependent control of the susceptibility to undergo plastic changes, known as 'meta-plasticity,' is a prediction of the influential Bienenstock–Cooper–Munro (BCM) model for neuronal plasticity. The BCM model posits that activity levels determine a sliding threshold of input strengths required for synaptic modification, above which synaptic strengthening occurs and below which synapses are weakened. Alternatively, an important role for inhibition may be to help sharpen the temporal precision of firing of postsynaptic neurons in response to sensory inputs. Spike timing-dependent plasticity, in which synaptic changes depend critically on whether a postsynaptic neuron fires before or after its presynaptic partner, may be facilitated by maintenance of an appropriate excitatory–inhibitory balance.

The increasing availability of useful transgenic mouse models for the study of activity-dependent developmental plasticity ensures that many more candidate genes will be found to participate in this process.

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NMDA Receptors and Development

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Developmental Expression of Diverse NMDAR Subunits

The *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) are tetrameric ion channels that are ligand-gated by glutamate and glycine and voltage-gated by postsynaptic depolarization. These properties make it an excellent coincidence detector of simultaneous presynaptic glutamate release and postsynaptic depolarization. The main pore-forming units of the NMDAR convey on it different kinetic and conducting properties that are changed through a maturational program of gene expression and synaptic activity. The channel pore is cation-selective and highly permeable to calcium, an important second messenger and the most intensely studied player in investigations of NMDAR function. Compared to other glutamate receptors, the time course of NMDAR activation and deactivation is relatively slow. These properties make the receptor a poor candidate for mediating high-frequency spike transmission but an excellent candidate for mediating long-term structural changes in response to the recent activity history of inputs to particular synapses.

Most NMDARs contain two obligate NR1 subunits, and any two of the four NR2 subunits, NR2A–2D. There are up to eight known splice variants of the NR1 subunit that are expressed heterogeneously in the brain and during development; however, virtually every brain region expresses at least one NR1 subunit by E19. Genes encoding the NR2 family of subunits can be regulated by a developmental program, activity, or both. Although detailed expression patterns of individual subunits vary greatly, consistent patterns have emerged (**Figure 1**). In most regions, NR2B and NR2D are expressed at highest levels in the embryo and early postnatal period, and they yield receptor currents with slow deactivation kinetics (ratios of deactivation time constants *in vitro* for NR2A : 2B : 2C : 2D are 1 : 3 : 3 : 40). NR2B expression usually persists in the adult brain, although at somewhat lower levels, whereas NR2D subunits are virtually eliminated. NR2A and NR2C are present in mature receptors, with NR2C expression largely confined to the cerebellum and thalamus.

The fact that immature NMDARs in most parts of the brain have much slower deactivation kinetics than mature NMDARs is thought to have important implications for receptor function in the young brain, where lower frequency activity patterns predominate. These patterns tend to correlate pre- and postsynaptic firing on a slow timescale (hundreds of milliseconds). Thus, a receptor with a correspondingly slow deactivation permits coincidence detection even at low frequencies. Additional mechanisms appear to exist in the immature brain to facilitate NMDAR activation because immature receptors in some regions lack voltage-dependent gating *in vivo*, a property that may arise from the presence of NR2D subunits. This could potentially eliminate coincidence detection by the NMDAR, and could contribute to the different effects of NMDAR activation observed in the immature brain, compared to the mature brain. Faster kinetics of receptors containing NR2A subunits could allow tighter temporal control in the detection of coincident activity in mature animals, where high-frequency activity predominates.

Two unusual, recently cloned subunits, NR3A and NR3B, are thought to be important in dampening NR2-containing NMDAR currents. They inhibit channel opening and calcium flux in response to glutamate when co-assembled with NR2 subunits both *in vitro* and *in vivo*. Whereas NR3A expression is widespread, NR3B subunits are restricted to motor nuclei of the brain stem and spinal cord.

In these regions and others with high fidelity, nonplastic synapses, NR2 and NR3A subunits are embryonically expressed and postnatally downregulated, often to undetectable levels. As they disappear, inhibitory NR3B subunits appear and persist into adulthood, accompanying the effective loss of detectable NMDA currents in these structures. This downregulation over development may be critical for the prevention of activity-dependent rearrangements in critical hard-wired circuits, but it does not preclude the possibility that the more plastic, immature forms of the receptor can return when circuit conditions are changed by trauma, disease, pharmacology, or behavior. Thus, the impetus for biomedical scientists to study the mechanisms of NMDAR regulation and the pathways it uses to effect synaptic change remains strong.

NMDARs in Early Brain Development

NMDARs have diverse but poorly understood roles in the immature brain. At embryonic stages, NMDAR

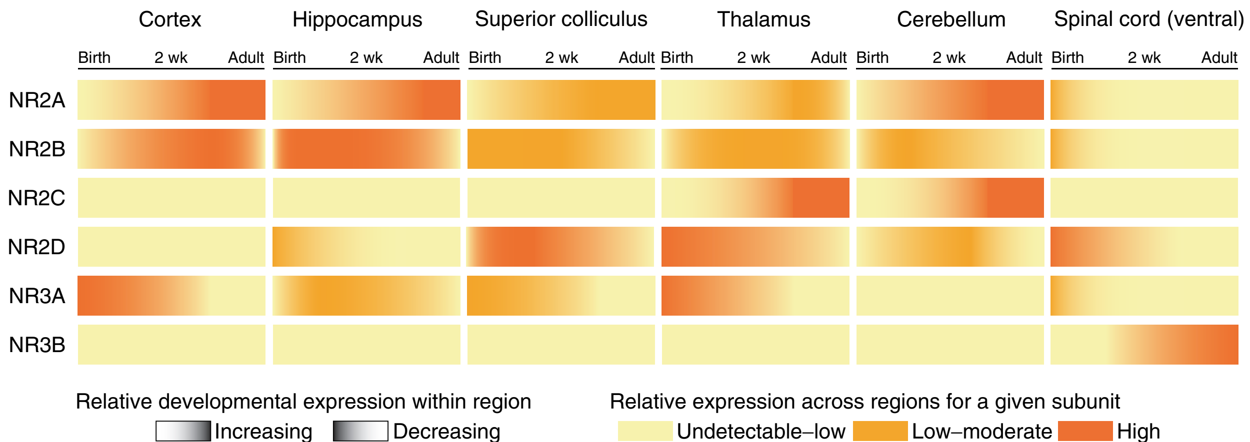

Figure 1 Developmental patterns of NMDAR subunit expression in the rodent brain. Summary of approximate expression levels of each of the known NMDA receptor subunits NR2A–2D and newly cloned subunits NR3A and NR3B over postnatal development. Where possible data from quantitative protein expression studies were used. Note the similar expression patterns of NR2B, NR2D, and NR3A, expressed early and downregulated in approximately the second postnatal week, and NR2A, NR2C, and NR3B, which appear after the first postnatal week. However, there is significant heterogeneity across different brain regions and even within brain regions, where subunits can be expressed in a laminar- and cell-specific manner. In addition, detection methods with lower sensitivity and spatial resolution (e.g., western blotting and histoblots) may not detect weak or sparsely expressed subunits in a given region.Data compiled from Dunah AW, Yasuda RP, Wang Y, et al. (1996) Regional and ontogenic expression of the NMDA receptor subunit NR2D protein in rat brain using a subunit-specific antibody. *Journal of Neurochemistry* 67: 2335–2345; Fagiolini M, Katagiri H, Miyamoto H, et al. (2003) Separable features of visual cortical plasticity revealed by *N*-methyl-D-aspartate receptor 2A signaling. *Proceedings of the National Academy of Sciences of the United States of America* 100: 2854–2859; Fukaya M, Hayashi Y, and Watanabe M (2005) NR2 to NR3B subunit switchover of NMDA receptors in early postnatal motoneurons. *European Journal of Neuroscience* 21: 1432–1436; Monyer H, Burnashev N, Laurie DJ, Sakmann B, and Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12: 529–540; Nishi M, Hinds H, Lu H, Kawata M, and Hayashi Y (2001) Motoneuron-specific expression of NR3B, a novel NMDA-type glutamate receptor subunit that works in a dominant-negative manner. *Journal of Neuroscience* 21: RC185; Quinlan EM, Olstein DH, and Bear MF (1999) Bidirectional, experience-dependent regulation of *N*-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. *Proceedings of the National Academy of Sciences of the United States of America* 96: 12876–12880; Sans N, Petralia RS, Wang Y, Blahos J, Hell JW, and Wenthold RJ (2000) A developmental change in NMDA receptor-associated proteins at hippocampal synapses. *Journal of Neuroscience* 20: 1260–1271; Shi J, Aamodt SM, and Constantine-Paton M (1997) Temporal correlations between functional and molecular changes in NMDA receptors and GABA neurotransmission in the superior colliculus. *Journal of Neuroscience* 17: 6264–6276; Sucher NJ, Akbarian S, Chi CL, et al. (1995) Developmental and regional expression pattern of a novel NMDA receptor-like subunit (NMDAR-L) in the rodent brain. *Journal of Neuroscience* 15: 6509–6520; Wang Y, Bosy TZ, Yasuda RP, et al. (1995) Characteristics of NMDA receptor subunit-specific antibodies: Distribution of MR2A and MR2B receptor subunits in rat brain and ontogenetic profile in the cerebellum. *Journal of Neurochemistry* 65: 176–183; Wenzel A, Villa M, Mohler H, and Benke D (1996) Developmental and regional expression of NMDA receptor subtypes containing the NR2D subunit in rat brain. *Journal of Neurochemistry* 66: 1240–1248; Wenzel A, Fritschy JM, Mohler H, and Benke D (1997). NMDA receptor heterogeneity during postnatal development of the rat brain: Differential expression of the NR2A, NR2B, and NR2C subunit proteins. *Journal of Neurochemistry* 68: 469–478; Wong H, Liu X, Matos MF, et al. (2002) Temporal and regional expression of NMDA receptor subunit NR3A in the mammalian brain. *Journal of Comparative Neurology* 450: 303–317.

function is probably independent of synaptic communication because most glutamatergic synapses form after birth. It is possible that in embryonic structures, ambient glutamate is secreted from glial processes or immature axons in a paracrine manner, and this is how early NMDARs are activated.

Migration

NMDAR activation stimulates neuronal migration in vertebrate embryos. Functional NMDARs are expressed on neurons in the embryonic cortical plate (prior to synapse formation) and on some tangentially migrating neurons during the formation of cortical layers. NMDA agonist stimulates chemotaxis in cultured embryonic cortical neurons. Cerebellar granule cells express functional NMDARs before they have begun migration through the molecular layer and prior to synapse formation with mossy fibers. The migratory movements of these cells are slowed up to 50% by NMDAR antagonists and are not affected by blockade of other glutamate receptors.

Differentiation

NMDARs are also involved in the tuning of local circuitry once the migrating neurons reach their targets. Prior to synapse formation, electrical and chemical signals among groups of neurons are coordinated by gap junction connections between them. In neonatal

mice, cortical neurons are spontaneously active and calcium responses are synchronized among groups of adjacent neurons. Mice deficient in NR2B, and hence most early NMDAR current, have fewer coupled groups of neurons in the cortex. Gap junctions have been investigated closely in the hypothalamus, where NMDAR-mediated activity downregulates the Connexin36 gap junction protein. Both NMDAR blockade and elimination of the NR1 gene extend the period of gap junction coupling in the developing hypothalamus; similar results have also been reported for spinal motor neurons. The data suggest NMDARs might be able to regulate the switch from gap junction-connected cells to synaptically connected circuits in multiple brain regions, and can either speed or slow the rate of that development depending on the region and, potentially, the form of the receptor complex that is expressed.

Sculpting Neural Circuits

After neurons have reached their final destinations and begun to differentiate, they extend axonal and dendritic processes and begin to form synapses. This early neurite extension and synaptogenesis proceeds in the absence of synaptic activity. However, subsequent neuronal survival and the refinement of axonal and dendritic connections are dependent on neuronal activation and synaptic release. Early activity is spontaneous, often in the form of slow bursts. Guidance cues initially set up connections between sensory organs and target structures, where incoming axons are organized in a topographical map. As the map is forming, grossly ectopic axons are eliminated, and competition between axons with different activity patterns within the same topographic location results in the further segregation of axons within the target tissue. With the onset of high-frequency sensory-driven stimulation comes the more precise organization of synapses between cells that enables high-resolution sensory detection in the young adult.

NMDARs are important during afferent development for both the removal of incorrectly positioned axons during the formation of topographically organized connections and for the later stages of input-specific segregation and refinement. NMDAR involvement in these activity-dependent processes has been most extensively studied by pharmacological blockade during the development of visual pathways and by targeted genetic manipulation in somatosensory pathways: vertebrate retino-recipient nuclei (the optic tectum of amphibians and the superior colliculus (SC) and dorsolateral geniculate nucleus (dLGN) of mammals), and somatosensory whisker afferent pathways in rodents.

Competition and Segregation – Visual Pathways

NMDAR involvement in the segregation of competing inputs was first described in the amphibian optic tectum. In a normal tadpole, retinal axons from each eye connect to the contralateral tectum, where they each form synapses with multiple tectal neurons (**Figure 2(a)**). Retinal axons are initially targeted to approximate anteroposterior topographic locations in the tectum using ephrin and Eph receptor

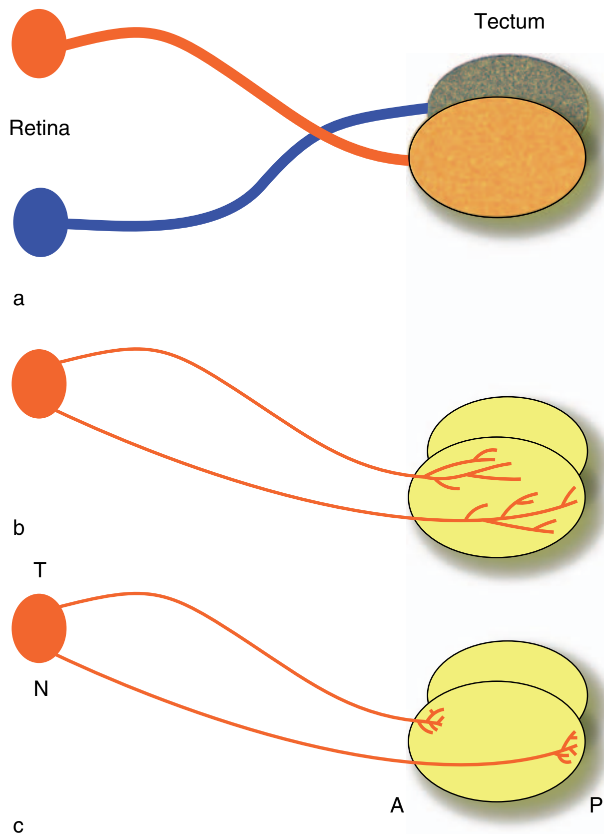

Figure 2 Development of the retinotectal pathway: (a) retinotectal pathway; (b) initial stage of topographical map formation; (c) final stage of topographical map formation. In (a), retinal ganglion cell axons from the retina of each eye travel to the brain as the optic nerve, cross to the other side of the brain at the optic chiasm, and make terminal arbors in the contralateral optic tectum of the brain. In (b), axons initially overshoot their target location in the tectum. Eph receptors expressed on retinal axons and ephrin ligands on postsynaptic membranes are required for this initial stage of map formation. Axons from the temporal side of the retina, destined for the anterior portion of the tectum, are repulsed from posterior tectum; axons from nasal retina are not as repulsed and terminate in posterior tectum. This process sets up a topographical map of the retina (and hence visual space) in the tectum. Ephrin-dependent cues must be present for the elimination of axon branches that are inappropriately topographically placed in the chick optic tectum, but do not affect the elaboration and refinement of branches in the appropriate location. In (c), eventually individual axonal branches become limited in both their mediolateral and anteroposterior extent.

guidance cues. They develop dense arbors in their target zone, through the maintenance of correctly placed branches and/or the elimination of ectopic ones (**Figure 2(b)**). In three-eyed tadpoles, produced by the embryonic addition of an extra eye primordium, retinal axons from two eyes innervate the same optic tectum, and the terminals of both sets of axons sort out from one another within a topographically appropriate location in a dynamic process called eye-specific segregation (**Figure 3**). Clusters of axons from the same regions of each retina have more similar activity patterns, and these axons sort within topographically appropriate tectal locations to optimally reproduce the point-to-point order of their cell bodies in each retina. In a tectum exposed to the NMDAR blocker AP5, axons from each eye desegregate and overlap within topographically appropriate regions (**Figure 4**). When AP5 is removed, the same axons resegregate.

Competitive NMDAR-dependent arbor rearrangement in the optic tectum is a form of the refinement process that keeps similarly active inputs together during normal development of many axonal projections within the brain. In the superior colliculus, NMDAR blockade disrupts the elimination of ectopic arbors during the formation of the retinocollicular map (**Figure 5**). Clearly, NMDARs are involved in the refinement of retinal axons, although they are not the sole mechanism by which the map refines. NMDARs perform a similarly modulatory function during retinal axon segregation in the developing lateral geniculate nucleus (LGN). In the ferret LGN, retinal axons sort within topographically appropriate regions according to their response properties to light (ON responds to light onset, OFF to light offset) within eye-specific laminae. Without NMDAR activity in the LGN during the refinement period, some axonal arbors spread across both laminae within an eye-specific layer, whereas others refine normally.

In vivo imaging of axonal dynamics during competitive arbor rearrangement provides some insight as to how this process might occur. Branches of a single axon growing into a tectal area dominated by the other eye are eliminated, and branches that grow

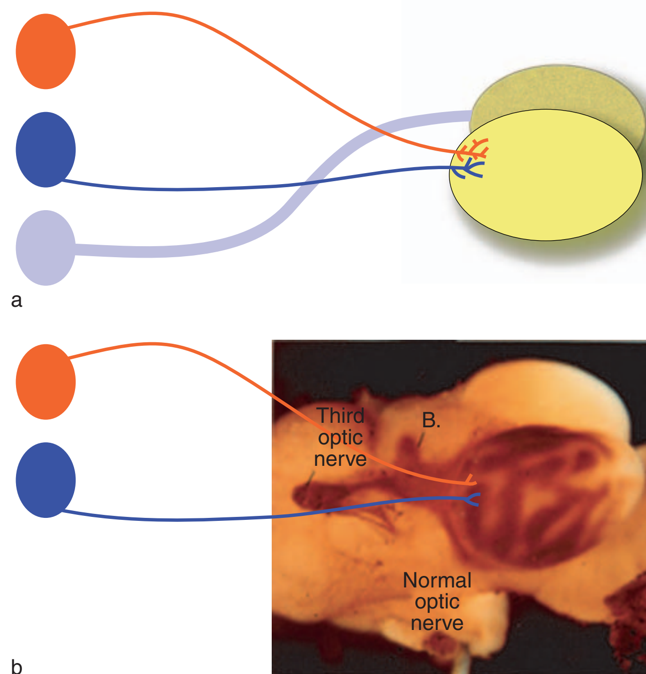

Figure 3 Eye-specific segregation in the optic tectum: (a) growth of axons when third eye is implanted; (b) pattern of axon terminals in the contralateral tectum. (a) When a third eye (dark blue) is implanted in the developing embryo, axons from this eye grow into one of the optic tecta at the same time as axons from the endogenous eye (red). Axons from both eyes undergo normal ephrin-dependent topographical refinement. Within a topographical location, however, individual axons sort out from one another, so that they occupy slightly different territory. In (b), when all the axons from one eye are labeled (here, the blue eye), the pattern of their axon terminals in the contralateral tectum can be observed. The image on the right is a whole mount showing the midbrain of a three-eyed frog in which one eye is labeled with the enzyme HRP, which is taken up and transported by retinal axons and catalyzes a colorimetric reaction in the tissue. Groups of axons from the two eyes segregate into dense stripes of clumped terminal arbors within appropriate topographic locations in the tectum. Retinal axon segregation can also be observed in the neuropil of Bellonci (B.), an amphibian structure most reminiscent of the dorsal lateral geniculate nucleus of mammals. Adapted from Constantine-Paton M and Law MI (1978) Anatomy and physiology of experimentally produced striped tecta. *Science* 202: 741–759, with permission from AAAS.

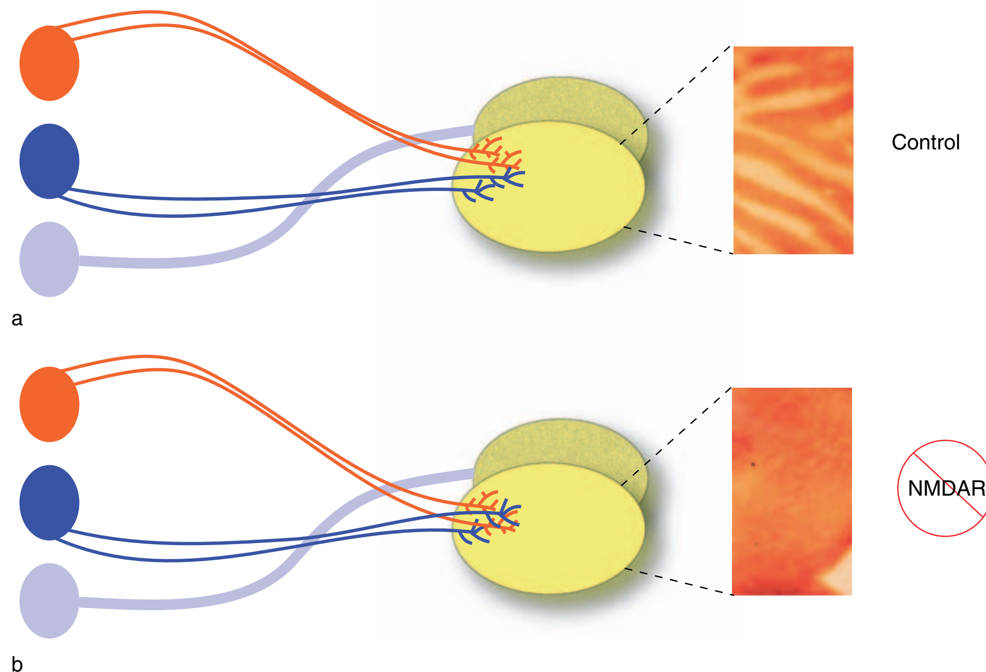

Figure 4 NMDAR-dependence of axon segregation in the optic tectum of a three-eyed frog: (a) control; (b) with NMDAR antagonist. In (a), eye-specific groups of axon terminals segregate into stripes in the three-eyed frog. In (b), when the NMDAR antagonist AP5 is applied chronically over the tectum, eye-specific axon terminals desegregate and are found in overlapping, although topographically appropriate, locations. The application of an agonist sharpens the stripe borders and the removal of the antagonist allows stripe recovery (not shown). NMDAR, *N*-methyl-D-aspartate receptor. Adapted from Cline HT, Debski EA, and Constantine-Paton M (1987) *N*-methyl-D-aspartate receptor antagonist desegregates eye-specific stripes. *Proceedings of the National Academy of Sciences of the United States of America* 84: 4342–4345.

into the area dominated by its own eye are elongated and stabilized. NMDAR blockade prevents the specific elimination of axon branches from areas dominated by the other eye (**Figure 6**). Thus, when an axon from a disparate retinal locus strays into a region where axons from a similar locus are concentrated, its inability to effectively stimulate tectal neurons results in its elimination. Clusters of axons from nearby retinal sites can cooperate to fire tectal neurons, and they continue to generate and stabilize axons in those areas in the absence of NMDAR activity. Thus, the specific elimination of these less efficient or incorrectly placed axons is NMDAR-dependent, after axons have already formed and elaborated using other (non-NMDAR) mechanisms.

The evidence suggesting that cooperative near-neighbor input activity is critical for activity-dependent refinement during development is strong. In the developing retina, slow cholinergic waves of activity sweep across local populations of ganglion cells and synchronize the firing of neighboring cells. Mice with a deletion of the $\beta 2$ nicotinic acetylcholine receptor subunit lack these spontaneous waves, and their ganglion cells fire in uncorrelated bursts. As a result, neighboring axons in the SC cannot cooperate to fire collicular neurons, and all axons are similarly inefficient at inducing their postsynaptic partners to fire. Retinal axons in the SC of these mice become abnormally dispersed across each collicular lobe. Postsynaptic glutamate receptors are probably involved in this refinement because retinal axons are glutamatergic; however, this has not been directly investigated.

NMDAR-induced synaptic weakening in the absence of correlated pre- and postsynaptic activity has been observed in the visual cortex at later stages of development. Axons of LGN neurons projecting to the cortex segregate into eye-specific domains early in the development of binocular animals, but remain plastic and sensitive to monocular deprivation by lid suture during a critical period. As a result, most cortical cells become responsive to the nondeprived eye, and responsiveness to the deprived eye is lost. When correlations between pre- and postsynaptic activity are disrupted by the suppression of postsynaptic action potentials, NMDAR-dependent depression of the connections between the open eye and the silenced postsynaptic neurons results. Thus, NMDAR-induced synapse weakening is an active process, in which high levels of synaptic activation are detrimental when the postsynaptic neuron cannot spike.

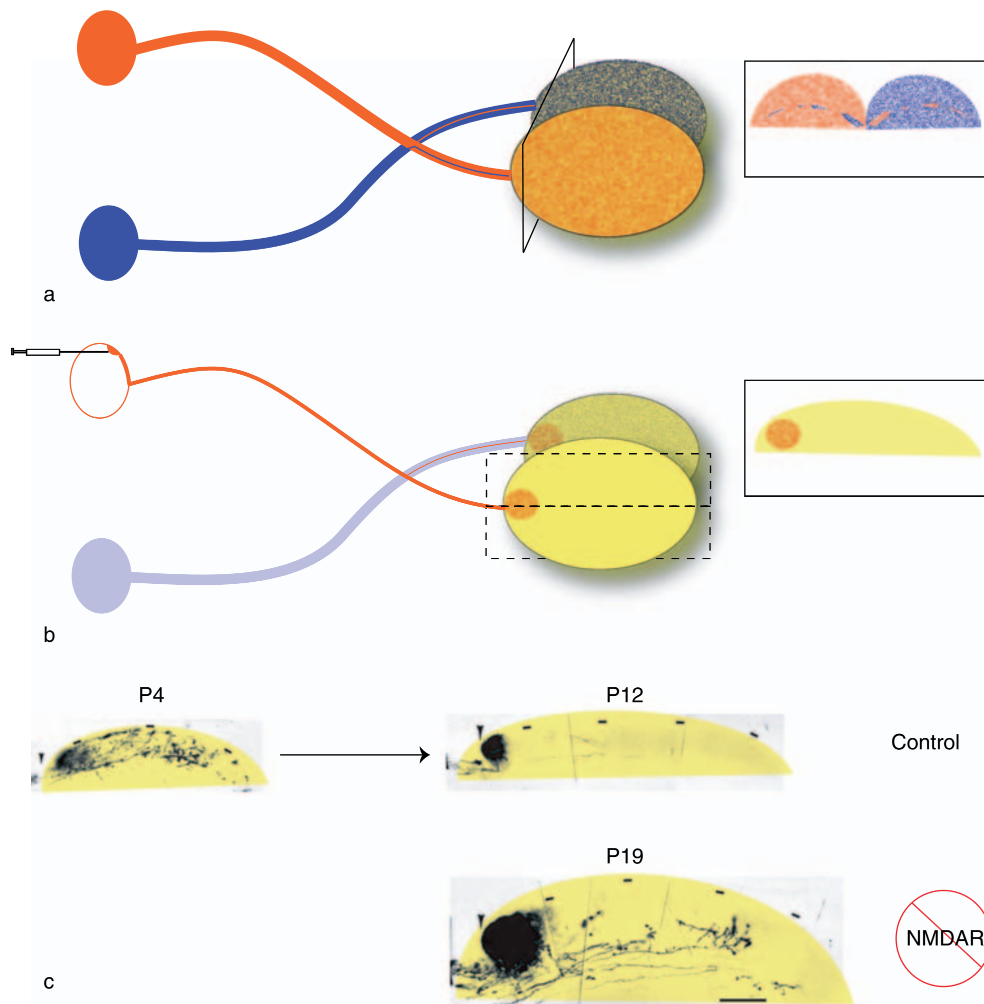

Figure 5 NMDAR-dependence of the elimination of ectopic axons in the mammalian superior colliculus (SC): (a) retinocollicular pathway; (b) dye labeling of a small population of axons; (c) in the control and with NMDAR antagonist. In (a), retinal axons project to the contralateral SC, with a minor sparse projection to the ipsilateral SC; the inset shows a coronal section of rat SC (only the superficial, retino-recipient layers of the SC are shown). In (b), dye is injected into a small portion of temporal retina labels retinal axons in the anterior SC; the inset shows a parasagittal section through the rat SC. In (c), dye injections are made at various times during the postnatal refinement of the topographical retinal map, which is largely complete by eye opening (postnatal day 13 (P13)). When NMDARs are blocked by chronic implantation of the NMDAR antagonist AP5 in a slow-release plastic implanted over the developing SC, some axons are found in inappropriate topographical locations, even at P19. The crude topography of most of the retinal axons is unaffected. NMDAR, *N*-methyl-D-aspartate receptor. Adapted from Simon DK, Prusky GT, O'Leary DD, and Constantine-Paton M (1992) *N*-methyl-D-aspartate receptor antagonists disrupt the formation of a mammalian neural map. *Proceedings of the National Academy of Sciences of the United States of America* 89: 10593–10597.

Competition and Segregation – Somatosensory Pathways

NMDARs are also crucial for the establishment of appropriate axonal and dendritic arbors in the developing somatosensory system, where genetic manipulations of the receptor have been applied. The patterning of rodent whisker-driven afferents in the pathways from the whisker pad to trigeminal nucleus to somatosensory thalamus to cortex have been extensively studied in genetically engineered mice with absent or reduced NMDAR activity. Mice lacking either the NR1 or the NR2B subunit of the NMDAR lack whisker-related patterns of sensory axons in the spinal trigeminal nucleus at birth. These mice die soon after birth from respiratory failure (NR1) and an inability to suckle (NR2B); thus, later stages of development cannot be studied. The addition of low levels of NMDARs to these mice with transgenesis permits their longer survival. In these NR1 knockdown mice, whisker patterns fail to form at all levels of the somatosensory pathway. They lack barrellettes in the trigeminal brain-stem nucleus, barreloids in the somatosensory thalamus, and whisker barrels in the somatosensory cortex (**Figure 7**).

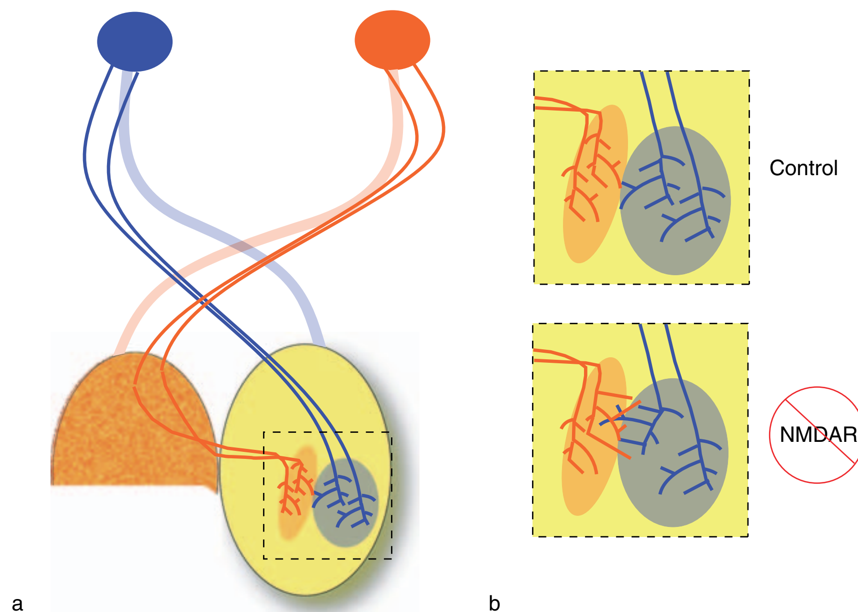

Figure 6 Ocular dominance: (a) partial lesion of one optic tectum inducing intereye competition; (b) NMDAR-dependence of the elimination of minority axons in areas dominated by other inputs. In (a), axons normally destined for a lesioned region of tectum (left) grow into the adjacent tectum and terminate in the appropriate topographical region. Ocular dominance patterns are set up by the two eyes in the doubly innervated region of the unlesioned tectum. In (b), the clear tadpole skull and proximity of the tectum at the top of the head make it possible to follow single axons over time in a living animal. Single axons were labeled with a fluorescent dye, and the pattern of branch additions and retractions over many hours was imaged by fluorescent microscopy. At the end of the experiment, whole retinal projections were labeled to determine the pattern of ocular dominance in the imaged tectum. This combined information revealed that axon branches are preferentially eliminated from territory dominated by the opposite eye. When the tadpole is raised with the NMDAR blocker in the growing media, axon branches are equally eliminated from same and opposite eye territories. These data are consistent with a role for the NMDAR in correlation-based branch elimination. NMDAR, *N*-methyl-D-aspartate receptor. (a) From Constantine-Paton M (1981) Induced ocular dominance zones in tectal cortex. In: Schmidt FO, Worden FG, and Denis SG (eds.) *The Organization of the Cerebral Cortex*, pp. 47–67. Cambridge, MA: MIT Press. Data from Ruthazer ES, Akerman CJ, and Cline HT (2003) Control of axon branch dynamics by correlated activity *in vivo. Science* 301: 66–70.

NR1 deletion restricted to excitatory cortical neurons has more subtle effects on cortical barrel patterns. In these mutant mice, gross whisker-related patterns form normally at all levels of the somatosensory pathway and there is a normal critical period for barrel fusion after one or several whiskers are removed. However, cortical barrel patterns are smaller and not well defined, suggesting later stages of activity-dependent refinement have been disrupted (**Figure** 7). Indeed, careful study of individual thalamocortical axonal afferents to the barrel shows that axonal arbors are not properly restricted to whisker boundaries (**Figure** 7). As in the visual system, NMDARs in the somatosensory pathways are required for the elimination of branches that are inappropriately positioned. In addition, in cortical barrels NMDAR activity also sculpts postsynaptic dendrite structure to orient dendrites within the appropriate target zone. In the cortex of the same mutant mouse, dendrites of cortical layer IV spiny stellate cells are improperly oriented and lack a normal inwardly polarized orientation with respect to the barrel walls (**Figure** 7).

Structural Refinement – Synapses

The exact mechanisms through which NMDAR stimulation is coupled to synaptic change and the eventual synaptic rearrangement that takes place are not yet determined. Disruption of NMDAR activity affects the development of excitatory synapses and dendritic spines, and also affects the overall axonal and dendritic structure. It is not known whether the proximal cause of these rearrangements is synaptic or extra-synaptic NMDAR activation or whether changes in synaptic strength that precede structural rearrangements are causal. However, in the immature brain there is growing evidence that the dominant effect of NMDAR activation is to weaken synapses, resulting in a net reduction of contacts and continued sprouting.

In developing pyramidal neurons of somatosensory cortex, refinement occurs via the elimination of dendritic spines, the sites of excitatory synapses on these neurons. Sensory deprivation slows this refinement in single neurons imaged over time *in vivo*, and NMDAR blockade mimics the effects of sensory

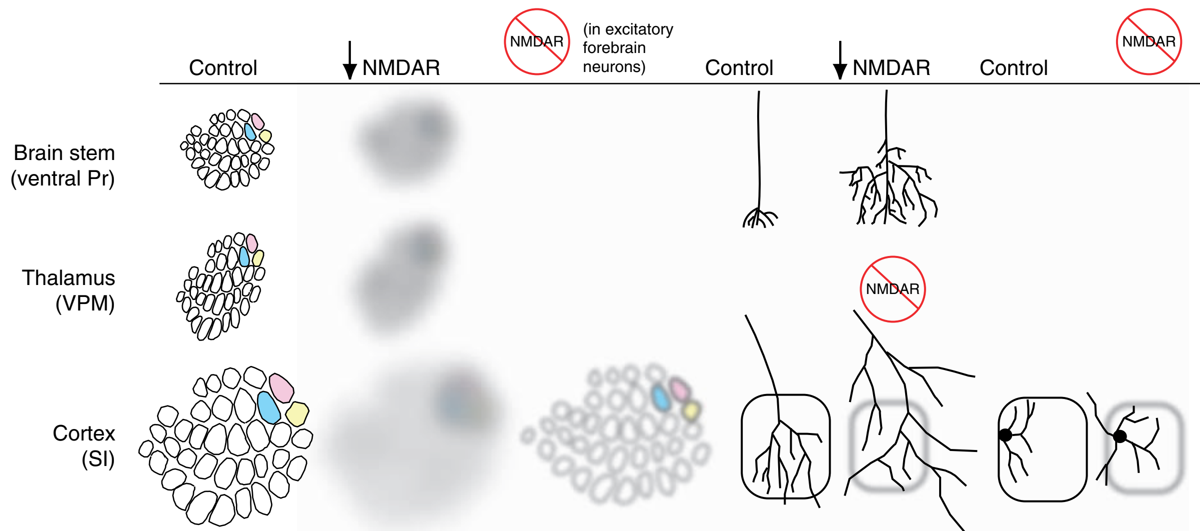

Figure 7 NMDARs as necessary for normal refinement of whisker afferents to somatosensory nuclei. Summary of generalized results from the genetic deletion of NMDARs in mutant mice. Three somatosensory nuclei are described: brain-stem nuclei (ventral Pr), thalamic nuclei (VPM), and somatosensory cortex (SI). Each barrel-shaped structure represents the organization of afferents from a single whisker. Barrels visualized with multiple anatomical methods yielded similar results, and a representative case has been drawn here. The Control column shows whisker maps in an adult, wild-type mouse, shown in similar orientation. There are sharp borders between adjacent whisker regions (first column), and whisker afferents to the brain stem have refined terminal arbors (fourth column). Thalamo cortical axons have terminal arbors that are contained within a single barrel structure in SI (fourth column), and cortical stellate cell dendrites are oriented toward the center of the barrel (sixth column). The ↓NMDAR column shows transgenic mice generated in a background strain containing an NR1 deletion, in which minimal NR1 expression has been restored by transgenesis. This permits survival beyond the first few days after birth (up to ~8 days). Whisker maps with distinct whisker regions are not detected in any somatosensory nuclei within a week after birth (second column). Reconstruction of single whisker afferent axons in the brain stem reveals that axonal terminals are larger than controls (fifth column). The knocked-out NMDAR column shows mice that had normal levels of NMDAR expression in the embryo and early postnatal life (columns three and seven). Postnatal deletion of NR1 that is restricted to excitatory forebrain neurons permits barrel formation in the SI of adult mice. However, the barrels are smaller than normal, and the borders are ill-defined (third column). Individual thalamocortical axons are not contained within the barrel walls (fifth column), and cortical stellate cell dendrites are not as polarized in orientation as cells from wild-type controls (seventh column). ↓, decreased; NMDAR, *N*-methyl-D-aspartate receptor; Pr, principal sensory nucleus of the trigeminal nerve; SI, primary somatosensory cortex; VPM, ventral posterior medial nucleus of the thalamus. Data from Iwasato T, Erzurumlu, RS, Huerta PT, et al. (1997) NMDA receptor-dependent refinement of somatotopic maps. *Neuron* 19: 1201–1210; Iwasato T, Datwani A, Wolf AM, et al. (2000) Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex. *Nature* 406: 726–731; Datwani A, Iwasato T, Itohara S, and Erzurumlu RS (2002) NMDA receptor-dependent pattern transfer from afferents to postsynaptic cells and dendritic differentiation in the barrel cortex. *Molecular and Cellular Neuroscience* 21: 477–492; Lee L, Iwasato T, Itohara S, and Erzurumlu RS (2005) Exuberant thalamocortical axon arborization in cortex-specific NMDAR1 knockout mice. *Journal of Comparative Neurology* 485: 280–292; Lee L, Lo F, and Erzurumlu RS (2005) NMDA receptor-dependent regulation of axonal and dendritic branching. *Journal of Neuroscience* 25: 2304–2311.

deprivation. Similarly, NMDAR blockade slows the elimination of spines from immature retinal ganglion cells. Thus, in retina and somatosensory cortex, NMDARs are required for developmental and sensory-dependent spine elimination.

Studies in the SC have found that NMDARs eliminate synapses and axonal branches only during a transient developmental period while retinal axons are refining and synaptic depression dominates. Chronic NMDAR blockade in the SC from birth increases the density of glutamatergic synapses and glutamate release sites on incoming retinal axons, until the middle of the second postnatal week (**Figure 8(c)**). NMDAR blockade has no net effect on synapse number or axonal sprouting in older colliculus, where there is balanced potentiation and depression, and the neuropil has a high synaptic density. Pushing the balance back toward depression with the application of an NMDA agonist pushes synapses toward functional depression in older colliculus and reduces the number of retino-collicular synapses (**Figure 8(c)**).

If, early in development, NMDAR-mediated synapse elimination is the primary mechanism by which axonal arbors retract, the expectation is that axonal branch elimination will follow synapse loss. Indeed, in the zebra fish and amphibian tecta, in the absence of a mature presynaptic site (identified as clusters of vesicle-release proteins), axon branches retract to the nearest synaptic site on the terminal, where they become stabilized. This is evidence that NMDAR-dependent

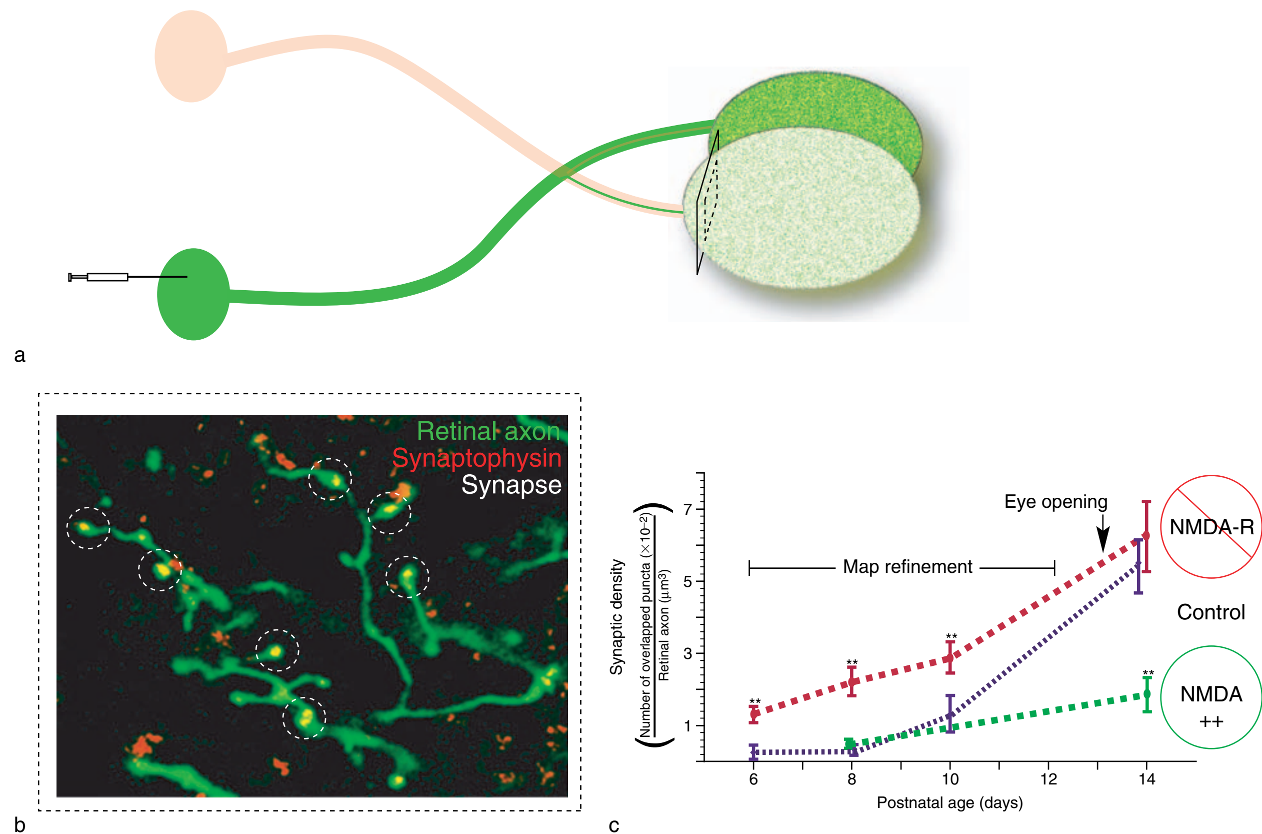

Figure 8 NMDA receptor activity eliminating synapses in the young SC: (a) dye labeling of contralateral and ipsilateral retinal axons; (b) identification of synapses on retinal axons; (c) NMDAR-dependence of synapse elimination during refinement. In (a), complete labeling of all retinal ganglion cells by intravitreally injected cholera toxin subunit B conjugated to fluorophores allows the visualization of both the ipsilateral and contralateral retinal projection from one eye. In (b), confocal microscopy and immunohistochemical localization of clustered presynaptic (or postsynaptic) markers and quantitative overlap analysis allows visualization of synapses along labeled retinal terminals. In accord with observations from electron microscopy, presynaptic release sites occur frequently at swellings or terminal bulges on the axon. In (c), the chronic blockade of NMDARs from birth increases the synaptic density along retinal axons during the first ~10 days. Synaptic density is already low at this time, and treatment with the agonist (NMDA) has no effect. By postnatal day (P)14, treatment with agonist reduces synaptic density and the antagonist ceases to have an effect. NMDAR, *N*-methyl-D-aspartate receptor; SC, superior colliculus. Adapted from Colonnese MT and Constantine-Paton M (2006) Developmental period for N-methyl-D-aspartate (NMDA) receptor-dependent synapse elimination correlated with visuotopic map refinement. *Journal of Comparative Neurology* 494: 738–7351.

sculpting of axon and dendrite structure may result from its proximal effect on synapse formation/elimination. Cytoskeletal organizing pathways involving Rho-family GTPases translate NMDAR activity into rearrangements of the cytoskeleton in dendritic processes; however, it is unknown whether this involves or is preceded by NMDAR-driven synaptic elimination.

Refinement of receptive fields and response properties

Given the importance of NMDA receptors in structural and synaptic refinement, modifications of receptive fields and response properties would also be expected when NMDAR activity is manipulated. Indeed, NMDAR activity is required for the compression of collicular receptive fields after a partial lesion limits the amount of target space available for retinal axon arborization. Similarly, enlarged receptive fields result when NMDARs are blocked during auditory map formation. The map of auditory space does not refine normally, and more neurons are broadly tuned. Finally, visual cortical neurons in NR2A knockout mice show dramatically reduced orientation preferences. Due to the complicated contribution of afferent innervation, dendritic structure, and local inhibition to receptive field responses, the locus and precise nature of these deficits are unknown. All are consistent, however, with a model in which NMDAR activity refines circuits by modifications of synaptic strength, followed by synaptic elimination or stabilization.

NMDAR Subunits and Developmental Long-Term Depression and Long-Term Potentiation

Near-simultaneous activation of pre- and postsynaptic neurons is believed to modify synaptic strength via NMDARs through mechanisms such as long-term potentiation (LTP), long-term depression (LTD), and spike-timing-dependent plasticity (STDP). These are important models of NMDAR function in the developing nervous system because nascent synapses are thought to be stabilized or eliminated by repeated synaptic potentiation or depression, respectively. STDP is a more specific form of LTP/LTD that also requires NMDARs. STDP causes potentiation if the postsynaptic cell responds within $\sim<40$ ms of a presynaptic stimulation (effective inputs), and causes depression if the postsynaptic cell responds between ~ 40 and 100 ms after stimulation (ineffective inputs). STDP can be induced by sensory stimuli, and may therefore represent the natural mechanism by which long-lasting synaptic changes are induced via NMDAR activation. However, in developing systems the effects of STDP can be rapidly reversed by spontaneous activity, indicating that a stabilizing process must occur before STDP permanently shapes connectivity.

NMDAR-driven synaptic modifications are bidirectional, in that either LTP or LTD of synaptic strength can be induced by receptor activation. LTD tends to be easier to induce in the young brain, and as the brain matures, the induction of LTP becomes possible. This switch from LTD to LTP induction correlates with the developmental progression of NMDAR subunit expression from slowly deactivating NR2B-rich receptors to mixed slowly and rapidly deactivating receptors and with other subunit-specific changes in the gating properties of these receptors that occur with development. Given this correlative evidence, it has been tempting to functionally link NR2B-rich receptor complexes to LTD and NR2A-rich complexes to LTP. At certain ages, a carefully controlled blockade of receptors containing NR2B eliminates hippocampal LTD, without affecting LTP. Mice lacking NR2A show a progressive deficit in LTP in the hippocampus with age that parallels the normal increases in hippocampal NR2A expression in wild-type mice.

However, there are many regions of the brain where tight correlations between LTD/NR2B and LTP/NR2A do not exist. It is unknown how mixed NR2A/NR2B receptors contribute to LTP and LTD. It seems reasonable that the dynamics of the calcium signal through these receptors may differ at different synaptic sites, due to the inherent subunit-specific differences, location, and association with different kinases and phosphatases that regulate calcium flux. Thus the NMDAR is likely sending mixed signals through the same synapses, and the net effect is a result of this diverse network of signals.

Changes in NMDAR subunit composition are also correlated with changes in the expression of scaffold proteins associated with the NMDAR at postsynaptic sites. These proteins may prove to be critically important in linking calcium influx through NMDARs to specific signaling pathways by tethering the necessary molecules close to the channel pore. In this way, both the electrophysiological responses of the NMDAR channel and the effector proteins it binds might interact to control the direction of synaptic strength changes during development.

A Developing NMDA Receptor Complex

Membrane-associated guanylate kinases (MAGUKs) are important components of the NMDAR protein complex in the postsynaptic density (PSD). Recent studies have begun to explore their contribution to the divergent functions of the NMDAR during synaptogenesis and developmental plasticity. These intracellular NMDAR-binding proteins contain multiple protein–protein interaction domains and bind to both scaffolding and signaling molecules at the PSD. In this way, NMDARs are connected to other transmembrane receptors in the PSD through a dynamic horizontal lattice of protein interactions. This complex links the receptor to the cytoskeleton, to receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), G-protein-coupled glutamate, and inositol triphosphate receptors (which release calcium from intracellular stores). Signaling molecules that drive cytoskeletal rearrangements and other calcium-dependent processes are also tethered near the NMDAR channel pore by MAGUKs.

Two MAGUKs in particular, synapse-associated protein (SAP-)102 and postsynaptic density protein (PSD-95, are differentially expressed during development. SAP-102 is the only MAGUK present at the young ages when immature NMDARs predominate. Like NR2B, SAP-102 expression does not disappear, but continues to be expressed in the adult, along with the mature MAGUK, PSD-95. They appear to have some specificity for NMDAR complexes containing NR2B or NR2A, respectively. PSD-95 has recently been shown to produce synapse potentiation by anchoring stargazin AMPA receptor (AMPAR) complexes in the PSD, and the activation of NMDARs containing NR2A have been reported to promote insertion of AMPARs containing GluR1 (a critical step in

LTP induction). With eye-opening in the developing visual pathway of rodents, PSD-95 is rapidly inserted into visual synapses, and synaptic potentiation and a second wave of synapse refinement follows soon after.

Calcium-sensitive proteins couple NMDAR activation to downstream transcription/translation, cytoskeletal rearrangements, changes in synaptic strength, and cell death pathways. The exact mechanism linking calcium influx through NMDARs with the activation of these pathways is not well defined. Guanine exchange factors (GEFs) and small GTPases are known to be downstream of NMDAR activation and to drive bidirectional changes in synaptic strength and cytoskeletal rearrangements. MAGUK scaffold proteins may be critically important in localizing these calcium-responsive proteins and their effector proteins to NMDARs, although some, such as Ras GTPase, are also independently targeted to membrane microdomains, and some, such as CaMKII-α, are able to bind the NMDAR directly. One GEF, RasGRF1, binds the NR2B subunit directly. Thus, subunit-specific MAGUK binding may not entirely account for the differential effects of NR2B versus NR2A activation. Existing data on subunit-specific protein interactions and developmental expression do suggest that entirely different NMDAR signaling complexes exist, depending on the subunit composition of the receptor and the proteins that are available to bind. Such distinctions are exceptionally difficult to establish with biochemistry and molecular biology alone because each synapse can contain a heterogeneous array of receptor-signaling complexes, but they may hold the key to the mechanisms of NMDAR function through development.

Summary

Data from the visual and somatosensory systems support the concept of the NMDAR as a causal effector of changes in synaptic strength and structural rearrangements during the activity-dependent development of sensory pathways. Mechanisms similar to LTP and LTD appear to be involved, where NMDARs detect the coincident activation of pre- and postsynaptic neurons and strengthen or weaken synapses based on this information. Early in development when immature subunits permit synaptic depression, NMDARs eliminate synapses. In many cases of NMDAR function during development, synaptic potentiation or depression precedes the structural reinforcement or withdrawal of connections. The subunit-specific interaction of NMDARs and scaffold proteins may direct changes in synaptic strength that underlie developmental refinement of neuronal connectivity and structure; however, this has not yet been directly examined. The heterogeneity of the receptor subunits and associated proteins permits a modifiable NMDAR complex that is exquisitely sensitive to changing patterns of activity in the developing brain.

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Optic Tectum: Development and Plasticity

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Introduction

The term 'optic tectum' is generally used in lower vertebrates to describe the homolog of the mammalian superior colliculus. In fish, amphibians, reptiles, and birds, it is represented by a single pair of dorsally situated dilatations in the mesencephalon, which are their principal visual structure in the brain. The optic tectum contains a visual map that is activated by its principal input, retinal axons. It responds through descending efferent pathways to brain stem and spinal motor areas giving a movement reaction to visual stimuli. At progressive levels of vertebrate phylogenetical transformation, the optic tectum becomes more structurally compartmentalized, giving rise in eutherian mammals to both the inferior and the superior colliculi, the latter maintaining more of the visual functions ascribed to the original optic tectum.

Nevertheless, as the cerebral hemispheres begin to play a more important role in the processing of neural information, the optic tectum is reduced in size relative to the rest of the brain. During the evolutionary process of 'telencephalization,' the cerebral hemispheres are thought to take over several of the visual tasks performed by the primitive optic tectum, and greatly extend the analyses and associative discrimination of visual stimuli. Although simplified, the human superior colliculus still preserves a laminar organization, receiving in its most superficial layers input from the retina and visual cortex, and in its deeper layers input from the auditory and somatic sensory systems. These different types of sensory information are arranged in three separate spatial maps, parallel to the surface of the colliculus, and integrated to direct eyes and head movements toward an external stimulus in a coordinated response. Functions of the superior colliculus also include aspects related to attention and pattern discrimination, working in closer interdependence with the geniculo-cortical system. Thus, in correlation with its physiological importance in the nonmammalian brain, the primal optic tectum shows a higher degree of anatomic complexity than its equivalent, the superior colliculus in the primates. Because of its behavioral relevance, and its convenience in the study of laminar neuronal organization, the optic tecta of fish and amphibians have been popular models in the elucidation of (1) axonal growth and targeting during development and regeneration of the central nervous system; (2) neuronal plasticity following injury; and (3) the neuroanatomic basis for visual-related behavior.

Cytoarchitecture

The optic tectum in lower vertebrates is composed of two prominent structures, which are symmetrically situated along the midline of the brain, and between the olfactory lobes and the diencephalons anteriorly, and the cerebellum posteriorly. It displays a lamination pattern made by a series of alternating layers of neural fibers and cells. Some variations exist in the histological organization of the optic tectum in different species. Here we will exemplify using the optic tectum of teleost fish, although most tecta follow a similar general plan. A commonly accepted nomenclature divides the optic tectum, from surface to depth, into the following: stratum marginale, stratum opticum, stratum fibrosum et griseum superficiale (SFGS), stratum griseum centrale (further subdivided into the inner plexiform layer and the inner gray layer), stratum album centrale, and stratum periventriculare. Each of these layers extends parallel to the surface throughout most of the optic tectum. Information from different regions in the visual field is integrated in different locations along the optic tectum's homogeneous preservation of laminar organization (**Figure 1**).

The stratum marginale is characteristic of fish and contains three elements: (1) the dendritic trees of pyramidal neurons (their somas are situated in the SFGS), which make synaptic boutons; (2) the marginal fibers originating in the torus longitudinalis, running parallel and laterally among glial elements; and (3) the stratum marginale ascending dendrites, which originate in neurons in the stratum griseum centrale.

The stratum opticum (SO; **Figure 1**), directly underlying the stratum marginale (SM; **Figure 1**), contains myelinated axons constituting part of the retinal afferents (contralateral), and large axons of tectal origin. These fibers make synaptic contacts with dendritic arbors from neurons whose somata are located in the stratum griseum centrale, the stratum album centrale, and the stratum periventriculare. They also synapse with dendrites of horizontal neurons whose cell bodies are located within the stratum opticum itself. Horizontal neurons show a spheroidal soma and dendrites that extend horizontally. The next deeper lamina is the stratum fibrosum et griseum superficiale, which is conformed by abundant myelinated

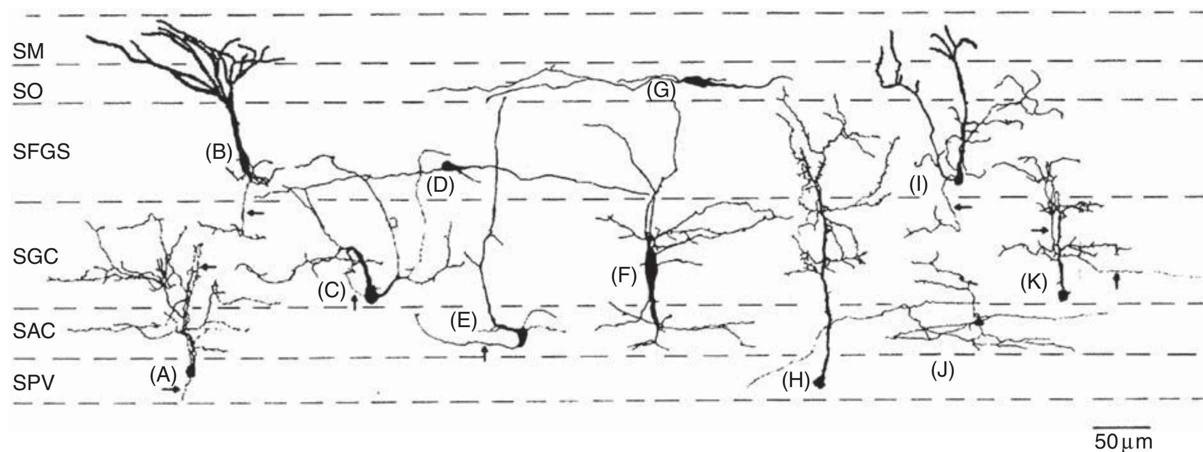

Figure 1 Composite diagram illustrating the principal neuronal types in different layers of the goldfish optic tectum. A, H, and K are pyriform neurons; B and I are pyramidal neurons; C, E, and J are nonoriented neurons; D and G are horizontal neurons; F is a fusiform neuron. SM, stratum marginale; SO, stratum opticum; SFGS, stratum fibrosum et griseum superficiale; SGC, stratum griseum centrale; SAC, stratum album centrale; SPV, stratum periventriculare.

axons and conspicuous cell bodies. Most of these axons are retinotectal afferents, whose terminal arbors synapse with dendrites extended by neurons located in deeper layers, and also with dendrites of pyramidal and bipolar neurons, located within this stratum. The pyramidal neurons are conspicuous in this layer. Horizontal neurons are present in this layer as well. The stratum griseum centrale (SGC; **Figure 1**) is divided into the outer plexiform layer and the inner gray layer. Most abundant in this lamina are the vertical dendritic arbors of neurons located deeper in the tectum (large piriform and other cells). Also present are the descending axons of pyramidal neurons. Several neuronal types have been found in this stratum: large fusiform neurons, extending dendrites into the SFGS, and an axon which leaves the tectum through the stratum album centrale; small pyriform neurons, giving rise to the stratum marginale ascending dendrites; small multipolar neurons, some of which extend axons that leave the tectum; and horizontal neurons with dendrites arborizing locally. The stratum album centrale (SAC; **Figure 1**) contains mostly bundles of myelinated axons, although unmyelinated fibers can also be recognized. The majority of these axons are of tectal origin, but some are originated in the contralateral retina, and the ipsilateral and contralateral telencephalon. Neurons here are the ganglionic and the large pyriform types, having large ascending dendritic arbors. Branches of the latter have been shown to be contacted by retinotectal terminals. Nonoriented neurons can be found here as well, representing a less defined type. The deepest laminae are the stratum periventriculare (SPV; **Figure 1**). It contains the 'periventricular neurons,' which extend dendritic processes into the overlying strata. However, the most characteristic cell type in this layer is the ependymoglial cell, which gives rise to an ascending process that reaches the uppermost aspect of the tectum while extending profuse branches along the way.

Afferents into the tectum are axonal terminals originating from (1) the retina, (2) the telencephalon, (3) the nucleus isthmi, (4) the dorsolateral and dorsomedial thalamic nuclei, (5) several pretectal nuclei, (6) the nucleus preglomerulosus, (7) the torus longitudinalis and the torus semicircularis, (8) the contralateral tectum and the dorsolateral tegmentum, and (9) diverse brain stem cell groups. Fibers originating in the contralateral eye course through the optic nerve, reaching the tectum in a retinotopic distribution. They terminate in the stratum opticum and the SGFS, and scarcely between the stratum album centrale and the stratum periventriculare. They synapse with horizontal neurons, pyramidal neurons, small bipolar neurons, fusiform neurons, and large fusiform neurons. In addition, optic tectum also receives a strong input from the auditory system and contains map of auditory space especially in owl and pigeons.

Tectal efferents originate from (1) the small bipolar neurons of the SFGS, (2) the fusiform neuron in the stratum griseum centrale, (3) the large multipolar neurons of the stratum griseum centrale and the stratum album centrale, and (4) large pyriform neurons of the stratum griseum centrale and the stratum album centrale. Tectal efferents form three efferent fiber groups: (1) the ascending group, running mostly toward the ipsilateral pretectum and thalamus, but some toward contralateral structures (including tecto-ocular fibers); (2) the medial group, coursing toward the contralateral tectum (forming the tectal

commissure, and terminating mostly in the stratum griseum centrale), but a few reaching the torus longitudinalis; and (3) the descending group, which extends into the ipsilateral and contralateral tegmentum and lower brain stem, including the nucleus isthmus (the nonmammalian homolog of the parabigeminal nucleus).

The optic tectum displays visually elicited electrical activity. Discrete areas of the retina receiving light from punctiform stimuli in the visual field project to discrete regions of the contralateral tectum (although species having binocularity project also ipsilaterally, mainly through an isthmotectal relay).The visual projections are arranged in a topographic manner, such that: nasal retina is represented in the caudal region of the tectum, and temporal retina in the rostral region; the dorsal retina projects to the lateral region of the tectum, and the ventral to the medial. This retinotopic organization extends across all laminae of the optic tectum. The topographic organization of axonal terminations is conserved also in the isthmotectal and tectoisthmal projections. There is orderly point to point correspondence between the retina and the tectum. The nasotemporal axis is represented along the caudorostral axis and the dorsoventral retinal axis is represented on the lateromedial axis of the tectum (**Figure 2**).

The mechanism of retinotectal map formation has been studied in mature fish as their optic nerves have a capability of regeneration. Seminal work was done by Roger Sperry who proposed that during development cells of the retina and tectum acquire cytochemical labels denoting their position within the retina or tectum. The growing retinal axons carrying the unique positional specificity on them are guided toward the tectal cells having corresponding labels; thereby, a selective and appropriate contact is made. By creating retinotectal mismatch or size disparity, Attardi and Sperry showed the regenerating optic axons reached their appropriate terminal sites by passing the inappropriate sites (**Figure 3**) Further research led to many aspects of neuronal specificity providing evidences of neuronal plasticity in which retinal fibers either 'compress' or 'expand' their projection depending on the available sites. In all such situations one element that remained constant was

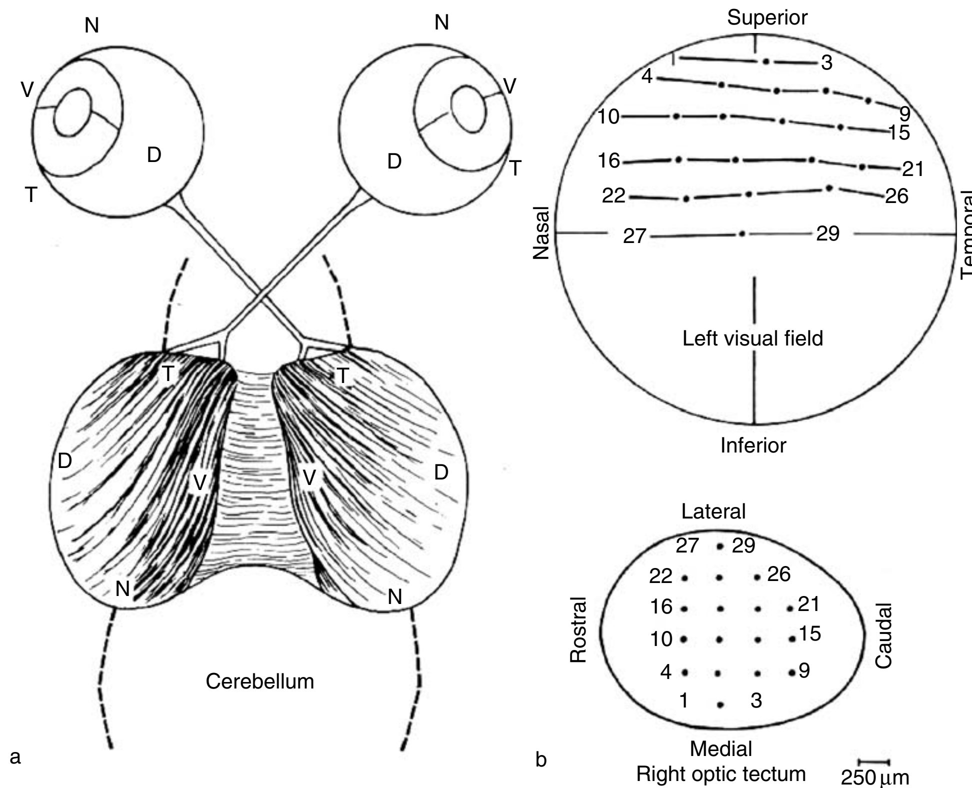

Figure 2 (a) Schematic representation of the projection of the retina onto the optic tectum in teleosts, showing the complete crossing of the optic nerves, and the division of the optic tracts into medial and lateral bundles before reaching the tectum. The optic nerve fibers spread in a caudolateral direction across the surface of the tectum from their entrance near rostromedial pole. Nasal retinal fibers (N) terminate in caudal tectum, temporal fibers (T) in rostral tectum, dorasal fibers (D) in lateral and ventral tectum, and ventral fibers (V) in medial tectum. (b) Visuotopic map from a normal left eye to the right optic tectum. Each number in the visual field shows the position of a visual stimulus that gave a localized response at the electrode positions indicated by the same number on the tectum.

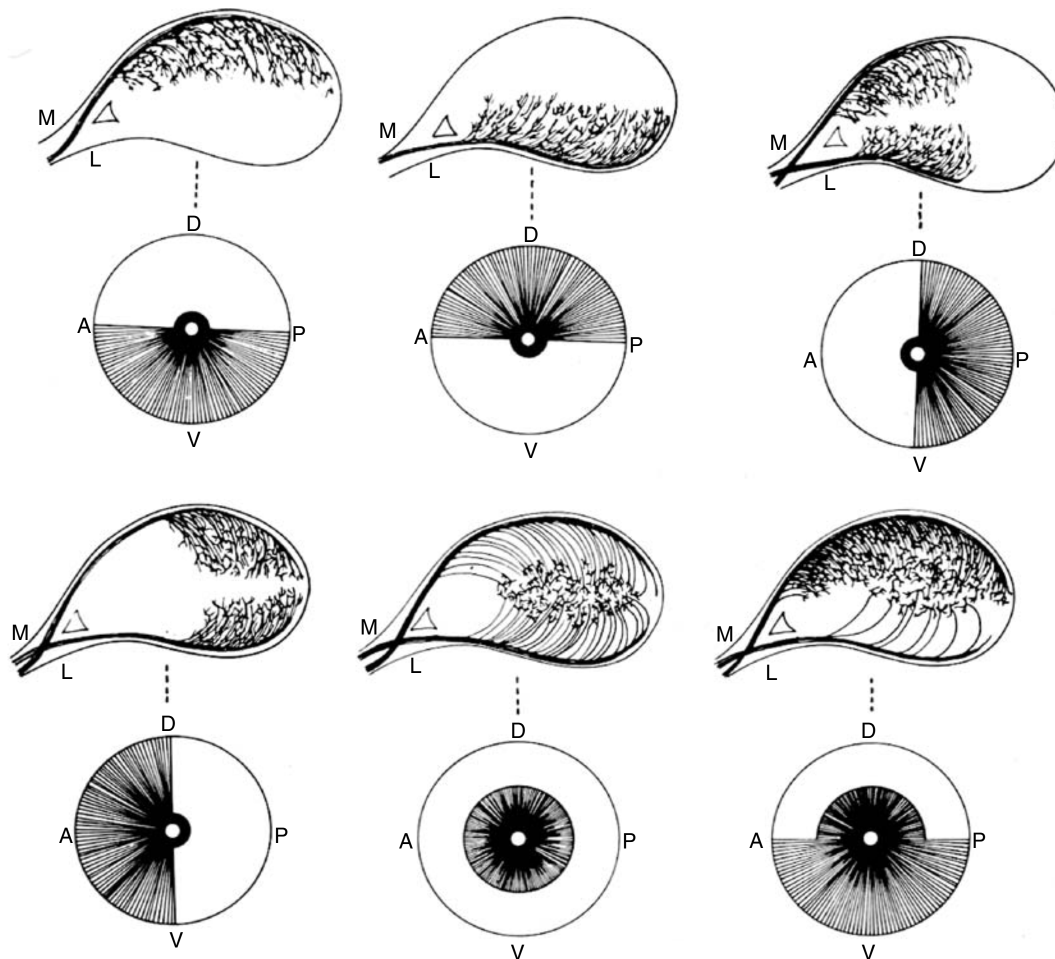

Figure 3 Schematic drawings of the regenerated optic fiber patterns observed in the tectum using Bodian protagrol method after optic nerve section and various partial retinal ablations. In each retina diagram, the white area denotes the ablated region. On the retinal diagrams: A, anterior; P, posterior; D, dorsal; V, ventral. On the tectum diagrams: M and L, medial and lateral branches of the optic tract. Redrawn with permission from Dr. RW Sperry.

the order of topography. These and other relevant aspects have been summarized by Sharma and Fawcett.

Retinotectal terminal arborizations show electrophysiological segregation among different tectal layers. While no light-evoked responses have been detected in the stratum marginale, unitary responses are predominantly 'on–off' and transient in the stratum opticum and the superficial-SFGS. Units recorded in the deep-SFGS give sustained responses and are either on-center or off-center type. Electrically active tectal dendritic elements with their highly organized spatial arrangement are an important component of the visual processing apparatus, rather than just relays of action potentials. Similar to the mammalian visual cortex, neuronal elements in the optic tectum display a high degree of specialization for different components in which visual information is separated. Studies of tectal field potentials show a directional selectivity to moving spots of light. Tectal neurons also show velocity sensitivities, since their firing rate depends on the stimulus velocity. There are also specific tectal neurons in fish, which respond only to certain orientations of polarized light, as received by specialized photoreceptors.

There are several neuronal types in the optic tectum, which respond directly to photic stimuli because of direct synapses with retinal ganglion cell axons. Pyramidal cells located in the SFGS, which send dendrites within the SFGS, to the stratum opticum, and to the stratum marginale, have a short latency of response. These cells have strong spontaneous activity, display slow adapting responses with movement directionality, and are perhaps involved in the orientation circuit. Pyriform neurons located in the stratum periventriculare, which send dendritic trees in the upper tectal strata, respond in a delayed form to retinal input, and they are thought to receive direct connections from retinal ganglion cells, via slow conducting fibers reaching the deep tectum. These cells

have a wide range of electrical activity, although smaller pyriform neurons tend to have stronger spontaneous activity and photic responses. Horizontal neurons represent a heterogenous population of tectal neurons arranged in several strata. Their electrophysiological responses have a wide range of variation. Many of them display strong spontaneous activity and short latencies to retinal stimulation, demonstrating their direct connections with retinal afferents.

Multiple types of molecules have been identified in recent years as playing a role in interneuronal communication in the optic tectum of nonmammalian species. Their variety serves as an indication of the complexities of tectal circuitry. Neurotransmitters and neuromodulators that have been shown in tectal neurons include: glutamate, γ-aminobutyric acid (GABA), acetylcholine, serotonin, somatostatin, substance P, neurotensin, neuropeptide Y, gonadotropin-releasing hormones, corticotrophin-releasing factor, and arginine vasotocin. Glutamate-positive retinal fibers terminate in the stratum opticum and the SFGS. Glutamate receptors of different types are present on tectal neuron dendrites. Glutamate is not only necessary for visual information relay, but in addition, *N*-methyl-D-aspartate (NMDA) receptor has been shown to be important in the electrical-activity-driven refinement of the retinotectal terminal arborizations. This process occurs during development and regeneration, and leads to sharpening of the retinotopic map. Glutamate mediates transmission into the tectum by marginal fibers projecting from the torus longitudinalis, and has been found in optic tectum neurons as well, including pyramidal, multipolar, fusiform, large pyriform, and periventricular. GABA, present in neurons and fibers scattered across the frog optic tectum, enhances the excitatory synaptic transmission of optic nerve terminals. Acetylcholine has an important role in the isthmotectal projections and in the intrinsic circuitry of the optic tectum. Cholinergic input modulates the transmission of these fibers via presynaptic nicotinic receptors. Two molecules of interest in the sexual dimorphism that the lower vertebrate brain displays are arginine vasotocin and galanin. They are present in higher levels in the optic tectum (among several brain regions) of male fish, frogs, and newts than in that of females, or sexually unresponsive males.

Development of Tectum and Axonal Guidance

The optic tectum develops simultaneously with the eye. It differentiates from the alar plate of the mesencephalon. Four phases can be considered: cell proliferation; migration; fiber growth and synaptogenesis; and a phase of regressive changes including cell death and neurite pruning. The development of the topographic connection of the retina with the tectum has been a focus of research for many years. In the prospective mesencephalon, the local anterior–posterior polarity is generated through the activity of a long-range signal from the posterior boundary with the rhombencephalon, the isthmus. Many of the successive genetic interactions that determine tectal evolution in the midbrain have been identified at the molecular level.

The development of the ectodermal midbrain/hindbrain organizer is induced by mesoderm. The spatially correct expression of the homeobox genes, Otx-2 (anterior) and Gbx-2 (posterior), is important for the development of the isthmic-signaling region. Otx-2 is expressed in the anterior neuronal tube down to the mid-hindbrain boundary. Gbx-2 is expressed in the rhombencephalon and borders with the expression of Otx-2. Then a diffusible substance, such as fibroblast growth factor 8 (FGF-8), emanating from the organizer determines midbrain polarity. Several genes (including En-1, Otx-2, Pax-2, and Wnt-1) regulate FGF-8 itself transcriptionally. Pax proteins are transcription factors that contain paired box and homeobox DNA-binding domains. Pax-2 covers the whole of the mesencephalon, whereas Pax-5 is expressed in high levels in the isthmic region. Positive inductions by FGF-8, in conjunction with inhibitory influences from the mesencephalic–diencephalic junction, produce a decreasing caudal–rostral gradient of engrailed (En-1 and En-2) expression along the posterior–anterior axis of the midbrain. Eventually, the engrailed distribution determines the positional specificity of tectal neurons. Ephrins and the Eph receptors are among the cell-surface molecules directly responsible for neural guidance of afferent retinal fibers toward specific tectal positions. Ephrin-A5 and Ephrin-A2 are the two molecules shown to be engrailed dependent *in vivo*. They distribute in gradients, increasing from anterior to the posterior poles, with Ephrin-A5 restricted to the caudal half of the tectum. Eph receptors are expressed in gradients across the early retina and on the growth cones of ganglion cells. Eph-A3 is the preferred receptor for Ephrin-A5 and Ephrin-A2. It is present in a retinal gradient, increasing from nasal to temporal. The tectal distribution seems to be a repellent guidance system that stops growing retinal axons at different anterior–posterior positions. Grg-4 is expressed early in the mesencephalon and causes repression of molecules, including FGF-8, Pax5, En-2, and Ephrin-A2. Thus, Grg-4 might contribute to setting the anterior limit of the tectum and its rostrocaudal polarity formation (**Figure 3**).

The dorsoventral patterning of the mesencephalon is determined by the Shh molecule, which is expressed in the floor plate of the neural tube up to the diencephalon. In the tectum, if Shh is expressed ectopically in the dorsal region, the dorsal tectum receives dorsal retinal fibers, which normally terminate at the ventral tectum. Furthermore, Shh represses the expression of the tectum-related genes (En-1, En-2, Pax-2, Pax-5, and FGF-8) and induces ventral markers, such as HNF3b and ptc. Ephrin-B ligands and their receptors appear to be the guidance cues for spatial segregation along the dorsal–ventral axis. Wnt3 is expressed in a medial–lateral decreasing gradient in chick optic tectum and mouse superior colliculus (Schmitt et al.). Retinal ganglion cell (RGC) axons from different dorsal–ventral positions showed graded and biphasic response to Wnt3 in a concentration-dependent manner. Wnt3 repulsion is mediated by Ryk, expressed in a ventral-to-dorsal decreasing gradient, whereas attraction of dorsal axons at lower Wnt3 concentrations is mediated by Frizzled(s). A classical morphogen, Wnt3, acting as an axon guidance molecule, plays a role in retinotectal mapping along the medial–lateral axis, counterbalancing the medial-directed EphrinB1-EphB activity.

Several molecules known to affect neurite behavior are concentrated in the retinorecipient laminae making them plausible candidates to participate in the regulation of laminar specificity. These include the adhesion molecule SC1/DM-GRASP/BEN, semaphoring/collapsing receptors and ephrin-B2; in addition, N-cadherin is concentrated in the synaptic cleft at retinotectal synapses. N-cadherin and glycoconjugates recognized by the plant lectin Vicia villosa agglutinin-B4 (VVA) guides retinal axons. Versican is an important layer-specific cue for presynaptic maturation of retinal ganglion cells.

Reelin (RELN) is an extracellular matrix protein largely related with laminar organization in several brain areas. In the optic tectum (OT) of trout, as in amniotes, RELN immunoreactivity increases within specific cell layers as lamination proceeds, and decreases when it is complete, except in the stratum opticum (SO), where RELN-immunoreactive cells are observed throughout life. Time-course expression of RELN in the OT suggests a role in the early modeling of synaptic contacts and the accommodation of new retinal arriving axons throughout life.

In summary, the spatial identity of the neurons within the optic tectum seems to be tightly regulated by the graded induction of a series of asymmetric genes, transcriptional factors, and receptors for signal transduction during early development. The exact sequences of different gene activation leading to the formation of adult tectum are not yet clear.

Behavioral Correlates

The optic tectum is involved in (1) perception of form (related to food catching and escape behavior); (2) responses that can be conditioned to visual stimuli; (3) ocular convergence movements; and (4) 'food searching' forward body movements. However, as evidenced in fish, there are visual responses in which the tectum is not directly involved. The optokinetic and dorsal light reflex appear to be mediated by nontectal targets (e.g., diencephalic and pretectal groups), since they persist after tectal ablation. The optic tectum might also be involved in visual memory, as long-term potentiation has been elicited in it. Nevertheless, visuo-spatial conditioning memory involves other brain structures and persists even after abolishing the tectum.

In both fish and frogs, electrical microstimulation of the optic tectum can elicit complex stereotypic body movements. It has been demonstrated that tectoreticular circuit helps in the generation of saccadic eye movements in goldfish. The optic tectum of goldfish, as in other vertebrates, plays a major role in the generation of orienting movements, including eye saccades. To perform these movements, the optic tectum sends a motor command through the mesencephalic and rhombencephalic reticular formation, to the extraocular motorneurons. These motor responses have been studied in the two primary behaviors of vertebrates, feeding and avoidance. Distance of the prey from the eye determines whether frogs will merely fixate on it, or snap at the stimulus. This difference seems to be related to the size of the retinal area stimulated by moving objects. As would be expected, tectotectal connections are involved in behavior involving conjugated eye movements, such as turning toward prey, as shown in studies of the effects of severance of these fibers. In contrast, hemisection of the tectal descending fibers eliminates the frog's ability to snap forward, while preserving that of turning. On the opposite end of the behavioral spectrum, the optic tectum of nonmammalian vertebrates is involved in the avoidance to possibly threatening objects. Both frogs and fish react by jumping or flipping away when they visually spot large dark objects approaching. This behavior is completely eliminated by tectal ablation. Smaller lesions to areas of the tectum abolish the above-mentioned responses to stimuli within specific corresponding regions of their visual field. Stimulation of the rostral tectum induces turns of the head and body toward the frontal field; activation of caudal tectum elicits body orientation toward the rear field.

The primitive optic tectum, like the superior colliculus of primates, integrates other sensory modalities

in addition to vision. In weakly electric fish, electrosensory stimuli (used in the detection of objects in their surroundings) are processed in the tectum. This type of input is directed to areas in the optic tectum in such a way that it overlaps with visual information coming from the corresponding space region. Processed information closes the stimulus-response circuit as the tectum projects to the diencephalic nucleus, which has efferents to the mesencephalic prepacemaker, connecting in turn with the medullary pacemaker nucleus, which finally controls the electric organ discharge (used in interactions with other fish, such as in courtship). In snakes, another sensorial type of information is important. Temperature changes in its surroundings are detected by the heat-focusing pit organ, which connects with the optic tectum to process these stimuli.

In spite of the enormous progress made during the past five decades, our knowledge of the functioning of the optic tectum leaves further opportunities for investigation. Among others, studies are needed to elucidate in further detail the electrochemical features of the tectal circuitries, and to advance our understanding of the integration mechanisms in which the optic tectum accomplishes simultaneous processing of different modalities of sensory information, in order to provide coherent responses to the environment.

See also: Optic Nerve Optic Chiasm and Optic Tracts; Retinal Development: An Overview.

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Dendrite Development, Synapse Formation and Elimination

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Introduction

In the central nervous system of adult animals, many neurons have functionally polarized architecture in which dendrites, which receive information from other neurons, are physically separate from axons, which send information to other neurons. In general, axons and dendrites both have complex treelike structures, called arbors. The area covered by the axons and dendrites and the density of branches within the dendritic and axonal arbors govern the type and number of neurons which are connected within a circuit. Consequently, the mechanisms that control the development and maintenance of neuronal structure critically affect the ability of the neuron to function within the brain circuit. For instance, the importance of neuronal structure in brain function is clear from studies of brains of children with mental retardation, in which neurons are dwarfed in size compared to those in healthy children.

The acquisition of mature neuronal structure is classically described as being governed by both 'intrinsic' and 'extrinsic' factors, although in fact extrinsic factors, such as growth factors and synaptic inputs, affect intrinsic events such as gene transcription and trafficking of guidance molecules to the cell surface. Several recent studies using modern molecular genetic, imaging, and electrophysiological methods now provide strong evidence that excitatory synaptic inputs control the development of neuronal structure in the intact brain.

Synaptogenesis and Synapse Maturation

The formation and maturation of synapses can be distinguished into several steps:

1. *Establishment of an adhesive contact.* Dynamic filopodial processes from growing presynaptic axons and postsynaptic dendrites come into contact and form an initial adhesive contact, possibly mediated by integrins, cadherins, or Wnt/Frizzled cell surface adhesive molecules.
2. *Conversion of the adhesive contact to a nascent synapse.* In the case of glutamatergic synapses in the vertebrate central nervous system, nascent synapses are characterized by the predominance of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. NMDA receptors (NMDARs) require postsynaptic depolarization at the same time as ligand (i.e., glutamate) binding in order to permit conductance through the channel. As such, the NMDAR acts as a coincidence receptor. In the context of synapse formation, this ensures that transmission at nascent synapses occurs when other inputs to the postsynaptic neuron surpass a threshold synaptic strength. NMDARs are permeable to calcium. Intracellular calcium signaling may be required for maturation of the developing synapse.
3. *Synapse maturation.* Glutamatergic synapse maturation is characterized by the recruitment of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type ionotropic glutamate receptors into synapses. This renders the synapses functional at resting potentials. The assembly of the complex postsynaptic density, including scaffolding proteins and signaling proteins, occurs as glutamate receptors are trafficked into developing synapses.

Features of Nascent and Mature Synapses

Studies in many experimental systems indicate that synaptic transmission at newly formed synapses is mediated by the NMDA type of glutamate receptors and that AMPA receptors (AMPARs) are added to synapses as they mature. Synapses with only NMDARs are 'silent' at resting potential due to the voltage-dependent block of the NMDAR channel, and the addition of AMPARs to synapses renders them functional at resting potentials. Similarly, the fraction of silent synapses, in which transmission is mediated solely by NMDARs, is high in early stages of synapse formation and decreases as synapses and neurons mature, due to the insertion of AMPARs at synaptic sites. Consequently, the fraction of silent synapses and the ratio of AMPA to NMDA receptor-mediated transmission can be used as indicators of synaptic maturity (see **Figure 1**). Although some glutamatergic synapses reportedly develop without NMDARs, a sequence in which transmission at new synapses is mediated principally by NMDARs followed by the addition of functional AMPARs to synaptic sites appears to occur at the majority of glutamatergic synapses. Indeed, trafficking of AMPARs into developing synapses is required for their maturation and is required for synaptic plasticity in adult animals under a variety of conditions, including experience-dependent sensory plasticity and fear-conditioned learning. Although these data suggest

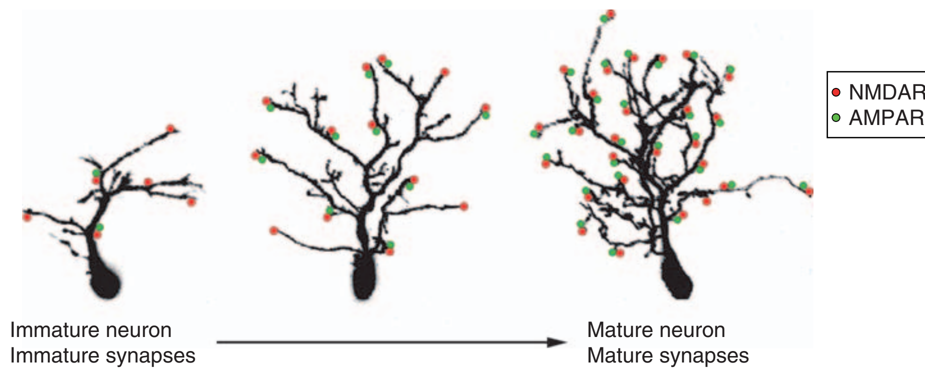

Figure 1 Synaptogenesis and dendrite development are concurrent. *In vivo* images of a neuron from the optic tectum of *Xenopus laevis* tadpoles were collected once a day over 3 days. The dendritic arbor increases in complexity over this time period through the net addition of arbor branches. Images collected over shorter intervals (not shown) demonstrate that net arbor growth occurs as a result of rapid branch addition and retraction. Synapses are located throughout the arbor. New excitatory synapses are mediated principally by *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, shown as red dots. α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type receptors, shown as green dots, are added to these synapses as they mature. Even after 3 days of growth, the dendritic arbor continues to show structural changes and to add new (NMDA receptor (NMDAR) only) synapses.

that AMPAR trafficking is the key element required for synapse maturation and synaptic plasticity, AMPAR trafficking into synapses may escort other proteins into the postsynaptic density, and it is possible that these other proteins in combination with AMPARs are required for synaptic plasticity.

Developing glutamatergic synapses can be distinguished from mature synapse by several other features. Synapse maturation includes changes in the presynaptic element, including recruitment of presynaptic machinery and proteins into the axon terminal and recruitment of synaptic vesicles. Ultrastructural studies indicate that developing synapses have few, sparsely packed synaptic vesicles, and that the most reliable ultrastructural indicator of synaptic maturation is the density of synaptic vesicles.

In vertebrate neurons the subunit composition of synaptic NMDARs switches during development, from receptors containing mostly NR2B subunits to those including NR2A subunits. This change in subunit composition can be detected pharmacologically and by a faster decay of the synaptic responses. Furthermore, immature synapses may show greater spillover of transmitter from the synaptic cleft to activate extrasynaptic receptors, due to relatively poor envelopment of nascent synapses by glia.

Synapse maturation and developmental plasticity of the strength of synaptic communication are thought to be regulated by changing the efficacy of transmitter release from the presynaptic terminal and by changing the response of the postsynaptic neuron to the input signal. The mechanisms underlying these changes are similar to those which occur during learning and memory in the adult animal. For instance, calcium influx through NMDARs, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation, and AMPAR trafficking underlie both synapse maturation and changes in synaptic strength associated with learning.

Process of Dendritic Arbor Development

In vivo time-lapse imaging experiments have shown that dendrites of central nervous system neurons grow by the highly dynamic addition and retraction of fine branches. Although most newly added branches retract within about 10 min of being added to the arbor, a small fraction of branches is maintained and these extend to become more stable components of the arbor. The newly added branches may sample the local environment for appropriate presynaptic contact sites. Establishment and maintenance of synapses may then confer a longer lifetime on the branches that form stable synapses and permit the dendritic arbor to enlarge over time (see **Figure 1**).

Classical studies using fixed anatomical preparations have led to the idea that axonal and dendritic arbors go through a period of exuberant overgrowth followed by pruning of branches based on competitive mechanisms (see **Figure 2**). This model of neuronal growth and pruning suggests that neurons in the adult brain have limited capacity to recover following injury, and this in turn has discouraged scientists and clinicians from exploring the potential of the brain to regain function after trauma.

Modern *in vivo* imaging methods demonstrate that neurons in the brain grow very differently than thought from classical studies. Specifically, branch additions and retractions are concurrent, so that the neuron has an ongoing capacity to add new branches and to refine its connections within the

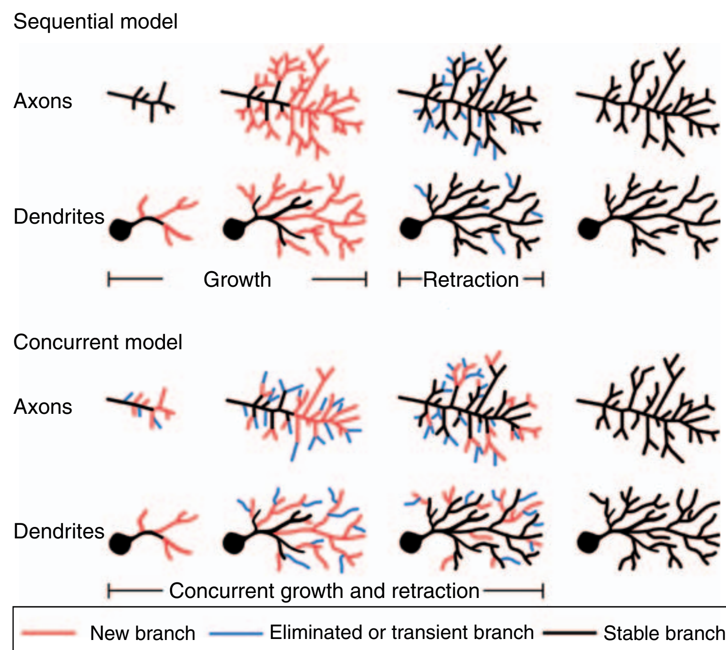

Figure 2 Models of dendritic and axon arbor elaboration. Dendritic and axon arbor elaboration and pruning are concurrent, not sequential. Top: Diagrams of the patterns of growth of axons and dendrites according to the traditional model, in which neurons undergo an exuberant growth phase followed by a temporally distinct phase of net branch retraction. Bottom: Diagrams of the patterns of growth of axons and dendrites in which branch additions and retractions are concurrent. Adapted from Hua JY and Smith SJ (2004) Neural activity and the dynamics of central nervous system development. *Nature Neuroscience* 7: 327–332.

circuit (**Figure 2**). The refinement of the arbor structure occurs in response to signals from the environment and from other neurons in the circuit. These observations provide critical insight into the cellular mechanisms governing dendritic and axonal arbor development and clearly indicate that learning and recovery from trauma can occur even in the adult brain by tapping into the cellular mechanisms that shape the neuronal structure during development of the brain.

Synaptic Inputs Increase Dendrite Arbor Growth

Several time-lapse imaging experiments now provide convincing support for the hypothesis that the formation and maturation of synaptic contacts stabilize dendritic arbor structure (**Figure 3**). Direct *in vivo* time-lapse imaging reveals that visual stimulation increases the growth of optic tectal dendritic arbors *in vivo* by promoting the stabilization of newly added branches. Pharmacological blockade of either AMPA- or NMDA-type glutamate receptors decreases dendritic arbor growth *in vivo* and blocks visual stimulation-induced dendritic arbor growth. Similarly, deafferentation or blocking inputs within the auditory system, as well as other sensory systems, has severe effects on dendritic arbor development of neurons that receive and process sensory information. *In vivo* imaging experiments in which the maturation of glutamatergic synapses was blocked show that AMPAR trafficking into developing synapses is required for the stabilization of newly added dendritic arbor branches and the cumulative elaboration of the complex dendritic arbor. Together, these data indicate that the iterative process of dendritic arbor development requires the coordinate formation and stabilization of glutamatergic synaptic inputs (**Figures 1–3**).

Conclusion

Synapse formation is characterized by the assembly of a complex protein machine that spans a specialized junction, the synapse, which forms between two neurons. Recent multidisciplinary experiments combining time-lapse *in vivo* imaging, molecular manipulations, and electrophysiological recordings demonstrate that synapse formation and maturation are required for the normal development of neuronal structure, including the axon and dendrite. These experiments have also shown that the development of axons and dendrites occur as a result of simultaneous addition and retraction of branches within the neuron. These observations overturn two previous ideas about brain development, that neuronal growth can occur in the absence of synapse formation and synaptic activity, and that neurons undergo a phase of exuberant growth followed by

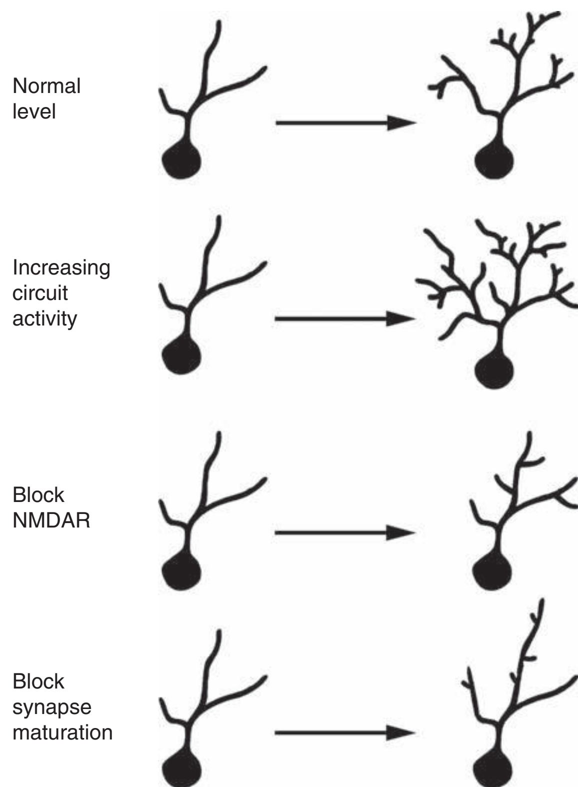

Figure 3 Synaptic input drives dendrite development. The normal rate of dendritic arbor development is increased by enhanced synaptic input and is decreased when glutamate receptor activity or synapse maturation is blocked. The extent of dendritic arbor growth under 'normal' levels of circuit activity in the brain is depicted at the top. The effect of increasing activity on dendritic arbor development is shown in the second row; the effects of blocking *N*-methyl-D-aspartate receptor activity or blocking synapse maturation are shown in the third and fourth rows. Based on data from Sin WC, Haas K, Ruthazer ES, et al. (2002) Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. *Nature* 419: 475–480; Rajan I and Cline HT (1998) Glutamate receptor activity is required for normal development of tectal cell dendrites *in vivo*. *Journal of Neuroscience* 18: 7836–7846; and Haas K, Li J, and Cline HT (2006) AMPA receptors regulate experience-dependent dendritic arbor growth *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* 103: 12127–12131.

a separate period of pruning. This modern view of neuron development suggests that brain activity will increase synapse formation as well as the establishment and maintenance of optimal neuronal circuits. This view also lends hope to the idea that brain exercises will aid recovery from trauma even in adults.

See also: Postsynaptic Development: Neuronal Molecular Scaffolds; Presynaptic Development and Active Zones; Topographic Maps: Molecular Mechanisms.

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Axon Guidance: Morphogens as Chemoattractants and Chemorepellants

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Introduction

The growth cones of developing neurons are guided to their targets by attractive and repulsive cues in the extracellular environment. Receptors on the growth cones recognize these cues and transduce signals that ultimately lead to changes in the direction of growth. Several families of molecules acting as guidance cues have been identified, including Netrins, Slits, Ephrins, and Semaphorins. In addition to these families, secreted signaling molecules from families of classical morphogens, known for their roles in controlling cell fates in a concentration-dependent manner, can act as axon guidance molecules. Sonic hedgehog (Shh) of the hedgehog family, bone morphogenetic protein 7 (BMP7) of the transforming growth factor-beta (TGF-β) family, and members of the Wnt family have all been found to function in the guidance of specific classes of neurons.

For each of the three morphogen families, the receptors and canonical signaling pathways through which they control cell fate are well studied and involve transcriptional regulation as their output. However, some members of these families can also signal through noncanonical pathways and control processes such as cell movements and cell polarity. The roles of these families in axon guidance, plus the finding that they likely act directly on the growth cone, argue for the activation of noncanonical signaling pathways that lead to cytoskeletal rearrangement.

Shh as a Chemoattractant for Commissural Neurons

Commissural neurons residing in the dorsal region of the spinal cord adjacent to the roof plate project axons through the floor plate at the ventral midline (**Figure 1(a)**). These commissural axons initially extend away from the roof plate and take a ventral and circumferential pathway through the dorsal spinal cord. Midway to the ventral midline, the axons change course and project ventrally and medially to the floor plate. After crossing the floor plate, they then make an abrupt turn and project anteriorly on the contralateral side of the spinal cord.

Commissural axons are guided to the ventral midline by Netrin-1, a chemoattractant secreted by cells of the floor plate and the adjacent periventricular zone. In *Netrin-1*$^{-/-}$ mutant mice, as well as in mice mutant for the Netrin receptor deleted in colorectal cancer (DCC), many commissural axons project abnormally, often failing to enter the ventral region of the spinal cord. However, some commissural axons in these mutants do manage to reach the floor plate, suggesting the existence of one or more additional factors in the floor plate. Indeed, the floor plate of *Netrin-1*$^{-/-}$ mice is still effective in reorienting commissural axons when juxtaposed to spinal cord explants, indicating the presence of another chemoattractant.

Shh was implicated as the additional factor since it is secreted by the floor plate and is known to have long-range effects within the spinal cord. In addition, Shh can cause rapid changes in the growth cone behavior of cultured retinal ganglion cells. An initial indication that Shh might be the missing floor plate factor was garnered by demonstrating the ability of Shh-expressing COS cells to reorient commissural axons in spinal cord explants. This reorienting effect of Shh is mediated through the Shh signaling component Smoothened (Smo), since the Smo inhibitor cyclopamine abolishes the effect.

Assessing the axon guidance role of Shh *in vivo* is more challenging because of its role in early patterning of the spinal cord. Shh acts as a morphogen to generate distinct neuronal cell types within the ventral spinal cord in a concentration-dependent manner. Although Shh is not directly required for generation of the dorsally located commissural neurons, these neurons do project their axons ventrally through regions that are patterned by Shh. This rules out the analysis of mice mutant for Shh, Smo, or the Shh receptor patched (Ptc) since other guidance cues in the ventral spinal cord of these mutants might be altered. However, using the Cre/loxP recombinase system and the *Wnt-1* promoter to drive expression of Cre recombinase, Smo can be selectively removed in commissural neurons developing within an otherwise normally patterned spinal cord. In these conditionally mutant mice, commissural axons project abnormally into the ventral spinal cord, sometimes failing to make the medioventral turn toward the floor plate and instead projecting along the edge of the spinal cord. Thus, Shh appears to be acting as a chemoattractant for commissural neurons. Ultimately, the commissural axons in these conditional Smo mutants do reach the ventral midline and form

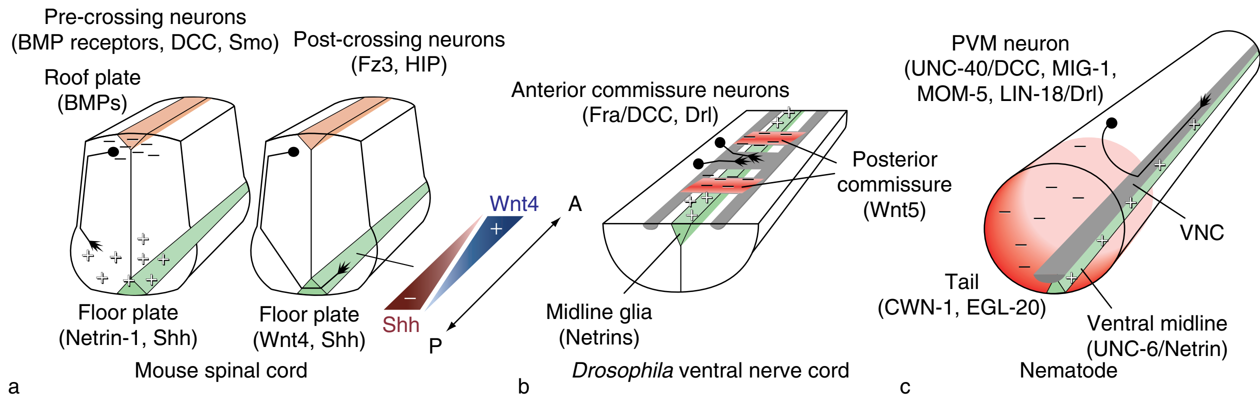

Figure 1 Morphogens as axon guidance molecules. Schematic of the axon projections of neurons in the developing mammalian spinal cord (a) and the ventral nerve cords (VNC) of *Drosophila* (b) and nematode (c). The floor plate (green) secretes Netrin-1 (+), which acts as a chemoattractant for commissural axons expressing the Netrin receptor DCC. The floor plate also secretes Shh, which functions in concert with Netrin-1 to attract commissural axons expressing the Shh signaling component Smo. The roof plate (orange) secretes BMP7 (−), which repels the initial growth cones of commissural neurons away from the dorsal midline, probably as a heterodimer with GDF7. The specific BMP receptors in commissural neurons mediating the repulsive response are not known. After crossing the floor plate, growth cones of commissural neurons turn anteriorly in response to an increasing posterior-to-anterior (P-to-A) gradient of Wnt4, plus a decreasing gradient of Shh, secreted by floor plate cells. Fz3 and HIP are thought to mediate the responses to Wnt4 and Shh, respectively. In *Drosophila*, the major axon tracts (gray) consist of the bilaterally symmetric longitudinal connectives running in the anterior/posterior axis and, connecting the two sides in each segment, an anterior and posterior commissure. Like the vertebrate floor plate, the midline glia (green) secrete Netrins (+) which attract commissural axons expressing the DCC homolog Fra. Commissural axons that project through the anterior commissure, all of which express the Drl receptor, are repelled from the posterior commissures of their segment of origin and the adjacent anterior segment by Wnt5 (−). In the nematode, migrating cells that end up at the ventral midline (green) secrete the Netrin homolog UNC-6 (+), as does the VNC (gray). Axons expressing UNC-40, the nematode DCC homolog, are attracted to the VNC. Wnt proteins CWN-1 and EGL-20 (−) are expressed by cells in the tail and act to repel PVM and other anteriorly projecting neurons expressing the Fz receptors MIG-1 and MOM-5. The Drl/Ryk homolog, LIN-18, acts redundantly in Wnt-mediated repulsion.

a normal-looking commissure, arguing that Netrin-1, in the absence of Shh, is sufficient to attract commissural axons to the floor plate. If Shh is the only additional guidance factor secreted by the floor plate, the expectation is that *Smo* mutant commissural neurons should not be able to reach the floor plate in a *Netrin-1*$^{-/-}$ mutant background.

Although a loss-of-function phenotype is the gold standard for demonstrating that a gene is required for a particular process, a phenotype does not necessarily reveal the underlying mechanism. For example, rather than acting directly on the growth cones of commissural neurons, Shh could be activating a retrograde signal within the neurons that regulates the expression of genes encoding other guidance receptors. The definitive test for a guidance molecule's direct action on growth cones is the *in vitro* growth cone turning assay. Here, the spatial and temporal application of a factor can be precisely controlled by pulsing it through a pipette, creating a gradient of purified protein to which isolated cultured neurons can respond. Importantly, in this context Shh has the ability to rapidly attract growth cones and this attraction is abolished by cyclopamine. Thus, Shh, signaling through Smo, can indeed act as a chemoattractant.

Shh as a Chemorepellant for Postcrossing Commissural Neurons

After crossing the midline, commissural axons turn anteriorly along the contralateral side of the floor plate. RNA interference (RNAi) experiments in chick embryos have implicated Shh in this guidance event. When Shh levels are reduced by injection of Shh dsRNA, commissural axons tend to either stall at the floor plate exit point or project posteriorly. A decreasing posterior-to-anterior gradient of Shh suggests that in this case Shh is acting as a chemorepellant, a notion supported by its ability to repel postcrossing commissural axons in spinal cord explants. Importantly, this repulsion is not mediated by Smo and Ptc, since neither is expressed by commissural neurons by the time they have crossed the midline. Furthermore, cyclopamine has no effect on turning behavior. Instead of Ptc, another Shh receptor, hedgehog interacting protein (HIP), has been implicated. Lowering HIP function by RNAi knockdown results in the same turning defects observed when Shh is downregulated.

A model has thus emerged that depending on the receptor employed, Shh can act as either a chemoattractant or a chemorepellant. However, in contrast

to Shh chemoattraction, it is not known whether HIP-mediated repulsion by Shh is a result of direct action on the growth cone rather than the transcriptional control of other guidance receptors, such as those involved in Wnt-mediated attraction of post-crossing commissural axons.

BMP7 as a Chemorepellant for Commissural Neurons

The early phase of commissural axon growth within the dorsal spinal cord is unaltered in either *Netrin-1*$^{-/-}$ mutants or mice lacking a floor plate, suggesting the existence of additional non-floor plate-derived factors in their guidance. The proximity of commissural neurons to the roof plate and the stereotyped projections of their axons away from the dorsal midline suggests a role of the roof plate in repulsion (**Figure 1(a)**). This idea is supported by experiments showing that when juxtaposed to a spinal cord explant, the roof plate can reorient commissural axons away from the side facing the roof plate. Furthermore, the reorienting activity can be mimicked by COS cells expressing BMP7, one of the BMPs secreted by the roof plate.

BMP7 functions most efficiently in the axon reorienting assay by forming heterodimers with GDF7, another BMP family member expressed by roof plate cells but which on its own has no axon reorienting activity. In *BMP7*$^{-/-}$ mutant mice, there is a significant increase over wild type in the number of commissural axons that initially project medially and dorsally instead of laterally and ventrally. Similar numbers are seen in *GDF7*$^{-/-}$ mutants. However, in each case, these defects are corrected such that later in development the projection pattern of commissural neurons resembles that of wild type. Thus, in addition to BMP7 and GDF7, other cues must be operating–repulsive ones from the roof plate and/or attractive ones from more lateral positions in the dorsal spinal cord. The former possibility is bolstered by the finding that roof plates from *BMP7*$^{-/-}$ *GDF7*$^{-/-}$ double mutants, although compromised in their ability to redirect commissural axons within spinal cord explants, still retain some residual axon-reorienting activity.

Similar to the patterning role of Shh in the ventral spinal cord, BMP family members function early in development to generate specific dorsal cell types. In fact, GDF7 is required to generate a specific subclass of commissural neurons, raising the question of whether the observed guidance effects of BMP7 are direct or indirect. Although the guidance role of BMP7 has not been cleanly separated from its inductive role, as was done for Shh using the conditional *Smo* mutation, several lines of evidence argue against an indirect mechanism involving changes in cell fate. First, *BMP7*$^{-/-}$ mutants show no obvious defects in dorsal spinal cord patterning, presumably due to redundancy with one or more of the other roof plate BMPs. Second, commissural neurons in spinal cord explants from *BMP7*$^{-/-}$ mutants are able to reorient to the same extent as those from wild type in response to a wild-type roof plate, suggesting that these neurons retain at least some of their wild-type properties, including the expression of an appropriate BMP receptor complex capable of transducing the BMP7 signal. Finally, although *in vitro* growth cone turning assays to test for repulsion have not been carried out, BMP7 alone, as well as BMP7:GDF7 heterodimers, is capable of causing rapid growth cone collapse of commissural neurons *in vitro*. Collectively, these studies advance the case for a direct role on the growth cone in axon guidance.

Wnt Proteins as Chemorepellants

Wnt5 in *Drosophila*

In addition to specifying cell fates, Wnt proteins play diverse signaling roles in the developing nervous system. These include presynaptic axon remodeling during vertebrate synaptogenesis, the maturation of the *Drosophila* neuromuscular junction, and the polarity of neurons along the anterior–posterior axis in the nematode *Caenorhabditis elegans*. As first shown in *Drosophila*, Wnt family members have also turned out to function as axon guidance molecules.

As in vertebrates, the large number of commissural neurons in the *Drosophila* embryonic ventral nerve cord (the fly counterpart to the spinal cord) project their axons across the midline to the contralateral side (**Figure 1(b)**). Analogous to the vertebrate floor plate, specialized cells at the midline, the midline glia, divide the two halves of the ventral nerve cord and play a critical role in axon guidance. Like the floor plate, midline glia secrete Netrins that act as chemoattractants for commissural axons expressing Frazzled (Fra), the fly homolog of the Netrin receptor DCC.

Once attracted to the midline, commissural axons do not cross randomly. Instead, in each segment they reproducibly choose one of two distinct tracts that connect the two sides, either the anterior or the posterior commissure. This choice of whether to project anteriorly or posteriorly into the appropriate commissure is controlled by one of the seven fly members of the Wnt family, Wnt5, and a receptor to which it binds, derailed (Drl). The Drl receptor is expressed on the growth cones and axons of all neurons that

project through the anterior commissure. In *drl* mutants, many of the anterior commissure axons abnormally project through the posterior commissure; conversely, when misexpressed by posterior commissure neurons, Drl switches their axonal projections to the anterior commissure. Drl is therefore not only required by anterior commissure neurons for their guidance but also sufficient to dictate the choice of the anterior commissure for crossing axons.

In *wnt5* mutants, as in *drl* mutants, anterior commissure axons project abnormally through the posterior commissure. Since Wnt5 is expressed by cells associated with the posterior commissure (and the Drl receptor by anterior commissure neurons), it is functioning as a chemorepellant to keep Drl-expressing axons from entering the posterior commissure. Such a repulsive activity was illustrated *in vivo* by misexpressing Wnt5 in the midline glia. In these embryos, anterior commissure axons are prevented from crossing the midline in a Drl-dependent manner, overriding Netrin-mediated attraction.

Although Drl binds Wnt5 and is required for Wnt5 function in guidance, it is not yet clear whether Drl transduces the Wnt5 signal within growth cones or whether it acts as a coreceptor with a member of the other family of Wnt receptors, the Frizzled (Fz) proteins. Fz receptors are good candidates for transducing such a signal since they have been shown not only to mediate canonical Wnt signaling resulting in transcriptional regulation but also to mediate noncanonical signaling involved in controlling cell movements. In support of the coreceptor hypothesis, the mammalian homolog of Drl, Ryk, can bind to the cysteine-rich domain of Fz proteins when the two are co-expressed in tissue culture cells, suggesting the ability of Fz and Ryk to form a complex. Regardless of which receptor(s) transduces the signal, it is clear that Wnt5 controls the guidance of anterior commissure axons and that the Drl receptor is essential for Wnt5 signaling.

However, does Wnt5 act directly or indirectly in these guidance events? The difficulty in purifying active Wnt proteins has hindered efforts to test Wnt5 for repellant activity in an *in vitro* growth cone turning assay. However, a key observation *in vivo* does suggest a direct role in guidance: Drl can dictate commissure choice in a Wnt5-dependent manner when misexpressed on growth cones shortly before they make the choice. This makes it less likely that a retrograde signal, followed by a round of transcription and translation, is required.

Mammalian Wnts

Wnt proteins, acting through the Ryk receptor, also control axon guidance events in the developing mammalian nervous system. Similar to Drl expression on anterior commissure axons in *Drosophila*, Ryk is expressed on axons that project across the midline within the corpus callosum. In *Ryk*$^{-/-}$ mutant mice, callosal axons reach and cross the midline but often fail to project away from it on the contralateral side. The Wnt ligand in this case appears to be Wnt5a, which together with Wnt5b is most closely related to *Drosophila* Wnt5. Wnt5a binds to Ryk and is expressed by midline glia known to be required for proper guidance of the callosal axons. The spatial and temporal expression pattern of Wnt5a suggests that it is acting as a chemorepellant to guide Ryk-expressing axons away from the midline, a notion supported by the finding that Wnt5a can repel cortical axons in explants from wild-type, but not *Ryk*$^{-/-}$, brains.

Like callosal axons, axons of corticospinal neurons also express Ryk. These axons project posteriorly from the brain through a dorsal region of the spinal cord that exhibits an increasing posterior-to-anterior gradient of a number of Wnts, including Wnt1 and Wnt5a. Anti-Ryk antibodies block both the posterior growth of corticospinal axons *in vivo* and the repellant activity of Wnts on cortical axons in cultured explants, providing good evidence that Ryk is required for posterior guidance by interpreting the Wnt gradient as a chemorepellant one. The striking similarities between mammalian Wnt-mediated corticospinal axon guidance and *Drosophila* Wnt5-mediated commissure choice argue for a deep-rooted conservation of function: both fly Wnt5 and mammalian Wnts are acting as chemorepellants that signal via the Drl/Ryk receptor to control guidance in the anterior–posterior axis.

Wnts in the Nematode

In the nematode *C. elegans*, the conserved cue Netrin (UNC-6 in nematode) guides axons to the ventral nerve cord at the midline (**Figure 1(c)**). Once they enter the ventral nerve cord, growth cones choose to project either anteriorly or posteriorly. For example, the axon of the posterior ventral microtubule cell (PVM) enters the nerve cord and projects anteriorly toward the head. Two Wnts, CWN-1 and EGL-20, are expressed in the posterior end of the embryo and act redundantly as chemorepellants to guide the PVM axon, as well as others, anteriorly. In *cwn-1:egl-20* double mutants, the PVM axon often projects posteriorly and this guidance defect is enhanced by misexpression of EGL-20 anteriorly. In contrast to *Drosophila* and mouse, Wnt repulsion of the PVM axon is mediated primarily through two Fz receptors, with LIN-18, the nematode homolog of Drl/Ryk, acting redundantly, presumably through a parallel pathway.

Wnt Proteins as Chemoattractants

After crossing the floor plate of the spinal cord, commissural axons turn anteriorly toward the brain (**Figure 1a**). Observations of cultured spinal cord explants provided the first indication that this guidance event might be controlled by a chemoattractant. Near the posterior cut end of spinal cord explants, commissural axons project anteriorly in a normal fashion, but near the anterior cut end, they either stall or project randomly along the anterior–posterior axis. This suggests that an increasing posterior-to-anterior gradient of a diffusible chemoattractant, which slowly escapes from the cut ends of explants, guides commissural axons anteriorly. A candidate molecule approach led to the Wnt family, particularly Wnt4, whose mRNA levels exhibit an increasing posterior-to-anterior gradient in the floor plate. Wnt4-expressing COS cells juxtaposed to the anterior cut end of explants can redirect postcrossing axons anteriorly, in effect rescuing the projection defects near the cut end. Further support for this guidance event being mediated by a Wnt protein comes from the analysis of mice deficient for Fz3, one of the Wnt receptors expressed by commissural neurons. In these mice, commissural growth cones emerge from the floor plate and tend to project randomly along the anterior–posterior axis of the spinal cord.

Similar to Wnt function in *Drosophila* and nematode, Wnt4 appears to control anterior–posterior guidance. However, in contrast to the repellant activity of Wnts in the guidance of *Drosophila* commissural axons, the nematode PVM axon, and of mammalian callosal and corticospinal axons, Wnt4 is acting as a chemoattractant in the guidance of postcrossing commissural axons. Notably, this guidance event does not involve Ryk since commissural neurons do not express the receptor. Instead, it seems that a member of the Fz family of Wnt receptors, acting in the absence of Ryk, mediates the guidance. A model similar to that for Shh has been proposed in which Wnts can act as either chemoattractants or chemorepellants depending on whether a Fz or Ryk receptor is involved. However, the relative roles of these receptors in Wnt-mediated guidance in nematode suggest that the situation may turn out to be more complicated.

Conclusion

Hedgehog, TGF-β, and Wnt family members not only have roles in cell fate specification and early embryonic patterning but also act as axon guidance molecules. For Shh and BMPs, morphogen gradients initially used to specify cell types in the spinal cord are reused later in development to attract or repel axons. There is evidence that Shh, BMP7, and Wnt proteins act directly on the growth cone rather than through canonical signaling pathways to the nucleus. The current challenge, and one which is faced generally in studies of guidance molecules, is to identify the signaling pathways engaged by the receptors and to understand how these signals are integrated within the growth cone to achieve changes in direction of growth.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axonal Pathfinding: Extracellular Matrix Role; Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh); Axonal Pathfinding: Netrins; Axonal Regeneration: Role of Growth and Guidance Cues; Growth Cones; Morphogens: History.

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Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh)

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Introduction

Over the past decade, genetic, biochemical, and molecular approaches have led to the identification of four major conserved families of guidance cues with prominent developmental effects: the netrins, slits, semaphorins, and ephrins. More recently, members from three other families of secreted signaling molecules have been shown to act as guidance cues: the wingless/Wnt, the hedgehog (Hh), and the decapentaplegic/bone morphogenetic protein/transforming growth factor-β (Dpp/BMP/TGF-β) families. In addition to their axon guidance properties, these molecules share a common characteristic of having been previously identified as morphogens controlling cell fate and tissue patterning. This discovery has opened the door to the study of an entirely new set of axon guidance cues.

This article focuses on the role of the morphogen Shh in axon guidance. After briefly introducing the role and the signaling pathway of Shh in patterning the neural tube, we discuss the emerging evidence that Shh is reused later in development to guide axons, both in the developing spinal cord and the retina. We conclude by discussing the implications for Shh signaling in axon guidance.

The Hedgehog Family in Cell Fate Specification and Tissue Patterning

Morphogens are signaling molecules produced in a restricted region of a tissue; they provide positional information by diffusing from their source to form long-range concentration gradients. A cell's program of differentiation in response to a morphogen is dictated by its position within the gradient and thus on its distance from the morphogen source. Two criteria have gained acceptance as the evidence needed to qualify a secreted signaling protein as a morphogen: it must have a concentration-dependent effect on its target cells and it must exert a direct action at a distance. In vertebrates, Sonic hedgehog (Shh) has been shown to fulfill these criteria and function as a morphogen to specify cell fate in the developing neural tube.

Hedgehog proteins are found in insects and vertebrates, but not nematodes. They are encoded by a single hedgehog gene in flies, and three in mammals: *Shh*, Indian hedgehog (*Ihh*), and Desert hedgehog (*Dhh*). In vertebrate embryos, one of the first steps in the development of the nervous system is the specification of the diverse neural cell fates of the neural tube. Shh is secreted by the notochord and by floor plate cells at the ventral midline of the neural tube, and functions as a graded signal for the generation of distinct classes of ventral neurons along the dorsoventral axis of the neural tube (**Figure 1(a)**). In agreement with its role as a morphogen, Shh is able to induce a range of ventral spinal cord cell fates in a concentration-dependent manner and has been shown to exert a direct action at a distance to specify neural tube cell fate.

Much evidence indicates that the cell fate specification and tissue patterning activities of Hhs are mediated by members of the Ci/Gli transcription factor family, but the signaling mechanisms that lead to activation of these transcription factors are not fully elucidated. Genetic and biochemical experiments have shown that Hhs activate signaling by binding to their receptor Patched (Ptc), which leads to the relief of Ptc-mediated inhibition of Smoothened (Smo), also a transmembrane protein, which can then activate downstream signaling (**Figure 2**). Smo associates directly with a Ci-containing complex, which contains the atypical kinesin Costal-2 (Cos2), the protein kinase Fused (Fu), and the Suppressor of Fused (Su(fu)). This complex constitutively suppresses pathway activity. Activation of Hh signaling reverses this regulatory effect and allows Ci to activate transcription of Hh target genes, thus specifying cell fate.

Roles of Shh in Axon Guidance

Shh Is a Chemoattractant for Commissural Axons

During spinal cord development, commissural neurons, which differentiate in the dorsal neural tube, send axons that project toward and subsequently across the floor plate, forming axon commissures (**Figure 1(b)**). These axons project toward the midline in part because they are attracted by netrin 1, a long-range chemoattractant secreted by the floor plate. In mice mutant for *netrin 1* or the netrin 1 receptor gene, *Dcc* (*deleted in colorectal cancer*), many commissural axon trajectories are foreshortened, fail to invade the ventral spinal cord, and are misguided. However, some of them do reach the midline, indicating that other guidance cues cooperate with netrin 1 to guide these axons. Further analysis of *netrin 1* knockout

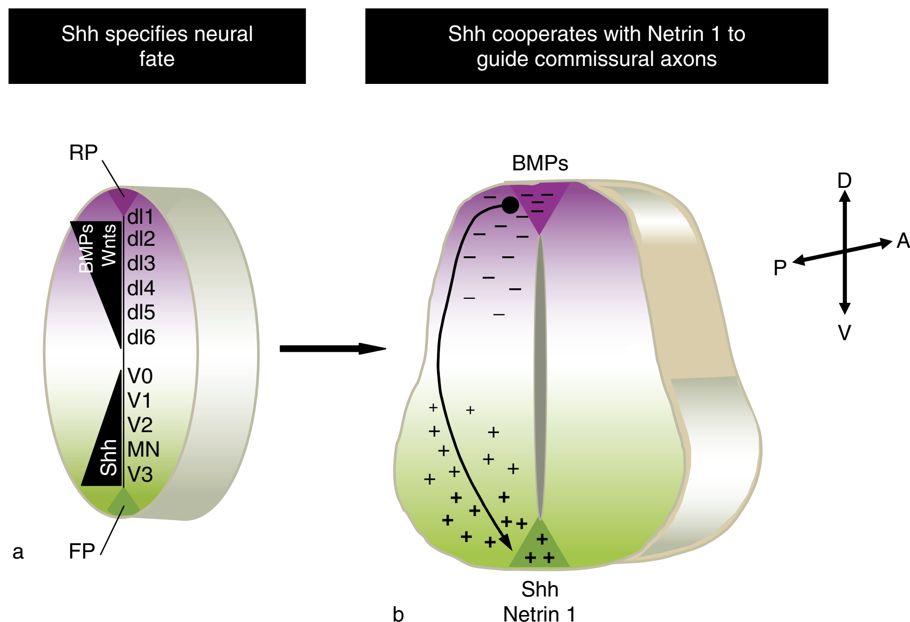

Figure 1 Neuronal cell fate specification and guidance of precrossing commissural axons by Sonic hedgehog (Shh) and netrin 1; cross-section representations of the developing neural tube. Shh is first used to pattern neural progenitors in the spinal cord and then appears to be reused as a guidance cue for commissural axons. (a) In the early neural tube, Shh – together with bone morphogenetic protein (BMP) and Wnt – protein concentration gradients act to specify neural cell fate in the ventral (and dorsal, for BMP and Wnt) spinal cord. (b) Later, the axons of differentiated commissural neurons are attracted to the ventral midline by the combined chemoattractant effects of netrin 1 and Shh. BMPs also contribute to commissural axon guidance by repelling axons from the dorsal midline. V0–V3, ventral interneuron subpopulations; dI1–dI6, dorsal interneuron subpopulations; MN, motor neurons; RP, roof plate; FP, floor plate; D, dorsal; V, ventral; P, posterior; A, anterior. Adapted from Charron F and Tessier-Lavigne M (2005) Novel brain wiring functions for classical morphogens: A role as graded positional cues in axon guidance. *Development* 132: 2251–2262.

mice suggests that the floor plate might actually express an additional diffusible attractant(s) for commissural axons.

Given its expression by the floor plate and its long-range effects in the spinal cord, Shh was a candidate for a midline-derived axonal guidance cue. Shh was indeed shown to function as an axonal chemoattractant that can mimic the netrin-1-independent chemoattractant activity of the floor plate in *in vitro* assays. The chemoattractant activity of Shh, like the chemoattractant activity of floor plate derived from *netrin 1* mutants, can be blocked by cyclopamine, which blocks the actions of Shh in cell fate determination by inhibiting the Shh signaling mediator Smo. This shows that Smo is required for Shh-mediated axon attraction and, importantly, that the netrin-1-independent chemoattractant activity of the floor plate also requires Hh signaling. Since Shh is the only Hh family member expressed in the spinal cord at this stage, these results suggest that Shh functions as a floor plate-derived chemoattractant for commissural axons.

While the reorienting effect of Shh could be due to a direct chemoattractant effect, an alternative explanation was suggested by the fact that Shh is a potent morphogen. Since in these assays commissural axon turning occurs within the spinal cord tissue explant, it seemed possible that Shh was not acting directly on the axons but rather was repatterning and altering the expression of guidance cues by cells within the explant, which then secondarily guided the axons to the Shh source. Arguing against this possibility was the finding that the spinal cord explants used to assess chemoattractant activity are at a developmental stage at which they have apparently lost the competence to be repatterned by Shh, as assessed using a battery of markers of dorsoventral patterning.

A direct action of Shh in attracting the axons was supported further by two sets of experiments. First, Shh was shown to attract the growth cones of isolated *Xenopus* spinal axons in dispersed cell culture in a cyclopamine-dependent manner, proving that Shh, acting via Smo, can function as a chemoattractant, at least for these *Xenopus* axons. A second way of providing evidence that Shh can act directly on commissural axons to guide them relied on blocking Shh signaling selectively in commissural neurons without blocking it in the terrain through which their axons course. This was achieved by conditional inactivation of a floxed allele of *Smo* using the Cre recombinase

expressed under the control of the Wnt1 promoter, which drives expression in the dorsal spinal cord (as well as in neural crest progenitors). When Cre, driven by this promoter, was used to delete a floxed *Smo* allele in the dorsal spinal cord, commissural axon trajectories were defective in the ventral spinal cord, where Cre is not expressed (**Table 1**). This result strongly implies that the axonal misrouting is not due to repatterning of the ventral spinal cord, and must instead reflect a guidance defect arising from loss of Smo function in commissural neurons. Taken together, these results suggest that Shh functions to guide commissural axons both *in vitro* and *in vivo* by acting directly as a chemoattractant on these axons through an Smo-dependent signaling mechanism.

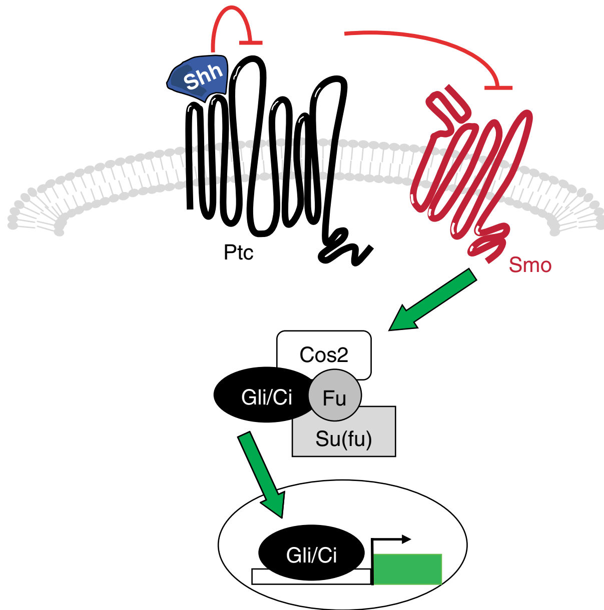

Figure 2 The Sonic hedgehog (Shh) signaling pathway. Genetic and biochemical experiments have shown that hedgehogs activate signaling by binding to their receptor Patched (Ptc; a 12-pass transmembrane protein), which leads to the relief of Ptc-mediated inhibition of Smoothened (Smo), a 7-pass transmembrane protein, which can then activate downstream signaling. Smo associates directly with a Gli/Ci-containing complex which contains the atypical kinesin Costal-2 (Cos2), the protein kinase Fused (Fu), and the Suppressor of Fused (Su(fu)). This complex constitutively suppresses pathway activity by leading to the proteolytic cleavage of Ci, which acts as a transcriptional repressor. Activation of hedgehog signaling reverses this regulatory effect and leads to the production of full-length Ci, which activates transcription of hedgehog target genes.

Boc Is a Receptor for Shh in the Guidance of Commissural Axons to the Floor Plate

Although Shh acts through Smo to attract commissural axons to the floor plate, Smo does not bind Shh, and the binding receptors acting with Smo to mediate the effect of Shh in axon guidance remain elusive. Cdon (cell adhesion molecule-related/downregulated by oncogenes) and Boc (biregional Cdon binding protein) have been implicated in enhancing muscle differentiation. They are type I transmembrane proteins consisting of four to five immunoglobulin (Ig) and two to three fibronectin type III (FNIII) repeats in the extracellular domain. This domain architecture is highly related to that of axon guidance receptors of the Robo and Dcc families. Both Cdon and Boc share a high degree of homology in their extracellular domains and are expressed during early stages of central nervous system (CNS) development. Interestingly, mice with homozygous mutations in Cdon display a microform of holoprosencephaly (HPE), a developmental defect of the forebrain and midface caused by a failure to delineate the midline. In both humans and mice, many forms of HPE are caused by disruptions in the Shh signaling pathway, suggesting that Cdon and Boc might regulate Shh signaling, a possibility supported by the finding that a Cdon/Boc homolog in *Drosophila*, iHog, is required for Hh signaling. Taken together, these observations suggested that Cdon and/or Boc might function in Shh-mediated axon guidance in vertebrates.

The ability of Boc and Cdon to bind Shh was tested and they were found to bind specifically and directly to Shh, suggesting that they could function as Shh receptors. These results are consistent with recent reports demonstrating that Cdon, Boc, and iHog bind and

Table 1 *In vivo* experiments supporting a role for Shh in axon guidance

Gene	*Species*	*Experiment*	*Phenotype*	*Reference*
Smo	Mouse	Conditional inactivation of *Smo* in commissural neurons	Commissural axons project abnormally and invade the motor columns	Charron et al. (2003)
Boc	Mouse	*Boc* inactivation	Commissural axons project abnormally and invade the motor columns	Okada et al. (2006)
Shh	Chick	Ectopic expression of Shh in the optic chiasm	Retinal axons are prevented from crossing the chiasm	Trousse et al. (2001)
Shh	Chick	Silencing of *Shh* by RNA interference	Commissural axons stall at the contralateral floor plate border, with some axons randomly turning caudally or rostrally	Bourikas et al. (2005)

signal through the Hh pathway. To explore their involvement in commissural axon guidance, the expression of Cdon and Boc was examined in the developing mouse spinal cord. Boc is expressed by differentiating commissural neurons and the Boc protein, but not Cdon, is expressed by growing commissural axons. To assess the functions of these receptors in commissural axon guidance, Boc and Cdon mutant mice were generated and sections of spinal cord from Boc and Cdon mutant embryos were analyzed. Although commissural axon projections appeared normal in Cdon mutant animals, abnormal projections of commissural axons were observed in Boc mutants: the axons were highly dispersed and invaded the ventral spinal cord, with ectopic projections extending over the motor columns. Analysis of various neural tube markers indicated that neuronal patterning is occurring normally in these animals. This phenotype is similar to that observed in mice following conditional removal of the Shh signaling mediator Smo in commissural neurons (using a Wnt1 promoter to drive Cre expression; see earlier) and suggests that Boc acts in the same pathway as Smo to guide commissural axons in response to Shh. Finally, using an *in vitro* commissural axon turning assay to test the role of Boc in Shh-mediated axon turning, it was shown that RNA interference (RNAi)-mediated knockdown of Boc impaired the ability of commissural axons to turn toward an ectopic source of Shh *in vitro*. These results indicates that Boc is required for commissural axons to respond to the chemoattractive effect of Shh. Collectively, these data suggest that Boc plays an essential role as a receptor for Shh in commissural axon guidance.

Consistent with a role for Boc in axon pathfinding, an earlier study showed defects in forebrain axon guidance in zebra fish treated with morpholinos directed against Boc. However, based on indirect evidence, these results were interpreted to reflect a role for Boc as a repulsive ligand. In the mammalian spinal cord, the aforementioned results argue that Boc functions as a receptor for Shh-mediated attraction; whether it can also function as a repulsive ligand in other contexts in mammals remains to be explored. Additionally, it will be of interest to determine whether Boc and Cdon also play a role in the guidance of other axonal tracts in the developing mammal and fish embryos.

Shh Guides Commissural Axons along the Longitudinal Axis of the Spinal Cord

After commissural axons have reached and crossed the floor plate, they make a sharp anterior turn toward the brain (**Figure 3**). The molecules involved in the dorsoventral projection of commissural axons to and at the floor plate have been well described, but it is only recently that cues controlling anteroposterior guidance have been identified. Remarkably, the guidance of commissural axons to the floor plate is not, apparently, the only effect of Shh on commissural axons: recent evidence suggests that Shh also guides postcrossing commissural axons in the rostral direction along the longitudinal axis of the spinal cord. Using a subtractive hybridization approach to identify guidance cues responsible for the rostral turn of postcrossing commissural axons in chick embryos, Bourikas and colleagues identified differentially expressed candidates whose function they investigated by RNAi-mediated *in ovo* gene silencing. Unexpectedly, one of their candidates turned out to be Shh. In agreement with these results, silencing of the *Shh* gene by a different RNAi construct or injection of a hybridoma producing a function-blocking Shh antibody led to axon stalling at the contralateral floor plate border, with some axons

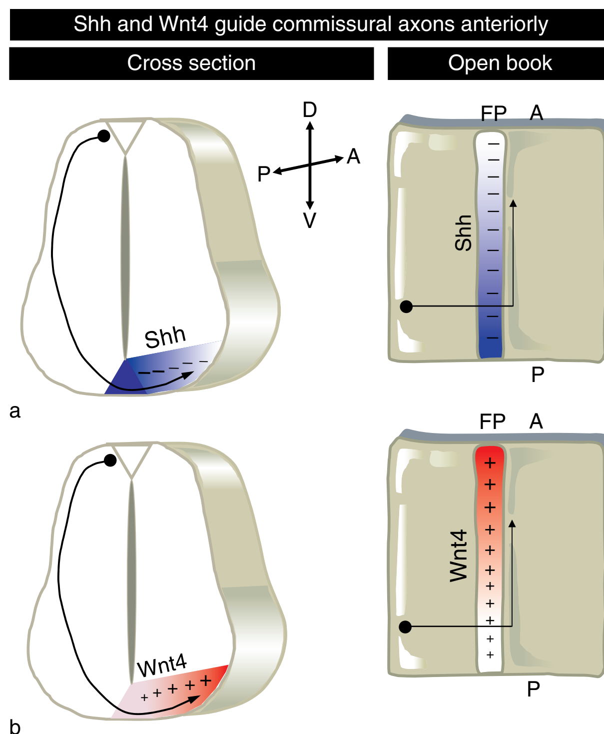

Figure 3 Guidance of postcrossing commissural axons by Sonic hedgehog (Shh) and Wnt4. (a) After crossing the floor plate, chick commissural axons are repelled from the posterior pole by an increasing anterior to posterior Shh gradient. (b) In rodents, commissural axons are attracted to the anterior pole by an increasing posterior-to-anterior Wnt4 gradient. Left panels in a and b are cross-section representations of the developing spinal cord, and right panels are open-book representations. FP, floor plate; D, dorsal; V, ventral; P, posterior; A, anterior. Adapted from Charron F and Tessier-Lavigne M (2005) Novel brain wiring functions for classical morphogens: A role as graded positional cues in axon guidance. *Development* 132: 2251–2262.

turning caudally or rostrally, apparently in a random manner. Importantly, marker analysis revealed that the patterning of the spinal cord was not apparently affected by these manipulations, suggesting that these experiments were performed after neural cell fate specification by Shh has occurred. Finally, postcrossing commissural axons were shown to avoid ectopic Shh *in vivo*. Together, these results provide strong evidence that Shh is essential for the normal guidance of commissural axons along the longitudinal axis of the spinal cord, at least in chick embryos.

An Shh gradient could guide commissural axons along the longitudinal axis directly, or could alternatively be acting only indirectly by controlling a graded distribution of a distinct guidance cue. Two lines of evidence, however, were provided for a direct role of Shh. The first came from an investigation of the receptor mechanism for this guidance. Interestingly, neither cyclopamine nor Smo RNAi interfered with the rostral turn of commissural axons along the longitudinal axis, suggesting that Smo might not be involved in this process. Instead, RNAi-mediated silencing of *Hip1*, a gene encoding an Shh-binding membrane protein transiently expressed in commissural neurons at the time when they cross the floor plate, as well as in the periventricular region, resulted in the same postcrossing phenotype as seen with Shh RNAi. These results, which contrast with the essential role of Smo in Shh-mediated attraction of commissural axons to the floor plate, suggest that Hip1 might be involved in transducing an Shh guidance signal in postcrossing commissural neurons. The relatively restricted expression of Hip1 mRNA to commissural neurons would be consistent with a direct action of Shh on these axons. A second line of evidence that supports a direct role for Shh was obtained by *in vitro* experiments, which showed that postcrossing commissural axons from spinal cord explants can be repelled by Shh beads *in vitro*. Taken together, these results suggest a model in which Shh could be functioning directly through Hip1 as a chemorepellent for postcrossing commissural axons.

Prior to the finding that Shh controls the anteroposterior guidance of commissural axons in chicks, Wnt4 was reported to play a role in this process in rodents. Using a novel *in vitro* assay, evidence was obtained that the activity responsible for the anterior guidance of postcrossing commissural axons in rodents is an increasing posterior to anterior gradient of a diffusible attractant. Although it is not yet known whether Wnt4 guides postcrossing commissural axons in chicks and whether Shh guides postcrossing commissural axons in rodents, it is nonetheless interesting to note that, if this is the case, the complementary Wnt4 and Shh gradients might act cooperatively in the rostral guidance of commissural axons. Additionally, since Boc plays a role in the guidance of precrossing commissural axons, it will be interesting to determine whether it also plays a role in the guidance of postcrossing commissural axons.

Shh Is a Negative Regulator of Retinal Ganglion Cell Axon Growth

Retinal ganglion cell (RGC) axons growing toward the diencephalic ventral midline are faced with the decision to project either contralaterally or ipsilaterally in response to guidance cues at the optic chiasm (**Figure 4**). Homozygous inactivation of the mouse *Pax2* gene alters the development of the optic chiasm, and RGC axons never cross the midline in these mice. Interestingly, whereas in wild-type mice Shh expression is downregulated in the chiasm as RGC axons are migrating toward this region, Shh expression is ectopically maintained along the ventral midline in *Pax2*$^{-/-}$ mice. These observations raise the possibility that the continuous expression of Shh at the ventral midline might contribute to preventing RGC axon crossing. In agreement with this idea, Trousse and colleagues found that ectopic expression of Shh in the midline region interferes with RGC axon growth and prevents them

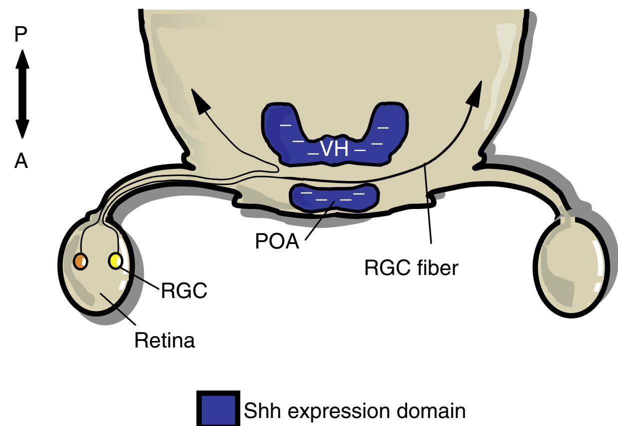

Figure 4 Sonic hedgehog (Shh) expression at the chiasm border defines a barrier within the ventral midline implicated in guiding retinal ganglion cell (RGC) axons. RGC axons growing toward the diencephalic ventral midline are faced with the decision to project either contralaterally or ipsilaterally in response to guidance cues at the optic chiasm. The Shh expression domain is shown in blue. Shh can inhibit retinal axons *in vitro*, suggesting that *in vivo* it may be acting on the axons directly rather than by altering the expression of distinct guidance cues in the chiasm, although conclusive evidence for this guidance function *in vivo* remains to be obtained. A, anterior; P, posterior; POA, preoptic area; VH, ventral hypothalamus. Adapted from Charron F and Tessier-Lavigne M (2005) Novel brain wiring functions for classical morphogens: A role as graded positional cues in axon guidance. *Development* 132: 2251–2262.

from crossing the midline (**Figure 4**). Consistent with the idea that Shh might be acting directly on RGC axons, it was shown that these manipulations do not affect patterning and neural differentiation in the eye. Further experiments will be required to determine whether the chiasm region is repatterned in these experiments, but *in vitro* experiments support the idea that Shh acts directly to control RGC axon migration: addition of exogenous recombinant Shh to retinal explants decreases the number and length of growing axons, without interfering with the rate of proliferation and differentiation of cells in the explant, and time-lapse analysis shows that addition of Shh to retinal explants rapidly causes growth cone arrest and subsequent retraction of RGC axons. Since the response of the growth cone to many extracellular guidance cues appears to be modulated and in some cases perhaps even mediated by intracellular cyclic nucleotide levels (cyclic adenosine monophosphate and cyclic guanosine monophosphate, cAMP and cGMP), the possibility was explored that the effect of Shh on retinal axons *in vitro* might be due to a change in cAMP levels. In agreement with this, addition of Shh to retinal growth cones was shown to decrease intracellular levels of cAMP, a finding consistent with the observation that lowering cAMP levels favors growth inhibition.

Taken together, these results provide evidence that Shh expression at the chiasm border helps define a barrier within the ventral midline that serves to guide RGC axons, and suggest that Shh may be acting on the axons directly, rather than indirectly by repatterning the chiasm. The finding that Shh acts directly to guide RGC axons is also supported by a recent study suggesting that Shh acts directly and rapidly on the growth cone of RGCs cultured *in vitro*. Proving that the effect *in vivo* is direct will, however, require additional studies, such as identifying the mechanism that mediates retinal growth cone responses to Shh and showing that cell-autonomous inhibition of this signaling pathway in the neurons results in guidance defects *in vivo*.

Molecular Mechanism underlying Shh-Mediated Axon Guidance

The aforementioned studies, summarized from several laboratories, have provided evidence that Shh plays a role in the guidance of at least two types of axons: commissural and RGC axons. In the case of precrossing commissural axons, Shh acts as a chemoattractant and its effect is mediated by Boc, a recently identified Shh receptor related to Dcc and Robo family members. In addition, the chemoattractant effect of Shh requires the canonical Shh signaling molecule Smo. After commissural axons have crossed the floor plate, evidence indicates that Shh then functions as a chemorepellent to direct their anterior migration. This later effect of Shh appears to require the Shh binding protein Hip1 and might occur independently of Smo activity. Finally, in the case of RGCs, Shh acts as a negative regulator of axonal growth, at least *in vitro*, but the receptor and the signaling molecules involved in this effect remain elusive.

Although the chemoattractant and chemorepellent effects of Shh might appear to be inconsistent with one another, many other guidance cues have been shown to be bifunctional and exert opposite effects, depending on the context. For example, extrinsic factors can convert netrin attraction to repulsion by modulating cyclic nucleotide levels. Thus, the opposite effects of Shh on pre- and postcrossing commissural and retinal axons might be due to an intrinsic or extrinsic factor that modulates cyclic nucleotide levels. Alternatively, as the molecular mechanisms underlying the effects of Shh on commissural and retinal axons are poorly understood, it is also possible that these pre- and postcrossing effects are mediated by distinct signaling pathways that result in opposite guidance effects – a possibility that also has a precedent in the case of netrins, which can attract axons by activating Dcc family receptors and repel them by activating UNC5 family receptors.

In all of the studies summarized here, Shh was shown to act rapidly (in an hour or less) to affect growth cone morphology. Although these results appear inconsistent with the model that the Shh axon guidance effect function through the transcriptional signaling pathway to the nucleus, this needs to be formally proved, as none of the studies described here has addressed this issue directly. Nonetheless, even if a transcriptional response is found to be required for their guidance effects, additional local signaling would still be required to be elicited in the growth cone in order to generate a polarized response leading to growth cone turning in a specific direction. Indeed, a purely transcriptional response consisting of a retrograde signal to the nucleus, followed by an anterograde signal back to the growth cone, cannot account for the polarized turning effect of a guidance cue. Further studies aimed at understanding the molecular mechanisms underlying growth cone turning by Shh will be necessary to identify the molecules linking Shh signaling to localized growth cone effects.

In this regard, despite many efforts, the canonical Hh signaling pathway is only beginning to be understood, and many intermediate signaling molecules remain to be identified and characterized. Thus, it

is possible that the signaling proteins eliciting the growth cone effects are simply components of the signaling pathway required for cell fate specification that are awaiting identification and further characterization to uncover their function in axon guidance.

Alternatively, Shh might be acting through entirely different signaling pathways in axon guidance and cell fate specification, including the use of a different receptor. For example, although Smo and Boc are required for Shh-mediated commissural axon guidance to the floor plate, it is not known whether Ptc, the canonical Shh-binding component of the Shh receptor, is involved. This finding contrasts with chick postcrossing commissural axon guidance, in which Smo does not appear to be required for the rostral turn away from the Shh gradient. Additional experiments on commissural and retinal axons will be required to determine the receptor components and intracellular signaling molecules mediating the effects of Shh on axon guidance.

Conclusion

The discovery that the morphogen Shh can be reused to guide axons has generated considerable excitement in the field, and it will be interesting to see to what extent its signaling components are conserved between the morphogenic and guidance responses. The characterization of the Shh chemotropic signaling pathway will open new avenues to study how guidance signals regulate the motility and steering of the growth cones, and will help elucidate the mechanisms directing the complex wiring of the nervous system.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Axonal Pathfinding: Netrins; Growth Cones; Sonic Hedgehog and Neural Patterning; Wnt Pathway and Neural Patterning.

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Axonal Pathfinding: Netrins

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Introduction

Directing growing axons to their targets is an essential step toward establishing appropriate connections in the nervous system. The growth cone, located at the motile tip of an axon, senses cues within its environment to guide extending axons. Extracellular guidance cues can attract or repel axons. They may be transmembrane, glycosylphosphatidylinositol (GPI)-linked, or secreted cues. Secreted cues have the unique capacity to function at a distance by diffusing away from their source. The netrin family contains both secreted members (netrin-1–netrin-4) and GPI-linked members (netrin-G1 and netrin-G2).

The discovery of the netrin family can be traced back to observations made in the late nineteenth century by the Spanish neuroscientist Santiago Ramón y Cajal. He was the first to propose that gradients of diffusible cues might guide axons. One location where he thought this may occur is in the developing spinal cord where commissural axons extend ventrally toward the floor plate (**Figure 1(a)**). Specifically, he proposed that a gradient of a diffusible cue, emanating from the floor plate at the ventral midline, would function to attract growing commissural axons. In the 1980s, co-culture studies using explants of embryonic neural tube provided experimental evidence for the presence of guidance cues secreted by the floor plate. Initial experiments demonstrated that axon bundles would extend from an explant containing the cell bodies of commissural neurons, through a collagen matrix toward a floor plate explant cultured at a distance (**Figure 1(b)**). Subsequent experiments placed an ectopic floor plate perpendicular to the normal trajectory of commissural axons, resulting in the axons being redirected within the neural epithelium toward the ectopic floor plate (**Figure 1(c)**). An activity that promoted commissural axon outgrowth was then identified in lysates of embryonic chick brains. Two related proteins were purified, their corresponding cDNAs cloned, and recombinant protein shown to mimic the ability of the floor plate to attract commissural axons. Sequence analysis identified homology to UNC-6, a protein required for the circumferential guidance of cells and axons in the roundworm *Caenorhabditis elegans*. These proteins were named netrins, based on the Sanskrit word *netr*, meaning 'one who guides.'

Netrin Family Members

Four secreted netrins (netrin-1–netrin-4) have been identified in vertebrates, along with two membrane-anchored forms, netrin-G1 and netrin-G2. Thus far, orthologs of netrin-2 have only been identified in birds and fish. Mammals express secreted netrin-1, netrin-3, and netrin-4. The netrin family can be divided into three subfamilies: netrins 1–3 (sometimes called 'classical netrins'), netrin-4, and netrin-G1 and netrin-G2. Based on sequence, netrin-4 and the netrin-Gs are more similar to laminins than to netrins 1–3, which exhibit a high degree of similarity to each other (**Figure 2(b)**). Another compelling argument for this subdivision is based on evolutionary conservation. Orthologs of netrins 1–3 have been detected in all bilaterally symmetrical animals studied so far, while orthologs of netrin-4 and netrin-Gs have only been found in vertebrate species (**Figure 2(c)**). Functional differences also support this distinction. Different receptors mediate the function of netrins 1–3 and netrin-Gs. Furthermore, netrins 1–3 similarly elicit axon outgrowth from embryonic spinal commissural neurons, while netrin-Gs do not.

Netrin Structure

Despite these differences, all netrins are classified into a single family on the basis of their size, approximately 600 amino acids, the presence of two characteristic conserved N-terminal domains, domains V and VI, and a more variable C-terminal domain, domain C. Domains V and VI in netrins are homologous to domains V and VI found at the N-terminal ends of the extracellular matrix protein laminin (**Figure 2(a)**). Laminins are large secreted heterotrimers made up of α, β, and γ subunits. Domains V and VI of netrin-4 and netrin-Gs are most similar to β subunits of laminin, while those of netrins 1–3 are more similar to the γ subunits (**Figure 2(b)**).

Domain VI, at the N-terminal end of netrins and laminins, is composed of approximately 300 amino acids. In laminins, this domain interacts with heparin, cell surface receptors, and extracellular matrix (ECM) proteins, and is required for calcium-dependent multimerization that generates a larger matrix of laminin molecules. Genetic studies in *C. elegans* have demonstrated that the highly conserved sequence SXDXGXS/

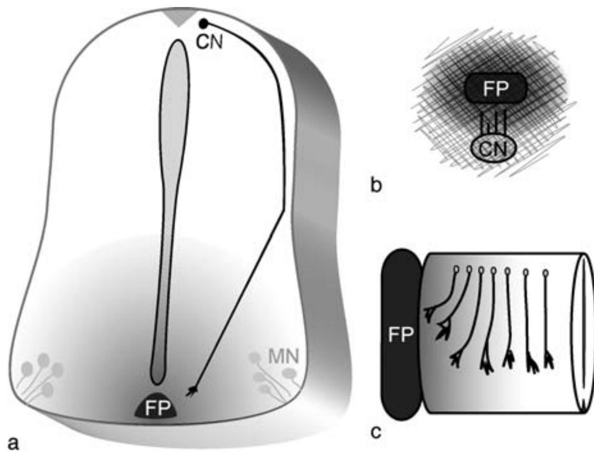

Figure 1 (a) The floor plate (FP) attracts commissural neuron (CN) axons, in part, by releasing netrin-1 (MN, motor neuron). (b) Commissural neurons will extend axon bundles from an explant of dorsal embryonic spinal cord through a collagen matrix when either floor plate or cells expressing netrin are placed within 200–300 μm. (c) An ectopic floor plate or a cell line expressing netrin-1 will attract commissural neuron axons when placed perpendicular to their trajectory.

TW within domain VI of the netrin UNC-6 is required for both axon attraction and repulsion. The middle netrin domain, domain V, is approximately 150 amino acids long and contains three cysteine-rich epidermal growth factor (EGF) repeat subdomains named V-1, V-2, and V-3. Mutation of the third EGF repeat (V-3) disrupts chemoattraction in *C. elegans*, whereas repulsion is lost following mutation of either V-2 or V-3 domain.

The C-terminal domain C of secreted netrins (netrins 1–4) exhibits relatively limited sequence similarity to domains found in the complement C3, C4, and C5 protein family (i.e., CC3, CC4, and CC5), secreted frizzled-related proteins (sFRPs), type I C-proteinase enhancer proteins (PCOLCEs), and tissue inhibitors of metalloproteinases (TIMPs). The majority of netrin protein in the embryonic or mature central nervous system (CNS) is not freely soluble. Structure–function analyses of domain C suggest that it contributes to binding netrins to cell surfaces or extracellular matrix. Domain C includes many basic amino acids that may bind negatively charged sugars associated with pro-

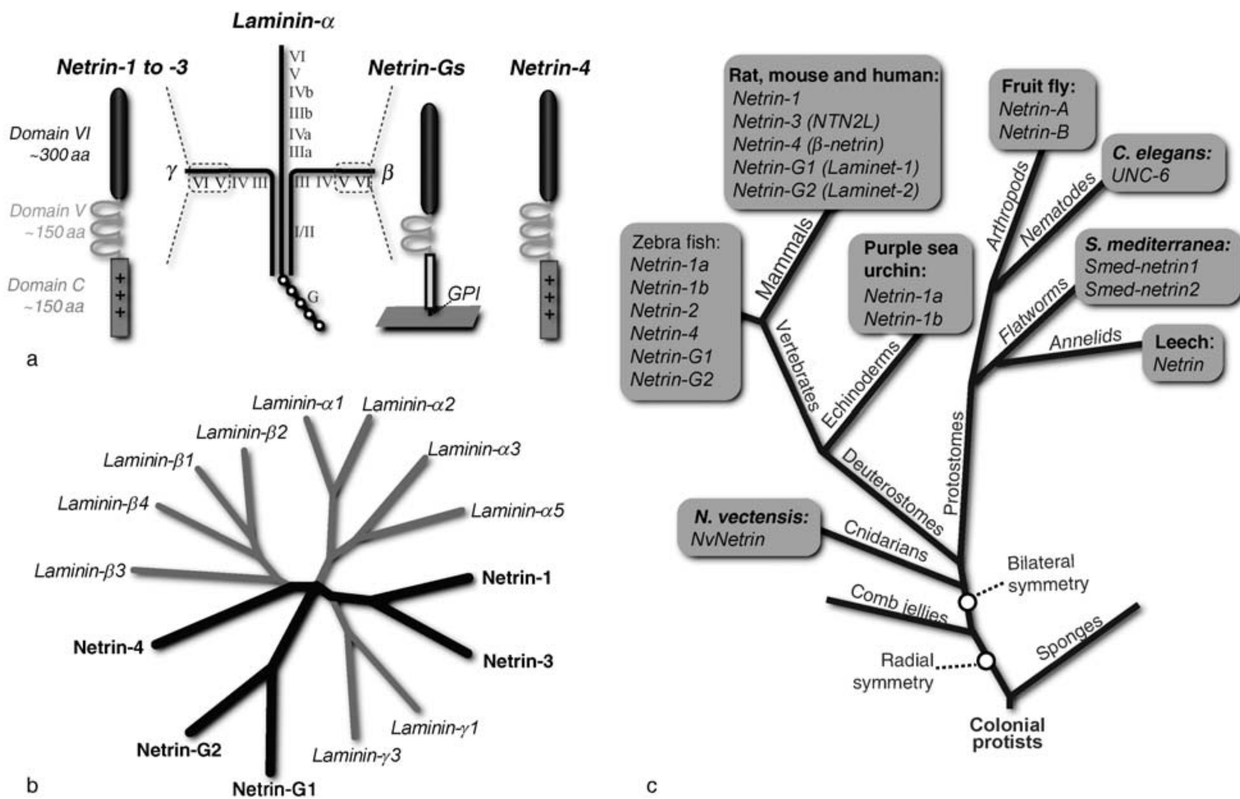

Figure 2 (a) Netrins contain N-terminal domains V and VI related to corresponding N-terminal domains of laminins. Domain V is composed of cysteine-rich epidermal growth factor repeats. Domain C in secreted netrins contains many positively charged, basic residues (GPI, glycophosphatidylinositol). (b) Phylogenetic tree based on the sequences of domains VI and V in human netrins and laminins. (c) Evolutionary tree diagram highlighting the presence of netrin homologs in a wide variety of bilaterally symmetrical organisms.

teoglycans, such as heparan sulfate proteoglycans and chondroitin sulfate proteoglycans. Presentation of netrins bound to surfaces may be a common mode of action in the netrin family; although the domain C is not conserved in the netrin-Gs, a C-terminal GPI link anchors them to cell surfaces.

Netrin Expression and Function during Development

Orthologs of netrin-1 have a firmly established, evolutionarily conserved role as secreted cues that direct axon guidance relative to the midline of developing bilaterally symmetric nervous systems. In fact, a netrin-1 ortholog has been identified in the stellate sea anemone *Nematostella vectensis* – an organism thought to exhibit some of the earliest hallmarks of bilateral symmetry (**Figures 2(c)** and **3(g)**). Multiple lines of evidence support the conclusion that netrin-1 attracts commissural axons toward and repels subsets of motor neuron axons away from the midline. Expression of a netrin-1 ortholog at the midline early in neural development is highly conserved (**Figure 3**). Ectopic expression of netrin-1 is sufficient to alter axon extension. Furthermore, netrin-1 ejected from a micropipette functions as a chemoattractant or chemorepellent, depending on the axons stimulated. Genetic disruption of the netrin UNC-6 in *C. elegans* disrupts circumferential axon extension toward and away from the ventral midline. Loss of netrin-1 function in mice generates major disruptions in multiple axon commissures, including the spinal ventral commissure, the corpus callosum, and the hippocampal commissure.

Netrin-1 also influences axon guidance away from the midline. It is required for retinal ganglion cell axons to exit the retina, for dopaminergic axon guidance in the ventral midbrain, and for thalamocortical projections. Netrin-1 also directs neural precursor cell migration, attracting cells that will become inferior olivary, pontine, luteinizing hormone-releasing hormone (LHRH), antidiuretic hormone (ADH), and oxytocin neurons, and repelling striatal neuronal precursors, cerebellar granule cells, spinal accessory neurons, and oligodendrocyte precursor cells. Although netrin-3 can mimic the ability of netrin-1 to attract spinal commissural axons and repel trochlear motor neuron axons *in vitro,* its expression in the spinal cord begins after the initial commissural axons have pioneered the path to the floor plate. Netrin-3 may, however, influence guidance of dorsal root ganglia axons to peripheral targets in the developing peripheral nervous system (PNS).

Very little is known regarding the role of netrin-4 in the development of the nervous system. It is widely expressed in the developing olfactory bulb, retina, and dorsal root ganglia, as well as by cerebellar granule, hippocampal, and cortical neurons. A relatively low level of netrin-4 expression has been detected adjacent to floor plate cells in the developing spinal cord, but only after the first commissural axons have crossed the midline. As discussed later, netrin-Gs do not appear to have a major role in the outgrowth and guidance of axons. They are expressed primarily by neurons, with very limited expression outside the nervous system. Netrin-G1 is expressed in the dorsal thalamus, olfactory bulb, and inferior colliculus, whereas netrin-G2 is expressed in the cerebral cortex.

Netrin-Induced Signal Transduction

Research investigating the signaling mechanisms elicited by netrins has focused on netrin-1, and there is little known about signaling by other netrins. Here we review current insight into netrin-1 signal transduction during axon guidance. The DCC ('deleted in colorectal cancer') family and the UNC5 homolog family are well-established receptors for netrin-1. In vertebrate species, these include DCC and neogenin, as well as UNC5A–UNCD. Both classes of receptors are single-pass transmembrane proteins that are also members of the large immunoglobulin superfamily.

Chemoattractant Signaling in Response to Netrin-1

DCC is required for axon chemoattraction to netrin-1. Although initially identified in vertebrates as a potential tumor suppressor, the importance of DCC in axon guidance was realized shortly after the discovery of netrin-1 as a result of mutational studies done in the roundworm *C. elegans*. While mutation of the *netrin* homolog *unc-6* affects both ventrally and dorsally directed neurons, mutations of the *C. elegans* homolog of *dcc*, *unc-40*, primarily disrupt ventrally directed axons. It was quickly realized that this phenotype was consistent with a potential role for UNC-40/DCC during axon attraction to netrins/UNC-6. Loss of DCC function in mice was then shown to cause neurodevelopmental defects very similar to loss of netrin-1 function, including disruption of the spinal ventral commissure, corpus callosum, and hippocampal commissure.

Structurally, the extracellular domain of DCC contains six fibronectin type 3 (FN3) repeats and four immunoglobulin (Ig) repeats. Netrin-1 appears to bind to the FN3 repeats of DCC, though reports conflict as to the exact domain involved. The DCC intracellular domain has no known catalytic activity; rather, it contains several putative phosphorylation

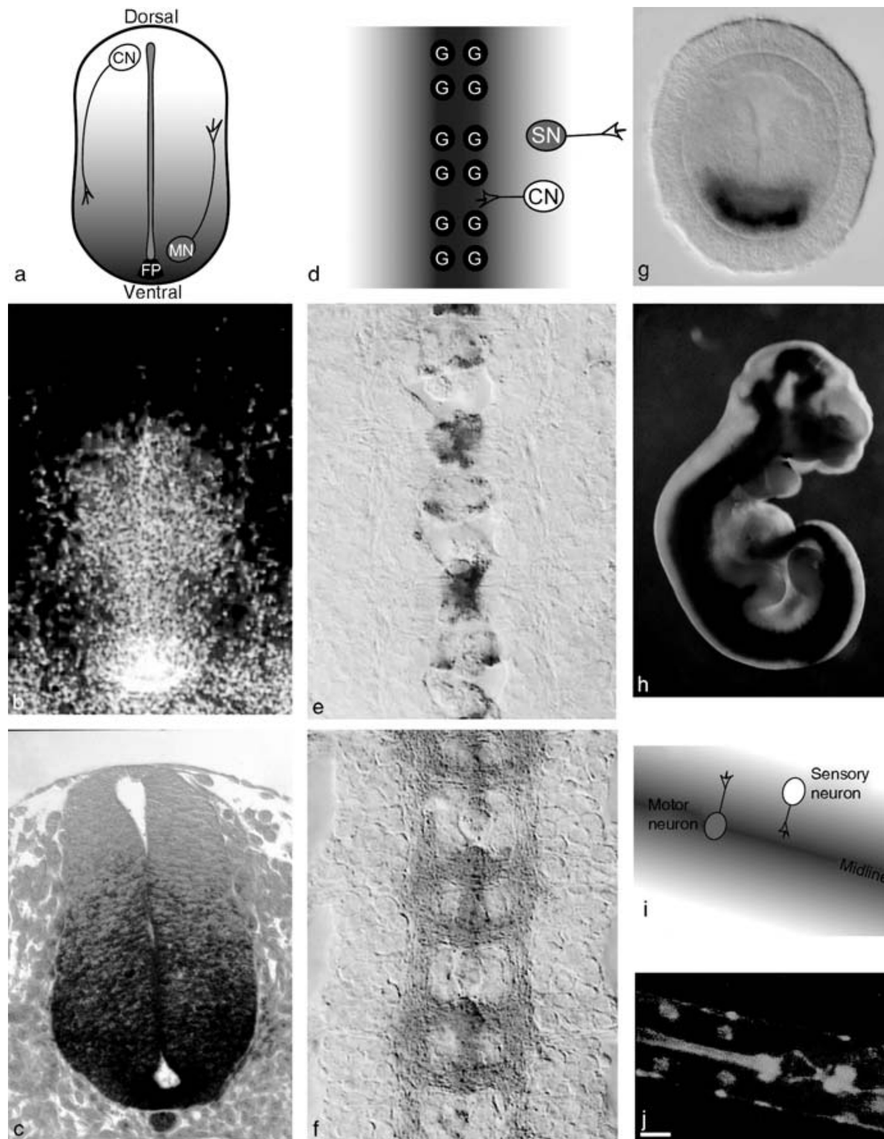

Figure 3 Midline expression of netrin homologs in a variety of organisms. The diagrams in panels (a), (d), and (i) illustrate simplified models of the distributions of netrin protein at the midline in the developing mouse spinal cord, fruit fly and *C. elegans* nematode worm, respectively. In each case, sensory neurons extend toward, while motor neurons extend away from, the source of netrin protein at the midline. *In situ* hybridization illustrates floor plate cells expressing netrin-1 in the embryonic day 9.5 mouse spinal cord (b). Whole-mount staining for expression of a β-galactosidase reporter gene shows netrin-1 expression in an entire E12.5-day-old mouse embryo, illustrating netrin-1 expression along the full rostrocaudal extent of the spinal cord, the developing brain, and the peripheral nervous system (h). *In situ* hybridization reveals netrin expression in the fruit fly (e) and the stellate sea anemme *N. vectensis* (g) during neural development. The distribution of netrin protein in the embryonic day 9.5 mouse spinal cord (c), as well as fruit fly (f) and *C. elegans* (j) embryos are also shown. CN, commissural neuron; MN, motoneuron; SN, segmental nerve. (b) Reprinted from Serafini T, Colamarino SA, Leonardo ED, et al. (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87: 1001–1014, with permission. (e, f) Reprinted from Harris R, Sabatelli LM, and Seeger MA (1996) Guidance cues at the *Drosophila* CNS midline: Identification and characterization of two *Drosophila* netrin/UNC-6 homologs. *Neuron* 17: 217–228, with permission. (g) Reprinted from Matus DQ, Pang K, Marlow H, et al. (2006) Deep evolutionary roots for bilaterality in the metazoa. *Proceedings of the National Academy of Sciences of the United States of America* 103: 11195–11200, with permission. (j) Reprinted from Wadsworth WG, Bhatt H, and Hedgecock EM (1996) Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in *C. elegans*. *Neuron* 16: 35–46, with permission.

and binding sites for intracellular proteins. Based on particularly strong evolutionary conservation, three domains (P1, P2, and P3) within the intracellular domain of DCC have been described. The P2 domain is rich in proline residues, containing four PXXP putative SH3 domain-binding motifs, while the P3 domain contains several highly conserved possible phosphorylation sites.

Numerous studies have now described signal transduction events implicated in the response to netrin-1, but our understanding of the mechanisms underlying how a growth cone responds to netrin-1 as a chemoattractant remains fragmentary. Netrin-1-induced multimerization of DCC via its P3 intracellular domain is thought to be essential for chemoattraction. Netrin-1 binding DCC activates the Rho GTPases Rac1 and Cdc42, key intracellular coordinators of cytoskeletal and adhesive interactions. Furthermore, intracellular proteins reported to associate with the intracellular domain of DCC include the adapter protein Nck1, the tyrosine kinases Fak and Fyn, the actin-binding proteins Ena/Vasp and N-Wasp, and the serine/threonine kinase Pak, a downstream effector of Rac1 and Cdc42 activation. Netrin-1 binding DCC also triggers generation of phosphoinositides and their breakdown by phospholipase C into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, leading to calcium release from intracellular stores and activation of protein kinase C. **Figure 4**(**a**) outlines a speculative model of the signal transduction events that occur during chemoattraction to netrin-1.

Chemorepellent Signaling in Response to Netrin-1

Axonal repulsion in response to netrin-1 requires a member of the UNC5 protein family. Four family members are present in mammals: UNC5A, UNC5B, UNC5C, and UNC5D. In *C. elegans*, in contrast to mutation of the *dcc* homolog *unc-40* that affects ventrally extending axons, mutation of *unc-5* predominantly causes defects in dorsally directed axons (i.e., those extending away from the source of UNC-6 netrin). Misexpression of *unc-5* is sufficient to redirect them dorsally, away from the ventral source of UNC-6. The extracellular domain of UNC5 homologs contains two membrane-proximal thrombospondin repeats and distally two immunoglobulin repeats. Netrin-1 binds to both immunoglobulin repeats. The intracellular domain of UNC5 contains three identified conserved domains: a ZU5 domain, a DCC-binding (DB) domain, and a death domain (DD). Although the specific function of the ZU5 domain is not known, a homologous domain is present in the scaffolding protein zona occludens-1 found at tight junctions, and deletion of the UNC5 ZU5 domain in *Drosophila* disrupts UNC5-mediated chemorepulsion.

Interestingly, depending on the distance of the growth cone from the source of netrin, different signaling mechanisms appear to be engaged. Long-range repulsion to netrin requires expression of both UNC5 and DCC, whereas short-range repulsion requires UNC5, but DCC is not essential. One hypothesis for this difference is that DCC and UNC5 together may form a more sensitive netrin receptor complex that is able to respond to the lower concentrations of netrin present at greater distances. At long range, the cytoplasmic domains of UNC5 and DCC associate directly. At short range, genetic studies in *C. elegans* have stressed the importance of an association between UNC5 cytoplasmic ZU5 and DD domains. Several proteins that interact with the UNC5 intracellular domain have been identified. These include the tyrosine kinase Src1, the tyrosine phosphatase Shp2, the F-actin anticapping protein Mena, the structural protein ankyrin, and the adapter protein Max1. Netrin-1-mediated growth cone repulsion triggers tyrosine phosphorylation of UNC5's intracellular domains at multiple sites. A speculative model outlining intracellular events that occur during short- and long-range netrin-mediated growth cone repulsion is illustrated in **Figure 4**(**b**).

Regulating Growth Cone Response to Netrin-1

Single growth cones have been shown to have the capacity to rapidly switch between responding to netrin as an attractant or a repellent. The mechanisms that control this shift in responsiveness have been the topic of intense investigation.

Altering UNC5 or DCC expression is one mechanism that influences how a growth cone responds to netrin. For example, the homeobox transcription factor 'even-skipped' promotes *unc5* expression in the fruit fly *Drosophila melanogaster*. Local protein synthesis within the growth cone also appears to contribute to the attraction of axons to netrin-1, though the exact mechanism of action is not clear. DCC expression can be downregulated through proteolytic degradation by either extracellular metalloproteinases or intracellularly, being ubiquitinated by an interaction with Siah-1, a RING domain-containing protein. The intracellular domains of UNC5 proteins are substrates for proteolysis by caspases.

The concentration of cyclic nucleotides within an axonal growth cone has a profound influence on how it responds to guidance cues. In particular, the intracellular concentration of cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA), can regulate the response to netrin-1. High levels of cAMP are associated with attractant responses to netrin-1, while growth cones with low concentrations are repelled. Although cAMP and PKA activation may regulate intracellular signal transduction, a specific mechanism regulating the direction of the response made by a growth cone has not been identified. It has been shown that activating PKA leads to the recruitment of DCC from an intracellular pool of vesicles to the growth cone plasma membrane, thereby enhancing chemoattraction to netrin-1. In addition, activating protein kinase C (PKC) leads to endocytosis of UNC5 proteins inducing neurons to

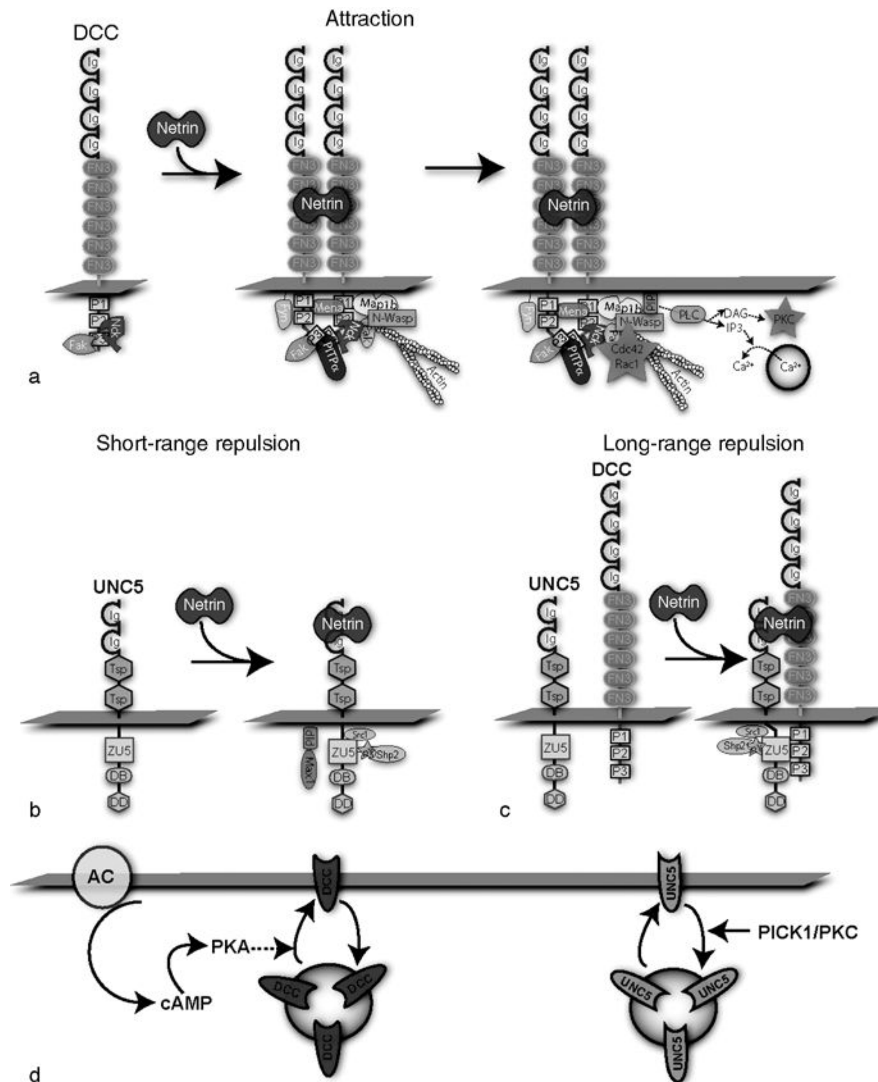

Figure 4 Speculative models outlining chemoattractive, chemorepellent, and modulatory signaling in response to netrin-1. (a) Chemoattraction to netrin-1 can be divided into three conceptually different stages: in the absence of netrin-1, during the initial response to netrin-1, and during cytoskeletal remodeling triggered by netrin-1. The adapter protein Nck1 and the tyrosine kinase Fak associate with the intracellular domain of the DCC ('deleted in colorectal cancer') protein in the absence of netrin-1. Upon netrin-1 binding, DCC multimerizes through association of its P3 domains. Phosphatidylinositol transfer protein-α (PITPα) can also bind the P3 domain of DCC and promote the generation of phosphoinositides (PIPs) by phosphatidylinositol 3-kinases (PI3Ks). PIPS can then be hydrolyzed by phospholipase C (PLC) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), leading to Ca^{2+} release from intracellular stores and activation of protein kinase C (PKC). Netrin-1 binding to DCC leads to phosphorylation of the intracellular domain of DCC and association of proteins such as Fak, Fyn, and Pak. Intracellular Ca^{2+} increases can lead to activation of the Rho GTPases Cdc42 and Rac, and cause remodeling of the cytoskeleton through proteins such as the Wiskott–Aldrich syndrome protein (N-Wasp), Ena/Vasp, and Map1b. (b) Short- and long-range repellent signaling to netrin-1. In the absence of netrin-1, Mena and ankyrin link UNC5 to the cytoskeleton. Upon binding netrin-1, UNC5 is tyrosine-phosphorylated, independently of DCC, by tyrosine kinases such as Src1. Netrin-1 induces recruitment of the tyrosine phosphatase Shp2 to a phosphorylated tyrosine residue in the ZU5 domain. PIPs have been proposed to regulate interaction of Max-1 with UNC5. (c) Long-range repulsion to netrin-1 requires association between the intracellular domains of UNC5 and DCC. (d) Both DCC and UNC5 can traffic between intracellular vesicular pools and the cell surface. Protein kinase A (PKA) can recruit DCC to the plasma membrane. PKC induces endocytosis of UNC5 from the cell surface. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PICK1, protein interacting with C kinase-1.

switch their response to netrin-1 from repulsion to attraction. These findings suggest that neuromodulatory factors that regulate PKA or PKC may influence axon outgrowth by altering which netrin receptors are presented by the growth cone.

Other Potential Netrin Receptors

Receptors for secreted netrins, other than DCC, neogenin, and UNC5 proteins, have been suggested. The G-protein-coupled adenosine A2B receptor has been

proposed as a receptor for netrin-1. Through an influence on intracellular cAMP concentration, it is possible that A2B may influence the response to netrin-1; however, contrary to an early report, A2B is neither expressed by nor required for spinal commissural axon guidance. Integrins, best known for their role as receptors for extracellular matrix components, have also been implicated as netrin receptors. Specifically, an adhesive interaction between netrin-1 and integrins $\alpha6\beta4$ and $\alpha3\beta1$ has been suggested to contribute during development of the pancreas; however, evidence of this *in vivo* remains to be demonstrated. Interestingly, the region of netrin reported to interact with these integrins is not in domains V and VI that are homologous to laminins, which are ligands for integrins. Rather, $\alpha6\beta3$ and $\alpha3\beta1$ integrins bind to a highly charged sequence of basic amino acids at the C-terminus of netrin-1.

Netrin Expression in the Mature Nervous System

Roles for netrins beyond directing axon and cell migration during development are beginning to be identified. Netrins and their known receptors are expressed in the adult nervous system: netrin-1 is expressed by neurons, Schwann cells in the PNS, and oligodendrocytes in the CNS. Subcellular fractionation of mature CNS white matter has determined that netrin-1 protein is enriched at the interface – known as periaxonal myelin – between axons and oligodendrocytes, suggesting that a function for netrin-1 in the mature CNS may be to regulate axon oligodendroglial interactions. Interestingly, as the mammalian spinal cord matures, DCC expression falls while UNC5 homolog expression increases. This suggests that UNC5 repellent signaling may be the dominant mode of responsiveness to netrin in the adult spinal cord. Although the functional significance of netrin-1 expression in the adult CNS remains unknown, an intriguing hypothesis is that netrins contribute to maintaining appropriate connections in the intact CNS by restraining inappropriate axonal sprouting.

The expression of netrin-1 by myelinating oligodendrocytes raises the possibility that it might function as a myelin-associated inhibitor of axon growth following injury. Netrin-1 does not appear to be a major component of an injury-induced glial scar in the mature spinal cord, but essentially normal netrin-1 expression persists on either side of the injury site. Although an influence of netrin-1 on axon regeneration in the adult CNS has not been demonstrated directly, these findings suggest that netrin-1 may be a component of CNS myelin that inhibits axon regeneration by neurons expressing UNC5 following injury. Such a role may explain, in part, why increasing cAMP in neurons promotes the ability of axons to grow in the adult mammalian CNS, as increasing cAMP recruits DCC to plasma membranes of growth cones and converts netrin-mediated repulsion to attraction. Interestingly, studies carried out in lamprey, a primitive vertebrate with the capacity for substantial axon regeneration following spinal lesion, correlated poor axonal regeneration following lesion with neuronal expression of UNC5 protein.

Netrin-3, netrin-4, netrin-G1, and netrin-G2 are also expressed in the adult brain. In humans, mutation of the netrin-G1 gene is a rare cause of the childhood neurodevelopmental disorder known as Rett syndrome. This disease is characterized by normal early development followed by loss of purposeful use of the hands, distinctive hand movements, slowed brain and head growth, gait abnormalities, seizures, and mental retardation. In mice, netrin-G1 deficiency does not lead to any obvious changes in neural circuitry, but does lead to altered synaptic responses and defects in sensorimotor gating behavior. Similarly, the netrin-G2 receptor NGL-2 influences the formation of glutamanergic synapses through an interaction with the postsynaptic scaffold protein PSD-95. Together, these findings indicate that netrin-G proteins have a role in the maturation, refinement, and maintenance of synapses, rather than in the guidance of axons.

Netrin Outside of the Nervous System

Netrins are expressed in many tissues. Netrin-1 is expressed in the heart, tongue, lung, inner ear, intestine, mammary gland, and pancreas, netrin-3 is expressed in the bowel, pancreas, and muscle, and netrin-4 is expressed in the intestine, pancreas, spleen, vascular networks, kidney, ovaries, and lung. Functional roles for netrins have been demonstrated in several developing tissues. Netrin-1 has been implicated in vascular patterning, although some disagreement remains regarding whether it functions principally as a repellent, acting via UNC5B, or an attractant, acting via undefined receptors, or both (in different vascular beds). Both netrin-1 and netrin-4 shape the developing lung through an influence on branching of the epithelial endoderm. In the developing mammary gland, netrin-1 is expressed by a layer of luminal cells, and an interaction with neogenin is required for proper organization of the terminal end buds.

Conclusion

Homologs of netrin-1 are evolutionarily conserved guidance cues that function as chemoattractants and

chemorepellents that direct cell and axon migration in the developing nervous system. The receptor DCC is essential for chemoattraction to netrin-1, and UNC5 proteins are required for chemorepulsion. The netrin-G subfamily contributes to the maturation, refinement, and maintenance of synapses. In addition to these roles in the CNS, netrins also influence the development of a variety of other tissues.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axonal Pathfinding: Extracellular Matrix Role; Growth Cones.

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Axonal Pathfinding: Extracellular Matrix Role

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Introduction

Normal behavior and other neural activities depend on the correct wiring of neural circuits during development. A critical step in forming neural circuits is the growth of axons from nerve cell bodies to the sites where synaptic connections are made. In spanning between neural somata and their synaptic targets, growing axons forge pathways that become the axonal tracts and peripheral nerves of the mature nervous system. The routes that axons take to reach their targets are determined by motile activities at their tips, called growth cones. Growth cones extend fine protrusions that adhere to nearby cells and surfaces. These adhesive contacts provide a toehold from which further protrusions are made. As growth cones crawl forward, they choose a path by detecting and responding to the spatial and temporal distributions of extracellular guidance molecules encountered in their local environments. Five major families of extracellular molecules – netrins, neurotrophins, semaphorins, slits, and ephrins – provide positive and negative cues that orient the migration of growth cones to their targets. These guidance molecules bind receptor proteins on growth cones and initiate cytoplasmic signals that regulate the motility and adhesive contacts that determine the advance, retreat, turning, branching, and stopping of growth cones. This article describes molecules that play a key role in axonal pathfinding by mediating the adhesive interactions necessary for growth cone migration.

Mechanism of Growth Cone Migration

Cytoskeletal Dynamics

Growth cone migration and axonal elongation involve the cytoskeletal components, microtubules and actin filaments. Axon elongation requires the advance and polymerization of microtubules, which are bundled in the axon but which spread apart in the growth cone, where individual microtubules dynamically probe forward to the front of a growth cone via polymerization and movement involving microtubule motor molecules (**Figure 1**). Axonal growth occurs where the main microtubule bundle and associated organelles advance in the growth cone, as determined by the positions and stabilization of these forward 'pioneering' microtubules. These dynamic microtubules project forward into an actin filament network that fills flattened dynamic projections, called lamellipodia, and fingerlike filopodia. This extensive filament system is continually remodeled, as actin filaments initiate and polymerize at the front margin and are then moved back to be fragmented and depolymerized, recycling subunits to the front. Multiple actin-binding proteins regulate this dynamic organization of actin filaments.

Growth cone migration is driven by forces produced within this actin filament domain. Actin polymerization creates protrusive forces that expand lamellipodia and elongate the tips of filopodia. Myosin motor molecules bind actin filaments and generate mechanical forces that move cargo bound to the myosin tail domains or pull on actin filaments to create tensions. Myosin II motor activity pulls actin filaments rearward, where they are depolymerized. Tensions generated by myosin II activity in the actin-rich leading margin can either direct or halt microtubule advance, depending on the situation. Myosin II-generated tensions produce the exploratory movements of lamellipodia and filopodia, whereas excessive levels of tension may sweep microtubules back in a contracting actin network that can collapse a growth cone. It is in the context of these dynamic cytoskeletal activities that adhesive interactions are critical to growth cone migration (**Figure 2**).

Adhesive Contacts of Growth Cones

Growth cone plasma membranes contain adhesion receptors that bind noncovalently to adhesion molecules on other cells or surfaces. Lamellipodia and filopodia initiate adhesive interactions as they explore their environment, and if these bonds persist, receptors cluster to form discrete adhesive contacts, which include intracellular adhesion complexes. Adhesion complexes remain in place or shift rearward as a growth cone advances. These adhesive complexes play two roles in growth cone migration. First, they include proteins that anchor actin filaments at adhesive sites. These links constitute a 'clutch' that stops the retrograde movement of actin filaments and permits the advance of microtubules and axonal organelles (**Figure 2**). Without stabilization provided by adhesive contacts growth cone migration fails, and tensions within the axonal cytoskeleton cause axonal retraction. Second, these complexes include proteins of signaling cascades, protein kinases, protein phosphatases, and Rho GTPases, which act on proteins that regulate the organization of actin filaments and microtubules. Thus, adhesive contacts provide points

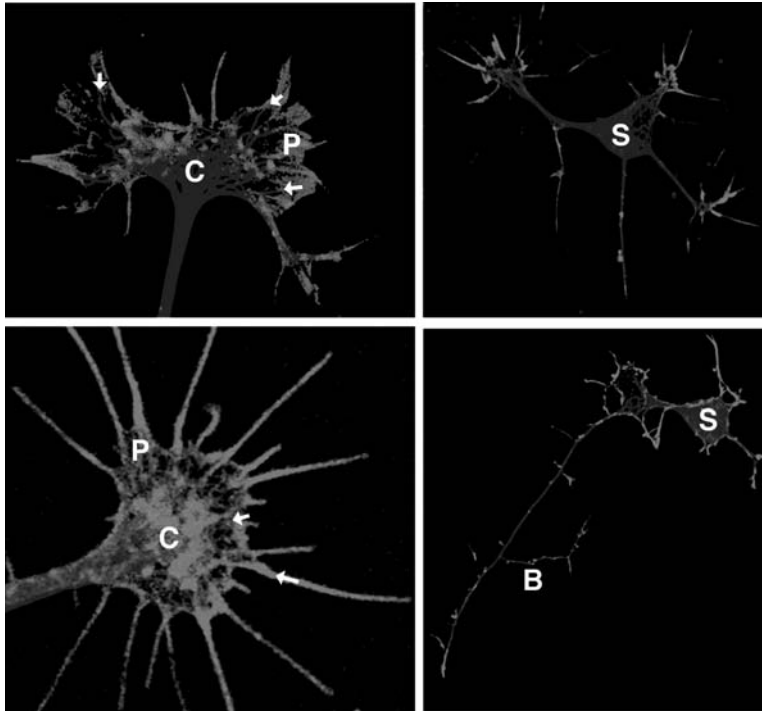

Figure 1 The distribution of microtubules and actin filaments in developing neurons and in axonal growth cones. Microtubules (green) are densely packed in the neuronal cell bodies (S) and are bundled in the axons and branches. Actin filaments are arrayed in filament networks and bundles in the peripheral domains (P) of the growth cones and along the shafts of the axons, where small areas of actin filament dynamics may give rise to collateral branches (B). In a growth cone, the microtubules from the central bundle of the central domain (C) splay apart and individual microtubules extend into the P domain and into filopodia (arrows).

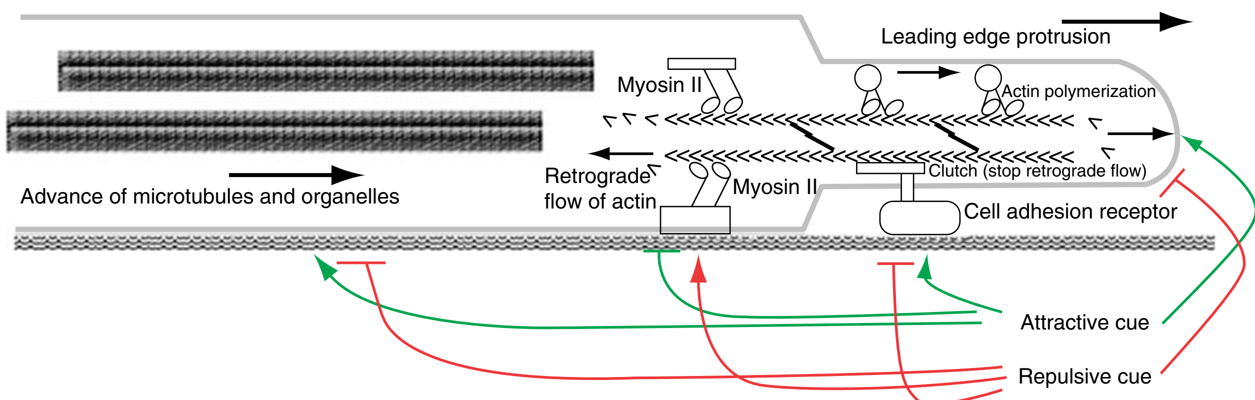

Figure 2 A model of the mechanism of growth cone migration. Actin polymerization pushes the leading margin of the growth cone forward. Forces generated by myosin II pull actin filaments backwards, where filaments are disassembled. When growth cone receptors make adhesive contacts with a surface, a 'clutch' links the adhesive contact to actin filaments of the leading edge, and the retrograde flow of actin filaments stops. This permits the advance of microtubules and organelles and promotes axonal elongation. Intracellular signaling generated by attractive and repulsive axonal guidance cues interacts with the molecular mechanisms of actin polymerization, myosin II force generation, adhesive contacts, and microtubule advance to regulate the paths of growth cone migration.

of stability that are essential to growth cone migration, and they are signaling centers from which regulatory activities promote growth cone motility.

The genetic regulation that determines neuronal phenotype also directs expression of receptors for adhesive ligands and guidance cues by the growth cones of neurons of a particular type. Extracellular positive and negative axonal guidance cues, whether surface bound or soluble, signal through their receptors to modulate an interacting set of pathways that regulate cytoskeletal and membrane dynamics. Thus, growth cone behaviors reflect a complex integration

of signaling events triggered at multiple receptors for guidance cues and adhesion molecules. By locally regulating the interplay of adhesive contacts and cytoskeletal dynamics within a growth cone, guidance cues determine the pathways of axonal growth (**Figure 2**).

Three major types of adhesive interactions promote growth cone navigation. Growth cones migrate within extracellular spaces that contain a complex mixture of glycoproteins, organized into an extracellular matrix (ECM) of fibers, protein aggregates, and basal laminae, which are discrete ECM layers at tissue interfaces. One major adhesive interaction of growth cones involves binding of integrin receptors to adhesive ECM proteins, especially the laminins. Two other major adhesive interactions involve growth cone contacts with cells or other axons along their pathways. These interactions involve two groups of adhesive molecules, the cadherins and the immunoglobulin superfamily of cell adhesion molecules (IgCAMs). Cadherins are expressed on all tissue types, including neurons and axons. Cadherin adhesions involve homophilic binding between like cadherin molecules on two interacting cells. Weaker heterophilic interactions between different cadherins can also occur. IgCAMs are also expressed on all tissues, including neurons. Adhesive interactions of IgCAMs can involve homophilic interactions, similar to cadherins, but also heterophilic interactions in which an IgCAM on a growth cone binds a different IgCAM on adjacent cells. Even heterophilic interactions of IgCAMs with non-IgCAMs occur.

Integrin adhesion receptors Integrin receptors are heterodimers of alpha and beta subunits. More than 20 integrin heterodimers have been identified in humans. The binding specificity for ECM components depends on the particular combination of alpha and beta subunits in a heterodimer. The 12 integrin dimers that are expressed in the mammalian nervous system include receptors for collagens, laminin-1 and laminin-5, fibronectin, tenascin, thrombospondin, vitronectin, and VCAM-1. A growth cone can express multiple integrins, allowing interactions with multiple ECM molecules. The cytoplasmic domains of integrins lack enzymatic activities, but when integrins bind adhesive ligands, conformational shifts in the cytoplasmic domains trigger formation of focal contacts that involve integrin clustering and creation of docking sites for proteins that initiate signaling and links to the cytoskeleton. When lamellipodia and filopodia of growth cones bind laminin-1, proteins that localize to the contact sites include paxillin, talin, vinculin, zyxin, and focal adhesion kinase (FAK). Vinculin and talin link actin filaments to the adhesive contacts, providing a clutch for growth cone migration (**Figure 3**). The presence of the adapter protein paxillin and activation of FAK initiates further protein interactions and signaling by Src family kinases, MAP kinases, and Rho GTPases. Activation of Rac1 and Cdc42 GTPases promotes actin polymerization by regulating actin-binding proteins and actin filament dynamics. Thus, when integrins on growth cones bind laminin-1, growth cone migration is stimulated by increased actin filament polymerization to protrude the leading margin and by the establishment of adhesions to stabilize these protrusions and promote the advance of microtubules.

Cadherins and IgCAMs Stimulation of N-cadherin and IgCAMs, such as NCAM and L1, by ligand binding between cells leads to activation of the FGF receptor tyrosine kinase, which triggers signals involving PLC-gamma, DAG lipase, cytoplasmic [Ca^{2+}] elevation, and activation of MAPK. IgCAMs also signal via Src kinases to activate Rac1, PI3K, and MAPK. Cadherin signaling is also reported to activate Rac1. Thus, several pathways activated by cadherins and IgCAMs promote actin filament polymerization. Adhesive binding of cadherins and IgCAMs provides anchorage for actin filaments, creating the clutch necessary for growth cone migration. The cytoplasmic tails of many cadherins, such as N-cadherin, bind catenins, which bind actin filaments and link N-cadherin adhesive sites to the actin cytoskeleton in growth cones (**Figure 4**). The cytoplasmic domain of L1 binds the cytoskeletal linker ankyrin, but L1–ankyrin interactions are involved in stable adhesive junctions, such as at nodes of Ranvier, and not in growth cone migration. Members of the ezrin–moesin–radixin (ERM) proteins mediate actin filament binding to membranes, and interactions of L1 (and other IgCAMs) with ERM proteins may serve as a clutch in growth cone protrusions that bind via L1 or other IgCAMs.

These adhesion receptors can be regulated in ways that are important to growth cone pathfinding. The expression levels of integrin receptors on growth cones are increased when laminin levels are low or when ECM proteins, such as proteoglycans, which interfere with laminin–integrin binding, are present. These responses would maintain growth cone adhesion and migration in environments when access to laminin is reduced. L1 is endocytosed from central regions and recycled to the leading margin of growth cones, increasing availability of L1 for adhesive contacts of lamellipodia and filopodia. The functions of adhesion receptors can also be modulated from the cytoplasm in an 'inside-out' manner or via *cis* interactions with other components of the plasma membrane. An important manner in which guidance

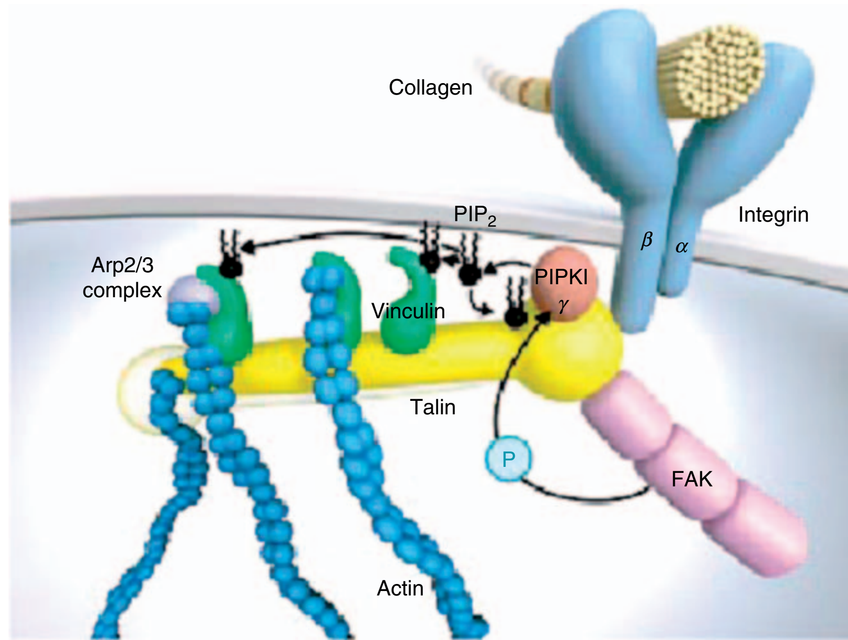

Figure 3 A model of integrin binding to ECM molecules and the formation of intracellular adhesive complexes. An alpha–beta integrin heterodimer is shown bound to a collagen fibril, and the intracellular adhesion complex is pictured, showing the proteins vinculin and talin, which are involved in linkage to actin filaments, and FAK kinase, which initiates signaling cascades. The Arp2/3 complex nucleates actin filament assembly. Reproduced from Brakebusch C and Fässler R (2003) The integrin–actin connection, an eternal love affair. *EMBO Journal* 22: 2324–2333, with permission from Nature Publishing Group.

molecules exert their positive and negative effects on growth cone pathfinding is by modulating the functions of adhesive receptors (**Figure 2**). For example, the negative cue semaphorin 3A may inhibit growth cone migration by blocking integrin-mediated cell adhesion. In addition, adhesion mediated by N-cadherin is inhibited by the negative guidance cue Slit protein via its receptor, Robo. Thus, the negative or repulsive effects of semaphorin 3A and Slit on growth cone pathfinding can involve these inhibitory effects on growth cone adhesion. On the other hand, the attractant netrin signals to activate the kinase FAK, which promotes integrin-mediated adhesion, suggesting that positive guidance cues activate adhesive interactions of growth cones.

Adhesion Molecules and Growth Cone Pathfinding

What are the roles of these adhesion molecules in the pathfinding behaviors of growth cones? Major pathways that are followed by many growing axons offer multiple adhesive ligands for growth cone migration, such as laminins, fibronectin and collagens in the ECM, and cadherins and IgCAMs on adjacent cells and axons. These multiple options for adhesion may provide redundancy, ensuring growth cones form sufficient adhesive contacts for effective migration. The first growth cones that 'pioneer' a pathway have limited options for binding to ECM or cell surface adhesion molecules on adjacent cells, whereas growth cones that enter an established pathway can track along previously extended axons by binding to cadherins and IgCAMs expressed on the surfaces of axonal shafts. Several *in vivo* examples of pathfinding roles of adhesion molecules are described in the following sections.

Laminins

Laminins are large adhesive glycoproteins (MW 1 000 000 Da) that consist of heterotrimers of alpha, beta, and gamma chains. Ten laminin chains are known, forming 11 known heterotrimers with widely varied expression throughout different tissues. The laminins present several domains that mediate laminin binding to several cell surface receptors and to other ECM components. The most common laminins are typically present in basal laminae, an ultrastructural ECM layer associated with epithelia, muscle cells, Schwann cells, and glia. Laminin-1, which has been studied the most, promotes axonal growth *in vitro* from virtually every type of neuron, indicating that laminins have broad roles in promoting growth cone migration. Examples of growth cone migration along basal laminae include growth cones of Rohon–Beard neurons in *Xenopus*, growth cones of retinal ganglion cells in the retina and optic nerve, and pioneer axons in

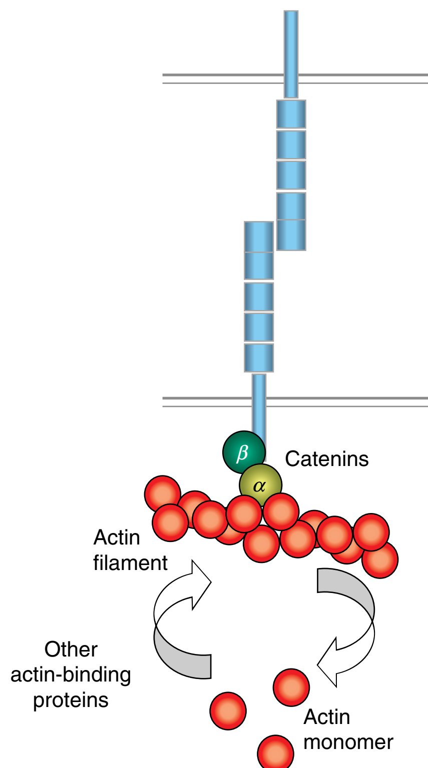

Figure 4 A model of homophilic adhesion between cadherin adhesion molecules and the intracellular binding to actin filaments. Cadherin molecules bind between cells and become linked to actin filaments by way of interactions with alpha and beta catenins. From Weis WI and Nelson WJ (2006) Re-solving the cadherin–catenin–actin conundrum. *Journal of Biological Chemistry* 281: 35593–35597.

the grasshopper limb bud. However, in addition to basal laminae, laminin is transiently expressed in the loose cellular environments of developing tissues, including the nervous system, on cell surfaces and associated with sparse ECM fibers. The growth cones that pioneer pathways, such as the corticofugal pathway of the neocortex or the medial longitudinal fasciculus from the brain into the spinal cord, migrate within loose extracellular spaces in the wall of the immature central nervous system (CNS), where the cells are labeled in a punctate manner by laminin antibodies. The expression of laminin on these cells is transient, and eventually laminin immunoreactivity is restricted to the basal lamina at the outer boundary of the CNS wall. In the developing peripheral nervous system (PNS), laminin is expressed in basal laminae and at early stages in the mesenchyme through which motor and sensory axons extend. Schwann cells express abundant laminin, forming the basal laminae that enclose axon–Schwann cell units. This punctate cellular expression of laminin diminishes during development, although laminin remains present in basal laminae.

In view of the wide distribution of laminins and the ability of laminin-1 to promote robust axonal growth from many neuronal types, it is thought that laminins function permissively, providing adhesion that is required for growth cone migration, but not in an instructive manner to influence pathfinding decisions. Laminins and other ECM molecules may broadly promote growth cone migration along a pathway, whose boundaries are defined not by the absence of adhesive ECM molecules but, rather, by the expression in adjacent tissues of negative guidance cues, such as slits or semaphorins. This 'surround repulsion' occurs in both developing CNS and PNS. Several mutational studies have reported specific errors in pathfinding when a laminin is absent or blocked. Laminin function is essential for growth cone turning in the grasshopper limb bud, and zebra fish with mutations in the laminin-alpha-1 chain exhibit multiple axon guidance defects throughout the CNS, but not in every location. These results suggest that laminin-mediated adhesion is essential for growth cone navigation in at least some instances.

Fibronectin

Fibronectin is a large adhesive glycoprotein (MW 250 000 Da) that is widely distributed in the ECM, including within ganglia and the endoneurium of the PNS. Like the laminins, the fibronectin molecule contains multiple domains that mediate binding to other ECM components and to multiple cellular receptors, including several integrin heterodimers. During development of the PNS and CNS, fibronectin is present in a punctate distribution in loose cellular spaces of immature nervous tissue, and eventually fibronectin expression diminishes as development ends, especially in the CNS. In tissue culture studies, fibronectin promotes axonal growth, but not as vigorously as does laminin. In addition, axonal growth by PNS neurons on fibronectin surfaces exceeds the responses of CNS neurons, probably because PNS neurons express higher levels of fibronectin receptors than CNS neurons. Evidence is lacking for a requirement for fibronectin in growth cone pathfinding.

Integrins

Because neurons express multiple integrin subunits and because many ECM components, such as collagen, laminin, or fibronectin, can bind more than one integrin heterodimer, the essential roles of particular integrins in growth cone pathfinding are not clearly defined. Mouse knockouts of α_1 and α_6 integrins, which are laminin-1 receptors, do not reveal clear defects, however, injections of anti-β_1 integrin, part of several neuronal receptors for ECM proteins, into

Xenopus embryos disrupts retinal axonal pathfinding. Similarly, conditional knockout of β_1 integrin in sensory neurons results in deficits in innervation of skin, where sensory axons extend through the dermal ECM and along the epidermal basal lamina. The $\alpha_4\beta_1$ integrin heterodimer is specifically implicated in the growth and arborization of sympathetic axons within cardiac muscle. In *Drosophila*, mutations in the integrins α-PS1 and-PS2 lead to pathfinding errors.

Cadherins

Cadherins are characterized as single-pass transmembrane proteins that contain an ectodomain of five cadherin repeats and a conserved cytoplasmic tail. Binding of calcium ion stabilizes an extended rodlike structure of the ectodomain, which is necessary for optimal adhesion by alignment of cadherin molecules on apposing cells. There are at least 100 cadherins, and most are expressed in the developing vertebrate brain on immature cells, neurons, and glia. Their functions are numerous, including cell sorting, boundary formation, target recognition, synaptogenesis, and synapse function. Regarding axonal pathfinding, the widely expressed N-cadherin stimulates *in vitro* axonal growth from a variety of CNS and PNS neurons. *In vivo* studies involving antibody injection or genetic mutation also implicate N-cadherin in axon growth and fasciculation. These results indicate that cadherins promote growth cone migration along axons in highly populated common pathways, but it is unclear whether cadherins play a role in the pathfinding of early pioneer growth cones. In some cases, a common pathway may be shared by several classes of elongating axons, which are distinguished by the expression of different cadherins. For example, the tectofugal projections of chickens express four different cadherins among different axon fascicles. These cadherins may mediate specific pathfinding, as the formation of homophilic adhesions of growth cones to axons expressing the same cadherin directs growth cones along specific axon fascicles toward their targets. Forced expression of specific cadherins causes growth cones to abnormally follow fascicles that express the same cadherin. Finally, growth cones often share expression of specific cadherins with neurons in their particular target. Thus, cadherins also have roles in target recognition and subsequent synaptogenesis.

L1 and NCAM IgCAMs

Proteins that contain an immunoglobulin (Ig)-like domain constitute the Ig superfamily, which makes up more than 2% of human genes, constituting the largest gene family. The neuronal Ig superfamily includes a large number of molecules, which have functions in axonal pathfinding not only as cell adhesion molecules but also as axonal guidance cues and as receptors of guidance cues. This discussion is restricted to two members of this large family, L1 and NCAM. The IgCAM L1 is widely expressed on axons in the developing CNS and PNS, and tissue culture studies show that substrates coated with L1 promote homophilic adhesion and axonal growth from many neuronal types. Spontaneous human mutations in the L1 gene and mouse L1 knockout studies both indicate important roles for L1 in brain development and function. Multiple anatomical and functional deficits result from human and mouse L1 mutations, including a failure of corticospinal axons to decussate in the hindbrain pyramids. Crossing defects were not found in other tracts or were not so extensive. L1 also interacts in *cis* with receptors for other guidance cues, including the semaphorin 3A receptor, suggesting that the defect in pyramidal decussation observed in L1 mutants could be due to disrupted pathfinding responses to semaphorin 3A and other guidance cues, as well as to reduced growth cone tracking along axons.

Another prominent neuronal IgCAM is NCAM, the first neuronal IgCAM identified. NCAM is widely expressed on immature neurons and glia and also on other embryonic cells, such as myoblasts. In tissue culture studies, NCAM mediates neuronal adhesion and axon growth. In addition to homophilic adhesive interactions, NCAM also forms heterophilic adhesive interactions. Antibodies against NCAM can induce axon defasciculation *in vitro* and *in vivo*. Several isoforms of NCAM are expressed, and in some situations NCAM carries a carbohydrate polysialic acid (PSA) moiety that reduces NCAM adhesion. In NCAM-deficient mice defects in fasciculation of hippocampal axons were observed, but in general only minor defects in development or behavior were observed. Perhaps, in the absence of NCAM, other cell adhesion molecules serve the same functions.

Adhesion Molecules and Axonal Regeneration

When the pathfinding phase of circuit construction ends, as growth cones reach their targets, the expression of neural cell adhesion molecules and their receptors is downregulated. However, injury or damage to nervous tissues can disconnect neural circuits, and axons must regenerate in order to reconnect neurons. When axons are injured in the PNS, axon regeneration is often robust, leading to varying degrees of functional recovery. Schwann cells, which ensheath all PNS axons, stimulate axonal regeneration by upregulating their expression of growth factors, and

laminins, fibronectin, and cadherin, as substrates for growth cones. Axon regeneration in the PNS is also promoted by increased expression of integrin receptors by regenerating neurons. In the CNS of adult mammals, regeneration of injured axons is poor, and recent research has focused on inhibitory components of myelin and glial scars that block growth cone adhesion and trigger signals that inhibit growth cone motility. In lower vertebrates, CNS regeneration is often successful, and this involves the upregulation of expression of adhesive ligands, such as L1 and cadherins, as demonstrated in regenerating zebra fish optic nerves and spinal cords.

Several strategies for improving axonal regeneration in mammalian model systems, and eventually humans, emphasize measures to improve the adhesive environment for growth cone migration. When stem cells that express L1 are transplanted into a mammalian CNS lesion, increased regeneration of corticospinal axons occurs. Purkinje cells transfected to express L1 and GAP43 show enhanced axonal regeneration. *In vitro* regeneration of axons by adult neurons on laminin and fibronectin is improved by transfection of neurons to express increased levels of the appropriate α integrin chains. Finally, many natural and synthetic bridges have been designed that include adhesion molecules to promote axonal regeneration across lesion sites. These studies demonstrate that strategies to increase the adhesive interactions of regenerating growth cones can stimulate axonal regeneration after injuries in adults. Probably, improved axonal regeneration in adults will also require an increase in the intrinsic ability of adult neurons to sprout and grow axons. This may involve upregulation of genes for adhesion receptors, for other guidance cue receptors, and for proteins that drive the dynamic cytoskeletal functions of immature neurons.

Summary

Growth cone adhesion is integral to the mechanism of growth cone migration and pathfinding. Adhesive interactions of growth cones provide stability for lamellipodial and filopodial protrusions of growth cones and also act as signaling centers that regulate actin and microtubule dynamics and organization in a migrating growth cone. The adhesive interactions of growth cones are also a target of guidance cues that determine where growth cones turn, branch, and stop migrating. Migrating growth cones make three kinds of adhesive contacts with ECM and with other cells. These contacts involve integrin receptors, which recognize laminin and other ECM components; cadherins, which form homophilic adhesions; and IgCAMs, which can form homophilic and heterophilic adhesive interactions. Major pathways of growth cone migration during development contain one or, perhaps more typically, multiple adhesive ligands available to growth cones. The navigational decisions of growth cone pathfinding are based on local differences in adhesive stability for growth cone protrusions and in dynamic protrusive activity, as based on adhesive signaling and the integration of signaling triggered from other guidance cues.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Axon Guidance: Guidance Cues and Guidepost Cells; Axon Guidance: Morphogens as Chemoattractants and Chemorepellants; Axonal Pathfinding: Guidance Activities of Sonic Hedgehog (Shh); Axonal Pathfinding: Netrins; Growth Cones.

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Postsynaptic Development: Neuronal Molecular Scaffolds

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Introduction

Neuronal synapses generally fall into two groups, excitatory and inhibitory. Excitatory synapses are mostly present in dendritic spines, which are thorn-like structures on dendrites. Inhibitory synapses are located on dendritic shafts and the cell body. Excitatory synapses are characterized by the presence of an electron-dense thickening in the postsynaptic side known as the postsynaptic density (PSD). The PSD is formed through the assembly of macromolecular postsynaptic protein complexes containing receptors, scaffolds, and signaling proteins.

Postsynaptic development at excitatory synapses is thought to involve an initial axodendritic contact, followed by localization of early postsynaptic proteins and recruitment of additional proteins to permit growth into mature postsynaptic structures. During the past decade, mechanisms underling the assembly and molecular organization of excitatory neuronal synapses have been thoroughly studied. Inhibitory neuronal synapses have received relatively less attention, partly because the wealth of proteins in the PSD has attracted scientific interest. This article focuses on postsynaptic differentiation at excitatory synapses. In particular, it discusses how postsynaptic scaffolds contribute to the assembly, organization, and plasticity of the PSD.

Postsynaptic Scaffolding Proteins

Components of the PSD have been identified by various experimental approaches including mass spectrometry and the use of protein interaction traps such as the yeast two-hybrid system. Proteomic approaches have identified hundreds of PSD components, including membrane proteins, scaffolding proteins, signaling proteins, and cytoskeletal proteins. Proteomic studies have further provided information on relative and absolute amounts the PSD proteins and their phosphorylation.

Here, postsynaptic scaffolding proteins are defined as proteins that are relatively more abundant than are other PSD proteins and that possess various domains for protein–protein interactions. The presence of such domains strongly implies that the proteins are implicated in the assembly and organization of the PSD. Many postsynaptic scaffolds contain the PDZ (PSD-95-Dlg-ZO1) domain, a 90-amino-acid-long module that interacts with the C-terminal PDZ-binding motif of other proteins. The PDZ domain is one of the most common protein domains known, being found in approximately 580 proteins encoded by the mouse genome. PDZ proteins exhibit 1–13 tandem arrays of PDZ domains. PDZ domain-binding motifs are found in a wide variety of proteins, including membrane proteins and signaling proteins. PDZ proteins, due to their ability to interact with other proteins and form macromolecular protein complexes, are found mainly in specialized cell-to-cell junctions, including neuronal synapses and tight junctions.

A well-known PDZ-containing postsynaptic scaffold is PSD-95, which is a family of proteins with four known members: PSD-95/SAP90, PSD-93/chapsyn-110, SAP97, and SAP102. PSD-95 family proteins contain various domains for protein–protein interactions, including three PDZ domains, one SH3 domain, and one GK domain. Splice variants of PSD-95, PSD-93, and SAP97 (PSD-95β, PSD-93ζ, and SAP97β) contain an additional domain, L27, at the N-terminus. Through these domains, PSD-95 binds to a wide variety of proteins and is involved in the assembly and molecular organization of the PSD. Proteomic studies have shown that PSD-95 is one of the most abundant proteins in the PSD. The four members of the PSD-95 family seem to differ in function, with PSD-95 and PSD-93 being more important in synapses and SAP97 and SAP102 playing roles in protein trafficking.

Functionally, transient overexpression of PSD-95 in dissociated neurons increases the number and size of dendritic spines and α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) glutamate receptor-mediated synaptic transmission. In hippocampal slices, PSD-95 overexpression drives GluR1 AMPA receptors into synapses, occludes long-term potentiation (LTP), and enhances long-term depression (LTD). Conversely, acute PSD-95 knockdown reduces the ratio of excitatory to inhibitory synapses and suppresses AMPA receptor-mediated synaptic transmission. Transgenic mice with truncated PSD-95 exhibit reduced LTD, enhanced LTP, and impaired spatial learning. These knockout mice show unaltered AMPA receptor-mediated currents, in contrast to acute PSD-95 knockdown, probably due to functional compensation by other PSD-95 family proteins. Indeed, double-knockout mice lacking both PSD-95 and PSD-93 show a markedly reduced ratio of AMPA/*N*-methyl-D-aspartate (NMDA) excitatory postsynaptic currents. Together, these results implicate PSD-95 in the

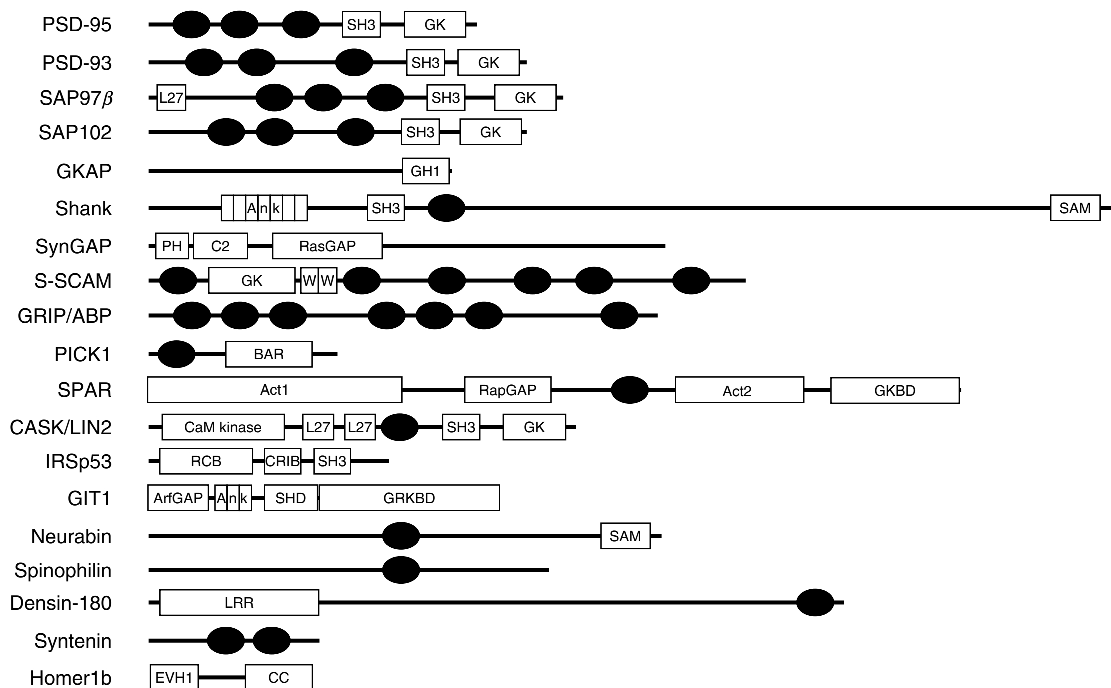

Figure 1 Schematic diagram of PSD proteins. Domain structures of selected PSD proteins. PDZ domains are shown as dark ellipses. Other domains are indicated: Act1, actin regulatory domain 1; Act2, actin regulatory domain 2; Ank, ankyrin repeats; ArfGAP, Arf GTPase-activating protein; BAR, Bin-Amphiphysin-Rvs domain; C2, calcium/lipid binding domain 2; CaM kinase, Ca^{2+}/calmodulin-dependent kinase (CaMK)-like domain; CC; coiled coil domain; CRIB, Cdc42/Rac-interactive binding; EVH1, ENA/VASP homology domain 1; GH1, GKAP homology domain 1; GK, guanylate kinase-like domain; GKBD, PSD-95 GK binding domain; GRKBD, GRK2 binding domain; kinase, serine/threonine kinase domain; L27, domain initially found in LIN2 and LIN7; PH, pleckstrin homology domain; RapGAP, Rap GTPase-activating protein; RasGAP, Ras GTPase-activating protein; RCB, Rac binding domain; SAM, sterile α motif; SH3, Src homology 3 domain; SHD, Spa2 homology domain; WW, domain with two conserved Trp (W) residues. Proteins: CASK/LIN2; vertebrate homolog of lin2; GIT1, GRK-interacting protein 1; GKAP, GK-associated protein; GRIP/ABP, glutamate receptor interacting protein/AMPA receptor binding protein; IRSp53, insulin receptor tyrosine kinase substrate p53; PICK1, protein interacting with C-kinase; PSD-93, postsynaptic density protein 93; PSD-95, postsynaptic density protein 95; SAP97, synapse-associated protein 97; SAP102, synapse-associated protein 102; Shank, SH3 and ankyrin repeat-containing protein; SPAR, spine-associated RapGAP; S-SCAM, synaptic scaffolding molecule.

regulation of excitatory synapses, dendritic spines, synaptic strength and plasticity, and learning and memory.

Other postsynaptic scaffolds implicated in postsynaptic development are listed in **Figure 1**. A large number of these scaffolds contain PDZ domains, as does PSD-95, suggesting that PDZ-based interaction is a widespread mechanism for postsynaptic assembly and organization.

Principles Governing Postsynaptic Differentiation

An important question in synapse formation concerns which side of the synapse initiates synapse formation. Some published studies indicate that the assembly of functional nerve terminals precedes postsynaptic differentiation. Time-lapse microscopy and retrospective immunostaining on cultured hippocampal neurons (11–14 days *in vitro* (div)) indicate that contact-induced formation of functional nerve terminals is followed by clustering of postsynaptic proteins including PSD-95 and glutamate receptors in approximately 45 min. Early stage hippocampal neurons (5–7 div) contain nonsynaptic mobile packets with postsynaptic scaffolds such as PSD-95, GKAP/SAPAP, and Shank/ProSAP. Some of these packets are fast moving and can be recruited to PSD-95/GKAP-negative nascent synapses apposed to functional nerve terminals, suggesting that the presynaptic side instructs postsynaptic assembly. However, a proportion of these mobile packets move slowly. These packets contain neuroligin-1, and when they contact axons, they induce functional nerve terminals by recruiting mobile synaptophysin clusters. This suggests that a certain degree of postsynaptic assembly also occurs prior to the formation of functional nerve terminals, and that synaptogenesis occurs in a bidirectional manner.

Presynaptic assembly is thought to be mediated by vesicular intermediates including synaptic vesicle (SV)

precursors and specialized dense-core vesicles known as Piccolo/Bassoon transport vesicles (PTVs). These two types of vesicles carry preassembled complexes of SV proteins and active zone components (scaffolds and plasma membrane proteins), respectively. Notably, active zones are assembled from a small number (typically two or three) of PTVs. A related question is whether postsynaptic assembly also occurs in this manner. Early stage (3 or 4 div) cortical neurons exhibit rapidly moving NMDA and AMPA receptor transport packets similar to the mobile packets of early hippocampal neurons (mentioned previously). This suggests that modular transport of postsynaptic proteins may play a role in early synaptogenesis. However, late-stage (8–13 div) hippocampal neurons exhibit a gradual recruitment of postsynaptic proteins, including NMDA receptors, PSD-95, and Shank. Although reasons for this discrepancy remain to be determined, a possible explanation is that neurons at different developmental stages may have distinct mechanisms of postsynaptic protein assembly.

Induction of Postsynaptic Differentiation

When the presynaptic side induces postsynaptic differentiation, what might be the initiating signals? Neurotransmitters, released from nerve terminals, are good candidates. Neurotransmitters may affect the morphology of dendritic spines and filopodia (long thin protrusions on dendrites), suggesting that the neurotransmitters may instruct synapse formation. However, transgenic mice that cannot release neurotransmitters, due to the absence of the presynaptic protein Munc13 or Munc18, exhibit normal synapse formation. In addition, blockade of neurotransmitter release in cultured neurons does not inhibit synapse development. These data suggest that neuronal activity is not required for synapse formation.

Another possible trigger is transsynaptic adhesion between presynaptic and postsynaptic cell adhesion molecules. Ideally, transsynaptic adhesions should be heterophilic to minimize homophilic adhesions between dendrites and axons. In addition, synaptic adhesion molecules need to permit coupling of adhesion events to induction of synaptic differentiation through the recruitment of various synaptic proteins to the sites of contact. A well-known example of heterophilic and synaptogenic transsynaptic adhesion is that between presynaptic neurexins and postsynaptic neuroligins. Neurexins were originally identified as receptors for α-latrotoxin, a potent neurotoxin from black widow spider venom that induces massive neurotransmitter release from nerve terminals. Subsequently, neuroligin was identified as an endogenous postsynaptic ligand of neurexins. In addition, neuroligin was found to directly interact with the PDZ domains of PSD-95 through the C-terminal tail, providing a novel mechanism of synapse formation.

The neurexin–neuroligin complex promotes synapse formation in a bidirectional manner. Neuroligin expressed in nonneural cells induces presynaptic differentiation in contacting axons of co-cultured neurons. Conversely, neurexin presented on nonneural cells or beads induces the clustering of key postsynaptic proteins in contacting dendrites. Direct aggregation of neuroligins on the surface membrane of dendrites induces similar clustering of postsynaptic proteins. Acute knockdown of neuroligins reduces the number and function of synapses. These results suggest that neuroligin is a key mediator of synapse formation. Interestingly, transgenic mice deficient in three neuroligins (mice with triple knockout mutations of neuroligin-1, -2, and -3) exhibit reductions in synaptic transmission, but their synapse number is not affected. This suggests that neuroligins regulate functional maturation of synapses rather than their formation, although further work in this area is required.

An interesting feature of the neuroligin family is that neuroligin isoforms differentially localize to excitatory and inhibitory synapses. Specifically, neuroligin-2 is mainly found at inhibitory synapses, whereas other neuroligin isoforms are detected at excitatory synapses. Consistent with these observations, direct aggregation of neuroligin-2 on dendrites induces the clustering of gephyrin, an inhibitory postsynaptic scaffold. Acute knockdown of neuroligin-1, -2, and -3 in cultured neurons results in a greater reduction in the function of inhibitory synapses than excitatory synapses. In addition, the neuroligin triple-knockout mice show an increase in the ratio of excitatory to inhibitory synapses. These results suggest that neuroligin-2 regulates inhibitory synapse formation and/or function.

SynCAM is another family of synaptic cell adhesion molecules implicated in excitatory synaptic differentiation. SynCAM family members, expressed in nonneural cells, induce the formation of functional nerve terminals in contacting axons. These terminals have release properties similar to those seen in regular synapses. The C-terminus of SynCAM contains a PDZ-binding motif and associates with synaptic PDZ proteins, including CASK/LIN2 and syntenin. This, together with data from studies on neuroligin (described previously), suggests that synaptic differentiation is mediated by synaptic scaffolds that couple synaptic adhesion events to the recruitment of various synaptic proteins.

When the great diversity of neuronal synapses is considered, synaptogenic adhesion molecules other than neuroligin and SynCAM may act in concert

with postsynaptic scaffolds. NGL, a family of adhesion molecules, interacts with PSD-95 through the C-terminus in a manner similar to the neuroligin–PSD-95 interaction. The extracellular domain of NGL associates with netrin-G/laminet, a GPI-anchored adhesion molecule. The complex of netrin-G, NGL, and PSD-95 is implicated in the regulation of excitatory synapse formation. In support of this idea, NGL presented on nonneural cells or on beads induces presynaptic differentiation in contacting axons. Direct aggregation of NGL on the dendritic surface induces postsynaptic protein clustering. The fact that both neuroligin and NGL associate with PSD-95 suggests that PSD-95 is one of the key postsynaptic scaffolds involved in adhesion-dependent postsynaptic differentiation. In addition, the dual association of PSD-95 with neuroligin and NGL suggests that these two adhesion molecules may have physical or functional interactions.

Extracellular factors capable of inducing aspects of postsynaptic differentiation at excitatory synapses include Narp, a secreted immediate early gene product upregulated by synaptic activity, and ephrin, a ligand of EphB receptor tyrosine kinases. Narp presented on nonneural cells induces the clustering of AMPA receptors, but not of NMDA receptors, in contacting dendrites. Narp interacts with the extracellular N-terminal domain of AMPA receptors. Narp induces AMPA receptor clustering at shaft synapses of aspiny neurons, and this leads to secondary NMDA receptor clustering, probably through stargazin and PSD-95. Ephrin activation of EphB receptor tyrosine kinases induces co-clustering of EphB and NMDA receptors. This interaction is mediated by their extracellular domains and does not require the kinase activity of EphB receptors.

Localization and Organization of Postsynaptic Scaffolds

If the clustering of postsynaptic adhesion molecules on dendrites is the beginning of postsynaptic differentiation, how might subsequent postsynaptic protein clustering occur? A possibility is that adhesion-induced primary clustering of neuroligin on dendrites may promote secondary clustering of PSD-95 though the C-terminal PDZ interaction, which would lead to additional recruitment of PSD-95-associated proteins. Against this, however, is the observation that while neuroligin-1 selectively binds to the third PDZ domain of PSD-95, synaptic localization of PSD-95 requires the first two PDZ domains but not the third. Notably, NGL, another adhesion molecule binding to both of the first two PDZ domains of PSD-95 but not to the third, enhances synaptic localization of PSD-95.

PSD-95 occurs in molar excess relative to other postsynaptic proteins. Therefore, instead of depending on its synaptic localization on the interaction with other proteins, PSD-95 might increase its cluster size at synapses by self-multimerization. In support of this notion, PSD-95 forms multimers through both N- and C-terminal domains. The expansion of PSD-95 multimers is more likely to be lateral than vertical because, ultrastructurally, PSD-95 mainly localizes to regions close to the postsynaptic membrane. The lateral expansion might be aided by PSD-95 interaction with membrane proteins and/or palmitoylation (a lipid modification promoting membrane attachment). Indeed, PSD-95 co-expressed with membrane proteins in heterologous cells forms large surface clusters in which both proteins are co-localized. In addition, mutations of PSD-95 that block palmitoylation eliminate membrane protein clustering by PSD-95.

PSD-95 is likely to recruit other postsynaptic scaffolds, including GKAP and Shank. The C-terminal GK domain of PSD-95 associates with GKAP, and the C-terminal PDZ-binding motif of GKAP further associates with Shank. GKAP and Shank are relatively abundant in deeper layers of the PSD and contain various domains for protein–protein interactions. Shank further associates with Homer, which is in turn linked to metabotropic glutamate receptors and IP3 receptors. Shank promotes spine maturation by mechanisms requiring synaptic Homer recruitment. In support of a possible role for PSD-95 in the recruitment of GKAP and Shank, a GKAP mutant lacking the ability to bind to PSD-95 induces the aggregation and degradation of Shank.

Synaptic Adhesion Molecules

Synaptically localized PSD-95 may reversely promote postsynaptic localization of adhesion molecules such as neuroligins or other postsynaptic adhesion molecules, further stabilizing synapse adhesion and promoting presynaptic differentiation. Indeed, overexpression of PSD-95 in cultured neurons concentrates neuroligin-1 and NGL at excitatory synapses. Importantly, PSD-95 induces the translocation of neuroligin-2 from inhibitory to excitatory synapses. Because neuroligin-2 induces presynaptic differentiation at both excitatory and inhibitory synapses, this translocation is likely to increase the number of excitatory synapses at the expense of inhibitory synapses. Therefore, the relative amounts of neuroligin-2 and PSD-95 in a single neuron may determine the ratio of excitatory and inhibitory synapses.

Although PSD-95 concentrates neuroligin at excitatory synapses, this does not seem to involve a direct interaction between the two proteins because a

neuroligin-1 mutant lacking the PSD-95-binding C-terminus is normally targeted to excitatory synapses. Instead, synaptic neuroligin-1 localization requires a membrane-proximal domain in the cytoplasmic domain. This suggests that neuroligin does not depend on its binding either to PSD-95 or to presynaptic neurexins for synaptic localization, and that there is a mechanism that precedes neuroligin localization for early postsynaptic differentiation.

Postsynaptic Receptors

Postsynaptic differentiation at excitatory synapses involves synaptic localization and local trafficking of NMDA and AMPA glutamate receptors. NMDA receptors are targeted to synapses at early stages of development, in contrast to AMPA receptors, thus forming NMDA receptor-only silent synapses. PSD-95 may contribute to synaptic localization of NMDA receptors by interaction with NR2 subunits. In support of this idea, C-terminal casein kinase II phosphorylation of NR2B on Ser1480 within the C-terminal PDZ-binding motif disrupts PSD-95 interaction with NR2B and decreases surface expression of NR2B. PSD-95 coexpression slows the internalization rate of chimeras of Tac (a surface membrane protein) containing the distal tail of NR2B. However, an NR2B mutant that lacks the ability to bind to both PSD-95 and AP2, a clathrin adaptor complex, is retained in the synapse, suggesting that PSD-95 binding may not be important for synaptic localization. In addition, mobile NMDA receptor transport packets in early stage neurons can be recruited to nascent synapses lacking PSD-95. In contrast to NR2B, NR2A does not depend on PSD-95 interaction for synaptic localization, suggesting subunit-specific rules for NMDA receptor trafficking.

Postsynaptic adhesion molecules may regulate synaptic NMDA receptor clustering. A mutant neuroligin-1 that lacks PSD-95 binding ability no longer induces PSD-95 clustering in cultured neurons but retains the ability to cluster NMDA receptors, although weaker than that afforded by wild-type neuroligin-1. This suggests that neuroligin is capable of recruiting NMDA receptors through PSD-95-independent mechanisms. SALM, a PSD-95-interacting family of synaptic adhesion-like molecules, exhibits NMDA receptor clustering activity. SALM1, a member of this family, directly associates with the NR1 subunit and promotes dendritic clustering of NMDA receptors through mechanisms requiring the PDZ-binding C-terminus. SALM2 forms a complex with both NMDA and AMPA receptors, and direct aggregation of SALM2 on dendrites induces co-clustering of NMDA and AMPA receptors.

Synaptic localization of AMPA receptors is regulated by stargazin/TARP (transmembrane AMPA receptor regulatory protein), which directly associates with both AMPA receptors and PSD-95. Stargazin traffics AMPA receptors to synapses via two distinct mechanisms. Stargazin induces surface expression of AMPA receptors and also facilitates synaptic docking of the stargazin–AMPA receptor complex. The latter mechanism depends on binding of the stargazin C-terminus to PSD-95. In support of a role for PSD-95 in synaptic localization of stargazin and AMPA receptors, a stargazin mutant that lacks PSD-95 binding rescues surface AMPA receptor responses, but not synaptic AMPA receptor responses, in cerebellar granule cells from stargazin-deficient stargazer mice.

Other postsynaptic proteins regulating synaptic localization and trafficking of AMPA receptors include GRIP/ABP, PICK1, and *N*-ethylmaleimide-sensitive factor (NSF). GRIP/ABP, a multi-PDZ protein, is implicated in the stabilization of AMPA receptors at the synaptic surface. Synaptic AMPA receptor localization is also regulated by various GRIP-associated proteins including liprin-α (a multidomain protein), GIT1 (a multidomain protein), and LAR (a receptor tyrosine phosphatase). PICK1, through its PDZ domain, associates with protein kinase Cα (PKCα) in addition to AMPA receptors, and it promotes synaptic delivery of PKCα. PKCα directly binds to GRIP, probably promoting the interaction between PICK1-bound PKCα and its substrate, GRIP-bound GluR2. PKC-dependent phosphorylation of GluR2 on Ser880 within the C-terminal PDZ-binding motif selectively disrupts GluR2 association with GRIP but not GluR2 association with PICK1, facilitating AMPA receptor endocytosis. NSF, an ATPase involved in membrane fusion, binds to a cytoplasmic region of AMPA receptors that is distinct from the GRIP/PICK1 binding site. NSF is implicated in maintaining synaptic AMPA receptors by disassembling the AMPA receptor–PICK1 complex and promoting AMPA receptor recycling/delivery. Interestingly, the NSF binding site in AMPA receptors overlaps with that of AP2, and the AP2–AMPA receptor interaction is required for regulated AMPA receptor endocytosis and LTD. In strong support of these *in vitro* results, cerebellar LTD is absent in GluR2 and PICK1 knockout mice and in two different strains of GluR2 mutant knockin mice (GluR2Δ7 and GluR2 K882A). GluR2Δ7 mice have a deletion of the last seven amino acid residues required for both GRIP and PICK1 binding, and GluR2 K882A mice carry a mutation to block PKC-dependent Ser880 phosphorylation. In addition to promotion of endocytosis, PICK1 and NSF regulate AMPA receptor exocytosis. PICK1 and NSF are required for activity-dependent

insertion of GluR2 (calcium-impermeable)-containing AMPA receptors in cerebellar granule–stellate cell synapses. In support of these *in vitro* observations, PICK1 knockout and GluR2Δ7 knockin mice lack this form of plasticity.

Other Membrane Proteins

Synaptic scaffolds concentrate and cluster interacting membrane proteins at the surface membrane. Stability of membrane proteins at the synaptic surface seems to be achieved through the inhibition of their endocytosis and promotion of their exit from intracellular pools and insertion into the plasma membrane. PSD-95 inhibits the endocytosis of NMDA receptors, Kv1.4 potassium channels, and β_1-adrenergic receptors. In addition, PSD-95 promotes the rate of membrane insertion of NMDA receptors. A C-terminal PDZ-binding motif in NR1-3, a splice variant of NR1, suppresses NR1-3 retention at the endoplasmic reticulum (ER), suggesting that a PDZ protein promotes the ER exit and surface expression of NMDA receptors. However, there are examples of the opposite situation, in which scaffolding proteins enhance endocytosis. S-SCAM/MAGI-2, a synaptic multi-PDZ protein, enhances the endocytosis of β_1-adrenergic receptors, and PICK1 is involved in AMPA receptor internalization, as described previously. Finally, functional properties of membrane proteins can be directly regulated by their interaction with scaffolds. PSD-95 suppresses single-channel conductance of the Kir2.3 potassium channel and increases the channel opening rate of NMDA receptors.

Signaling Pathways

Another key event in postsynaptic development is the establishment of signaling pathways in the PSD. A suggested role for synaptic scaffolds is to couple upstream receptor activations to downstream signaling pathways. PSD-95 associates with neuronal nitric oxide synthase, coupling NMDA receptor activation to nitric oxide generation. Similarly, PSD-95 associates with SynGAP, a neuronal GTPase activating protein (GAP) for Ras and Rap small GTPases. This interaction is not involved in synaptic localization of SynGAP but is implicated in functional coupling between NMDA receptors and the Ras-ERK signaling pathway, which regulates AMPA receptor trafficking and synaptic plasticity.

Synaptic scaffolds couple kinases and phosphatases with their specific substrates. PSD-95 and SAP97 associate with AKAP79/150, a neuronal A-kinase-anchoring protein interacting with protein kinase A (PKA), PKC, and protein phosphatase 2B (calcineurin). This interaction, in the context of SAP97 association with GluR1, promotes the PKA-dependent phosphorylation of GluR1 on Ser845, a modification implicated in AMPA receptor function and synaptic plasticity.

PSD-95 associates with Fyn, a nonreceptor tyrosine kinase, to promote Fyn-mediated tyrosine phosphorylation of NR2A. PSD-95 binds to another nonreceptor tyrosine kinase, Src, regulating NMDA receptor-dependent synaptic transmission and plasticity. Notably, this interaction is not involved in synaptic recruitment of Src but, rather, in the suppression of Src activity and Src-mediated NMDA receptor upregulation, reminiscent of the direct functional regulation of membrane proteins by scaffolding proteins.

F-Actin

F-actin is a key cytoskeletal component both in dendritic filopodia and in spines, and it is implicated in the regulation of spine morphogenesis and synaptic plasticity. F-actin is both physically and functionally associated with PSD components. For instance, stable maintenance of GKAP, Shank, and AMPA receptors at synapses requires F-actin integrity. Conversely, LTP- and LTD-inducing stimuli regulate F-actin polymerization. In this context, it is conceivable that PSD proteins may organize F-actin-regulatory signaling pathways.

F-actin polymerization in dendritic spines is regulated by small GTPases, including Rac, Cdc42, Rho, and Rap. Proteins acting upstream and downstream of these small GTPases associate with postsynaptic scaffolds, suggesting that these interactions may constitute related signaling pathways in the PSD. PSD-95 associates with and promotes synaptic localization of kalirin-7, a guanine nucleotide exchange factor (GEF) for Rac1. Shank binds βPIX, a GEF for Rac1 and Cdc42, and promotes synaptic localization of βPIX and βPIX-associated PAK, a kinase downstream of Rac1 and Cdc42 that regulates spine morphogenesis through LIMK-1 and MLC. Both PSD-95 and Shank associate with IRSp53, an abundant postsynaptic protein downstream of Rac1 that regulates spine morphogenesis. Shank associates with Abp1, α-fodrin, and cortactin, proteins that have F-actin binding, bundling, and nucleating activities, respectively. GRIP/ABP associates with EphB receptor tyrosine kinases, and ephrin activation of EphB receptors induces dendritic spine formation through the kalirin–Rac1–PAK pathway and the Cdc42 GEF intersection. Neurabin and spinophilin, which bind protein phosphatase 1 and F-actin and regulate dendritic spines, interact with Lfc, a Rho GEF. In

addition, NMDA receptors associate with and phosphorylate Tiam1, a Rac1 GEF that mediates NMDA receptor-dependent spine regulation.

Neuronal Transport

Synaptic proteins synthesized in the cell body must be transported to their target synapses. Kinesin is a microtubule (MT)-based motor protein implicated in this process. There are approximately 45 kinesin family proteins in the mouse and human genomes. Most kinesins move toward the plus end of MTs. MTs in axons are unidirectionally oriented, with their plus ends pointing toward nerve terminals, whereas dendritic MTs are bidirectionally arranged. Kinesin-dependent transport has been studied mainly in axons, although evidence supports kinesin involvement in dendritic transport.

An important question in neuron motor-dependent transport is how the limited number of kinesin proteins can transport a large number of cargoes. Studies raise the intriguing possibility that molecular scaffolds function as 'motor receptors,' linking motors to various cargoes through protein interaction domains. In accordance with this notion, synaptic scaffolds link kinesins to their specific cargoes. KIF1Bα, a kinesin motor, interacts with PSD-95 and S-SCAM, which are in turn linked to various synaptic proteins. KIF17 associates with the LIN2/7/10 PDZ protein complex that is coupled to NMDA receptors. KIF1A associates with liprin-α, a multidomain protein interacting with the GRIP–AMPA receptor complex. KIF5 interacts with GRIP/ABP, a binding partner of both AMPA receptors and EphB receptors, and disruption of the KIF5-GRIP interaction suppresses EphB trafficking and dendritic morphogenesis. Interestingly, GRIP drives KIF5 to dendrites, suggesting that a cargo regulates polarized transport of a motor.

Regulation of Postsynaptic Assembly

Synapses are dynamically formed and eliminated during development and plasticity. These processes are likely to involve rapid assembly and disassembly of the PSD. Indeed, neuronal activity regulates synaptic localization of PSD components, changing the overall molecular composition of the PSD. GluR1 and protein phosphatase 1 are delivered to synapses by LTP- and LTD-inducing stimuli, respectively. Phosphorylation of synaptic proteins regulates their synaptic localization by affecting, for example, protein–protein interactions. Phosphorylation of the N-terminal domain of PSD-95 by cyclin-dependent kinase 5 suppresses synaptic clustering of PSD-95, whereas SAP-97 phosphorylation in the L27 domain by CamKII enhances synaptic SAP97 localization. Phosphorylation of PDZ-binding ligands disrupts their binding to PDZ domains. Lipid modification also plays a role in postsynaptic assembly. Known examples include palmitoylation of PSD-95, PSD-93, GRIP, and AMPA receptors. The lipid addition regulates the trafficking and synaptic localization of these proteins. Synaptic activity depalmitoylates and disperses synaptic PSD-95 clusters. Enzymes that mediate protein palmitoylation (palmitoyl acyl transferases) of neuronal substrates have been identified. Lastly, protein degradation through the ubiquitin–proteasome pathway is involved in postsynaptic assembly. Neuronal activity regulates ubiquitination of key PSD proteins, including GKAP, Shank, and AKAP79/150. Mdm2, an E3 ubiquitin ligase, plays a role in ubiquitinating PSD-95. SNK, a polo-like kinase induced by synaptic activity, phosphorylates SPAR, a PSD-95-associated Rap GAP that regulates F-actin, and this in turn induces PSD-95 degradation and spine loss.

See also: Dendrite Development Synapse Formation and Elimination; Presynaptic Development and Active Zones.

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Postsynaptic Specialization Assembly*

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Introduction

Synapse are specialized contacts used by neurons to communicate with each other. Morphologically, they are cell junctions characterized by specialized membrane and submembrane regions, and their assembly takes place in various stages involving the accumulation of specific pre- and postsynaptic proteins at the contact site. Recent biochemical, genetic, and imaging studies have begun to elucidate the molecular mechanisms underlying their formation, growth, and maturation, and understanding the development and organization of neuron/neuron synapses is crucial to our understanding of the development of the nervous system, in which abnormalities contribute to most neurological and behavioral disorders. Moreover, it is now clear that the adult brain is constantly reorganizing itself in response to experience, and that at least some synaptic developmental mechanisms are used in mature brain during synaptic plasticity.

At the postsynaptic sites of excitatory synapses is localized a specific EM electron-dense organelle called the postsynaptic density (PSD) where the two main types of glutamate-receptor channels, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, cluster in the postsynaptic membrane. The PSD has attracted the interest of a number of scientists because it is now clear that it regulates synapse function (**Figure 1**).

Inhibitory synapse in brain are mainly mediated by γ-aminobutyric acid (GABA) neurotransmitters, whose receptor channels are localized on the postsynaptic membrane and are not associated with a clear PSD because the molecular composition of the postsynaptic compartment is much simpler.

Excitatory and inhibitory synapses are therefore clearly distinguishable on the basis of the morphology and molecular composition of the postsynaptic compartment. Each neuron receives both excitatory and inhibitory synapses, which are located in cross-proximity to dendrites in strictly controlled numbers and ratios in order to allow the correct functioning of brain circuits.

Our knowledge of the neuron/neuron synaptogenesis of both types of synapses has recently increased as a result of improvements in time-lapse microscopy and the molecular identification of specific proteins in neuronal synapses. This article concentrates on the structure and development of the synapses between central neurons in mammals, paying particular attention to the postsynaptic compartment. Emphasis will be placed on their molecular and structural composition of the excitatory synapses, and the early steps of synapse formation that underlie their molecular mechanisms.

Excitatory Synapses: Postsynaptic Organization

The PSD is a protein complex that forms a specialized organelle and adheres to the postsynaptic membrane in opposition to the active zones located in the axonal presynaptic terminal. A number of functions have been proposed for this structure, including the regulation of adhesion, the control of receptor clustering, and the regulation of receptor functions.

The PSD contains neurotransmitter and transmembrane receptors, various scaffold proteins, cytoskeletal elements, and regulatory enzymes, all of which are assembled together in a disk-like structure that is 30–40 nm thick and a few hundred nanometers wide.

PSD molecular architecture is designed to specialize in signal transduction, and its protein composition is highly modifiable in order to allow for the strengthening and weakening of glutamatergic synapses. Neural activity dynamically regulates both the function and composition of the PSD, and involves mechanisms such as protein phosphorylation, local translation, ubiquitination and degradation, and subcellular redistribution. Because of the central role of the PSD in synaptic transmission and plasticity, the protein composition of the PSD has been extensively studied in an attempt to understand synaptic mechanisms.

The protein components of the PSD have been gradually discovered over the years using two-hybrid yeast and biochemical approaches. The PSD is typically purified by means of differential centrifugation, sucrose gradient sedimentation, and detergent extraction, because the PSD structure cannot be solubilized using mild nonionic detergents such as Triton X-100. Kennedy et al. first discovered several PSD proteins (e.g., PSD-95, densin-180) by sequencing protein bands from one-dimensional gels of PSD preparations, and many other PSD proteins were later identified using the two-hybrid yeast system as binding partners of known postsynaptic proteins, such as

[*] I wish to dedicate this paper to the memory of Alaa Ei-Husseini, a great friend and an outstanding scientist.
[†] Deceased

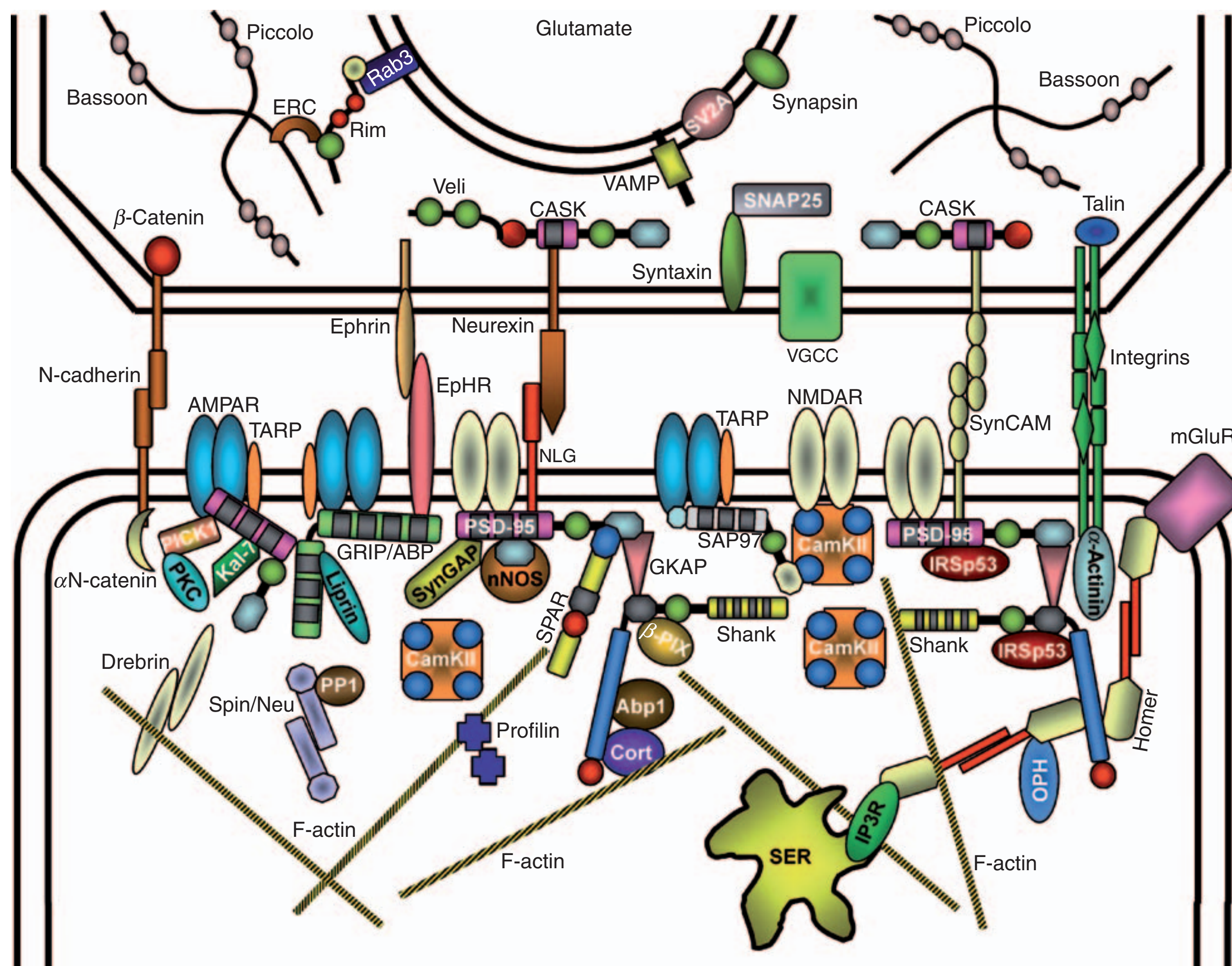

Figure 1 Molecular organization of glutamatergic synapses. Only the major components and relative interactions are shown. NLG, neuroligin; Kal-7, kalirin-7; Spin/Neu, spinophilin and neurabin I; Cort, cortactin; OPH, oligophrenin; PKC, protein kinase C; VGCC, voltage-gated calcium channels; nNOS, nitric oxide synthase; NMDAR, *N*-methyl-D-aspartate; GKAP, guanulate kinase-associated protein; IP3R, inositol-1,4,5-triphosphate; mGlur, metabotropic glutamate receptors; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazoll propronic acid receptor.

the PSD-95 family of PDZ-containing scaffolds that interact with the cytoplasmic tails of NMDA receptors. More recently, modern proteomic and imaging techniques have probably identified all of the proteins of the PSD, and quantified the relative stoichiometry of some of them.

The components of PSD proteins can be divided into four subgroups on the basis of their functions: receptor transmembrane proteins, scaffold proteins, signaling proteins, and cytoskeletal proteins. We will here discuss the major PSD components in each subgroup.

The PSD-95 Family and Other Major PSD Scaffold Proteins

The first and still most widely studied PSD scaffold protein was PSD-95. The PSD-95 family is encoded by four genes: PSD-95/SAP90 (synapse-associated protein 90), PSD-93/chapsyn-110, SAP102, and SAP97. The structure of these proteins is characterized by the assembly of three PDZ (PSD-95, Dlg, ZO-1 homology) domains, an SRC homology (SH3) domain, and a guanylate kinase-like (GK) domain. SAP97 also has a LIN2/LIN7 (L27) domain at the N-terminal. The PSD-95 family belongs to a protein superfamily called membrane-associated guanylate kinase (MAGUK), which is characterized by the presence of at least one PDZ and one GK domain.

Immuno-EM and tomography studies have indicated that PSD-95 is localized very close to the postsynaptic membrane (a mean distance of 12 nm), a good position for interacting with postsynaptic membrane proteins such as receptors, ion channels, and cell-adhesion molecules, as well as with cytoplasmic proteins (**Figure 1**). It has been suggested that these interactions are important for PSD-95 clustering

and the targeting of postsynaptic membrane proteins, but there is still disagreement as to whether PSD-95 is essential for the synaptic clustering of NMDA receptors in mammals, which is certainly not eliminated by the genetic disruption of PSD-95.

It now seems clear that the most important biochemical function of PSD-95 is to organize the signaling complexes at the postsynaptic membrane. PSD-95 interacts with a wide variety of cytoplasmic signaling molecules and, by physically bringing together cytoplasmic signal-transducing enzymes and surface receptors, may therefore facilitate signal coupling in the PSD. For example, the overexpression of PSD-95 in hippocampal neurons increases the number of dendritic spines, the maturation of excitatory synapses, and the accumulation of AMPA receptors, whereas PSD-95 knockout by RNA interference in developing hippocampal neurons modifies the excitatory/inhibitory synapse ratio in favor of inhibitory synapses. PSD-95 directly binds to the proteins involved in actin remodeling: these include kalirin-7, a guanine nucleotide exchange factor (GEF) for RAC1 that promotes spine formation possibly as a downstream effector of the EphB receptor; SPAR, an inhibitory GTPase-activating protein (GAP) for RAP that promotes the growth of dendritic spines; and IRSp53, a downstream effector of Cdc42 small GTPase. The ability of PSD-95 to recruit AMPA receptors seems to be related to its binding to the tetra-spanning membrane proteins Stargazin and its relatives TARP, which are associated with AMPA receptors and essential for receptor surface expression, and synaptic accumulation and function.

Three other major scaffold components of the PSD that directly interact with each other are Shank, guanylate kinase-associated protein (GKAP), and Homer protein families. Shank, which is also known as proline-rich synapse-associated protein (ProSAP), is a large scaffold protein whose multidomain organization consists of an ankyrin repeat near the N-terminal, followed by an SH3 and a PDZ domain, a long proline-rich region, and a sterile alpha motif domain (SAM) at the C-terminus. Shank proteins (codified by the three genes *Shank1–3*) molecularly link two glutamate receptor subtypes: NMDA receptors and type-I metabotropic glutomate receptors (mGluRs). The Shank PDZ domain binds to the C-terminal of GKAP. Homer interaction with the proline-rich domain ensures the association of Shank with type-I mGluRs.

Shank interacts with a number of actin-binding proteins. It binds to cortactin, Abp1, fodrin, the Rac1 and Cdc42 exchange factor βPIX, and the Cdc42-binding protein IRSp53. Other important Shank interactors are proteins that regulate dendritic morphology and formation, such as Dasm1 and Densin-180, whose activity also seems to be regulated by Shank. Finally, Shank proteins can potentially be produced locally because of the presence of its mRNA in dendrites. The observation that the haploinsunficency of Shank3 in humans probably causes the neurological symptoms of individuals affected by the 22q13 deletion syndrome indicates that Shank proteins play a major role in synapse functions.

The GKAP/SAPAP family of proteins is less well characterized, but its four members were originally identified as proteins interacting with the GK domain of PSD-95. GKAP has five repeats of 14 amino acids involved in interacting with PSD-95 and, as it binds to *S*-SCAM, nArgBP2, Dynein light chain, and Shank, may therefore function as a scaffolding protein that links PSD protein complexes to motor proteins.

Homer proteins are encoded by three genes (*Homer 1–3*), and typically consist of an N-terminal Ena/VASP homology 1 (EVH1) domain followed by a coiled-coil domain that mediates dimerization with other Homer proteins. The EVH1 domain of Homer1 binds to a PPXXF or very similar sequence motif in Shank, mGluR1/5, inositol-1,4,5-trisphosphate (IP_3) receptor, ryanodine receptor, and to different members of the TRPC family of ion channels. Through their ability to self-associate, Homer isoforms containing the coiled-coil domain (called 'CC-Homer' or Homer1b in the case of the Homer1 gene) can physically and functionally link the proteins and receptors that bind to the EVH1 domain. Homer1a is a short-splice variant of Homer1 that contains the EVH1 domain, but lacks the coiled-coil domain; it functions as a natural dominant negative because it cannot dimerize. It is important to note that Homer1a expression is induced at mRNA level by synaptic activity.

AMPA receptors are linked to a different set of scaffolding proteins, which includes GRIP/ABP (encoded by the two distinct genes *GRIP1* and *ABP/GRIP2*), and the protein interacting with C kinase 1 (PICK1); these interactions may account for the dynamic cell biological behavior of AMPARs at synapses.

It is believed that GRIP is involved in the synaptic trafficking and/or stabilization of AMPARs and other interacting proteins. GRIP has up to seven PDZ domains by means of which it interacts with many proteins, including Eph receptors and their ephrin ligands, an RAS guanine nucleotide exchange factor (RasGEF), liprin-α, the transmembrane protein Fraser syndrome 1 (FRAS1), and the metabotropic and kainite-type glutamate receptors. It can therefore participate in synaptic function not only by interacting with AMPARs, but also by associating with Eph receptors and their ephrin ligands, which have been

found to be involved in dendrites and dendritic spine morphogenesis and hippocampal synaptic plasticity. The widespread cellular distribution of GRIP, and its interaction with motor proteins (directly with conventional kinesin KIF5 or indirectly with KIF1A via liprin-α), suggest that multiple motor proteins may contribute to the transport of AMPARs.

PICK1 is located at neuronal synaptic and nonsynaptic sites. Its PDZ domain has relatively promiscuous binding properties, and both pre- and postsynaptic partners have been found: PKCα, GluR2/3, the netrin receptor UNC5H, various metabotropic glutamate receptor subtypes, the dopamine plasma-membrane transporter, and the erythroblastic leukemia viral oncogene homolog 2 (ErbB2) receptor tyrosine kinase. In most of these cases, the subcellular localization and/or surface expression of these partners seems to be regulated by interactions with PICK1.

Receptor and Transmembrane Proteins

The major transmembrane and receptor proteins are of course the glutamate receptors which, in the PSD, are organized into supramolecular signaling complexes by interacting with specific PDZ domain-containing scaffold proteins, such as PSD-95/SAP90 and their associated proteins for the NMDA receptors, and GRIP/ABP for the AMPA receptors. The distribution of glutamate receptors and content of synapses vary during development: immature synapses preferentially contain NMDA receptors and gradually acquire AMPA receptors, an observation that has led to the identification of what have been called silent synapses because the absence of AMPA receptors makes them less responsive to glutamate. AMPA and NMDA receptors seem to be homogenously mixed and distributed inside the postsynaptic membrane, but the mGluRs are mainly distributed at the leading edge of the synapses.

Behind the receptors, an important role is played by the transmembrane adhesion molecules that regulate synapse formation and structures. Neuroligin is a synaptic adhesion molecule that probably plays an important role in synapse formation: it interacts with PSD-95 and transsynaptically with β-neurexins, which in turn bind to the PDZ domain of CASK/LIN2 (another scaffold of the MAGUK superfamily of proteins), which is enriched on both sides of the synapse and interacts with other synaptic membrane proteins such as syndecan and SynCAM. The transsynaptic neuroligin/β-neurexin interaction seems to be important for inducing pre- and postsynaptic synaptic differentiation, and the amount of PSDS-95 regulates the balance between the number of inhibitory and excitatory synapses (see below). When heterologous cells overexpressing neuroligin are co-cultured with neurons, they are able to induce morphological and functional presynaptic specializations in contacting axons.

Cadherins are found on both the pre- and postsynaptic sides of neuron synapses. Dominant-negative studies of neuronal(N)-cadherin function indicate that the cadherin cell-adhesion system is important for synapse integrity and the morphological maturation of dendritic spines; however, it is unclear whether cadherins play an initiating role in synapse formation or a supporting role in synapse growth. Through their cytoplasmic tails, cadherins bind α- and β-catenins, which in turn bind to the actin cytoskeleton and thus anchor and consolidate the cadherin cell-adhesion complex.

Integrins are heterodimeric glycoproteins that contain α- and β-subunits. Many of the 16 α- and eight β-subunits that have been identified so far are expressed in different spatial patterns in the brain. Integrins function as cell-to-matrix or cell-to-cell adhesion molecules by respectively binding to extracellular matrix proteins or immunoglobulin-superfamily receptors on other cells. They interact through their cytoplasmic domains with the actin-binding proteins, talin and α-actinin, and also activate nonreceptor protein tyrosine kinases, such as the focal-adhesion kinase that affects cytoskeletal organization and intracellular signal transduction. One immunoglobulin domain-containing protein that is found in synaptic membranes is the synaptic cell-adhesion molecule (SynCAM), which can also induce presynaptic differentiation in contacting axons when it is expressed in nonneuronal cells. SynCAM shows homophilic cell-adhesion activity through its extracellular domain, and can bind to PDZ proteins such as calcium/calmodulin-dependent serine protein kinase (CASK) through its cytoplasmic tail.

Finally, synapses contain the important tyrosine kinase EphB (ephrin B) family of RTKs and their cognate ephrinB ligands. These proteins play well-known roles in axon guidance and topographic map formation in the nervous system. The ephrinB/EphB receptor system is also involved in the development of excitatory synapses and dendritic spines by means of the phosphorylation of a cell-surface heparan/sulphate proteoglycan Syndecan and the recruitment of kalirin, a guanine-nucleotide exchange factor for the Rac GTPase. In addition, EphB2 seems to interact physically with NMDA receptors and enhance their channel activity, and ephrinB ligands can induce the clustering of NMDA receptors.

Signaling Proteins

A number of signaling proteins are associated with PSD-95. One of these is nitric oxide synthase (nNOS), a Ca^{2+}/calmodulin-activated enzyme that produces the

nitric oxide involved in regulating neurotransmission and excitotoxicity. Interestingly, the ternary NMDAR/PSD-95/nNOS complex may functionally couple NMDAR gating to nNOS activation, as is suggested by the fact that disrupting the NMDAR/PSD-95 interaction by means of a synthetic peptide mimicking the last nine residues of NR2B reduces NMDAR-induced excitotoxicity without affecting NMDAR function.

The most abundant signaling protein in the PSD is its predominant kinase CaMKII, whose multiple functions include regulating receptor targeting and function at synapses. The substrates for CaMKII have not yet been completely identified, but they include several PSD proteins.

Another abundant PSD signaling molecule that binds to PSD-95 is the synaptic Ras GTPase-activating protein (SynGAP), a GAP for the Ras small GTPase which, after activation by CaMKII, suppresses the ERK pathway regulating synaptic plasticity. PSD-95 also associates with the nonreceptor tyrosine kinases of the Src family and their upstream activator, proline-rich tyrosine kinase 2 (Pyk2), both of which are thought to be important for synaptic plasticity. It is therefore possible that it may localize the Pyk2–Src signaling cascade close to NMDARs, although the importance of PSD-95 scaffolds in synaptic regulation by tyrosine phosphorylation has not been directly investigated.

Smaller amounts of several other signaling proteins are associated with the PSD, including a number of kinases and phosphates whose functions have not been completely clarified.

Cytoskeletal Proteins

It has been demonstrated that a number of actin-binding proteins (usually Rho and Rac GTPase effectors regulated by Ca^{2+}) control the actin cytoskeleton in dendritic spines and excitatory synapses. Most of them are formed by the concatenation of different protein interaction domains and thus function as scaffolds assembled in multimolecular complexes that are enriched in the PSD.

One of the most abundant cytoskeletal proteins is drebrin, an F-actin-binding protein that is mainly expressed in neurons and highly concentrated in the PSD. In cortical neurons, drebrin overexpression increases the length of dendritic spines and, in hippocampal neurons, drebrin promotes actin assembly and the synaptic clustering of PSD-95 in the PSD. Importantly, drebrin (but also profilin II and αN-catenin) are redistributed to dendritic spines with an increased F-actin content after the induction of LTP in the dentate gyrus, or general synaptic activation and NMDA receptor activation, which suggests their importance in inducing F-actin polymerization and/or stabilization upon synaptic stimulation.

Profilin is a small actin-binding protein that promotes actin polymerization by positioning the actin monomers at the barbed end of the growing F-actin. Profilin II stabilizes spine morphology in a mature state, and suppresses dendritic spine motility by reducing actin dynamics.

αN-catenin is a cadherin-associated protein that, together with β-catenin, links adhesion molecules to the cytoskeleton and actin. In the absence of αN-catenin, dendritic spines are more motile and their filopodia rapidly protrude and retract from the spine heads, a sign of unstable synaptic contacts. Conversely, the overexpression of αN-catenin accelerates dendritic spine maturation and decreases spine motility, thus suggesting that it promotes spine morphogenesis and stabilization.

Spinophilin (or neurabin II) and neurabin I are two related F-actin-binding proteins with similar domain structures containing a PDZ and a coiled-coil domain that form homo- and heterodimers. Via its actin-binding domain, spinophilin is predominantly localized on the dendritic spines of pyramidal neurons (as its name suggests); it seems to be required for the correct maturation of dendritic spines because knockout mice have more filopodia and immature spines, and show altered glutamatergic transmission. On the contrary, neurabin I overexpression induces the formation of dendritic filopodia in immature cultured hippocampal neurons, and promotes the enlargement of dendritic spines in older neurons. It is not clear how spinophilin and neurabin I have opposite effect on spines, although they both bind a similar set of synaptic proteins.

Two other abundant actin-binding proteins localized at the PSD are α-actinin-2 and IRSp53. α-Actinin is a member of the spectrin/dystrophin family of actin-binding proteins, and binds to the NR1 and NR2B subunits of NMDA receptors, thus providing a cytoskeletal bridge between NMDA receptors and actin.

IRSp53 not only interacts with Shank, PSD-95 and PSD-93, and all of these interactions are required for its spine localization: its overexpression in cultured neurons increases the density of dendritic spines, while its siRNA-mediated knockdown reduces spine density, length, and width. IRSp53 plays a role in linking PSD-95 to activated Rac1/Cdc42 and downstream effectors of actin regulation in spines.

Inhibitory Synapses: Postsynaptic Organization

The composition of inhibitory postsynaptic components is much simpler. Fast GABAergic neurotransmission is mediated by ionotropic GABA receptors (principally

the $GABA_A$ receptor subtype), which are ligand-gated chloride channels that are segregated from glutamate receptors and concentrated in the postsynaptic membrane of inhibitory synapses. Unlike the subunits of ionotropic glutamate receptors, each subunit of a $GABA_A$ receptor has its carboxyl terminus on the extracellular side of the membrane. The only major cytoplasmic domain of $GABA_A$ receptors is the loop between the third and fourth transmembrane segments. Reasoning that this loop is likely to form the site of GABA receptor interactions with intracellular proteins, two-hybrid yeast screens have been used to search for interacting gene products using these loops, and two proteins associated with microtubules have been identified: GABARAP, which binds to the intracellular loop of the abundant $\gamma 2$ subunit of $GABA_A$ receptors, and the MAP1B heavy chain that specifically binds to the intracellular loop of the $\rho 1$ subunit of ionotropic $GABA_C$ receptors, almost exclusively in the retina. In addition to these proteins, gephyrin, neuroligin-2, and the dystroglycan/dystrophin complex are other identified components of some mature GABAergic postsynaptic sites. Gephyrin and neuroligin-2 have been respectively implicated in GABA receptor localization to synapses and the formation of inhibitory synapses, but the mechanisms underlying these activities remain elusive: for example, neuroligin-2 is mainly localized at inhibitory synapses, but a possible C-terminal interactor (like PSD-95 at excitatory synapses) has not yet been identified.

Synapse Development: A Morphological View

Over the last few years, a number of studies have described how synapses are formed morphologically: for example, the first rudimentary synapses in the rat hippocampus can be observed on postnatal day 1, after which the density of synapses in the CNS gradually increases during the following weeks with the number doubling in the second postnatal week and from day 15 to adulthood. A similar time course can be observed in neurons *in vitro*, although the maximum number is generally smaller in cultures.

As previously mentioned, adhesion molecules play an essential role in synapse development, but it is not known how many neuronal cell-surface molecules really participate in this interaction, or how they signal to effect downstream events in synapse differentiation.

Synapse assembly has been more directly visualized using cultured neurons transfected with synaptic proteins tagged with fluorescent proteins, and these time-lapse studies have shown that many components of the synaptic junction are rapidly assembled after the initial contact of axons and dendrites in a timescale of tens of minutes (**Figure 2**). Some studies have shown that presynaptic markers such as Bassoon (a scaffold protein of the presynaptic active zone) and synaptophysin (a synaptic-vesicle membrane protein) can accumulate at new synapses before postsynaptic NMDA receptors and PSD-95, which may indicate that presynaptic differentiation precedes postsynaptic development.

These findings support the hypothesis that presynaptic specialization is constructed from preassembled 'packets' of vesicles and/or proteins, and at least two types of precursor complexes have so far been identified, one including VAMP, synaptic-vesicle protein 2 (SV2), synapsin I, and voltage-gated calcium channels, the other containing Piccolo, Bassoon, Rab3-interacting molecule (RIM), syntaxin, and SNAP-25 and N-cadherin.

The mechanisms of assembling postsynaptic specialization are less well characterized than those on the presynaptic side. Postsynaptic assembly may depend either on the gradual recruitment of individual proteins, or on the delivery of prefabricated protein complexes to the PSD. For example, a study has identified in dendrites the presence of a class of vesicles that carry NMDA receptors associated to proteins including Veli/MALS, CASK, and the microtubule-dependent motor KIF17. In a more recent publication it has demonstrated that a complex of postsynaptic scaffold proteins consists of two populations with different content, mobility and involvement in synapse formation. One subpopulation is mobile and relies on actin transport for delivery to nascent and

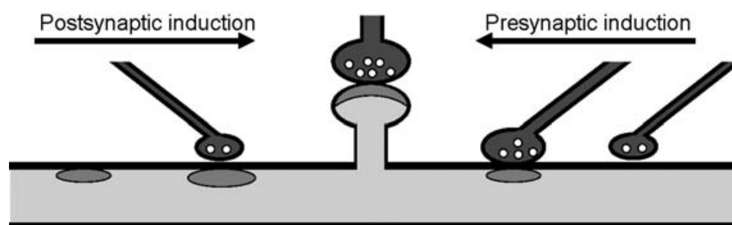

Figure 2 Schematic representation of the temporal sequence accumulation of specific pre- and postsynapse compartments based on time-lapse imaging observations of developing neurons in culture. Synapse formation is initiated by contact between dendrites and axons. These studies suggest that both synaptic sides can differentiate before the other, and that there are probably contact-induced signals triggering synapse assembly on both the pre- and postsynaptic sides.

existing synapses: these mobile clusters contain the PSD-95, GKAP, and Shank scaffolding proteins, and a slow-moving and short-traveling proportion contain neuroligin-1. The second group consists of stationary nonsynaptic scaffold complexes that mainly contain neuroligin-1, and can recruit synaptophysin-containing axonal transport vesicles that are rapidly transformed to functional presynaptic contacts that recycle the vital dye FM 4-64. These results postulate a mechanism whereby preformed scaffold protein complexes serve as predetermined postsynaptic hot spots for the establishment of new functional excitatory synapses. Interestingly, it has been found that the assembly of the scaffold protein complex requires PSD-95, because interfering with PSD-95 expression by siRNA in young hippocampal neurons disrupts the clustering of GKAP and Shank, reduces the number of excitatory synapses, and increases the number of vesicular GABA transporter (VGAT) puncta that are positive for neuroligin-1, thus indicating a shift in neuroligin-1 localization from excitatory to inhibitory contacts. The main conclusion of this study indicates that this preformed complex plays a role in inducing presynaptic maturation.

Other time-lapse imaging studies performed in dissociated hippocampal cultures indicate that PSD proteins accumulate gradually at nascent synapses with kinetics that have time constants in the range of minutes. Although not well understood, the gradual clustering of these proteins indicates that PSD proteins are recruited to nascent synaptic sites from diffuse cytoplasmic or membranal pools or, in some cases, are recruited to extrasynaptic sites, from where could then move laterally and merge with nearby synaptic clusters. It is possible to postulate that during the early stages of synaptic formation, postsynaptic preassembled protein complexes play a major role in the PSD assembly by catalyzing the initial step of synapses formation, whereas in a later stage gradual recruitment from diffuse intracellular pools regulates synaptic grow and maintenance.

Overall, these studies suggest that there are probably contact-induced signals triggering synapse assembly on both the pre- and postsynaptic sides and that both synaptic sides can differentiate before the other.

See also: Axonal Pathfinding: Extracellular Matrix Role; Postsynaptic Development: Neuronal Molecular Scaffolds.

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Presynaptic Development and Active Zones

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Introduction

Chemical synapses are specialized sites of cell–cell contact designed for the transmission of signals between neurons and their targets – muscles, glands, or other neurons. Synaptic transmission depends on the tightly regulated secretion of neurotransmitters by the presynaptic cell and the reception of this signal by postsynaptic neurotransmitter receptors. The directional nature of synaptic transmission is manifested in the asymmetric structure of pre- and postsynaptic compartments. Structurally, presynaptic compartments are characterized by the presence of hundreds to thousands of neurotransmitter-filled synaptic vesicles (SVs) and by active zones (AZs) – specialized regions of the presynaptic plasma membrane where SVs dock, fuse, and release neurotransmitter into the synaptic cleft (**Figure 1**). The AZ is characterized by an electron-dense matrix or lattice of proteins (the cytoskeleton of the active zone (CAZ)), arranged into regular arrays of electron-dense tufts linked together by fine filamentous material. This structure, known as the presynaptic web or grid, is thought to define the AZ as the site of SV docking and fusion. The postsynaptic reception apparatus is also characterized by an electron-dense thickening referred to as the postsynaptic density (PSD), the central function of which is to confine receptors of the appropriate type beneath the AZ. The PSD and CAZ are held in register by transsynaptic cell adhesion molecules and extracellular matrix proteins.

Although presynaptic specializations can contain hundreds to thousands of SVs (the so-called 'reserve pool'), at any given moment only a few are 'tethered' or 'docked' at the AZ. A depolarizing action potential and the consequential opening of voltage-dependent calcium channels leads to an influx of Ca^{2+}, triggering the fusion of some docked vesicles with the presynaptic plasma membrane. SV membrane proteins are then retrieved by clathrin-mediated endocytosis in a periactive zonal region or possibly by direct retrieval of vesicles at AZs. Endocytosed vesicles are refilled with neurotransmitter, possibly after passage through specialized endosomal compartments, and returned to the reserve pool.

The formation of a new functional presynapse is a demanding process that calls for the recruitment of numerous components. These include molecules involved in SV docking, fusion and endocytosis; structural AZ proteins; Ca^{2+} channels; proteins that confine SVs to the presynaptic bouton; and the SVs themselves. In addition, presynapse formation must be tightly coordinated with the formation of the appropriate postsynaptic receptive apparatus. Here, the current state of knowledge on cellular and molecular mechanisms involved in presynapse development is summarized.

Presynaptic Development: Initial Events

Most presynaptic sites are formed during development as axons grow out and establish connections with their targets. Much of our understanding of presynapse formation comes from studies on the vertebrate neuromuscular junction (NMJ). These synapses, formed between motor neurons and skeletal muscle cells, begin to form within a few hours after the closure of the neural tube. Motor neuron axons, guided by growth cones at their tips, establish contacts with muscle targets in their final stages of differentiation. These axons grow a short distance down the midline of the differentiating muscle, giving rise to side branches in the process. Eventually, these growth cones develop into bulbous enlargements that possess a rudimentary capacity for spontaneous and evoked release of neurotransmitter. With time (many hours to days), these structures differentiate into typical presynaptic terminals and gradually acquire mature functional characteristics.

Studies concerning NMJ formation have traditionally emphasized the roles of axonal growth cones in setting off the series of events that ultimately lead to NMJ differentiation. This neurocentric view seemed natural given the motility of axonal growth cones compared to the relative immobility of developing muscles, their ability to release neurotransmitter before they contact their targets, and their capacity for inducing postsynaptic receptor clustering when placed in nerve–muscle co-cultures. Recent evidence, however, reveals that not all instructive cues are axonally derived: it has been known for many years that small neurotransmitter receptor clusters form spontaneously in muscle cells even in the absence of innervation, although the role of such receptor clusters was not clear. Recent *in vivo* experiments, however, reveal that elongating axons tend to grow toward preformed receptor clusters concentrated along the midline of differentiating muscles, and

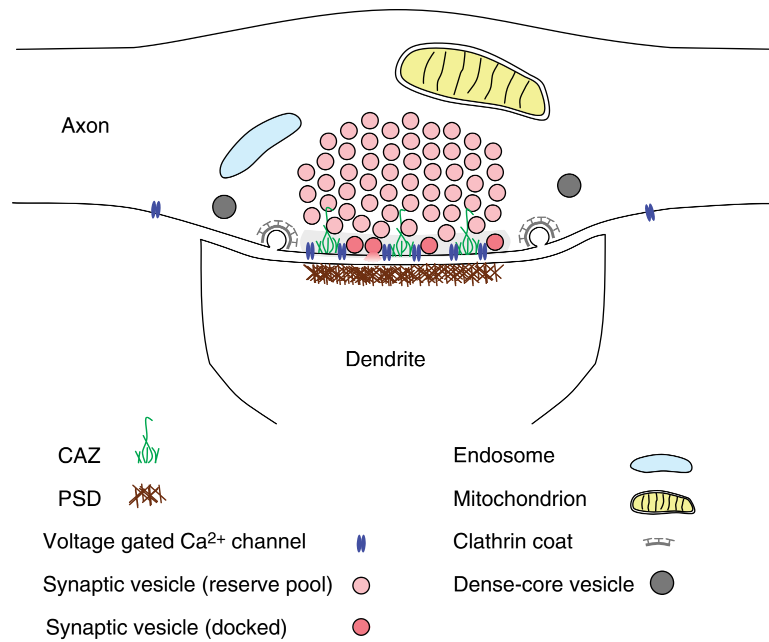

Figure 1 A schematic illustration of a vertebrate CNS, *en passant* spine glutamatergic synapse. The active zone, including CAZ molecules, voltage-gated Ca^{2+} channels, and docked vesicles, is shaded in gray. Clathrin-dependent endocytosis of synaptic vesicles is shown to occur along the margins of the active zone (the periactive zonal area). Distal to the active zone is the reserve pool, composed of numerous synaptic vesicles enmeshed and bound to a matrix of filamentous actin (cytoskeletal elements are not illustrated for purposes of clarity). Presynaptic compartments often contain mitochondria, a small number of dense core vesicles, and additional organelles such as endosomes.

these axons subsequently develop presynaptic specializations at these sites. This phenomenon serves to indicate that presynaptic differentiation is most likely specified by postsynaptic membrane factors. Interestingly, these factors are probably not the receptors themselves. Instead, studies suggest that components of the extracellular matrix, confined to muscle midline regions, may facilitate this process. This phenomenon also serves to highlight the fact that signals that specify pre- and postsynaptic differentiation are bidirectional, and thus the historic question (that is still raised occasionally) whether synaptic differentiation is induced by pre- or postsynaptic members is somewhat irrelevant.

In contrast to motor neuron axons which do not form synapses en route to their final destinations, axons of the vertebrate central nervous system (CNS) establish presynaptic sites along their lengths in the form of small swellings, or varicosities, known as presynaptic boutons. This arrangement allows one axon to form *en passant* synaptic connections with many dendrites along its route. *En passant* presynaptic boutons are often formed behind advancing axonal growth cones as they weave their way through the developing neuropil. However, the potential to form presynaptic boutons is not limited to the terminal growth cones and seems to be distributed along the entire axonal membrane. *In vitro* and *in vivo* studies indicate that presynaptic boutons may form along existing axonal segments in response to axodendritic contacts initiated by dendritic protrusive structures, namely growth cones at the tips of elongating dendritic branches or filopodia extended laterally from dendritic shafts. Axons also extend highly protrusive lateral structures that might also give rise to presynaptic boutons at positions far behind leading-edge growth cones. Thus, new presynaptic boutons can be formed from axonal growth cones, immediately behind advancing axonal growth cones and along established axonal segments.

Recent progress in imaging techniques, such as the introduction of green fluorescent protein (GFP) and the advent of multiphoton confocal microscopy, has provided unprecedented information on the cellular dynamics associated with presynapse development, both in cell culture and in the intact developing CNS of tadpoles, zebra fish, and mice. A consistent observation made in such imaging studies is that presynapse development is an extremely dynamic process. Specifically, these studies reveal that (1) many contacts formed between axons and dendrites are transient, resulting from exploratory-like growth and retraction

of axonal and dendritic processes; and (2) many, perhaps most, nascent presynaptic structures are transient in nature and often disappear within hours or less of their initial formation. These dynamics may be taken to indicate that the formation of a persistent presynaptic site is at odds with opposing mechanical forces that act to 'tear apart' the nascent axodendritic junctions – forces generated by axons and dendrites as these extend through the developing neuropil and by dendritic and axonal filopodia as they undergo cycles of extension and retraction. In this respect, it is interesting to note that *in vivo* imaging of axon and synaptic vesicle dynamics of retinal ganglion neurons in developing zebra fish embryos and *Xenopus* tadpoles indicates that the formation of a presynaptic site along an axonal branch stabilizes that branch and protects it from subsequent retraction, as if synapses form 'spot welds' between axons and dendrites. Furthermore, new axonal branches were observed to emerge preferentially from sites of presynaptic specializations. It was proposed that iterative rounds of branch extension from existing presynaptic sites and selective branch stabilization by synapse formation at tips of new branches would guide axon arbor growth in a manner reminiscent of the 'synaptotrophic hypothesis' proposed by James Vaughn several decades ago (**Figure 2**).

Maturation of Nascent Presynaptic Sites

The differentiation of growth cones or patches of axonal membrane into well-formed functional presynaptic compartments is associated with significant structural changes. Unfortunately, these are mostly beyond the resolving power of light microscopy, including the cutting-edge microscopy techniques that have been so informative in documenting the choreography of synaptogenesis. Electron microscopy (EM) has therefore been used to examine the fine structure of synapses in samples from developing tissue in an attempt to determine the sequence of structural rearrangements that takes place as nascent contacts evolve into mature presynaptic specializations. Collectively, these studies suggest that nascent CNS synapses have parallel, apposed membranes, with varying degrees of structural specializations (electron-dense thickenings). Initially, their presynaptic compartments are very simple, essentially composed of a patch of electron-dense membrane (considered to be the AZ) associated with only a small number (two or three) of SVs. Reserve pools of SVs are virtually nonexistent, as are the mitochondria that are commonly observed in mature presynaptic boutons. On the other hand, pleomorphic vesicular structures, dense core vesicles, some with spicules projecting from their surface, as well as coated vesicles are often observed at nascent presynaptic sites. With development, SV numbers increase and boutons become larger, and the presynaptic membrane becomes more complex.

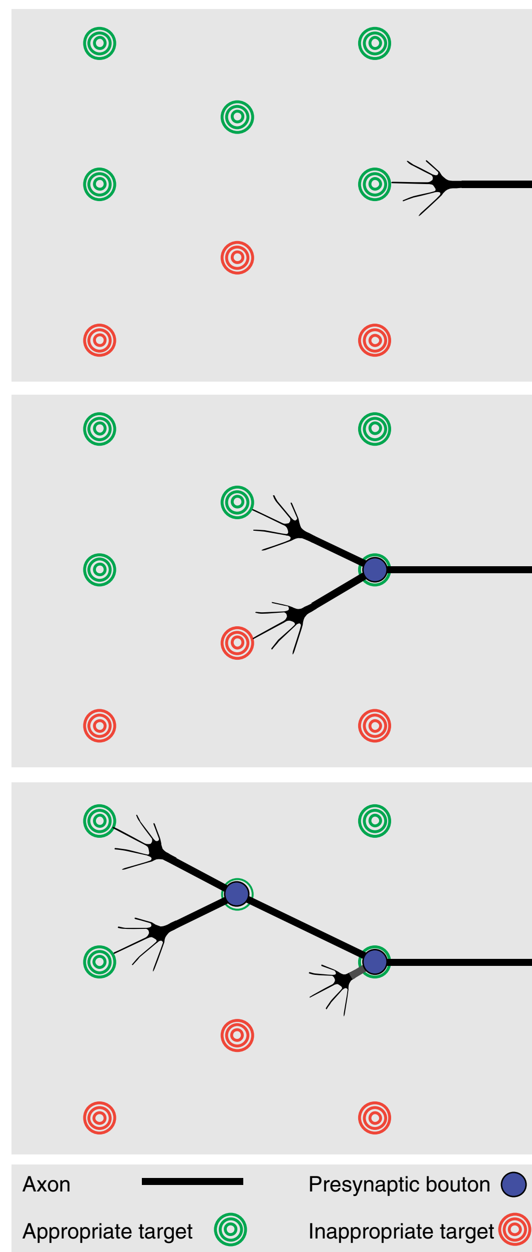

Figure 2 The synaptotrophic hypothesis as applied to axonal growth and presynapse formation. Axonal growth is governed by two principles: (1) the formation of a presynaptic specialization by an elongating axonal branch stabilizes this branch, whereas the failure to form such a connection entails subsequent branch retraction; and (2) new branches form preferentially from sites of newly formed presynaptic specializations. Iterative rounds of growth/retraction and branching according to these principles consequently result in the preferential establishment of presynaptic specializations within appropriate postsynaptic target regions.

In addition to structural changes, presynaptic maturation is also associated with changes in functional characteristics. For example, SV exocytosis becomes confined to the presynaptic AZ region; changes occur in the types and subunit composition of voltage-dependent calcium channels involved in evoked neurotransmitter release. Moreover, the sensitivity of SV exocytosis to tetanus toxin is increased, whereas the sensitivity to the vesicle budding inhibitor Brefeldin A is reduced. Finally, changes in the probability of neurotransmitter release are observed. Although details of these maturational changes differ from one type of synapse to another and from one organism to another, it is of note that such changes are protracted, taking place over days or even weeks, at least in vertebrates.

Relationships between structural and functional aspects of synapse maturation are not well understood. However, a combined analysis based on optical methods (recycling of styryl dyes), electrophysiological recordings, and EM indicates that presynaptic boutons of rat hippocampal neurons in primary culture pass through three distinct states. At the onset of detectable synaptic function (about 5 days after plating), synapses lack readily releasable vesicles, although they possess a pool of recycling vesicles that can release neurotransmitters under strong stimulation. During the next 2 days, small pools of docked, readily releasable vesicles begin to appear (presumably reflecting the formation of AZs). Subsequently, reserve pools of SVs start forming, increasing their size concomitantly with a parallel increase in the docked, readily releasable pool size over the rest of the maturation period (several more days).

The long maturation process described previously is seemingly at odds with light microscopy-based observations made in developing neurons that indicate that functional presynaptic sites can form surprisingly fast – within 1 h or less of the establishment of a new axodendritic contact. In these studies, however, new presynaptic sites were recognized as such on the basis of (1) morphological criteria, such as the clustering of a fluorescently tagged presynaptic or SV proteins at the prospective presynaptic site; (2) functional assays, usually the capacity for evoked endocytosis and exocytosis of fluorescent endocytosis tracers such as the styryl dye FM4–64; (3) cytochemical data – that is, retrospective immunohistochemistry using antibodies against synaptic molecules; or (4) combinations of these. These criteria are rather crude in comparison to EM-based ultrastructural analysis, and so, in most of these studies, it remains unknown if the new presynaptic sites had acquired the structural characteristics typical of mature presynaptic terminals or, perhaps, represent nascent structures that are yet to undergo a prolonged maturation process over the days and weeks to come.

Ideally, one would like to monitor the formation of individual synapses in living neurons, determine their functional characteristics, and then examine their ultrastructure by EM. To date, such experiments have been performed in model systems: Experiments performed in *Xenopus* spinal neurons co-cultured with muscle cells revealed that excitatory postsynaptic potentials as well as increases in intra-axonal calcium concentrations can be recorded within seconds to minutes of nerve–muscle contact. During the next few hours, marked increases were observed in the frequency of spontaneous synaptic currents and in the amplitude of both spontaneous and evoked synaptic currents. Surprisingly, practically no well-formed synaptic specializations were found when the same nerve–muscle contact sites were scrutinized by EM, despite their marked capacity for synaptic transmission. In particular, no obvious AZ-like structures were detected. Instead, presynaptic regions contained scattered SVs, some dense core vesicles, and endocytotic profiles. More mature-like presynaptic specializations were observed only a day or so after nerve–muscle contact. Similar experiments performed in nascent CNS synapses formed between hippocampal neurons in primary culture reveal a similar situation – the absence of well-formed AZs or numerous typical SVs at nascent presynaptic sites within 2 or 3 h of their formation, even though a capacity for stimulation-evoked vesicle recycling was demonstrated at the same sites before fixation. Instead, numerous pleomorphic and tubulovesicular structures as well as dense core vesicles were observed. These findings, as well as others, indicate that the ultrastructure of nascent CNS presynaptic sites might be significantly different from that of mature presynaptic boutons.

When considering the issue of presynaptic maturation, it is important to remember that periods of synaptogenesis can last days, weeks, or even months (depending on the organism). As discussed previously, this period is characterized by enormous cellular dynamics during which axons and dendrites grow, branches extend and retract, resulting in the formation and elimination of synaptic contacts at great rates. This means that the population of presynaptic sites is heterogeneous in terms of synaptic age, making it unlikely that all new synapses progress synchronously through well-defined developmental stages such as those described previously. It is more likely that much of this maturation process reflects genetically programmed global changes in neuronal maturational states. The maturational state would dictate the availability and cellular distribution of synaptic building blocks, the dynamics exhibited by

axons and dendrites, the competence of axons and dendrites to evolve into mature synaptic structures, and the functionality of molecular pathways that are involved in coordinating and assembling mature presynaptic structures.

This point is illustrated by several examples. *Xenopus* spinal neurons form rudimentary synapses with co-cultured muscle cells within 1 day in culture. However, synapsin I, a very prominent presynaptic molecule, is not detected before day 3 in culture, nor is it detected *in vivo* during early phases of synaptogenesis. It was therefore suggested that the onset of presynaptic maturation is causally related to the onset of synapsin I expression. Similarly, the expression of dynamin and amphiphysin, two molecules that have crucial roles in SV endocytosis, is delayed considerably in comparison to SV proteins such as synaptotagmin and SV2. Given their roles in presynaptic endocytosis, it is conceivable that the delayed expression of these molecules could be related to the late formation of SV reserve pools at new presynaptic sites.

Another global change that occurs with development is a gradual reduction in axonal and dendritic motility: In fact, *in vivo* imaging in mice (and in macaque monkeys as well) reveals that in adult animals, overall dendritic and axonal structure is remarkably stable, particularly when compared to the incredible dynamics observed during periods of intense synaptogenesis. Conceivably, this reduction in cellular dynamics may promote presynaptic maturation by increasing the likelihood that nascent presynapses will persist and evolve into mature presynaptic structures.

A phenomenon often observed during synaptogenesis is the presence of presynaptic structures that exhibit many ultrastructural or morphological hallmarks of bona fide presynaptic sites but exhibit practically no capacity for evoked neurotransmitter release. The reasons for this dysfunctional state are not known, although it is likely to reflect the presence of incompletely formed AZs or the lack of components essential for SV exocytosis. Interestingly, these presynaptically silent or mute synapses can be converted rapidly to functional presynaptic sites by elevating cAMP levels, by manipulating extracellular Ca^{2+} and Mg^{2+} levels, or by high-frequency stimulation of presynaptic neurons. This unsilencing seems to involve rapid actin polymerization mediated by brain-derived neurotrophic factor (BDNF) and the small GTPase cdc42 (a critical regulator of cytoskeletal rearrangements). Interestingly, the fraction of presynaptically silent synapses gradually diminishes over time, at a rate that parallels the stabilization of axonal and dendritic arbors, indicating that structural stabilization and presynaptic maturation are tightly interconnected developmental processes.

Cellular Mechanisms of Presynaptic Differentiation

Studies based on live imaging microscopy techniques have led to the realization that presynaptic development is a highly dynamic process, occurring over a time scale of minutes to hours. Some of the cellular mechanisms that underlie these dynamics have been characterized and will be discussed next.

As mentioned previously, axons of immature neurons display a capacity for evoked SV recycling along axonal segments, even in the absence of obvious targets. Closer examination has revealed the presence of mobile packets of vesicles or vesicular material that travel along axons at rates of up to 0.1–1 $\mu m\,s^{-1}$. These packets move intermittently in both directions, often pausing, sometimes splitting into smaller packets or merging into larger clusters that sometimes exhibit a capacity for depolarization-evoked exocytosis and endocytosis similar to bona fide synapses. In one study, carried out in cultured hippocampal neurons, mobile SVs (visualized by means of a GFP-tagged variant of the SV molecule VAMP2/Synaptobrevin2) were observed to accumulate rapidly at new axo-dendritic contacts sites that subsequently exhibited a capacity for stimulation-evoked SV recycling. Intriguingly, retrospective immunohistochemical analysis revealed that these packets were associated with additional presynaptic molecules, including voltage-dependent calcium channels, SV2, synapsin I, and amphiphysin. These findings have led to suggestions that the mobile packets observed in axons might be preassembled precursors (‘prototerminals’) involved in the rapid formation of true presynaptic structures.

The concept that vesicular structures might be used for presynapse formation was originally put forward several decades ago. As mentioned previously, electron micrographs of developing spinal cord synapses revealed the presence of dense core vesicles with spicules projecting from their surface. On basis of their structural similarity to AZ material and their proximity to presynaptic membranes, it was suggested that these might be involved in the delivery of materials necessary for presynaptic differentiation. In agreement with this idea, a previously unknown 80 nm dense core vesicle was shown to specifically contain multiple AZ components, including the CAZ scaffolding molecules Bassoon, Piccolo, and Rim, but not characteristic SV molecules. Later studies showed that these Piccolo/Bassoon transport vesicles (PTVs) are Golgi derived and that the formation of new functional SV release sites is preceded by the recruitment of two to five mobile Piccolo/Bassoon packets to these sites. These findings suggest that at

least some AZ proteins are incorporated into nascent presynaptic sites in a unitary fashion.

Time-lapse microscopy of SVs labeled with styryl dyes such as FM1–43 revealed that functional release sites occasionally give rise to small mobile vesicle clusters that moved along axons, sometimes merging with nearby presynaptic sites. Interestingly, such mobile clusters often display a capacity for SV release en route, even when they are not juxtaposed to postsynaptic structures or even dendrites, although they do contain the CAZ molecule Bassoon. These features suggested that these mobile structures might represent 'orphan' SV release sites. Intriguingly, orphan release sites were sometimes observed to give rise to new presynaptic sites at axodendritic contacts, indicating that new presynaptic compartments might sometimes be formed from 'units' of SV release machinery that 'bud off' preexisting synapses.

Presynaptic proteins are synthesized in the cell body and then 'shipped out' to axons by means of various axonal transport mechanisms. In principle, components of new presynaptic sites could arrive directly from somatic biosynthetic sources. However, studies such as those mentioned previously indicate that such components can also be 'pilfered' from nearby synapses. Intriguingly, studies show that typical SV membrane proteins (e.g., synaptobrevin-2/VAMP-2 and synaptotagmin-1) are also exchanged among neighboring presynapses by another route that involves SV fusion with the presynaptic membrane, lateral diffusion along the axonal membrane, and endocytosis at nearby presynaptic locations. These findings suggest that presynaptic proteins, SVs, and SV membrane proteins are, in fact, shared resources that are dynamically redistributed among or even competed over by neighboring presynaptic boutons, and that these pools may be drawn upon for the establishment of new presynaptic sites.

The finding that SV proteins are present in significant amounts on the axonal plasma membrane is relevant to another facet of presynaptic differentiation – the source of the numerous SVs found in presynaptic compartment. There seem to be two distinct sources for SVs. The first is the Golgi apparatus in the cell body, where SVs are formed and then transported into axons. These vesicles differ in several respects – both morphologically and in their molecular contents – from the typical clear core vesicles found at mature presynaptic sites, and they have thus been called SV precursors. It seems that such vesicles fuse with the axonal plasma membrane in growth cones and along its entire length in a relatively nonspecific manner. These fusion events, possibly mediated by the exocyst (a conserved protein complex essential for trafficking secretory vesicles to the plasma membrane), deposit SV membrane proteins in the axonal plasma membrane. This pool of SV membrane proteins is then used for the biogenesis of typical SVs by rounds of membrane protein sorting, clathrin-mediated endocytosis, endosomal processing, and local confinement to presynaptic compartments. This scenario may explain the presence of pleomorphic and tubulovesicular structures (SV precursors?) and the paucity of typical SVs observed at nascent presynaptic sites (as described previously) and also explain why the delayed expression of presynaptic molecules involved in SV endocytosis, biogenesis, and confinement would delay the formation of large SV pools at developing presynaptic sites.

The cellular events involved in presynapse formation may be summarized as follows (**Figure 3**): During early stages of neuronal differentiation, as axons are growing toward their targets, an inherent capacity for rudimentary SV recycling exists at axonal growth cones and along the axonal plasma membrane. Mobile packets of SVs, SV precursors, and AZ precursors travel up and down axons. As axons reach their target regions, fleeting axodendritic contacts, initiated by axonal and dendritic growth cones and filopodia, induce the clustering of such mobile packets at contact sites. Most of these contacts break up, and the transport packets disperse and renew their migration. As neurons mature, an increasing proportion of transient contacts do not break up and instead are stabilized by cytoskeletal rearrangements and cell adhesion interactions with postsynaptic membranes. These subsequently develop into functional presynaptic boutons, presumably by the fusion of PTVs with the presynaptic membrane, the recruitment of SV precursor transport packets, the recruitment of SV packets, and the local biogenesis of SVs. The new presynaptic sites are not completely stable, however, and occasionally 'orphan release sites' are uncoupled from their nascent postsynaptic partner and migrate to adjacent presynaptic sites or participate in the formation of new ones, presumably at sites of contact with dendritic structures. With time, genetically controlled processes stabilize axonal and dendritic arbors, reduce protrusive activity, and promote the structural and functional maturation of presynaptic structures.

Molecular Mechanisms of Presynaptic Differentiation

Mature presynaptic specializations are almost always found at sites of contact with postsynaptic targets, implying an exchange of signals that either induce the formation or promote the stabilization of presynaptic structures at these locations. Decades of research has resulted in the identification of numerous molecules

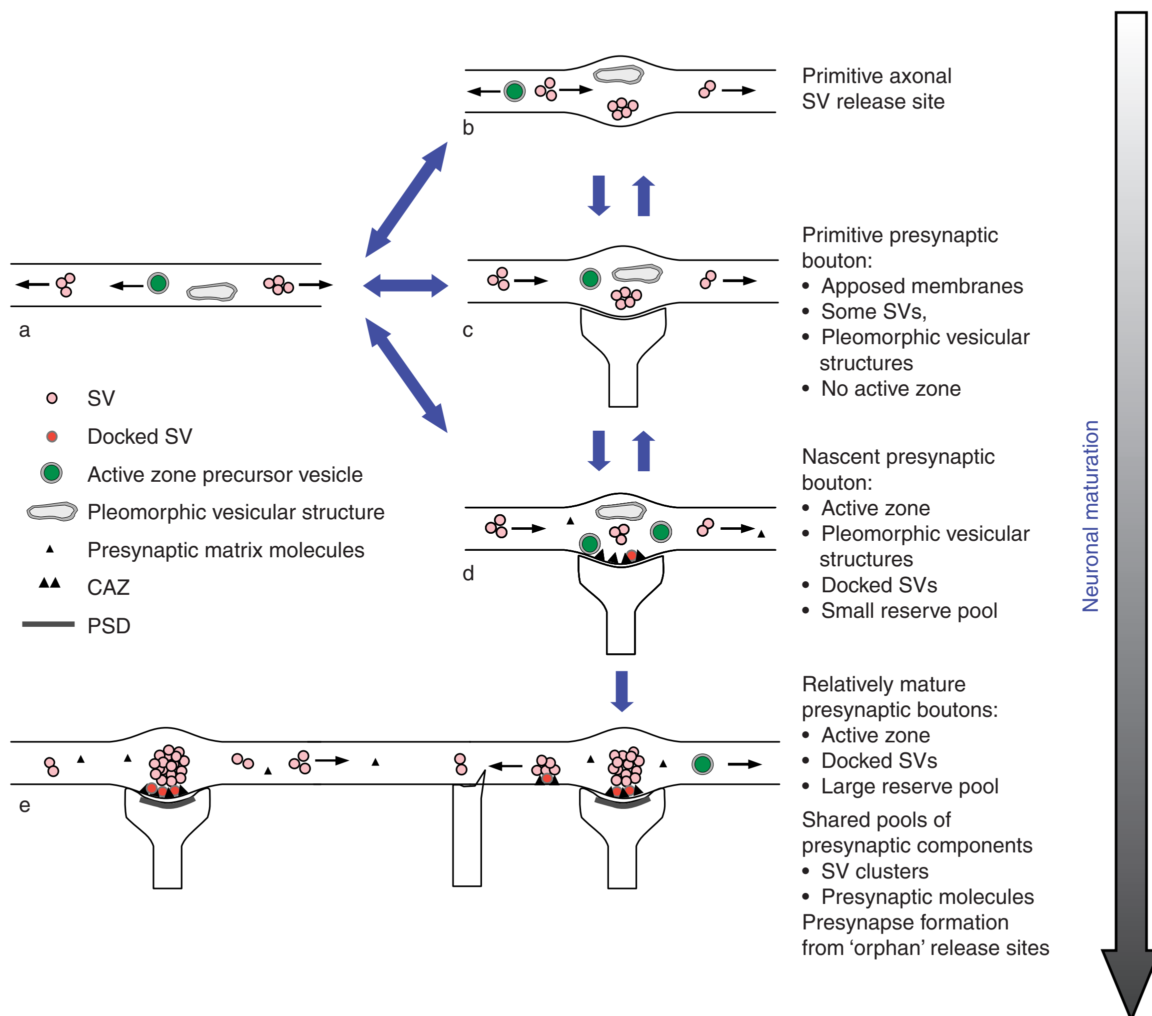

Figure 3 A model for presynaptic development. The formation of presynaptic specializations is envisioned to occur by multiple processes that take place over several time scales. (a) The axons of developing neurons contain several types of transport packets that are used for the assembly of nascent presynaptic structures. These include synaptic vesicle packets, pleomorphic tubulovesicular structures (synaptic vesicle precursors?), and active zone precursor vesicles such as PTVs. (b) In immature axons, primitive sites of synaptic vesicle recycling form spontaneously along axonal segments. (c) Contacts between axons and targets lead to the accumulation of various transport packets at contact sites, resulting in the formation of primitive presynaptic boutons. These sites display some capacity for synaptic vesicle recycling but lack the ultrastructural features of mature presynaptic boutons. (d) Alternatively, the establishment of an axodendritic contact might lead to the rapid formation of an active zone by the fusion of active zone precursor vesicles (such as PTVs) and the subsequent recruitment of synaptic vesicles. It is possible that this scenario becomes more common as neurons mature. (e) With time (days), synapses form larger synaptic vesicle reserve pools and gradually acquire the structural and functional characteristics of mature synapses. During this period, synaptic vesicles, plasma and synaptic vesicle membrane molecules, presynaptic matrix molecules, and even units of active zone material with cognate synaptic vesicles clusters (orphan release sites) are exchanged among nearby presynapses, sometimes giving rise to new presynaptic sites at nascent axodendritic junctions. It should be noted that uncertainty exists concerning some details of this scheme. For example, it is not known whether presynaptic sites form with a precise temporal order of events. Primitive presynaptic sites (c) could form first, followed by active zone formation (arrow from (c) to (d), or primitive sites could fall apart (arrow from (c) to (a) and reform later according to scheme (d) (arrow from (a) to (d). Similarly, axonal release sites (b) might form first and then become synaptic (arrow from (b) to (c) or eventually fall apart (arrow from (b) to (a). Adapted from Ziv NE and Garner C (2004) Cellular and molecular mechanisms of presynaptic assembly. *Nature Reviews Neuroscience* 5(5): 385–399.

involved in such bidirectional signaling. Yet, at the same time, it has become apparent that presynaptic differentiation is a dynamic, multistep process controlled by a host of molecular pathways. Thus, not all of the molecules identified so far are directly involved in specifying presynapse differentiation at specific locations and times, even though they do play roles in processes essential for the ultimate formation of presynaptic specializations, such as appropriate timing of presynaptic protein expression, axonal pathfinding, target recognition, arresting axonal growth motility, or stabilizing initial axon–target cell contacts.

Unfortunately, a comprehensive description of all molecules identified to date is beyond the scope of this article, and thus the focus is on a small number of relatively well-characterized molecules that do seem to be directly involved in presynapse formation induction.

Earlier work on NMJ formation led to the identification of Agrin, a large proteoglycan secreted by motor neurons that exhibits an ability to induce acetycholine receptor clustering beneath presynaptic axon terminals. This molecule was thus considered to be a major inducer of NMJ postsynaptic differentiation. Less is known about the reciprocal pathway – that is, the molecules responsible for inducing AZ formation in motor neuron terminals. However, laminin β_2, a component of the NMJ basal lamina, was shown to play an important role in presynaptic organization. Furthermore, it was shown to exert its effects via interactions with extracellular domains of presynaptic voltage-gated calcium channels, particularly P/Q and N type, the major subtypes found in NMJ AZs. Interestingly, however, laminin β_2 appears to be dispensable for the initial formation of AZs but is required for their subsequent stabilization. In this respect, it is also interesting to note that recent work implies that Agrin too is not required for acetylcholine receptor clustering *per se*, but it is required for counteracting neurotransmission-induced dispersion of these receptors. These findings highlight the importance of studying synaptic differentiation in a framework of molecular dynamics and stabilizing forces, rather than rigid molecular interactions and assembly processes.

Whereas secreted molecules clearly play crucial roles in NMJ formation, there are good reasons to believe that many of the bidirectional signals that pass between prospective pre- and postsynaptic partners rely on interactions between extracellular domains of axonal and target cell membranes proteins, particularly interactions mediated by various classes of cell adhesion molecules (CAMs). The cadherin family of calcium-dependent adhesion molecules has long been suspected of playing important roles in inducing presynaptic development, and there is evidence that this may be the case for some invertebrate synapses, such as those formed between photoreceptor cell axons and their targets in *Drosophila*. At vertebrate CNS synapses, however, classical cadherins do not seem to possess the capacity to induce presynaptic specialization on their own and appear to be more important for the stabilization of nascent presynaptic structures.

Several other classes of CAMs, however appear to perform similar functions. The best characterized is neuroligin, a member of the Ig superfamily of CAMs. Its presynaptic binding partners include members of the α- and β-neurexin families of cell surface proteins. Of these, the α-neurexins have been implicated in mediating the recruitment of voltage-gated calcium channels into the presynaptic AZ, whereas β-neurexins (essentially truncated α-neurexins) have been implicated in inducing AZ formation and SV clustering by actin polymerization promoted by their cytoplasmic, C-terminal tails or by interactions of these domains with presynaptic scaffolding molecules, such as CIPP, CASK/Lin-2, and Mint/Lin-10/X11α. When expressed in nonneuronal cells, Neuroligin can induce the formation of functional SV release sites in axons growing along these cells and this activity appears to require β-neurexin. When overexpressed in cultured hippocampal neurons, a large increase in morphologically identified synapses is observed. Conversely, RNAi-based knockdown of Neuroligin1–3 (the three functional forms in mice) in cultured hippocampal neurons reduces the number of morphologically identified synapses. Surprisingly, however, such increases or reductions in morphologically identified synapse number are not always associated with similar changes in functional synapse numbers as determined by miniature excitatory/inhibitory synaptic potential frequency. This puzzling discrepancy may indicate that additional molecules are required for inducing the formation of functional presynaptic compartments, a conclusion supported by the finding that normal numbers of synapses are formed in triple knockout mice lacking all three forms of murine neuroligin. It is worth mentioning, however, that the mice die soon after birth due to respiratory failure, which seems to be a consequence of reduced GABAergic/glycinergic and glutamatergic synaptic transmission in brain stem centers that control respiration.

A second molecule that exhibits a capacity to induce presynaptic specialization when expressed in nonneuronal cells is SynCAM, a member of the Ig superfamily of CAMs. Here, induction is via homotypic interactions with SynCAM present on both pre- and postsynaptic membranes. Interestingly, SynCAM overexpression in cultured hippocampal neurons leads to a large increase in functional synapse number but not an increase in morphologically identified synapse number – exactly the opposite of the phenotype observed for Neuroligin1. Furthermore, SynCAM effects seem to be specific for excitatory synapses, whereas Neuroligins seem to have a larger effect on inhibitory synapse formation.

Other cell adhesion molecule families, such as N-CAM and γ-protocadherins, have been implicated in controlling various aspects of presynapse development in vertebrate synapses. However, their roles and mode of action are not completely clear. In contrast, fascinating information concerning cell adhesion

molecules and presynaptic development has been obtained in invertebrate models, namely *Drosophila* and *Caenorhabditis elegans*, by harnessing the powerful genetic methods these systems offer. In one striking example from *C. elegans*, the expression of Syg2 in vulval epithelial cells (which are neither pre-nor postsynaptic cells) was shown to instruct hermaphrodite-specific neurons (HSNs) to form presynaptic specializations on specific motor neurons at the site where the three cells intersect. The effects of Syg2 on the HSNs is mediated by interactions with Syg1, both members of the Ig superfamily of CAMs, that are similar to the vertebrate molecules Nephrin and NEPH1. However, apparently normal presynaptic specializations still form in the absence of Syg1 or Syg2, albeit at erroneous locations, pointing to the difficulty in separating presynapse induction from more subtle roles such as a confinement of presynaptic structures to preferred locations.

Cell surface molecules that induce presynaptic differentiation do so by means of direct interactions with intracellular scaffolding/cytoskeletal molecules and by setting off various intracellular signaling cascades. Progress in identifying such molecular pathways has come from genetic screens in *Drosophila* and *C. elegans*. Such screens have led to the identification of several genes from several families that affect the morphology of presynaptic structures during development. For example, sad-1, a member of the microtubule affinity-regulating kinase (MARK) family, was found to be important for confining SVs to active zonal regions, whereas α-liprins were shown to control AZ size and complexity. Similarly, Pam/Highwire/Regulator of Presynaptic Morphology-1 (RPM-1) was found to play essential roles in organizing AZ and presynaptic structure, perhaps by direct interactions with other molecules but more likely by regulating multiple signaling cascades, including ubiquitination-dependent degradation of synaptic growth-promoting molecules and the MAP kinase pathway. Despite this progress, a satisfactory understanding of presynaptic differentiation in molecular terms is yet to be attained.

In summary, it seems that the induction of presynaptic differentiation involves multiple molecules and molecular cascades that act in parallel but probably differ in subtle ways. Even for molecules intimately linked to individual presynapse formation, it is not always clear if these actually trigger new presynapse formation or, rather, selectively promote the stabilization of presynaptic specializations that form spontaneously or migrate up and down axons in partially of fully assembled form. Further work is necessary to clarify the roles, interactions, and functional overlaps of these signaling cascades.

See also: Dendrite Development Synapse Formation and Elimination; Postsynaptic Development: Neuronal Molecular Scaffolds.

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Presynaptic Development: Functional and Morphological Organization

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Structural Features of Central Synapses

Chemical synapses are intercellular junctions critical for information transfer and processing in the nervous system. They consist of two compartments physically juxtaposed within several nanometers of each other: presynaptic terminals and postsynaptic specializations. Presynaptic terminals store and release neurotransmitter substances in membranous organelles named synaptic vesicles, whereas postsynaptic structures contain signaling molecules responsible for generation of neuronal responses to released neurotransmitters. Presynaptic terminals are highly organized subcellular structures. At the electron microscopic level, clusters of synaptic vesicles around the plasma membrane regions called active zones can readily distinguish them from other structures within a neuron. Synaptic vesicle exocytosis is thought to take place exclusively at the active zone, whereas synaptic vesicle endocytosis may occur within the vicinity of this region. Active zones are characterized by enrichment of scaffolding molecules, and enable assembly of proteins required for regulated vesicle fusion and recycling. Juxtaposed to the presynaptic terminal, the postsynaptic site is characterized by electron-dense material called the postsynaptic density. Postsynaptic density is enriched in scaffolding molecules that anchor neurotransmitter receptors and organize signaling in response to second messenger cascades activated by the neurotransmitter receptors. The pre- and postsynaptic sides of the synapse are held together with adhesion molecules spanning the synaptic cleft (**Figure 1**). The size of the active zone and the number of docked vesicles are critical determinants of the functional responses of a presynaptic terminal. These structural markers are continually modified during synapse maturation. Depending on the type of synapse, presynaptic terminals in a given synapse contain varying number of synaptic vesicles, some of which are physically attached or docked at the plasma membrane.

The most striking difference of synapses from other cell–cell junctions is the asymmetry of structures on both sides of the synaptic junction. Such asymmetry implies that two compartments must respond differently to the signal(s) that initiate synaptogenesis. This asymmetry is partially achieved through differential distribution of synaptic components to axonal and dendritic compartments within a neuron. Asymmetric interaction of cell adhesion molecules can also account for triggering divergent cascades of downstream events and induction of pre- and postsynaptic sites. However, it is important to note that despite this asymmetry the sizes of the structures on both sides of the synaptic cleft are all correlated, suggesting that the structural synapse assembly is significantly coordinated across the cleft.

In a mature presynaptic terminal, vesicles can be divided into two pools. The first pool contains a relatively small fraction of vesicles close to release sites. These vesicles can be released by brief Ca^{2+}-dependent stimuli or by hypertonic stimulation, which is Ca^{2+} independent. This release-ready pool of vesicles is referred to as the immediately releasable pool or the readily releasable pool (RRP). RRP vesicles are considered to be in a morphologically docked state, although not all morphologically docked vesicles are necessarily release competent at any given time. A priming step in addition to the morphological docking is required to make vesicles fully release competent. A secondary pool of vesicles, the reserve pool, is spatially distant from the release sites and constantly replaces the vesicles in the RRP that have been exocytosed. The rate of replenishment of RRP vesicles from the reserve pool is a critical parameter that determines the response of synapses to repetitive stimulation. Recent evidence indicates that intrasynaptic Ca^{2+} can facilitate the rate of replenishment. The number of vesicles contained in the RRP is a critical parameter that regulates the probability of release, which is defined as the probability that a presynaptic action potential can result in an exocytotic event. Therefore, the number of vesicles in the RRP and the rate and pathways by which they are replenished is a crucial determinant of presynaptic efficacy and of several forms of short- and long-term synaptic plasticity. Several lines of evidence support the presence of the non-recycling pool of vesicles in the synapse. Mechanisms that can render this pool functional remain to be determined.

Multiple Stages of Synapse Assembly

In the mammalian central nervous system (CNS), synapse formation is a precisely timed process. Synapses appear within days in a given brain region. At later stages of development, synapse proliferation

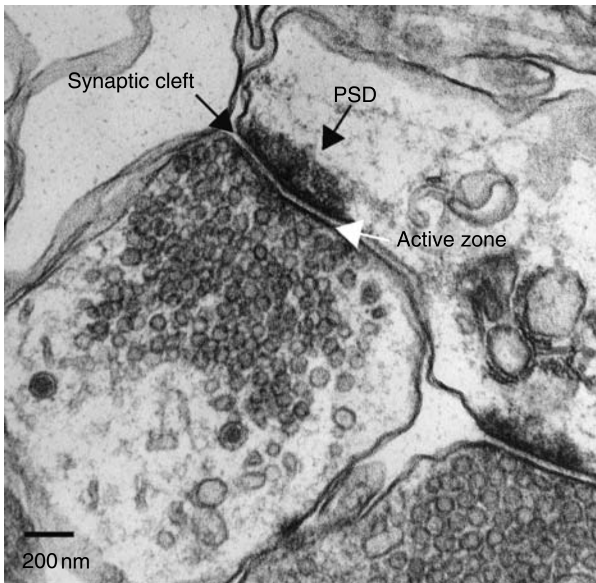

Figure 1 Structural organization of a central synapse. In electron micrographs, synapses can be readily distinguished by the cluster of synaptic vesicles around protein-rich (thus electron-dense) regions called the active zone. The size of the active zone is tightly correlated with the size of postsynaptic density, another protein-rich region associated with the postsynaptic dendrite. Synaptic cell adhesion molecules structurally linking the two sides of the synapse span the synaptic cleft between the presynaptic and postsynaptic regions. These molecules are thought to coordinate synapse maturation in pre- and postsynaptic compartments and also enable functional interactions between the two sides of the synapse. PSD, postsynaptic density.

is thought to be balanced by synapse elimination and pruning of synaptic contacts through activity-dependent mechanisms. Several lines of evidence suggest that synapse formation *per se* does not require neuronal activity. Initial events that establish immature synaptic contacts in neuromuscular junctions involve the interaction of axonal growth cones with target muscle membrane. In the central synapses, however, initial synapse formation is thought to take place between the axonal shaft and filopodial processes that extend from the dendrites. This type of interaction results in the formation of *en passant* synaptic boutons along the axonal shaft, which is a common feature of most CNS synapses. Retraction and stabilization of these filopodial processes together with contacted axonal regions or nascent presynaptic terminals marks the beginning of synapse maturation. Further maturation involves structural modifications that increase the anatomical complexity of the synaptic boutons, including an increase in the number of synaptic vesicles, the size of the synaptic boutons, and, in some cases, the number of active zones. An interesting aspect of synapse maturation is the matching change in the pre- and postsynaptic regions that results in a strong correlation between the size and complexity of both sides of a synapse.

The sequence of events leading to synapse formation has recently been studied in detail using time-lapse imaging techniques in dissociated hippocampal cultures. These studies took advantage of action potential-dependent, rapid synaptic vesicle recycling as the earliest indicator of synaptogenesis following the initiation of axo-dendritic contacts. These findings support the scenario that prepackaged presynaptic molecules are rapidly released at sites of axo-dendritic contact, forming functional presynaptic terminals. However, it has been previously shown that isolated synaptic vesicles in axons can also recycle in an activity-dependent manner prior to target contact. This immature form of synaptic vesicle recycling proceeds with slower kinetics compared to mature synapses. In some cases, this immature form of synaptic vesicle recycling has been shown to be resistant to tetanus toxin implicating the requirement for a vesicular SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) protein other than synaptobrevin-2 (VAMP-2), which is tetanus toxin sensitive. In the developing neuromuscular junction, unlike mature junctions, synaptic vesicle recycling is highly sensitive to Brefeldin A, which disrupts synaptic vesicle trafficking thorough endosomal intermediates.

Maturation of presynaptic terminals occurs in structurally and functionally distinguishable stages (**Figure 2**). During early synapse maturation, synapses are unresponsive to action potential stimulation or hypertonicity (which normally induces swift fusion of docked vesicles), although they can release neurotransmitters and recycle synaptic vesicles during strong stimulation such as induced by elevated extracellular potassium. This form of vesicle recycling detected in these nascent synapses is mechanistically analogous to the form observed in the absence of critical components of the synaptic SNARE machinery. This is consistent with the observation that synaptic vesicle recycling at this stage is tetanus toxin insensitive. Following this initial stage, synapses undergo a transition to become responsive to action potential stimulation and rapidly recycle synaptic vesicles. Examination of electron micrographs of nascent synapses reveals a strong correlation between this functional switch and the formation of the active zone, leading to the assembly of the RRP.

Indeed electron microscopic analysis of immature hippocampal cultures shows that synaptic vesicles are not as closely associated with plasma membrane as they are in mature synapses. These vesicles recycle in a calcium-dependent manner as they travel in the axon, indicating that basic machinery needed for

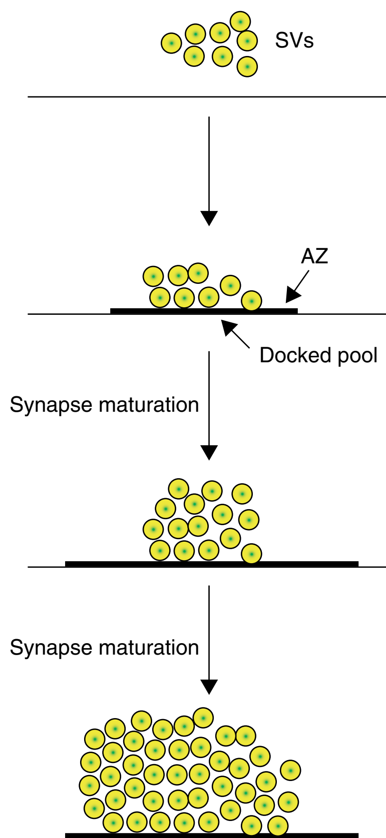

Figure 2 Sequence of events leading to maturation of presynaptic terminals during synaptic development. Nascent synapses typically contain synaptic vesicles that recycle only in response to strong stimulation. However, at these early stages of synapse assembly, vesicles are not tightly associated with the plasma membrane, presumably due to the immature state of active zones. These synapses lack a set of readily releasable vesicles; thus, they do not effectively respond to presynaptic action potentials. Formation of an active zone coincides with functional maturation of a presynaptic terminal. This stage may also involve actin cytoskeleton increasing association of the synaptic vesicle clusters with the active zone and the surface membrane release machinery. After these initial stages, synapse maturation involves a gradual increase in the size of the total vesicle pool, which also reflected an increase in the number of vesicles available for release. SV, synaptic vesicle; AZ, active zone.

docking and fusion is distributed loosely along the axon. Nevertheless, these hot spots of glutamate release sites may have an important role during the initial stages of synaptogenesis. The released glutamate can stimulate filopodial motility of both dendrites and axons, thereby increasing the chance of axo-dendritic encounter. However, in more mature cultures, glutamate strongly inhibits filopodial motility and stabilizes connections. These contradictory observations can be reconciled if during development changes in protein expression make filopodia less responsive to glutamate, or, alternatively, there is a concentration threshold for glutamate above which its activity has opposite effects.

Besides glutamate, many other secreted molecules increase the number and motility of dendritic and axonal filopodia prior to contact. These include brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), which have been shown to promote axonal arborization, dendritic growth, and synapse maturation. BDNF-coated beads can increase Ca^{2+} levels and trigger neurotransmitter release in contacting axons in a protein synthesis-dependent manner. Some members of the fibroblast growth factor (FGF) family of proteins (e.g., FGF22, FGF7, FGF10) can induce presynaptic organization via FGF receptor-2. In a recent study, Sanes and colleagues used a combination of chromatographic steps to isolate factors that cause axon branching and vesicle aggregation in chick motorneurons and purified FGF22 as an active component. Secreted Wnt proteins (Wnt-7a, Wnt-3) can also induce remodeling of growth cones and accumulation of synaptic vesicles. Wnt-7 knockout mice show a delay in maturation of multisynaptic glomerular rosettes formed between mossy fibers and granule cells in the cerebellum. Deficiency of a Wnt homolog in *Drosophila*, Wingless (Wg), causes abnormal pre- and postsynaptic differentiation indicating possible conservation of function.

The peak of synapse formation in the mammalian brain coincides with extensive proliferation of glia, in particular astrocytes. Astrocytic protrusions engulf potential contact sites between axonal and dendritic processes, implicating their possible role in synaptogenesis. Astrocytes release several factors that impact synaptic differentiation. Initial studies by Pfrieger and Barres showed that in the absence of glia the number of synapses formed between retinal ganglion cells in culture are reduced and individual synapses are less efficient in neurotransmitter release. Later work by Pfrieger and colleagues led to isolation of Apolipoprotein E (Apo-E) as the factor upregulated in the presence of glia. Apo-E is a carrier for cholesterol. In these experiments, application of cholesterol to retinal cultures caused a massive increase in spontaneous excitatory postsynaptic currents and the number of presynaptic nerve terminals. In parallel, Barres and colleagues identified thrombospondins 1 and 2 as additional glia-derived synaptogenic molecules, which specifically cause an increase in the number of synapses. Thrombospondins are multidomain extracellular matrix proteins, initially identified in platelet activation.

Role of Synaptic Cell Adhesion Molecules in Synapse Assembly

Whereas soluble factors play an important role in mediating the initial stages of vesicle aggregation and

priming axons for synapse assembly, synaptic cell adhesion molecules mediate the physical contact and functional communication between axonal and dendritic protrusions leading to the formation of synaptic junctions. These molecules are composed of several large families, which include N-cadherins, protocadherins, neural cell adhesion molecules (NCAMs), nectins, neurexins, and neuroligins. In most cases, extensive alternative splicing and differential glycosylation patterns create enormous variability in the possible repertoire of protein products. This high level of variety in individual protein products and the large number of combinatorial possibilities for intermolecular interactions between these molecules may contribute to the specificity of synaptic connections in the brain. However, the process of synapse formation itself seems to be somewhat promiscuous as evidenced by three observations. First, injury or degeneration in the brain can trigger extensive synaptic rewiring, which leads to formation of ectopic synapses between cells that do not normally make synapses with each other. Second, neurons in dissociated cultures form synapses rather promiscuously, where, in some conditions, cells can even form autapses with themselves regardless of their proper *in vivo* partners. Finally, axonal contacts onto polylysine-coated glass beads can induce assembly of presynaptic specializations. Taken together, these results indicate that the basic mechanism for synapse formation is inherent to all neurons. Furthermore, these observations suggest that synaptogenesis is not an event triggered by a single molecule, but rather a vast repertoire of molecular interactions that can lead to synaptogenesis between neurons. Activity-dependent processes test these connections over time and direct stabilization of the most resilient connections.

Neuroligin is the first adhesion molecule identified to be a direct inducer of presynaptic terminal assembly. When expressed in nonneuronal cells, they can cause an accumulation of presynaptic vesicle clusters on contacting axons. These synapses are fully functional as demonstrated by fluorescence imaging of synaptic vesicle recycling and electrophysiological detection of neurotransmitter release. The soluble extracellular domain of neurexin can mask this effect of neuroligin, indicating that neurexin is the mediator of the synapse-inducing activity of neuroligins within the presynaptic axon. An attractive part of the neurexin–neuroligin system is that it satisfies the necessary asymmetry required to induce different signaling events on pre- and postsynaptic sides of the synapse. Neurexins are primarily associated with the presynaptic site and intracellularly bind to calcium/calmodulin-dependent serine protein kinase (CASK) and syntenin. In contrast, neuroligins are located on the postsynaptic site and their C-termini interact with PSD-95.

SynCAM is the only other molecule shown to be sufficient to induce synapse formation when expressed in nonneuronal cells. It is a member of the immunoglobulin (Ig) superfamily, and mediates homophilic interaction through extracellular Ig domains. Similar to neuroligin, synapses induced *in vitro* by SynCAM are fully functional. Overexpression of SynCAM in dissociated cultures has a dominant positive effect in the functioning and formation of synapses. In contrast, the overexpression of dominant negative SynCAM lacking extracellular domain impairs presynaptic assembly. Unlike the neurexin–neuroligin interaction, SynCAM mediates a homophilic interaction, indicating that different downstream cues are present on different sides of the synapse (**Figure 3**).

How does the interaction of cell adhesion molecules translate into changes within the cell? Following initial contact, assembly of synapses takes 1–2 h and may occur in either of two ways. First, following contact, each molecule could be captured from the stream of axonal cytoplasm, and a synapse can be built depending on protein–protein interactions. A second model suggests that synaptic molecules are pre-assembled in small units in other parts of the neuron and transported to the axon. The speed of assembly favors a pre-assembled trafficking model. The evidence for the existence of cytoplasmic transport packages came from studies of Garner and colleagues. They have been able to isolate large dense vesicles from developing axons containing active zone proteins such as piccolo, bassoon, and RIM. Pre-assembled packages of active zone vesicles fuse with the axonal plasma membrane to create a scaffolding framework for other components. In addition to the active zone components, synaptic vesicles also assemble as clusters in a unitary fashion. Nevertheless, these two components do not seem to travel together along the axon. There is also evidence that at the end of this initial stage of synapse assembly, synaptic vesicles switch to a docked state and associate more closely with the plasma membrane in a synapsin-dependent manner.

Analysis of the intracellular interactions of neurexin and SynCAM reveals CASK as a converging downstream target. CASK is a member of the membrane-associated guanylate kinase family (MAGUK) and strongly interacts with neurexin and SynCAM cytoplasmic tail. Upon interaction with neuroligin, neurexin oligomerizes and recruits CASK. Given the multidomain structure of CASK, it is usually envisaged as a recruiter to the newly formed contact sites for both neurexin and SynCAM. CASK also

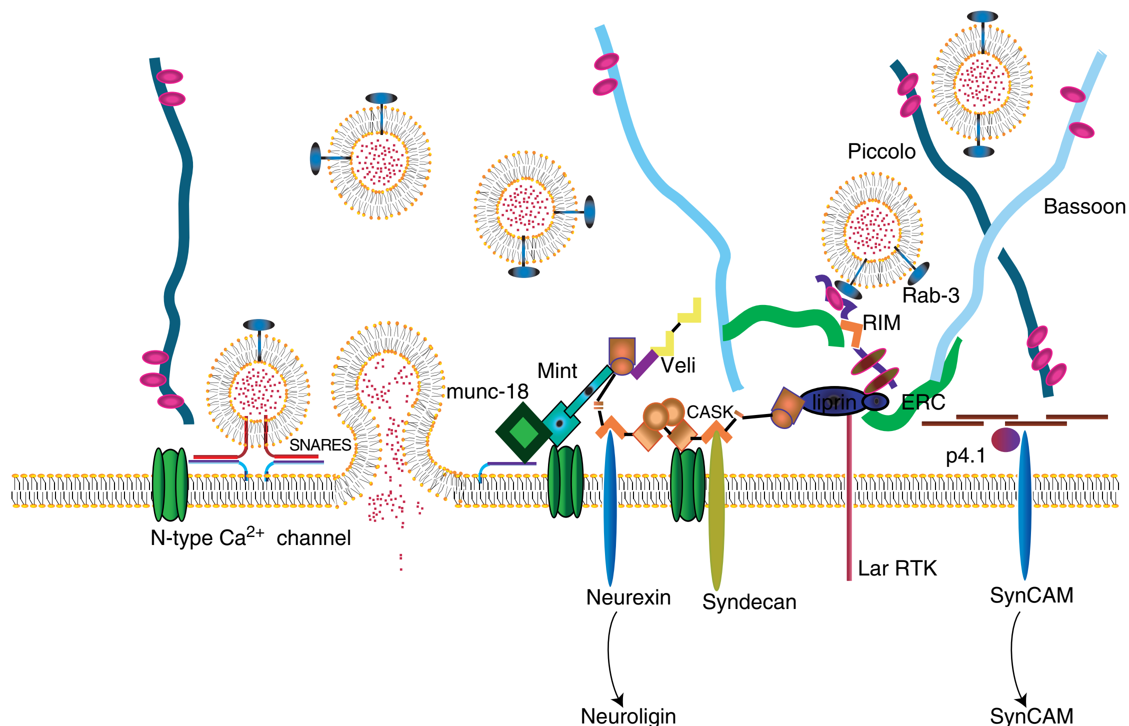

Figure 3 Putative molecular interactions that link synaptic cell adhesion molecules to the active zone and to the neurotransmitter release machinery. Cytoplasmic tails of neurexins and SynCAM can interact with a modular adaptor protein CASK which in turn binds another adaptor protein Mint interacting with the release machinery (munc-18 which interacts with syntaxin) as well as the voltage-gated calcium channels. Active zone components such as RIM can interact with synaptic vesicle-associated molecules, which include rab3, thus bridging synaptic junctional scaffold to the synaptic vesicle cluster.

forms a well-conserved tripartite complex with Mint and Veli, multidomain PDZ molecules. This complex is proposed to be responsible for the recruitment of vesicle fusion machinery. CASK has also been shown to interact with liprin, which organizes the presynaptic active zone in *Caenorhabditis elegans*. By tightly interacting with the active zone proteins RIM and ERC, liprins constitute the insoluble backbone of the active zone.

As mentioned above, synaptic vesicles are tethered at the vicinity of the active zone by the actin cytoskeleton. Actin depolymerizing agents have a strong disruptive effect on nascent synapses but not on mature synapses, implying a role for actin during synapse formation. CASK can polymerize actin on the neurexin C-tail and stabilize it by interacting with protein 4.1. In this way, synaptic adhesion molecules neurexin and SynCAM (and possibly other CASK-interacting adhesion molecules such as syndecans) can induce local polymerization of actin at contact sites and trap traveling synaptic components.

As discussed above, free-moving vesicle clusters have different cycling properties than mature ones. How maturation changes vesicle identity is not known. It could be achieved through transport of mature vesicles or conversion of the identity of existing vesicles. One can speculate that these initial synaptic vesicles are still present in mature synapses but in a reduced capacity for synaptic vesicle recycling. This may explain the presence of the enormous number of vesicles at synapses, while only a fraction of them are functional.

There are a multitude of possible pathways that can lead to eventual assembly of synaptic terminals. This redundancy can increase the robustness of the synapse assembly process and also contribute to the functional and structural versatility of synapses.

Stabilization of Synapses

Synapse formation is rather error prone at the initial stage, and a certain degree of mismatch often occurs. Therefore, initial promiscuous synapses either are usually secured by help of additional and more specific adhesion molecules, or are eliminated. Like previous stages, this process also occurs in a hierarchical manner. The synaptogenic molecules neurexin and neuroligin could be good candidates for helping to achieve this specificity. Neurexins undergoes extensive splicing, and recently Boucard and colleagues demonstrated that they interact according to a splice code. However, several other cell adhesion molecules

are also postulated to play a role in late stages of synaptic development.

One of the best-characterized synaptic cell adhesion proteins is N-cadherin. Similar to neurexins, they link the extracellular adhesive function to actin cytoskeleton via α- and β-catenins. Even though the function of cadherins at the synapse is not clear, several recent experiments provide significant insights. Overexpression of an N-cadherin construct lacking the extracellular domain while maintaining the ability to bind cytosolic partners markedly reduced the number of presynaptic boutons, indicating the importance of the adhesive function. In another set of experiments, expression of mutant α-N-catenin prevented the interaction of cadherin with the actin cytoskeleton but did not strongly affect presynaptic assembly. These experiments imply that cadherins have a rather adhesive function during early synaptogenesis. Therefore, they could act at an intermediate stage between initial contact and final maturation by prolonging the brief lifetime of axo-dendritic contacts. Disruption of cadherin function in mature synapses, however, does not have a strong effect. This finding raises the possibility that some of the cadherin functions are redundant with protocadherins, a subset of the cadherin superfamily. Protocadherins are composed of nearly 60 members expressed by three gene clusters that are expressed in distinct patterns in the nervous system and undergo extensive splicing.

Molecular Components of the Presynaptic Active Zone and the Cytomatrix

Active zones are the principal sites of synaptic vesicle fusion in synapses. The molecular components of the active zone are thought to serve a structural role by clustering synaptic vesicles around the active zone and increasing proximity between molecules on the synaptic vesicle membrane and the plasma membrane. Active zone proteins are also involved in priming the vesicles for release and perhaps in vesicle retrieval after fusion. Proteins, such as Bassoon and Piccolo, are recruited to activate the synapses during synaptogenesis. For instance, in experiments conducted by Garner, Ziv, and colleagues, Bassoon was detected in nascent synapses capable of action-potential-dependent uptake and release of FM dyes. In addition, dense core active zone precursor vesicles contain multiple synaptic proteins, including the active zone proteins, Bassoon and Piccolo. Fusion of these vesicles with the plasma membrane can rapidly assemble active zone. Despite extensive data on their localization, the functional properties of these active zone proteins are still unclear. However, for RIM1 and CASK, there are several well-characterized biochemical interactions with multiple proteins. As discussed above, in the case of CASK, these molecular interactions suggest a central role in the bridging of neurexins to munc-18, a critical component of presynaptic release machinery. Recent mouse knockouts of munc-13 and RIM1 uncovered critical functional roles for these molecules. Synapses deficient in munc-13-1 are severely impaired in their function. The remaining munc-13-2-dependent synaptic transmission displays marked synaptic facilitation. RIM1 knockout mice, on the other hand, have a less severe but significantly altered properties of short- and long-term plasticity. Interestingly, loss of these molecules does not lead to structural alterations in the synapse, which are presumably due to the redundancy of molecular interactions that assemble synapse structure.

How do the active zone proteins regulate synaptic function? This regulation is likely achieved by the ability of active zone proteins to recruit the components of fusion machinery, such as SNAREs and munc-18. An important step in the chain of events leading to vesicle fusion is the formation of the SNARE core complex between target membrane SNARE proteins (i.e., syntaxin and SNAP-25) and the synaptic vesicle SNARE, synaptobrevin/VAMP. Active zone proteins can exert significant functional effects by regulating the formation and dissociation of SNARE complexes. Replenishment of vesicles released at the active zone requires SNARE core complex assembly and disassembly. This assembly process in the synapse is much faster than the rates of SNARE core complex assembly *in vitro*. Therefore, the assembly process is most likely facilitated by protein–protein interactions between the components of the presynaptic active zone and synaptic vesicles. For instance, munc-18, a protein required for fusion, could be recruited to the active zone through its interaction with Mint (munc-18 interacting protein), which in turn binds to CASK.

Functional Maturation of Presynaptic Terminals and the Role of Activity

Following the initial assembly of synaptic terminals, a large number of synapses are functionally silent. In some cases, these functionally silent synapses can be rendered operational in response to activity. The most commonly studied models of silent synapses propose a postsynaptic mechanism that underlies this silence. According to this model, a fully functional presynaptic terminal may exist but the postsynaptic site does not possess α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors although it contains *N*-methyl-D-aspartate (NMDA) receptors. Activity, in turn, induces the insertion of functional AMPA receptors, making silent synapses functional under physiological conditions. In contrast,

studies in dissociated hippocampal cultures have also identified a developmental stage where synapses are presynaptically silent after assembly. This model possesses the same apparent features of an NMDA-only synapse, but the NMDA-only nature of these synapses is explained by a presynaptic mechanism and not by lack of postsynaptic AMPA receptors. According to this scheme, activation of postsynaptic AMPA or NMDA receptors can be determined by the kinetics of fusion pore opening and the release profile of glutamate. In young nerve terminals, neurotransmitter release occurs through a narrow fusion pore leading to exclusive activation of NMDA receptors as they have a higher affinity for glutamate. Synapse maturation in turn leads to an increase in preponderance of full fusion events, thus activating NMDA as well as AMPA receptors. An alternative model suggests that immature synapses do not readily respond to action potential stimulation leading to a full presynaptic neurotransmitter failure due to some inadequacy in fusion competence or localization of synaptic vesicles. This model is consistent with the previously discussed findings on the gradual reorganization of synaptic vesicle clusters after synaptogenesis. In a recent study, Shumin Duan and colleagues showed that a burst of action potentials can rapidly awaken these silent synapses by increasing the availability of synaptic vesicles for fusion through BDNF-triggered presynaptic actin remodeling mediated by the small GTPase Cdc42.

In most synapses, the initial structural assembly and functional unsilencing is followed by a gradual maturation process that typically involves alterations in short-term plasticity. Studies conducted in acutely isolated brain slices have described functional alterations that are solely of presynaptic origin. One finding of these experiments was an apparent decrease in release probability during synaptic development. This result is rather surprising, given the prevalent structural observation that the number of vesicles within a synapse increases during maturation implying an increase in synaptic reliability and release. Another interesting observation in cortical as well as hippocampal mossy fiber synapses is target-dependent alterations in short-term and some long-term forms of plasticity during the course of development. Cellular mechanisms underlying these developmental changes in short-term plasticity are postulated to involve altered Ca^{2+} dependence of fusion and regulation of vesicle mobilization in presynaptic terminals. It is tempting to speculate that synaptic cell adhesion molecules either individually or in combination may regulate these target specific functional alterations in the output of single neurons.

As exemplified by the presynaptic unsilencing process discussed above, several aspects of synaptic functional maturation during early development can be influenced by activity and neuromodulators. Most neuronal networks exhibit spontaneous action potential firing patterns and synaptic potentials in the absence of extrinsic influences. The background activity that arises from the properties of individual neurons and their characteristic synaptic connections has been shown to be critical for the refinement of synaptic connectivity within the nervous system. Most of the signaling cascades that play a role in synapse maturation can be physiologically activated or regulated by the background activity. These include several signal transduction pathways, including Ca^{2+}-signaling mechanisms and the activation of protein kinase C (PKC) and protein kinase A (PKA). For example, direct involvement of cyclic adenosine monophosphate (cAMP)-dependent signaling in synaptic development was demonstrated in hippocampal slices, as well as at the level of individual synapses in culture. Activation of Ca^{2+}, cAMP, or diacylglycerol second messenger cascades can be triggered either directly by neuronal activity through Ca^{2+} influx or indirectly by the release of glutamate and activation of metabotropic glutamate receptors.

Recent studies have shown that chronic alterations in spontaneous activity levels modify several synaptic properties including the size of postsynaptic responses, probability of neurotransmitter release, as well as the number of synapses. These experiments strongly support a role for background activity in regulating the proper functional maturation of individual synapses. Although activity is an indispensable component of synaptic development, the mechanism through which it influences synapse maturation and the elimination process is still elusive.

See also: Presynaptic Development and Active Zones.

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Development of Drosophila Neuromuscular Junctions

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Introduction

The *Drosophila* neuromuscular junction (NMJ) ranks as one of the best-understood genetic model-systems for examining synaptic development and function. Most research involves the motor neurons and muscle fibers of the embryonic and larval body wall. The connections are precise and relatively invariant, simplifying the analysis of mutant phenotypes. Furthermore, the NMJ's peripheral location aids experimental access. Developing motor neurons may be observed *in vivo,* and electrophysiological studies are possible at all developmental stages.

Drosophila generates its motor systems twice, first as an embryo and then again as a pupa. The adult motor system is more complex than the larval one, with segment-specific specializations for flight, walking, and reproductive behavior. Although many useful insights have emerged from studies of the developing pupa, most current research is focused on the more accessible embryonic and larval synapses.

General Features of the Neuromuscular Junction

The *Drosophila* larval body-wall muscles generate the movements involved in locomotion and postural control. Most research has examined the first seven abdominal segments (A1–A7). These share the same muscle pattern of 30 muscle fibers on each side, with only a minor difference in segment A1 (**Figure 1(a)**). The terminal (A8) segment has a distinct muscle pattern, as do the three thoracic segments (T1–T3).

The major source of innervation for the hemisegment is the ganglionic peripheral nerve (PN), which emerges laterally from each central nervous system (CNS) segment. In segments A1–A7, the PN contains between 80 and 90 axons, of which fewer than half are efferent. The PN elongates throughout development, reaching 3 mm by the end of larval life. At the body wall the PN divides into five branches. All dorsal and most mid-body-wall muscle fibers are innervated by the intersegmental nerve branch (ISN), whereas the remaining mid-body-wall muscles are innervated by the dorsal branch of the segmental nerve (SN)a. All ventral longitudinal muscle fibers are innervated by the SNb branch (also referred to as the ISNb), whereas the remaining oblique and superficial ventral muscles are innervated by the SNc and SNd (or, alternatively, ISNd) branches. A minor source of innervation is provided by the transverse nerve (TN), which exits from the dorsal midline of each abdominal CNS segment to innervate ventral and mid-body-wall targets.

All motor neurons are glutamatergic. A subset also expresses synaptic cotransmitters, including the neuropeptides proctolin and leukokinin I, and the neuromodulatory transmitter octopamine. Unlike many invertebrates, there are no inhibitory GABAergic motor neurons. The NMJ also responds to neuromodulatory molecules that circulate in the hemolymph, such as the FMRFamide peptides.

The neuromuscular junction consists of multiple branches bearing variously sized synaptic boutons. The NMJs lie on the muscle fiber side facing the animal's interior (**Figure 2**). Individual boutons contain multiple neurotransmitter release sites, characterized by T-bar ribbons with synaptic vesicles and soluble *N*-ethylmaleimide-sensitive factor attached protein receptor (SNARE) proteins. Surrounding each release site is a periactive region where vesicle endocytosis occurs. By the end of larval life, each bouton may contain up to 40 release sites. The postsynaptic membrane opposite the bouton is enriched for glutamate receptors (GluRs), adhesion proteins, and ion channels. The proteins are anchored by multiple adaptor proteins, including the postsynaptic density (PSD-)95 homolog Discs large (Dlg) and the 4.1 superfamily protein Coracle. The postsynaptic site has a highly folded membrane known as the subsynaptic reticulum (SSR).

There are two postsynaptic ionotropic GluRs at the NMJ. These multimeric receptors are assembled from the five receptor subunits expressed by muscles. The two receptors differ by whether they contain the GluR-IIA or GluR-IIB subunit, but both have GluR-IIC (also referred to as dGluR-III), GluR-IID, and GluR-IIE subunits. The GluR-IIA and GluR-IIB subunits form the channel pore and confer the single-channel conductance, antagonist sensitivity, and desensitization characteristics. On the presynaptic side, there is also a single GluR-A metabotropic GluR (also referred to as DmGluR-A) that contributes to synaptic augmentation and facilitation.

The Motor Neurons and Their Projections

There are approximately 35 motor neurons that innervate each abdominal hemisegment. Of these, some 30 type Ib motor neurons synapse with either a single muscle fiber or muscle fiber pair. The Ib neurons provide direct control of individual muscle fibers during locomotion. At the NMJ, the Ib motor

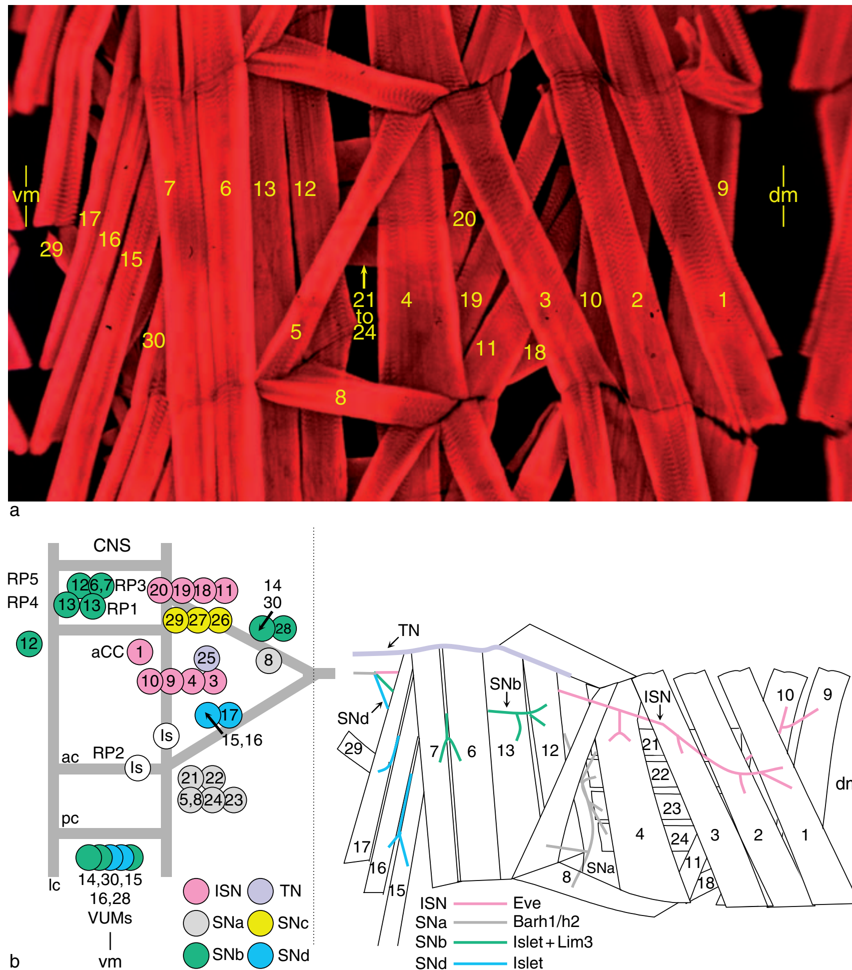

Figure 1 The motor neurons and muscle fibers of the abdominal body wall: (a) muscles of a single abdominal hemisegment displayed from the ventral midline (vm) to the dorsal midline (dm) (rhodamine phalloidin fluorescence; anterior at the top); (b) diagram of the central nervous system (CNS) (left) and muscle map showing the major nerve branches (right). In (a), each muscle fiber is designated by a number from 1 to 30 (several superficial muscle fibers are not visible in this dissection). In (b), left, the diagram shows the motor neurons that innervate a single abdominal hemisegment. The gray bars indicate the anterior commissures (ac), posterior commissures (pc), and the longitudinal connectives (lc). Each motor neuron's preferred muscle target is indicated by the number within the cell body. All the motor neurons shown are type Ib, except for two type Is cells. The color code indicates the nerve branch the axon takes. In (b), right, the muscle map is colored to indicate the transcription factor code that defines motor-neuron-targeting preference. ISN, intersegmental nerve; RP, radula protractor motor neuron; SN, segmental nerve; TN, transverse nerve; VUM, ventral unpaired median motor neuron. Panel (b, left) on Chiba A (1999) Early development of the Drosophila neuromuscular junction: a model for studying neuronal networks in development. *International Review of Neurobiology* 43: 1–24.

neurons have large boutons with diameters ranging from 2 to 6 μm (**Figure 2**). There are, in addition, two type Is motor neurons that function as common excitors. These cells each innervate large subsets of dorsal and ventral muscles, respectively, and have medium-sized 1–4 μm round boutons. Bouton diameters for both classes remain fairly constant throughout larval development. The size differences between Ib and Is motor neuron boutons may be due to differences in action potential firing rates. Type Ib boutons become smaller and resemble Is boutons when action potential firing rates are experimentally reduced using either Na^+ channel mutations or sublethal doses of tetrodotoxin (TTX) during development.

There are also two type II efferent neurons that express octopamine and are probably neuromodulatory. They each innervate multiple dorsal and ventral muscle fibers, respectively, with boutons smaller than 1 μm. Finally, a single type III motor neuron with oblong boutons innervates muscle fiber 12 in four abdominal segments. A typical muscle fiber receives three inputs: from a type Ib, a type Is, and a type II motor neuron.

Each type Ib motor neuron synapses with its target muscle fibers with high fidelity. The motor neuron RP1 innervates the ventral longitudinal muscle fiber 13 with only a 3% error rate. The preference of a motor neuron for a specific muscle fiber is retained

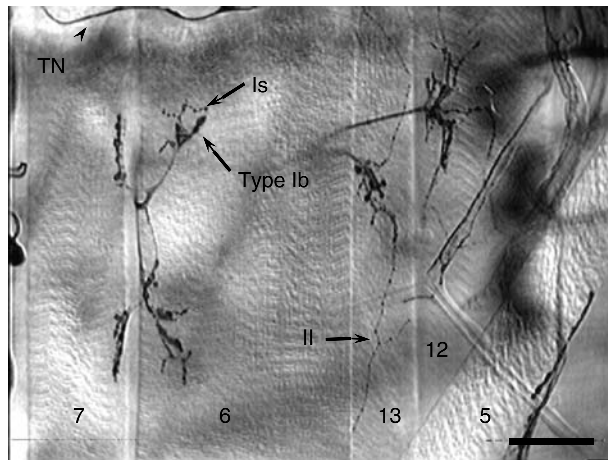

Figure 2 The neuromuscular junctions of the ventral longitudinal muscle fibers 7, 6, 13, and 12 of a third instar larva, visualized with neuron-specific labeling (anti-HRP). The three major motor neuron types (Ib, Is, and II) are distinguishable by their characteristic bouton morphology. Scale bar = 50 μm. From Chang TN and Keshishian H (1996) Laser ablation of Drosophila embryonic motoneurons causes ectopic innervation of target muscle fingers. *Journal of Neuroscience* 16: 5715–5726.

even when muscle patterning is altered. When muscle fiber 13 is duplicated, the RP1 motor neuron faithfully innervates both duplicated cells, whereas motor neurons to neighboring muscle fibers remain unaffected. This implies the presence in motor neurons of a cell-specific muscle-recognition mechanism.

Cellular Determination and Axon Guidance

Motor neuron growth cones make multiple guidance decisions during development. They must first take the correct neuropilar tracts to exit the CNS and then follow an appropriate nerve branch to either a dorsal or ventral muscle field. These choices require multiple receptors operating at specific times during development. The dorsal or ventral muscle field choice is governed by the combinatorial expression of several transcription factors, which, in turn, control the expression of specific receptors and signaling pathways. Significantly, some of the transcription factors involved in ventral muscle field preference are conserved evolutionarily between insects and mammals.

Factors Regulating Motor Neuronal Identity

Two transcription factors, Hb9 and Nkx6, contribute to ventral muscle targeting (**Figure 1(b)**). The proteins regulate the expression of at least two additional transcription factors, Islet and Lim3. Both Islet and Lim3 are expressed by motor neurons that take the SNb nerve branch to ventral longitudinal muscle fibers, whereas Islet alone is expressed by motor neurons projecting to the nearby ventral oblique muscle fibers innervated by the SNd branch. In *lim3* loss-of-function mutants, the SNb motor neurons behave like those of SNd. Conversely, overexpression of Lim3 in SNd motor neurons switches them to SNb targets. The homeodomain transcription factors Barh1/h2 play a similar essential role for SNa-projecting axons to the mid-body wall (**Figure 1(b)**).

Dorsally projecting motor neurons express the transcription factor Even skipped (Eve). Mutations of *eve* cause dorsal motor neurons to project to ventral regions. When Eve is expressed in ventral motor neurons, the neurons switch their identity and project to dorsal targets. Because Eve functions as a transcriptional repressor, it is probable that this guidance switch is due to the inhibition of factors required for ventral identity, such as Hb9 and Lim3.

The identity of the downstream effectors regulated by these transcription factors remains incompletely understood. For the ventrally projecting motor neurons, Islet and Lim3 positively regulate the expression of the Ig superfamily homophilic adhesion molecule Fasciclin III (FasIII). They also negatively regulate the IgSF protein beaten path (Beat1c), a molecule involved in axonal defasciculation at specific choice points along the body wall. Among the dorsal class of motor neurons, Eve indirectly regulates the expression of the Ig superfamily homophilic adhesion molecule Fasciclin II (FasII) and Unc5, one of the receptors for the Netrin chemotropic factors.

The Decision to Defasciculate

The next task faced by a motor neuron is to decide where to leave the axon fascicle. The decision to defasciculate depends on a balance between interaxonal adhesion and repulsion. All motor neuron axons express FasII, as well as the chemorepulsive molecule Semaphorin Ia (SemaIa) and its receptor PlexinA. Subsets of motor neuron axons also express additional cell adhesion molecules, such as Fasciclin I, FasIII, and Connectin (Con). Increased expression of FasII in motor neuron axons suppresses defasciculation, as does the reduction of either SemaIa or PlexinA. The *SemaIa* mutant phenotype can be suppressed by reducing levels of either FasII or Con. This is consistent with the idea that defasciculation is regulated by the relative levels of adhesion and repulsion between axons.

Defasciculation is also regulated by locally secreted molecules, such as Beaten path. *Beat* mutants fail to defasciculate, bypassing their muscle targets. Genetic interaction studies indicate that the Beat

protein tips the balance toward defasciculation by selectively interfering with FasII-mediated axonal adhesion, possibly by directly binding to the FasII ectodomain.

Additional insight into defasciculation has come from examining the neurally expressed receptor tyrosine phosphatases (RPTPs) dLAR, DPTP66D, and DPTP99A. In general, mutation of the RPTPs leads to a failure to defasciculate, although with mild phenotypic penetrance. The phenotype is more severe when multiple RPTPs are eliminated, suggesting redundancy or cooperative functions among the proteins. Although the identity of the ligands and intracellular targets of most RPTPs remains unresolved, dLAR is thought to interact with the extracellular heparin sulfate proteoglycans Syndecan and Dally-like. The intracellular targets of dLAR include the cytoplasmic tyrosine kinase Abelson (Abl), and its phosphorylation target Enabled (Ena). dLAR may contribute to a growth cone's chemotropic response by regulating actin cytoskeleton assembly.

Embryonic axons fail to defasciculate when muscle fibers are genetically eliminated from the body wall, suggesting that defasciculation also depends on muscle-derived cues. One candidate for a muscle-derived signal is Sidestep (*side*), a chemotropic ligand that is expressed by all muscle fibers. In *side* mutants, motor neurons bypass their muscle targets, but unlike *beat* mutants, the phenotype is not suppressed by reduced levels of FasII. This suggests that defasciculation depends on at least three mechanisms: (1) a local reduction in interaxonal adhesion, combined with (2) elevated interaxonal repulsion, and possibly (3) one or more chemotropic signals derived from the musculature.

Target Selection

As growth cones explore the body-wall musculature, they extend rapidly moving filopodia that sample cell surfaces. A filopodium may exist for as little as a few minutes and extend as far as two to three muscle fibers ahead of the growth cone. Time-lapse studies in *Drosophila* embryos show that the efferent growth cones direct themselves toward their destined targets by following trajectories pioneered by specific filopodial contacts. The earliest contacts between the motor neuron growth cone and the target muscle fiber occur at muscle protrusions known as myopodia. These processes resemble neuronal filopodia and increase the probability of interactions between the growth cone and muscle fiber. The myopodia may also present key cell-surface molecules to the motor neuron, promoting target recognition.

Molecular Recognition of Synaptic Targets

The molecular recognition of specific muscles by motor neurons involves both attractive and repulsive signaling (**Figure 3**). Some key molecules are expressed by all or large subsets of motor neurons and muscles, whereas others have narrower, cell-specific expression patterns. The molecules include secreted as well as cell-surface proteins. During embryonic synaptogenesis, all muscle fibers and motor neurons express FasII. In addition, all muscle fibers secrete the chemorepellant protein Semaphorin IIa (SemaIIa), and all motor neurons in turn express PlexinB, the SemaIIa receptor. Gain- and loss-of-function tests indicate that the

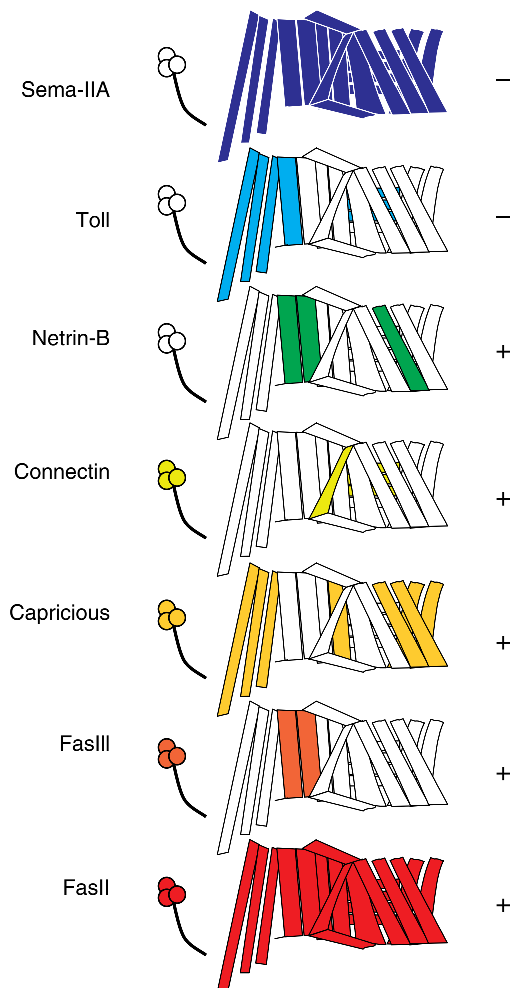

Figure 3 Multiple attractive (+) and chemorepulsive (–) molecules are expressed by individual muscle fibers and motor neurons at the time of innervation. A motor neuron's affinity for a specific muscle target depends on balancing multiple attractive and repulsive molecular cues. From Nicholson N and Keshishian H (2006). Metamorphosis and the formation of the adult musculature. In: Sink H (ed.) *Muscle Development in Drosophila,* pp. 113–120. New York: Springer.

affinity of motor neurons for muscle fibers is adjusted in part by balancing the relative levels of FasII, SemaIIa, and/or PlexinB. Although these broadly expressed proteins cannot define specific target preferences, their balance modulates the stability of neuromuscular contacts as synaptogenesis proceeds.

Specific targeting decisions depend on the expression of molecules within subsets of motor neurons and/or muscles (**Figure 3**). For example, the IgCAM FasIII is expressed by both motor neuron RP3 and its two synaptic targets, the muscle fibers 7 and 6. In loss-of-function *FasIII* mutants, RP3 shows low-frequency but statistically significant targeting errors. This indicates that other guidance molecules are available to guide RP3 to its targets with some accuracy. However, RP3 can be misdirected when adjacent muscles are made to express FasIII. Significantly, the ectopic expression does not degrade or alter the guidance of other motor neurons, indicating that muscle-expressed FasIII is a specific recognition cue for the RP3 motor neurons. Results similar to these have been obtained for several other proteins involved in target selection, including Connectin and Capricious. Probably every muscle fiber has a distinct and characteristic profile of molecules involved in recognition and targeting. For example, muscle fibers 7 and 6 express FasII, FasIII, NetrinB, Toll, and SemaIIa, whereas the nearby ventral longitudinal muscle fiber 12 expresses FasII, SemaIIa, and Capricious (**Figure 3**).

A combinatorial model involving attractive versus repulsive signaling has been tested by analyzing the phenotypes of *netrinB* (*netB*), *FasII*, and *SemaIIa* mutant embryos. In *netB* mutants, the RP3 motor neuron fails to reliably innervate muscle fibers 7 and 6, suggesting a role for NetrinB in guiding the growth cone to those muscles. The guidance defect is largely suppressed when S*emaIIa* mutations are combined with *netB*, suggesting that NetrinB-mediated attraction is in balance with repulsive signaling by SemaIIa. Because adjacent nontarget muscle fibers do not express NetrinB, this favors RP3's innervation of its NetrinB-positive target muscle fibers over neighboring ones. Similar scenarios exist for motor neurons projecting throughout the body wall, involving a balance between attractive and repulsive signals to define specific muscle targets against a generally repulsive nontarget background.

Synaptic Development and the Role of Activity

Although relatively little is known about how synaptogenesis is initiated, mutations of genes that delay this step have been informative. Tetraspanins are evolutionarily conserved proteins with widespread functions in cell signaling and motility. Three tetraspanin genes are transiently expressed by motor neurons as synapses form. In mutants of the tetraspanin gene Late bloomer, growth cones correctly identify and contact their target muscle fibers, but then delay synaptogenesis for hours. The phenotype is enhanced when all three tetraspanins are deleted, suggesting that the proteins function in a cooperative fashion to trigger synapse formation. How this happens remains unknown.

The transformation of an embryonic growth cone to an anatomically recognizable presynaptic terminal takes only 3–4 h. On the presynaptic side, the immature contact forms multiple round varicosities that prefigure synaptic boutons. Electron microscopy (EM) studies show that postsynaptic electron-dense regions arise exclusively at the contacts made between the growth cone and its preferred muscle fiber.

Early Roles for Activity at the Neuromuscular Junction

Both the localization of postsynaptic receptors and the refinement of synaptic connections depend on neural activity. Small glutamatergic potentials can be recorded from muscle as soon as the motor neuron growth cone arrives on site. GluRs are initially expressed throughout the muscle fiber. Following growth cone contact, the receptors accumulate at the site of the developing synapse. Although it is not known whether this early localization is due to the trapping of existing receptors (as occurs at larval synapses), the localization to the synapse is disrupted when motor neuron activity is suppressed.

From the time motor neurons first contact the musculature to the time of hatching, the embryo executes 1–2 strong peristaltic contractions/min. The contractions are driven by motor neuron action potentials activating NMJs throughout the body wall. The significance of this periodic, wavelike motor activity has been tested by suppressing motor neuron electrical activity. Normally, motor neurons withdraw contacts made with nontarget muscle fibers during the first few hours of synaptic development. The loss of evoked NMJ activity results in a failure to withdraw these incorrectly placed contacts throughout the body wall. The ectopic contacts are removed only if activity is restored during an early critical period that ends soon after the embryo hatches. The remaining contacts mature into functional but miswired NMJs. The mechanisms governing activity-dependent refinement remain largely unknown.

The Growth of the Neuromuscular Junction

After hatching, the larval NMJ undergoes dramatic growth. In hatchling larvae, the NMJs on muscle fibers 7 and 6 have fewer than 20 synaptic boutons, with each bouton averaging only two release sites. By the end of larval development 100 h later, the muscle fiber surface area has increased 100-fold and the NMJ contains 80–100 boutons, with up to 40 release sites per bouton.

Neurotransmission is also modified as the NMJ grows. Spontaneous miniature currents are initially small and infrequent, but become steadily larger and more regular as the embryo develops. By the time of hatching, these quantal events are skewed toward larger sizes. Quantal size (the postsynaptic response to a single vesicle) thereafter remains constant as the larval NMJ grows. The amplitude of the evoked excitatory junctional potential (EJP) also remains constant despite a fall in membrane resistance as the muscle grows. By contrast, quantal content (the average number of vesicles released per action potential) steadily increases as the NMJ enlarges. This is due to the large increase in the number of release sites at the NMJ and probably from increases in release efficiency.

In vivo imaging of NMJ development indicates that more than half of new boutons are added by intercalation within existing strings of boutons. In the remaining cases, the boutons are added at branch tips. The new boutons have the same density of vesicles, active zones, and T-bar ribbons as mature boutons and precede the development of postsynaptic specializations and SSR folding. Synaptic boutons are also eliminated from the NMJ. Because the SSR develops only after boutons have formed, isolated SSRs that lack boutons probably indicate elimination events. Nearly 18% of muscle fiber 7 and 6 NMJs in late first and early second instar larvae show these tell-tale bouton retraction footprints, compared to 5% in the third instar. Whereas bouton formation can occur anywhere along an NMJ branch, elimination is usually restricted to branch termini.

Time-lapse studies of green fluorescent protein (GFP)-tagged GluRs show that larval GluRs first appear in the muscle membrane as extrasynaptic hot spots. Fluorescence recovery studies of tagged GluR-IIA indicate that the extrasynaptic receptors are preferentially recruited into the expanding PSD that develops opposite the presynaptic boutons. After they enter the PSD, the receptors become immobilized, presumably by binding to adaptor proteins, with only a 20% turnover every 24 h.

The periactive zone surrounding the release site is enriched for the adhesion molecule FasII, a protein that plays an important role in NMJ growth. Newly budded boutons have lower levels of FasII than parental boutons. This is an intriguing observation because mutations that reduce FasII levels generally have enlarged NMJs. The actions of FasII on NMJ expansion depend in part on presynaptic signaling via the amyloid precursor protein (APPL) and through interactions with the PSD-95, Dlg and ZO1 (PDZ)-containing adaptor protein dX11/Mint/Lin-10. The periactive zone is also enriched for Bruchpilot (also referred to as NC82), a coiled-coiled domain protein with homology to the human active zone protein ELKS/CAST. Bruchpilot has a key role in defining the boundaries of the active zone and in the clustering of Ca^{2+} channels.

The Roles of Activity in Neuromuscular Junction Growth and Function

The NMJ's physical size is correlated to the level of neuromuscular activity. Mutations that increase membrane excitability, as well as treatments that increase locomotor activity, result in larger and more highly branched NMJs. These NMJs have up to twice as many boutons as controls and also have larger EJPs and more frequent spontaneous activity.

In genetically hyperactive larvae, there is a substantial reduction of the cell-adhesion molecule FasII at the NMJ. Hypomorphic *FasII* mutations, which directly reduce FasII to the levels observed in hyperactive larvae, have similarly enlarged NMJs. This implies that the activity-dependent downregulation of FasII is sufficient to account for the NMJ expansion seen with neuromuscular hyperactivity. Significantly, the expected NMJ expansion of hyperactive larvae is effectively blocked when FasII levels are held constant.

Cyclic adenosine monophosphate (cAMP) levels also influence NMJ growth. *dunce* mutants have elevated levels of cAMP and significantly expanded NMJs. When *dunce* is combined with hyperactivity mutations such as *Shaker,* there is a strong synergizing effect that further promotes NMJ expansion. Conversely, mutations that reduce cAMP levels suppress activity-dependent NMJ growth. Significantly, FasII levels at the NMJ are reduced in *dunce* mutants, and the overgrowth caused by increased cAMP levels is blocked when FasII levels are held constant. Thus, FasII is a common endpoint in multiple regulatory cascades affecting NMJ expansion.

Activity-dependent NMJ growth is also dependent on transcriptional regulation. The expression of the cAMP responsive element-binding protein (CREB) transcriptional activator enhances the NMJ expansion phenotype of FasII mutants, whereas the expression of

a negative repressor of CREB blocks the increase in presynaptic neurotransmission seen in *dunce* mutants. This indicates that presynaptic transmitter release and NMJ structural plasticity may be regulated in different ways. This idea is supported by the actions of AP-1, an immediate-early transcription factor composed of a Fos and Jun heterodimer. AP-1 functions through CREB to regulate NMJ growth but independently, via Jun kinase, to modulate synaptic function.

Activity influences the expression and localization of GluRs by increasing local subsynaptic translation during larval development. The NMJ expansion associated with elevated motor neuron activity is partially blocked by mutations that decrease the number of available receptors or suppress their translation. This indicates that receptor availability limits the formation of new boutons, which is supported by the finding that reducing GluRIIs to relatively low levels leads to smaller NMJs.

Finally, functional plasticity at the NMJ can arise soon after short bouts of hyperactivity; 40 min of vigorous locomotion is sufficient to cause long-term facilitation of evoked potentials at the NMJ. This is due to an increase in neurotransmitter release, surprisingly through the recruitment of physically larger vesicles to the release sites, resulting in increased quantal size. The effect depends on presynaptic protein kinase A activity and is independent of postsynaptic receptor activation. Similarly, when postsynaptic sensitivity is acutely decreased using GluR toxins, there is a rapid compensatory elevation of quantal release, possibly through the modulation of presynaptic Ca^{2+}-channel activity. The relationships between these acute forms of physiological plasticity and long-term growth changes at the NMJ are not yet known.

Transsynaptic Signals Involved in Neuromuscular Junction Development

Retrograde signaling, from muscle to motor neuron, plays several important roles during NMJ development. For example, GluR-II mutations lead to a compensatory increase in the number of release sites, elevating quantal release. This homeostatic mechanism holds the amplitude of postsynaptic potentials constant despite changes in muscle receptor sensitivity. Similarly, when muscle depolarization is suppressed by experimentally elevating the potassium leak, the motor neuron responds by increasing transmitter release. Elevating CREB transcriptional activity in muscle, achieved by expressing the CREB co-activator dCBP, also results in a retrograde inhibitory signal. The motor neuron responds by reducing both evoked neurotransmitter release and short-term facilitation.

The best-understood retrograde signaling system regulating NMJ growth involves the transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) ligand Glass bottom boat (Gbb). Gbb is expressed in body wall muscle fibers and regulates presynaptic development through both canonical and noncanonical pathways (**Figure 4**). In the canonical mode, Gbb binds to a presynaptic TGF-β receptor complex containing the type II BMP receptor Wishful thinking (Wit) and at least one of two type I BMP receptors. These kinases stimulate transcription by activating the R-Smad transcription factor Mad and its co-Smad Medea. Disrupting any of these results in smaller NMJs with reduced neurotransmission. Presynaptic TGF-β/BMP signaling also depends on proteins in the early and late endosomal compartments, as well as on retrograde axonal transport, suggesting

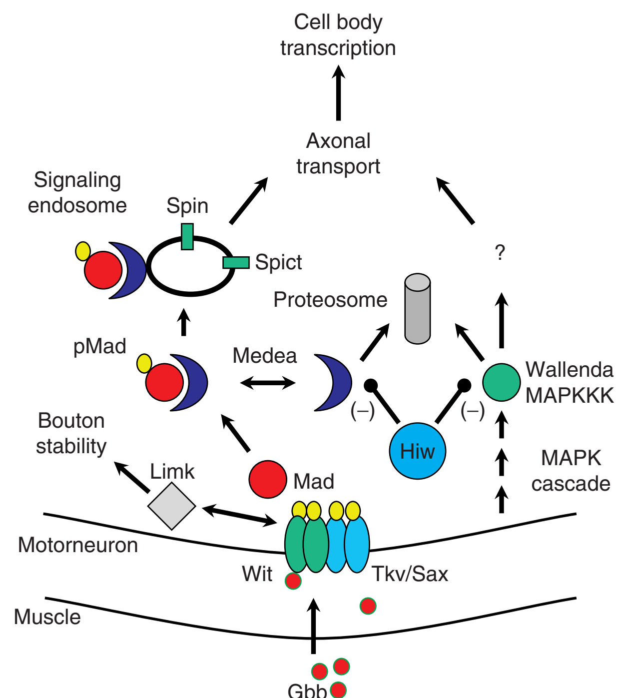

Figure 4 Retrograde signaling at the *Drosophila* NMJ. The TGF-β/BMP growth factor Gbb is expressed by muscle and is required for normal NMJ growth and bouton formation. The ligand stimulates a motor-neuronal receptor complex composed of the type II BMP receptor Wit and the type I receptors Tkv and/or Sax. The activated complex phosphorylates the R-Smad transcription factor Mad. Phospho-Mad associates with the co-Smad Medea and stimulates transcription at the cell body. Regulation is, in part, by ubiquitination of Medea via the E3 ligase (Hiw) and by regulation within the signaling endosome. Hiw also negatively regulates a MAPK cascade that promotes bouton addition through its action on the MAPKKK Wallenda. Gbb binding also stimulates a noncanonical signaling pathway involving Limk, leading to the stabilization of new boutons. BMP, bone morphogenetic protein; MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase kinase; NMJ, neuromuscular junction; TGF, transforming growth factor.

the physical transport of one or more proteins from the NMJ to the cell body.

The mechanisms that regulate Gbb release from muscle remain largely unknown. Inhibition of calmodulin-dependent protein kinase (CaMK)II in the muscle fiber stimulates a retrograde signal (possibly Gbb) that depends on the Wit receptor to increase the number of T-bar ribbons and the quantal content of motor neurons. Similarly, postsynaptic Dystrophin regulates a retrograde signal that influences presynaptic short-term facilitation, again through the activation of Wit in the motor neuron.

Finally, there is evidence that Wit can activate noncanonical signaling pathways independent of the Smads. For example, the C-terminus of Wit binds directly to presynaptic Lim kinase (Limk), leading to the stabilization of newly formed boutons. The loss of function mutation of *limk* results in a substantial expansion of the NMJ. Surprisingly, the increased number of NMJ boutons in *limk* mutants does not result in increased EJP amplitudes, as is the case when NMJs expand following elevated neuromuscular activity.

Mutations affecting Highwire *(Hiw)*, a large multifunctional protein with E3 ubiquitin ligase and scaffolding activities, reveal something of the complexity of presynaptic signaling during NMJ growth. Mutant larvae have significantly more boutons than normal, suggesting that Hiw functions to inhibit bouton addition. Hiw negatively regulates at least two signaling systems. It inhibits the TGF-β/BMP cascade involving Wit through a direct interaction with the transcriptional cofactor Medea, and it attenuates a mitogen-activated protein kinase (MAPK) cascade involving the MAPK kinase kinase (MAPKKK) protein Wallenda, the MAPK c-Jun N-terminal kinase (JNK), and the transcription factor Fos (**Figure 4**).

NMJ development also requires anterograde signaling from motor neuron to muscle. The Wnt family morphogen Wingless (Wg) is secreted by motor neurons, and its receptor is located on both pre- and postsynaptic membranes. In *wg* mutants, bouton formation is suppressed. The boutons have unbundled microtubules, as revealed by labeling the microtubule binding protein Futsch. In addition, the terminal NMJ branches do not elongate, resulting in less separation between boutons. *wg* larvae also have extensive postsynaptic phenotypes, including aberrant expression of the GluR-IIA, the Dlg adaptor protein, and overall reduced development of the SSR. Significantly, similar phenotypes are observed when a dominant negative Wg receptor is expressed in the body-wall muscles. These results suggest that Wg may function as both an anterograde as well as an autocrine signal to regulate NMJ growth and development.

Concluding Observations

Several areas of NMJ formation and maturation remain especially promising for further study. These include: (1) to decipher the combinatorial transcription factor codes that define motor neuronal identity, which control the receptors and signaling cascades involved in axon guidance; (2) to define the molecular signals that trigger synaptogenesis, transforming a growth cone into a synapse; and (3) to determine how multiple transsynaptic signals are integrated to control synaptic growth and function on time scales ranging from minutes to days. Finally, we need to acquire a deeper understanding of the motor system from a functional standpoint, including the roles that cotransmitters and circulating substances play in modulating motor output. Fortunately, the molecular-genetic and physiological tools are available to illuminate these areas of neuromuscular development and function in a highly accessible system of synapses.

See also: Neuromuscular Junction (NMJ): Mammalian Development; Neuromuscular Junction: Neuronal Regulation of Gene Transcription at the Vertebrate; Schwann Cells and Plasticity of the Neuromuscular Junction.

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Neuromuscular Junction (NMJ): Mammalian Development

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Introduction

The neuromuscular junction (NMJ) is an example of a highly specialized, asymmetric, cell-cell junction. The speed with which fast chemical transmission occurs requires that the specializations of the postsynaptic muscle cell surface that allow it to respond to the transmitter released from the nerve are less than a micrometer from the presynaptic release sites. An important question concerning the development of the NMJ is therefore, what are the mechanisms that account for the alignment and proximity of the distinctive specializations of its pre- and postsynaptic components? A second important question concerns the specificity of the mature nerve-muscle contacts. What part does the process of NMJ formation play in achieving functional matching of individual motor neurons to the muscle fibers they innervate?

This article gives an overview of the way mammalian NMJs develop. Most of the information has come from rats and mice, in which the most detailed studies of NMJ formation in vertebrates have been made. The article starts with the origin of motor neurons and skeletal muscle fibers and then considers how the NMJ itself is formed.

Development of Motor Neurons

Birth

Motor neurons are among the earliest nerve cells to be born, that is, to complete their final round of DNA synthesis. Soon after cell birth, the motor neurons begin to extend an axon that leaves the spinal cord in the nascent ventral roots. In mammals, the motor neurons that innervate an individual muscle are usually grouped into a longitudinally oriented column that extends over two to three spinal segments.

Axon Outgrowth and Motor Neuron Identity

The immature motor axons leave the spinal cord even before their target muscles have formed. As they grow, the axons select paths that lead to the muscles they are destined to innervate. The ability of an immature motor neuron to make such decisions indicates that it has some knowledge of its identity, and that different motor neurons therefore have different identities. In adults, motor neurons that innervate slowly contracting nonfatigable muscle fibers tend to be rich in oxidative enzymes that can be visualized by appropriate histochemical techniques. Well before the first NMJs are formed, embryonic motor neurons already differ in their oxidative enzyme profiles. This supports the idea that the motor neurons that innervate a single muscle differ in their properties and that those differences arise before any interaction with the muscle occurs.

Once contact with the appropriate premuscle mass has been established, but not before, the axons branch extensively. In rats and mice, functional contacts with newly formed limb muscles are first present around embryonic day 14 (E14), a week before birth. A similar stage occurs in humans at about week 9 of gestation.

Release of Acetylcholine from Growth Cones

The terminals of cultured motor neurons can release acetylcholine (ACh), the chemical transmitter at neuromuscular junctions, even before they make contact with muscle. This suggests that motor neurons *in vivo* also synthesize ACh and have the necessary specializations for its activity-dependent release at an early stage of their development.

Development of Skeletal Muscle Fibers

Myotube Formation

Most vertebrate skeletal muscle fibers arise during development from the fusion of many mononucleated postmitotic myoblasts. These spindle-shaped cells line up and fuse to form the primitive myotubes. As the myotubes grow and mature into muscle fibers, they incorporate additional myoblasts. In adult muscle fibers, there is typically one nucleus for every 10 μm of length. Thus, a single fiber in a large human muscle, such as *vastus lateralis*, which has fibers up to 20 cm long, has up to 20 000 nuclei.

Kinetics of Primary and Secondary Myotube Formation

In mammalian muscles, myotube formation occurs in two phases. The first involves the formation of an initial cohort of relatively few primary myotubes. As the muscle elongates, further myoblasts line up along the primary myotubes and eventually fuse to form secondary myotubes. These form initially roughly midway between the ends of the primary myotubes and then grow rapidly in length as they add new myoblasts. The two populations of myotubes, and the mature muscle fibers that form from them, have

somewhat different functional properties. The great majority of fibers in the adult muscles are derived from secondary myotubes.

Origin of Muscle Fiber Types

Most adult mammalian muscles contain fibers of different functional types. Some are specialized for relatively slow, sustained contraction whereas others are specialized for fast, high-powered contractions. At birth, the muscle fibers in rats and mice contract uniformly slowly. Differentiation into faster and slower contracting fibers begins soon after birth and is well established 2–3 weeks later. Most of the muscle fibers that develop from primary myotubes end up as slow in the adult, while secondary myotubes give rise to both fast and slow fibers. These distinctive properties arise from the pattern of genes expressed by the fibers. Most of the nuclei in each fiber express the same set of genes, raising the as yet unanswered question of how that homogeneity of gene expression comes about.

Early Appearance of Delocalized Postsynaptic Properties

Even before they are innervated, immature muscle fibers begin to express the receptor proteins that allow them to respond to ACh (acetylcholine receptors, or AChRs). AChRs of the fetal form, containing $\alpha_2\beta\delta\gamma$ subunits, are initially present over the whole fiber surface at a density of about $500\,\mu m^{-2}$. Other molecules that play roles in AChR localization and gene expression, such as rapsyn and muscle specific kinase (MuSK; see 'AChR aggregation' below), have a similar pattern of expression. As the muscle fibers become increasingly active, their activity suppresses the expression of all these proteins away from the NMJ.

Early Development of the NMJ

Initial Nerve–Muscle Encounters

Muscle fibers become innervated very soon after they first form. In rats and mice, signs of functional innervation can be detected within a day or two of the earliest myotube formation at about E12–14, depending on the muscle. The early nerve-muscle contacts lack many of the structural features of mature NMJs but are characterized by a high density of AChRs in the muscle fiber membrane. Whether the AChR clusters form before or after nerve contact is a matter of continuing debate (see below). Indeed, there is evidence that NMJs in different muscles in the same species form by different sequences of events.

Polyaxonal Innervation

A distinctive feature of the early motor innervation of vertebrates is that several motor neurons initially innervate each muscle fiber. This innervation occurs at a single postsynaptic site that is thus contacted by the terminal axons of several motor neurons (**Figure 1**). Myotubes cultured *in vitro* can acquire polyneuronal innervation, but such multiple inputs are normally distributed over the myotube surface rather than focused on a single site. The forces that initially restrict the immature nerve terminals to this single site *in vivo* are not known.

AChR Accumulation

A high density of AChRs is a hallmark of the postsynaptic membrane of the vertebrate NMJ. A distinct cluster of AChRs, detectable after labeling with fluorescent conjugates of the snake toxin α-bungarotoxin, is present from a very early stage of NMJ formation (**Figure 2**). Within the immature cluster, AChRs are often gathered into microclusters less than 1 μm across. The mean density of AChRs within the plaque

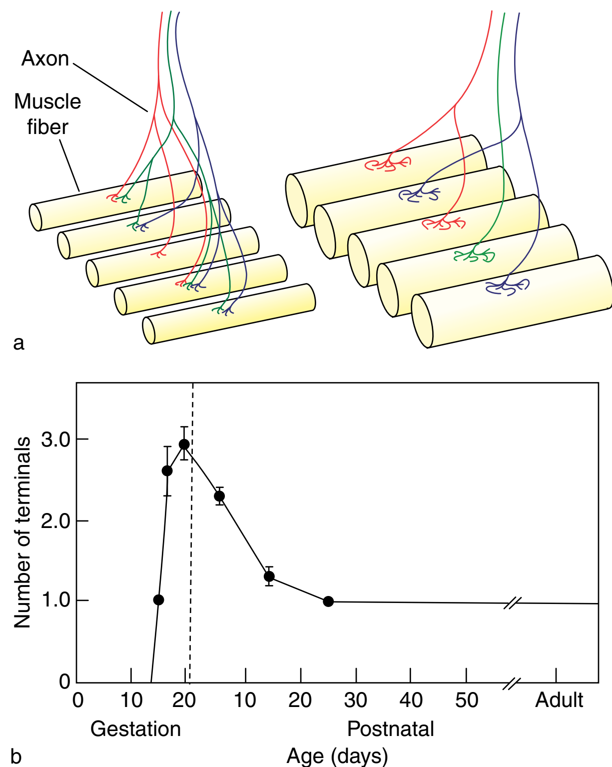

Figure 1 Development of muscle innervation. (a) Comparison of polyneuronal innervation of muscles in newborn rats or mice (left) with the adult state (right). (b) Changes in the number of axon terminals innervating neuromuscular junctions in rat diaphragm with age. (b) Reproduced from Bennett MR and Pettigrew AG (1974) The formation of synapses in striated muscle during development. *Journal of Physiology* 241: 515–545, with permission from Blackwell publishing.

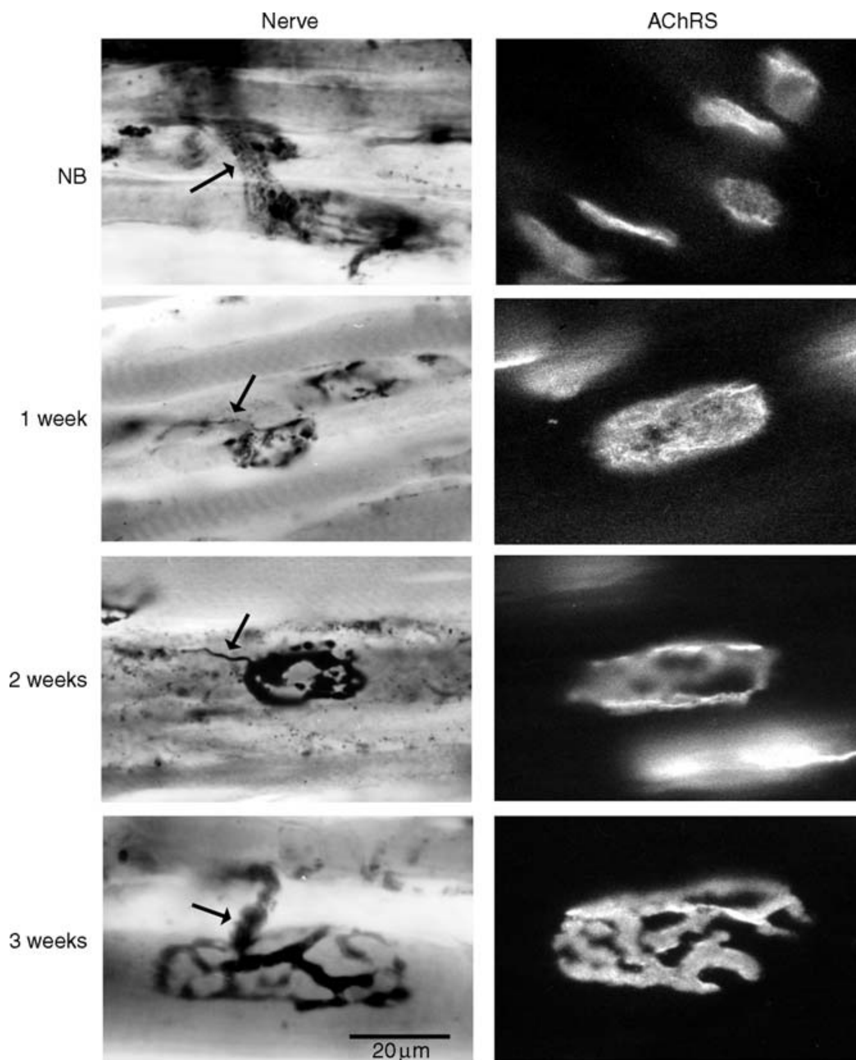

Figure 2 Structural maturation of mouse neuromuscular junctions (NMJs). Nerve terminals in postnatal mouse extensor digitorum longus muscles were labeled with zinc iodide-osmium, and acetylcholine receptors (AChRs) were labeled with R-α-bungarotoxin. Arrows point to the preterminal motor axon(s). Several axons are present at birth, but between 1 and 2 weeks after birth, all but one are withdrawn. The surviving axon becomes myelinated 2–3 weeks after birth. As the axon terminal enlarges, the initially uniform granular distribution of AChRs breaks up, leaving areas with low AChR density that come to match the branches of the nerve terminals. NB, newborn.

is about 3000 μm^{-2}. How the AChR cluster forms initially and is subsequently maintained are topics of extensive current research. There appear to be at least two components of this process: the aggregation of AChRs within the membrane of the muscle fiber and the enhanced expression of the genes encoding the AChR subunits by the myonuclei closest to the site of nerve contact.

AChR aggregation AChRs can exist in a form that is mobile in the plane of the cell membrane. In the case of cultured immature frog muscle precursor cells, such mobile AChRs aggregate at sites of contact with motor axons, suggesting that the nerve induces aggregation of AChRs in the muscle cell. Much evidence suggests that it is a protein, agrin, which is made by and released from motor axons, that triggers AChR aggregation. Agrin function is associated with its activation of a muscle specific kinase (MuSK), which triggers downstream signaling cascades in the muscle. A second key player in the process of agrin-induced AChR aggregation is the 43 kDa protein rapsyn. Rapsyn links AChRs to the plasma membrane and to components of the membrane skeleton. In the absence of rapsyn, no aggregation of AChRs occurs.

There is also much evidence that AChRs can self-aggregate, and on suitable substrates they can form clusters similar to those at mature NMJs. Some recent

studies have shown that in some species and muscles, AChR clusters form in the region of presumptive NMJs before nerve-muscle contacts are made. In these cases, neuromuscular contacts may form selectively on the presumptive postsynaptic sites. On balance, it seems likely that while the muscle fiber surface has an inherent tendency to form AChR clusters, agrin released from the nerve can either trigger clustering *de novo* or stabilize preexisting clusters.

Upregulation of postsynaptic gene expression The second process that contributes to AChR accumulation at the mature NMJ is the enhanced expression of the genes that encode the AChR subunits. This process is discussed below.

Within a few days of nerve contact, acetylcholinesterase (AChE), the enzyme that terminates ACh-action, accumulates in the synaptic basal lamina that forms between the nerve and muscle cells. As for AChRs, this accumulation is associated with upregulation of the genes encoding AChE.

Schwann Cells: The Motor Neurons' Companion

Throughout most of their life, motor axons are closely accompanied by Schwann cells. There is increasing evidence of a mutual dependency of motor axons and their companion Schwann cells during NMJ development. Thus, if the motor axons are cut at birth, the Schwann cells associated with their degenerating distal portion die. This can be prevented by addition of neuregulin, a growth factor normally produced by the motor neuron. Consistent with a role for neuregulin in promoting Schwann cell survival is a reduction of Schwann cell numbers in mice in which neuregulin expression is reduced by genetic manipulation.

NMJ Maturation: Presynaptic

At birth, both the pre- and postsynaptic components of the NMJ of rats and mice are immature in structure, function, and molecular makeup. Nonetheless, neuromuscular transmission is adequate for the simple movements of the neonate. The adult form of the NMJ arises in concert with increasing motor activity during the next 3–4 weeks as the result of a program of coordinated events affecting the presynaptic nerve terminal and the postsynaptic surface of the muscle fiber.

Myelination of the Axon

In rats and mice, myelination of the peripheral nerves begins a day or two after birth. Within a further week or so, the extramuscular parts of the nerves are well myelinated. By contrast, although the fine intramuscular branches leading to individual muscle fibers remain in close contact with Schwann cells, they remain unmyelinated until the elimination of supernumerary innervation is complete.

Synapse Elimination

During the first 2 weeks or so after birth, all but one of the axons that initially innervate each muscle fiber withdraws, leaving a sole survivor (**Figure 1**). A similar process occurs at most of the vertebrate NMJs that have been investigated. In humans, synapse elimination is complete by about 14 weeks of gestation. This important process has been extensively investigated, both at the NMJ and in the CNS.

A very different pattern of muscle innervation occurs in the muscle of fish, some muscles in other lower vertebrates, and in a few muscles of mammals. In these muscles, the mature muscle fibers are innervated at multiple sites by numerous axons. Although the development of the innervation pattern in these multiply innervated muscles has not been studied in detail, it is clear that the immature nerve–muscle contacts are not restricted to a single site and that the local competition that occurs in most mammalian muscles does not occur. A common feature of these muscle fibers is that instead of generating action potentials, the contraction is triggered by the summed effects of the local depolarizations at the multiple sites of innervation.

Maturation of the Nerve Terminal

Once synapse elimination is complete, the terminal of the sole surviving motor axon expands significantly. This involves a broadening of the regions of contact with the muscle and an overall increase in the area of synaptic contact (**Figure 2(a)**). As the nerve terminal expands, it retains its close contact with the terminal Schwann cells, which increase in number as a result of continuing cell divisions.

The factors that limit or determine the size of the mature terminal are poorly understood. Zones of postsynaptic specialization can be induced in a variety of experimental conditions and may adopt a size and appearance remarkably similar to those at mature NMJs. This suggests that factors within the muscle play an important role in determining NMJ size and its well-established correlation with muscle fiber caliber. This view has found recent support from the discovery that a class of mutations of the gene encoding Dok-7, a postsynaptic protein that regulates MuSK activity, causes a reduction in human NMJ size without significant alteration in muscle fiber size or the local density of AChRs. It remains to be seen whether neuromuscular synaptic size–strength homoeostasis in vertebrates is regulated by mechanisms

similar to those recently shown to operate in *Drosophila* larvae.

Transmitter release While growing motor axons may be able to release ACh in response to depolarization (see above), evidence from *in vitro* studies indicates that contact of the nerve terminal with muscle cells greatly increases the efficacy of quantal release. The structural basis of this enhanced release is unclear since active zones, the sites of nerve-induced quantal release at mature NMJs, have not been described until the NMJs acquire their mature form.

Even at immature NMJs, stimulation of any of the several axons innervating each muscle fiber is often adequate to trigger muscle contraction. However, the quantal content (the number of ACh quanta released by a single nerve impulse) is very low, initially about 5% of the adult value. During the 2–3 week period of synapse elimination, quantal release increases at some of the terminals at each NMJ while it declines at others. In general, greater quantal release is associated with a greater chance of a terminal's surviving the competitive process. Since transmitter release correlates strongly with synaptic size, and it is the smallest synapses that are ultimately eliminated, this association is perhaps not surprising. After synapse elimination is complete, the quantal content of the sole surviving input increases progressively, reaching its adult value at about 2 months of age. Part of this increase reflects the increasing size of the nerve terminal. In addition, there is an increase in the quantal release per unit area of membrane. This may be related to the maturation of the population of active zones.

Ca^{2+} Channel Switch

The changes in the effectiveness of quantal release are accompanied by a change in the type of calcium channels that trigger release. At adult mammalian NMJs, release is mediated by P/Q-type channels, as can be shown using selective blockers. In contrast, at the NMJs of newborn mice, both P/Q-type and N-type channels contribute to release. The contribution of the N-type channels is lost within 1–2 weeks after birth.

NMJ Maturation: Postsynaptic

Maturation of the nerve terminal is paralleled by numerous changes in the postsynaptic region of the muscle fiber that contribute to an increase in the speed and efficacy of neuromuscular transmission. This occurs in the context of a substantial increase in muscle fiber diameter. Immature muscle fibers typically have a diameter of 8–10 μm and relatively high electrical input resistance. As a result, relatively little ACh-induced current is required to depolarize them to the action potential threshold. This helps ensure effective neuromuscular transmission even when ACh output from the nerve is still low. As the muscle grows, the diameter of the fibers also grows, and their input resistance falls. A variety of changes in the postsynaptic region help to ensure and enhance the efficacy of transmission in spite of this.

Remodeling of the Postsynaptic Zone

As synapse elimination proceeds, important changes take place in the distribution of key synaptic molecules and in the conformation of the postsynaptic membrane.

Redistribution of AChRs and AChE At birth, the AChRs and AChE occupy more or less uniformly the oval plaque that defines the NMJ. With time, holes in this plaque appear where the density of postsynaptic molecules is relatively low. As synapse elimination is completed and the one surviving terminal begins to expand, the regions with a high density of postsynaptic molecules become closely associated with the nerve terminals, mirroring their pretzel-like shape (**Figure 2(b)**). In the case of AChRs, this redistribution is associated with a substantial increase in the local density, rising to about $10\,000\,\mu m^{-2}$. A number of molecules associated with the AChRs, including rapsyn, utrophin, and syntrophin, undergo a similar redistribution. This suggests that there are close links between them and that these molecules may help to stabilize the AChR cluster by cross-linking to the cytoskeleton.

Appearance of voltage-gated sodium channels channels and postsynaptic folds At the mature NMJ, the postsynaptic membrane is highly folded, with the folds extending into the muscle fiber. The voltage-gated sodium channels that account for the action potential, of a type termed Na_V1, are highly concentrated in the depths of these folds and in a perijunctional zone a few micrometers wide (**Figure 3(a)**). During development in rats, these channels are first detectable around the time of birth, about a week after the AChRs. At this time, the Na_V1 channels are present in highest density in a diffuse band some $200–300\,\mu m^2$ wide, centered on the AChR cluster but extending well beyond it and lacking its distinct boundary (**Figure 3(b)**). Thus, the two types of ion channel that are central to the postsynaptic response to the nerve have very different developmental patterns of expression.

Formation of the folds The postsynaptic folds begin to develop soon after birth (**Figure 4**). The factors that

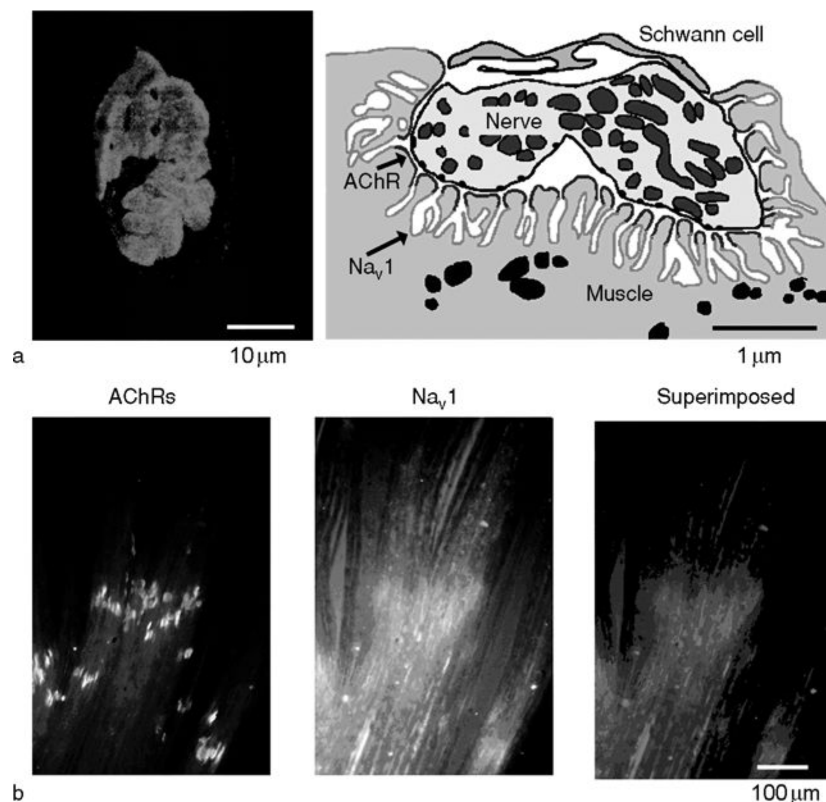

Figure 3 Voltage-gated sodium channels (Na_V1) at the mammalian neuromuscular junction (NMJ). (a) Adult rat. Na_V1s, shown in red, occupy the depths of the folded postsynaptic membrane and in the light microscope (left panel) appear as a red fringe surrounding the acetylcholine receptors (AChRs), shown in green. (b) Newborn rat. AChRs are concentrated in discrete spots at the immature NMJs while the Na_V1s, which are first detected at this age, have a much more diffuse distribution. (a) Reproduced from Slater CR (2003) Structural determinants of the reliability of synaptic transmission at the vertebrate neuromuscular junction. *Journal of Neurocytology* 32: 505–522, with the permission from Springer.

control their distribution and growth are poorly understood. It has been suggested that the opening of the fold represents a site of reduced nerve-muscle adhesion, possibly related to the presence of the active zones in the nerve terminal. However, folds also form in a variety of situations in which AChRs form clusters in the absence of the nerve. These include both denervated and regenerating muscle and muscle exposed to exogenously applied agrin, where AChR clusters associated with folds form at sites away from the NMJ.

From an early stage in fold formation during normal development, while AChRs are concentrated at the crests of the folds, nearest the nerve, the Na_V1 channels occupy the depths of the folds. This is likely to result from a high concentration of Na_V1 channels in the new membrane added during the process of fold formation combined with a barrier to diffusion of Na_V1 channels into the region of high AChR density.

Molecular Differentiation of the Postsynaptic Zone

The remodeling of the postsynaptic region is accompanied by changes in the patterns of isoforms of the ion channels expressed within it. These come about as a result of the combined effects of increasing muscle activity and signaling molecules released from the nerve on gene transcription in the subsynaptic myonuclei.

Changes in AChR expression After birth, the density of AChRs away from the NMJ declines so that in the adult it is generally undetectable. This is the result of suppression of AChR subunit gene transcription by muscle activity, mediated by the binding of myogenic regulatory factors to an E-box sequence in the genes of several AChR subunits. It raises a central question: How is it that AChRs remain at the NMJ itself? In brief, the answer is that agrin released from the nerve and bound to the synaptic basal lamina, through its activation of MuSK, induces activity-resistant transcription of the genes encoding both AChR subunits and a number of other postsynaptic proteins, by the myonuclei in the vicinity of the NMJ (see below).

In addition to the activity-mediated suppression of AChR expression away from the NMJ, there is a change in the type of AChRs expressed at the NMJ

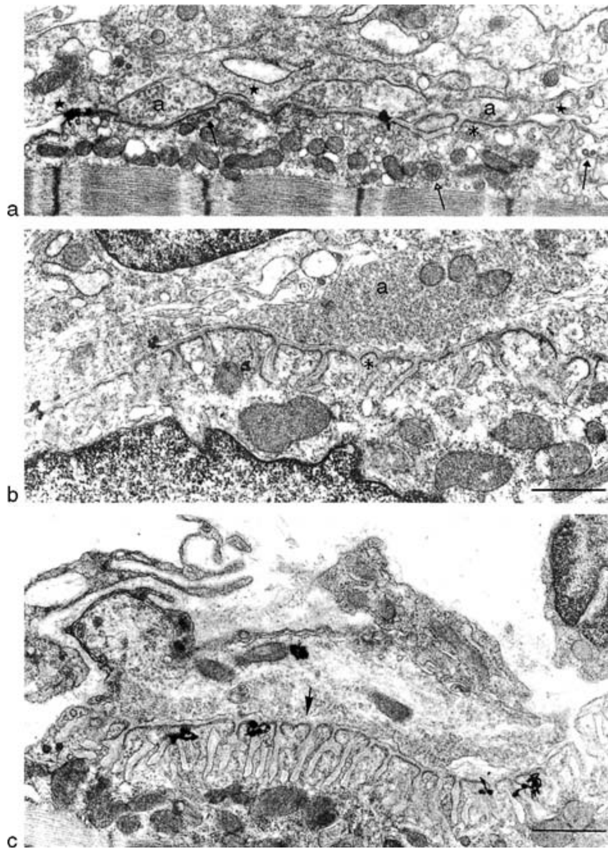

Figure 4 Ultrastructural maturation of mouse neuromuscular junctions (NMJs); development of postsynaptic folds at NMJs in postnatal mouse extensor digitorum longus muscles. (a) In the newborn, few folds are present. Solid arrows, subjunctional cytoplasm rich in coated vesicle and coated pits; open arrows, multivesicular bodies. (b) At 2 weeks, folds are present but less well developed than in adult. (c) In the adult, folds are well formed and closely packed. Solid arrow, junctional folds. a, axon terminal; star, Schwann cell cytoplasm; asterisk, thickened postsynaptic membranes, reflecting accumulation of AChRs and associated protiens. Scale bars = 1 μm. Reproduced from Matthews-Bellinger JA and Salpeter MM (1983) Fine structural distribution of acetylcholine receptors at developing mouse neuromuscular junctions. *Journal of Neuroscience* 3: 644–657. Copyright 1983 by the Society for Neuroscience, with permission.

itself. At birth the predominant fetal form of the AChR has a subunit composition of $\alpha_2\beta\delta\gamma$. During the first 2 weeks after birth, the γ subunit is replaced by an ε subunit to produce the adult form. Expression of the ε subunit is not suppressed by muscle activity. The two forms of AChR differ in their channel properties: The immature form has a longer mean open time (typically 7–8 ms) and a lower conductance (40–50 pS) than the adult form (1–2 ms; 50–60 pS). As a result, a single opening of average duration of a fetal AChR channel allows 3–4 times as much charge to enter the cell as an adult channel. This makes the fetal channels more efficient at converting bound ACh to charge entry. However, the slower kinetics of the currents mediated by fetal AChRs mean that immature NMJs are poorly suited to transmit the high-frequency repetitive activity that occurs in the mature animal. It is thus relevant that the changes in AChR properties occur at about the same time as the speeding up of the firing patterns of the motor neuron (see below) and the myelination of the most distal axons that allows those patterns to reach the NMJ.

Changes in Na_V1 expression An analogous change in isoform expression occurs for the Na_V1 channels. The first Na_V1 channels to appear are of an immature form, designated $Na_V1.5$. These differ from the adult form ($Na_V1.4$) in that they open at more-negative membrane potentials. As a result, less depolarization is required to trigger an action potential in an immature muscle than in a mature one. Expression of the gene encoding $Na_V1.5$, like that of the γ-AChR subunit gene, is suppressed by muscle activity, both at the NMJ and away from it. As a result, expression of $Na_V1.5$ declines to an undetectable level during the first 2–3 weeks after birth. Expression of the gene encoding $Na_V1.4$ is not sensitive to activity. Its expression first becomes detectable at birth and increases during the next few weeks, both away from the NMJ and at a higher level at the NMJ. The factors that control the onset of $Na_V1.4$ expression and its enhancement at the NMJ are not known.

mRNA accumulation The developmental increases in the concentration of AChR and Na_V1 at the NMJ are accompanied by localized increases in the levels of the mRNAs that encode them. At the mature NMJ, there is an increase in the concentration of mRNA encoding a number of critical postsynaptic proteins, including AChR subunits, AChE, and $Na_V1.4$, as well as supporting proteins including rapsyn and utrophin. These increases are apparent soon after birth and are probably induced by agrin acting via MuSK.

Accumulation of Myonuclei

The upregulation of expression at the NMJ of the genes encoding important components of the postsynaptic membrane is now believed to be induced by agrin acting via MuSK. At the NMJ, agrin is fixed in place by association with the synaptic basal lamina. This ensures that the effects of agrin are confined to the vicinity of the NMJ. The effective sphere of influence of this immobilized agrin is not more than about 100 μm, so myonuclei further than this from the NMJ do not have a synaptic profile of gene expression. At many NMJs, there is an accumulation of 5–10 myonuclei within this sphere of influence.

This amplifies the effect of the agrin–MuSK signaling system. The clustering of myonuclei begins soon after birth and is complete by about 1 month of age. The events leading to myonuclear accumulation are not yet understood.

Mammalian Motor Unit Maturation

The formation and maturation of the mammalian NMJ occurs in the context of the development of the motor unit as a whole. The maturation of the motor neuron, and in particular its pattern of firing, has an effect on the development of the NMJ itself. In turn, the increasing ability of the NMJ to transmit faithfully the patterns of activity arising in the motor neuron to the muscle fibers plays an important part in the acquisition of mature muscle properties. It may also lead to retrograde regulation of motor neuron properties.

Firing Patterns

In adult mammals, motor neurons vary considerably in their functional properties. Most notably, some fire action potentials in long trains at a frequency of 10–20 Hz while others fire in short bursts of 5–10 action potentials at frequencies of up to 100 Hz. Motor neurons in neonatal rats and mice fire at a uniformly low frequency and are often synchronized as a result of electrical coupling by gap junctions. The first evidence of faster, more adultlike firing patterns, and of differences between the firing patterns of different motor neurons, is seen about 2 weeks after birth, by which time electrical coupling is lost. The onset of these more adult firing properties coincides with the time of myelination of the most distal intramuscular nerve branches. It is likely that before this happens, these small branches would be unable to fire at high frequencies and could therefore not transmit the features of activity that distinguish different motor neurons in the adult.

Efficacy of Neuromuscular Transmission

During the early stage of polyaxonal innervation, more than one input to the muscle fiber is often capable of triggering contraction. As the competitive process unfolds, some inputs, and ultimately only one, come to have a much stronger impact on the muscle fiber than others. There is evidence that each branch of a motor axon competes locally at a given NMJ and that axons with the strongest input to a given NMJ are more likely to survive the competitive process than weaker ones. However, the evidence on this point is not clear cut, and neither of these findings fully explains the final outcome of the competition, in which a single motor axon, with appropriately matched properties, innervates each muscle fiber.

As the NMJ matures, both the pre- and postsynaptic components increase in size. For the muscle fiber, this results in a decrease in input resistance and therefore the need for a greater synaptic current to reach the action potential threshold reliably. This is achieved by the parallel increase in the size of the motor nerve terminal and with it, the quantal content. This, together with the folding of the postsynaptic membrane, results in the great reliability of neuromuscular transmission in the adult. The nature of the feedback mechanisms that regulate synaptic size and strength is intriguing, however, because one hallmark of the normal adult neuromuscular junction is a three- to fivefold excess of transmitter release over that required to trigger an action potential in the muscle fiber. This high safety factor ensures that every action potential entering a motor nerve terminal will normally trigger an action potential in all the muscle fiber it innervates.

Development of Muscle Fiber Homogeneity

An important consequence of the process of synapse elimination is that each motor neuron ends up innervating muscle fibers with very similar properties. Clear signs of increasing functional homogeneity of the muscle fibers within motor units are seen in mice 1–2 weeks after birth, as synapse elimination nears completion but before the distinctive patterns of activity of different motor units are well developed. It therefore seems unlikely that differences in activity patterns between motor neurons play a decisive role either in selecting which input survives at a given NMJ or in matching the properties of motor neurons to the muscle fibers they innervate. A possible alternative is that the matching of nerve and muscle cells is achieved by a molecular recognition system that involves activity-dependent expression of surface and/or diffusible molecules that interact so that during the process of synapse elimination, the most compatible nerve-muscle pairs survive at each developing NMJ. Such a mechanism could depend on activity as a driving force without the pattern of activity determining the specific outcome of the competition. As yet, however, no likely candidates for such a molecular recognition scheme have been identified.

It is also not yet clear how the properties of a mature muscle fiber are determined. There is good evidence for at least two very different, though not mutually exclusive, mechanisms. On the one hand, there is evidence that myoblasts are predetermined to make fast and slow muscle fibers even before myotube formation occurs. If correct, this mean that myoblasts of the same predisposition may fuse more or less selectively to make myotubes containing nuclei with intrinsically similar properties. On the other

hand, there is very strong evidence, particularly in mammals, that the properties of adult muscle fibers are sensitive to the patterns of activity they experience. This is consistent with the idea that the properties of a muscle fiber are modified after innervation by the pattern of activity of the motor neuron that innervates it. Both schemes raise important questions about how the muscle fibers innervated by a single motor neuron come to have the same properties.

Conclusions

The many changes in the structural, functional, and molecular properties of the NMJ that occur during its maturation adapt it for reliable high-frequency activation of mature muscle fibers. These changes are matched to complementary changes in the nerve and the muscle. Their overall effect is the conversion of an immature system that is good at generating slow muscle contractions in response to low-frequency activity in the nerve to a much faster system, adapted to the needs of a freely moving and increasingly independent animal.

The events that give rise to the mature NMJ are part of a coherent developmental program that defines the patterns of expression of a number of proteins, such as ion channels, that play central roles in neuromuscular transmission. In addition, it determines the size and conformation of the NMJ. Both aspects of the program have important consequences for the efficacy and reliability of the mature NMJ.

See also: Neuromuscular Junction: Neuronal Regulation of Gene Transcription at the Vertebrate; Neuromuscular Junction (NMJ): Postsynaptic Basal Lamina; Neuromuscular Junction: Synapse Elimination; Schwann Cells and Plasticity of the Neuromuscular Junction.

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Neuromuscular Junction (NMJ): Postsynaptic Basal Lamina

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Introduction

The primary function of skeletal muscle is to produce force. This force is controlled by the motor neuron that contacts the cell. This cell-to-cell contact was best described by Ramón y Cajal as a 'protoplasmic kiss.' The synapse between the motor neuron and the skeletal muscle cell has many names, including motor endplate, neuromuscular synapse, and neuromuscular junction. We will refer to this synapse as the neuromuscular junction. There are three cellular components of the neuromuscular junction – the skeletal muscle cell, the motor neuron terminal (NT), and the terminal Schwann cell (SC) (**Figure 1**).

Surrounding the muscle cell is an extracellular matrix. The extracellular matrix also covers the Schwann cell. The focus of this article is the specialized extracellular matrix that is found between the nerve terminal and the skeletal muscle cell; this specialized extracellular matrix is called the synaptic basal lamina. The synaptic basal lamina contains many molecules that are commonly found in all basal laminae throughout the organism, but it also contains many molecules that are unique to the neuromuscular junction.

The extracellular matrix is a vital and regulated extension of the cell. The fact that the extracellular matrix is part of the cell is easily understood by the fact that the matrix attaches to a number of membrane-bound structures. These membrane-bound structures in turn attach to the cytoskeleton of the cell. The matrix thus provides mechanical support for the cell. This is particularly important when considering the fact that the muscle fiber is twitching and moving large distances. The motor neuron is very well attached to the surface of the muscle cell that it is innervating. For example, in **Figure 1** the motor nerve terminal innervates the muscle cell seen at the bottom of the image. On top of the motor neuron sits the terminal Schwann cell. On top of the Schwann cell is another muscle cell that is regulated by another motor neuron. Thus, the two muscle cells in this image will twitch and move at different times. The extracellular matrix keeps the motor neuron precisely aligned with the muscle cell it innervates.

The precision at which the extracellular matrix aligns the motor neuron and muscle cell is astounding. The active zone is the site on the motor nerve terminal, where the synaptic vesicles fuse with the cell membrane to release the contents of the synaptic vesicle. This active zone is also the site where the voltage-gated calcium channels are concentrated in the membrane of the nerve terminal. The active zones are found precisely opposite the junctional folds in the skeletal muscle cell. The junctional folds are the invaginations in the membrane of the muscle cell. The acetylcholine receptors (AChRs) are concentrated on the crests of the junctional folds, immediately adjacent to the active zones from which ACh is released. The molecules in the synaptic basal lamina lie between these two membranes, and undoubtedly are intimately involved in both the mechanical linking of the two cells, and the cell-to-cell signaling that is essential for maintaining the structure and function of this synapse.

To provide a basis for understanding the synaptic basal lamina, in the following sections we first discuss the molecules that are found in all basal lamina. An important point to emphasize is that in a functional sense, the synaptic basal lamina extends across the nerve and muscle cell membranes to make contact with the cytoskeletons of both cells, thus accounting for the strong mechanical coupling between them. After considering all the extracellular molecules, we therefore discuss the multimolecular complexes that are found in the cell membrane and how they, in turn, are attached to the intracellular cytoskeleton. Finally, we discuss how the synaptic basal lamina is altered by the activities of proteases that cleave the matrix components. Altogether, these features reveal that the synaptic basal lamina is a dynamic cellular component that plays a critical role in the structure and function of the synapse.

Synaptic Basal Lamina Components

Collagen IV

The type IV collagens are critical components of basal laminae throughout the organism, and particularly of the synaptic basal lamina. There are features of collagen IV that are common to all basal laminae, and also specializations that are unique to the synaptic basal lamina.

The α chains of collagen IV have a long collagenous tail that has a glycine at every third amino acid. Characteristic of other collagen proteins there is a non-collagenous domain at the C-terminus; this region is called the NC1 domain. There are six genes that code for collagen IV α chains (*COL4A1* to *COL4A6*). These α chains can form either homo- or heterotrimers.

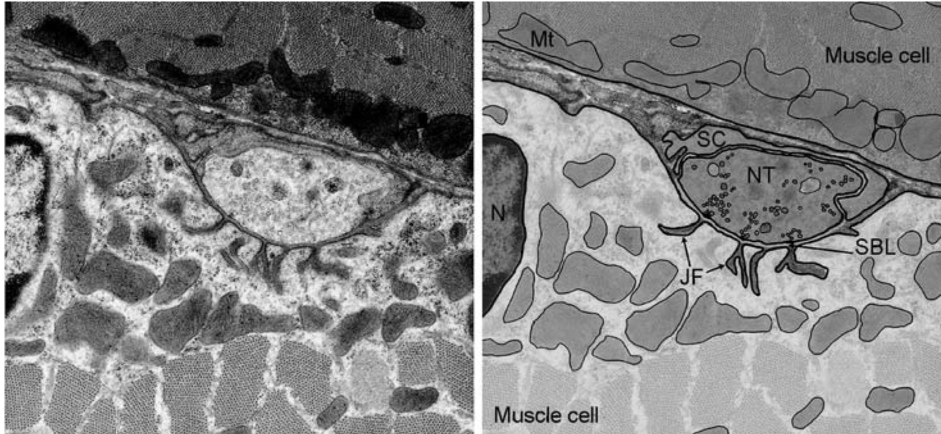

Figure 1 Electron micrograph of a neuromuscular junction from a mouse diaphragm. The left panel is an electron micrograph of a normal mouse neuromuscular junction. The right panel is the same image with the structures colorized and labeled. There are two muscle cells (red and light red). The muscle cell on the bottom is the cell that is innervated by the nerve terminal (green; NT). There is a separate muscle cell that sits on top of the nerve terminal; on top of the nerve terminal is a Schwann cell (yellow; SC). Also seen are the subsynaptic nucleus of the muscle cell (dark red; N) and the mitochondria (blue) in the muscles and in the nerve terminal. Also within the nerve terminal are clusters of synaptic vesicles (dark green). These synaptic vesicles are focused on the active zones (dark black). The active zones are found opposite the mouths of the junctional folds (JF). The synaptic basal lamina (SBL; red) is found in the area between the nerve terminal and the muscle cell on the bottom.

There could therefore be a large number of different combinations, but the only identified groupings are (two $\alpha 1$ and one $\alpha 2$); ($\alpha 3$, α, $\alpha 5$), and (two $\alpha 5$ and one $\alpha 6$). $\alpha 1$ and $\alpha 2$ are found everywhere in the basal laminae, but $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ are concentrated at the synaptic basal lamina.

The type IV collagens form a meshwork that is critical to the structure of the basal lamina. Three individual collagen IV α chains will combine to form a flexible trimer. The trimer is organized such that the NC1 domains are bundled together at one end, and the N-terminal 7S domains are bundled at the other end. These collagen IV trimers can further associate into larger structures. The N-terminal 7S domains from four trimers will bind to form an X-shaped structure, with the NC1 domains pointed outward (**Figure 2**). The NC1 domains on collagen IV trimers can then bind head to head with the NC1 domains on a separate collagen IV trimer. The resulting structure is a well-organized and regular meshwork of collagen IV.

The type IV collagens can also associate with other components of the synaptic basal lamina, and with proteins on the surface of the muscle, nerve terminal, and Schwann cell. In the synaptic basal lamina, the most common binding partners are the nidogens, which link the collagen IV meshwork to the laminins. On the cell surfaces, the NC1 domains can interact with integrins, proteoglycans, and the dystroglycan complex. Matrix metalloproteinases can cleave the type IV collagen, and this cleavage results in the release of the NC1 domains. The soluble NC1 domains are known to have potent effects on vascular formation, but the role of the NC1 domain at the neuromuscular junction is still largely unknown.

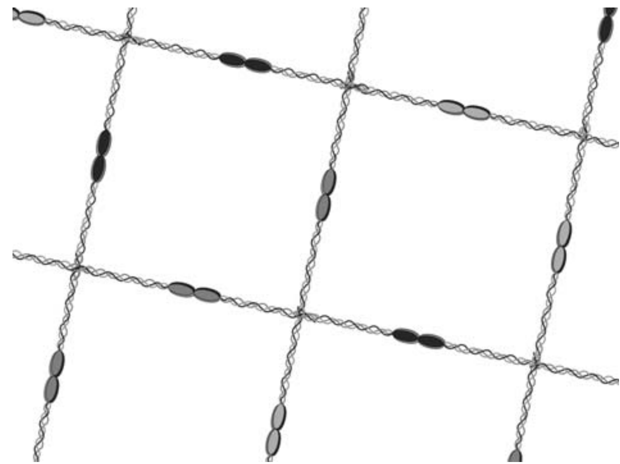

Figure 2 Highly schematic diagram of the type IV collagen meshwork in the basal lamina. Each collagen IV protein has an NC1 domain that is depicted as an oval, and a long collagenous tail. Three subunits assemble into a trimer, with the NC1 domains grouped together, and the collagenous tails intertwined. The NC1 domains from two trimers will interact head to head, and the 7S domains from four trimers will interact to form an X-shaped structure. The individual collagen IV proteins are depicted as either red, green, or blue. This diagram is overly simplified to show a single sheet of the collagen IV matrix.

Laminins

Laminins are heterotrimeric glycoprotein structures. Each laminin is composed of one α subunit, one β subunit, and one γ subunit. There are five genes

that code for α subunits, four genes that code for β subunits, and three genes that code for γ subunits. Of the myriad of possible combinations, currently there are 15 identified laminins. The laminins are important components of basal laminae throughout the organism, and play a special role at the neuromuscular junction.

The skeletal muscle cell produces the laminins found surrounding the skeletal muscle cell in the extrajunctional regions, and also the laminins found in the synaptic cleft. There are strong differences in the distribution of the various laminin subunits, revealing that the release of the laminins by the muscle cell is controlled. The subunits produced by the skeletal muscle cell are predominantly the $\alpha2$, $\alpha4$, or $\alpha5$, plus the $\beta1$, $\beta2$, and $\gamma1$ subunits. Of particular importance to the neuromuscular junction is the $\beta2$ subunit. The $\beta2$-containing laminins are highly concentrated at the synaptic cleft. Since the skeletal muscle cell produces the laminins, the production and release of the $\beta2$ subunit must be tightly controlled. The mechanism is still unknown. The $\beta2$-containing laminins do play an important role in the structure and function of the synapse. Mice that lack the $\beta2$ subunit have defects in synaptic release. The mice die at an early age due to a combination of problems associated with the loss of synaptic function, and also the loss of kidney function. One of the main features observed is the infiltration of the Schwann cell into the synaptic cleft. Thus one of the cellular mechanisms performed by the $\beta2$ subunit may be to stop the Schwann cell from wrapping the nerve terminal. In addition, the $\beta2$ laminin null mutants have disrupted active zones. Thus, the $\beta2$ subunit plays an important role in both the structure and function of the synapse.

The distribution of the α subunit is also controlled by the skeletal muscle cell. The $\alpha2$ subunit is predominantly found in the extrasynaptic basal lamina. The $\alpha5$ subunit is found concentrated at the neuromuscular junction, and it extends into the junctional folds. The $\alpha4$ subunit is found concentrated in a small, specialized region of the synaptic basal lamina. Of particular interest are recent findings that the $\alpha4$ subunit will bind to the voltage-gated calcium channels on the nerve terminal. The distribution and binding of the $\alpha4$ subunit reveal that this laminin chain is likely to play an important role in the communication between the muscle and the nerve terminal.

Nidogens (Entactins)

There are two nidogens in mammalian species. Nidogen-1 is a 150-kDa glycoprotein. It is a common component of all basal laminae, where it acts as a link by binding to both type IV collagens and laminin. Nidogen-2 is found in the basal lamina of skeletal muscle and its distribution mirrors that of nidogen-1. Nidogen-2 will bind to perlecan and collagen IV. Nidogen-2 also weakly binds to laminin-1. Interestingly, mice deficient in nidogen-1 have an apparently normal phenotype, and nidogen-2 is upregulated in the skeletal muscles of these animals. At the neuromuscular junction there is a uniquely glycosylated form of entactin. The size (150 kDa) and the activity of this uniquely glycosylated form of entactin are consistent with its identification as nidogen-1. There is also recent evidence revealing that nidogen can play a role in central nervous system (CNS) synaptic release, thus it is likely that the nidogens play a role at the neuromuscular junction.

Fibronectin

Fibronectin is a large glycoprotein that plays an important role in linking integrins to the extracellular matrix. Fibronectin will bind to a large number of matrix components, and substrates. The important binding partners in the synaptic basal lamina are the collagens and heparan sulfate proteoglycans (predominantly agrin). Fibronectin will then bind to the integrins on the surface of either the muscle cell or the nerve terminal. Fibronectin binding has been shown to alter synaptic release from the motor nerve terminal in culture, and to influence AChR aggregation in cultured myotubes. This activity has been shown to be dependent upon the activation of protein kinases in the cells. Thus, fibronectin acts as a mechanical link between matrix proteins in the extracellular matrix. It also acts as a mechanical link between the matrix and the cell cytoskeleton via the integrin. The binding to integrin also serves as a cell-signaling ligand that increases protein kinase activity. The protein kinase activity has been shown to work predominantly through protein kinases A and C, but it is likely that other protein kinases are also implicated in this signaling.

Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans (HSPGs) are a family of glycoproteins that share unique features. The molecules contain a protein core with large carbohydrate chains. The carbohydrate chains are sensitive to heparitinase. HSPGs are negatively charged. In their native forms the HSPGs run in bands as an extremely large smear (>200 kDa) when separated by apparent molecular mass in polyacrylamide gels. After heparitinase treatment the core protein is much smaller in size. HSPGs are found in all basal laminae and also associated with the cell surfaces. The three

major HSPGs found in skeletal muscle extracellular matrix are perlecan, agrin, and collagen XVIII.

Perlecan Perlecan has a widespread distribution. It is found in all basal laminae, and plays an important role in the storage and release of growth factors. There are three glycosaminoglycan (GAG) side chains extending form the N-terminal region.

Collagen XVIII Collagen XVIII has been recently shown to be a HSPG and to be present in the basal lamina of skeletal muscle. Loss of collagen XVIII in *Caenorhabditis elegans* has been shown to disrupt neuromuscular junction formation. There are no reports showing that collagen XVIII is concentrated at the vertebrate neuromuscular junction, so it remains unclear whether it has a function there.

Agrin Agrin is the most extensively studied HSPG at the neuromuscular junction, and it plays a pivotal role in the structure and function of the neuromuscular junction. Early experiments performed by UJ McMahan and colleagues were instrumental in revealing the fact that cell-signaling molecules were found in the extracellular matrix. When muscle fibers were damaged, in a way that left the extracellular matrix intact, the damaged portions of the muscle cells would be removed. In addition, the nerve terminal could be removed, leaving behind an empty basal lamina sheath. When the muscle cell regenerated by the proliferation and fusion of skeletal muscle satellite cells, the skeletal muscle would reform within the basal lamina sheath. The regenerated muscle cells would aggregate AChRs on their surfaces precisely at the spot that they contacted the previous synaptic site on the basal lamina. These experiments clearly showed that molecules stably bound to the synaptic basal lamina could direct the aggregation of AChRs on the surface of the muscle cell. Subsequent experiments revealed that the protein agrin could direct the aggregation of AChRs in culture. Mice that lack agrin do not develop neuromuscular junctions, and these mice die at birth.

Agrin is a multifunctional component of the synaptic basal lamina. The C-terminal region of agrin has been most extensively studied, since this is the region that is sufficient to induce the aggregation of AChRs. The number of other molecules that interact with agrin at the neuromuscular junction is extensive. The N-terminal region of agrin has been shown to bind to laminin. Agrin will also bind to α-dystroglycan and thus helps to link the dystroglycan complex to the extracellular matrix. Agrin will also bind to integrins, and to neural cell adhesion molecules (N-CAMs) on the surface of the motor neuron and muscle cell. In addition to these interactions, agrin will also bind to nidogen and collagen. Agrin clearly plays an important role in the structure and function of the neuromuscular junction, and this role is both structural (linking other proteins in the intracellular matrix together) and functional (acting as a cell-signaling molecule).

AChE

The main form of acetylcholinesterase (AChE) at the neuromuscular junction and the synaptic basal lamina is the collagen-tailed form of AChE, ColQ-AChE. ColQ-AChE is bound to the heparan sulfate proteoglycan perlecan and will also interact with the muscle-specific tyrosine kinase receptor. Its main function is to remove ACh from the synaptic cleft. In fact, the ACh released from the nerve terminal must first run the gauntlet of AChE that is bound to the synaptic basal lamina, before it can bind to the AChR concentrated on the crests of the junctional folds.

Cell Surface and Membrane Receptors

Dystroglycan Complex

The main components of the dystroglycan complex are α- and β-dystroglycan. These two forms are from a single gene, but have very different sizes and properties. The α-dystroglycan is 156 kDa in size; it is an extracellular protein that binds noncovalently to β-dystroglycan in the dystroglycan complex, on the one hand, and to laminin and/or agrin in the extracellular matrix, on the other. β-Dystroglycan is a 43 kDa glycoprotein and is an integral membrane protein. Numerous other proteins associate with the dystroglycans and together they form a large structure called the dystroglycan complex. The other members of the dystroglycan complex are the sarcoglycans (α-, β-, δ-, and γ-sarcoglycans), α-dystrobrevin, neuronal NO synthase (nNOS), and the syntrophins (α, β1, and β2). In addition, and of critical importance, the dystroglycan complex will bind to dystrophin or utrophin.

On the intracellular side, the dystroglycan complex will bind to the proteins dystrophin or utrophin. In turn, dystrophin and utrophin will bind to the actin cytoskeleton. The protein utrophin is shorter than dystrophin, and utrophin is concentrated at the neuromuscular junction. The dystroglycan complex is found throughout the entire length of the skeletal muscle cell and is particularly concentrated at the neuromuscular junction and the myotendinous junction. The dystroglycan complex is greatly reduced in Duchenne muscular dystrophy patients, who suffer from muscle fiber breakdown and degeneration. Thus, the dystroglycan complex plays an important

role in linking the intracellular actin cytoskeleton to the extracellular matrix. This link is undoubtedly important in the structural integrity of the muscle cell membrane and is probably critical to protect the membrane from tears that would result from the contractions of the skeletal muscle cell. At the neuromuscular junction the dystroglycan complex may also play an important role in cell-to-cell signaling. The binding of agrin to dystroglycan may be an important feature of the processes that regulate the induction and maintenance of the postsynaptic apparatus in muscle. The exact role of the agrin/dystroglycan complex interaction in postsynaptic signaling is still unknown.

MuSK

The aggregation of AChRs induced by agrin depends absolutely on the presence of a muscle-specific tyrosine kinase (MuSK). MuSK is clearly a downstream mediator of agrin, and MuSK knockout mice have virtually the same phenotype as those lacking agrin. However, the direct binding of agrin to MuSK has not been demonstrated, and the exact choreography of the events that start with agrin and end with the activation of MuSK has not been determined. MuSK has a large extracellular domain and an intracellular kinase domain. More recently it has been found that MuSK is required for the anchoring of the ColQ-AChE complex to the synaptic site. It is also been shown that the Abl kinases (Abl1 and Abl2) are downstream of activated MuSK. Thus, MuSK plays a central role in the events leading to the proper formation of the neuromuscular junction.

Integrins

Integrins are transmembrane protein complexes that form from heterodimers of α and β chains. Both chains are important for ligand binding. There are 18 α subunits and eight β subunits known, and currently 24 unique combinations of these have been identified. In addition, many of the subunits are alternatively spliced so that there are multiple variants of many subunits. Of particular interest is the $\alpha 7$ subunit, since it is found concentrated at the neuromuscular junction. The $\alpha 7$ subunit interacts with the $\beta 1$ to form the $\alpha 7\beta 1$ integrin. This integrin binds to laminin via the RGD (arginine-glycine-aspartate) domain on the $\beta 2$ arm of the synaptic laminin. The $\alpha 7$ subunit is alternatively spliced, and there are at least six variants of the $\alpha 7$ integrin.

While the binding of the $\alpha 7\beta 1$ integrin to laminin is a structural link between the matrix and the cell, the integrins also participate in intracellular signaling. On the intracellular side the integrins play a key role in linking to the actin cytoskeleton. This linkage is very similar to the molecular linkage found in a focal adhesion. Namely, the integrin binds to vinculin, α-actinin, and talin. All of these components are found aggregated at the postsynaptic site. In addition, integrins play an important role in regulating the activities of kinases. Of particular interest are the Src kinase family members Abl and Fyn, which have been shown to play a role in the assembly and maintenance of the postsynaptic apparatus. Thus, the integrins play an important structural and signaling role in the postsynaptic apparatus.

Integrins also are likely to play a presynaptic role, since treatment of frog muscles with RGD-containing peptides will reduce the increase in miniature endplate potential release upon muscle stretch. In addition to the $\alpha 7\beta 1$ integrins, the $\alpha 3\beta 1$ integrins are found at the presynaptic active zones. The $\alpha 3\beta 1$ integrins also interact with laminins in the synaptic basal lamina and can activate intracellular kinases. The precise role of the $\alpha 3\beta 1$ integrin is still largely unknown, but it is clear, at least in frogs, that the binding of integrin to matrix also has an important presynaptic cell-signaling function.

Cadherins

Cadherins are membrane-bound proteins that require calcium for their activity. They play an important role in the early development of the skeletal muscle cell. There are many cadherins, but the main ones produced in adult skeletal muscle are neural (N)-cadherin and muscle (M)-cadherin. Immunolabeling studies have revealed that both N-cadherin and M-cadherin are concentrated at the neuromuscular junction. The role of cadherins at the neuromuscular junction has been largely uninvestigated. N-Cadherin promotes neurite outgrowth *in vitro* and it is of interest that this effect is blocked by agrin. Both N-cadherin and M-cadherin play an important role in the formation of multinucleated muscle cells. They also may play a role at the synapse. The cadherins will form calcium-dependant bonds with cadherins on adjacent cells. Cadherins also interact on the intracellular surface with catenins. The α-, β-, and δ-catenins have been detected at the neuromuscular junction. In other systems it is known that the α- and β-catenins form a link with the actin cytoskeleton. In addition, the liberation of β-catenin from cadherins can result in nucleocytoplasmic shuttling to alter gene transcription via the interaction of β-catenin with T cell factor (TCF). Similarly, δ-catenin has also been shown to be present at the neuromuscular junction and interacts with the promoter region of rapsyn. Altogether, these results argue that the cadherin system functions at the neuromuscular junction to regulate cell structure and function.

Proteases at the Neuromuscular Junction

Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) are a large family of enzymes whose main function is to degrade the extracellular matrix. There are over 25 metalloproteinases, each with preferred matrix substrates. Most MMPs are released from cells in a pro form and must be first activated by other proteases. While it is clear that the MMPs play a role in matrix development and remodeling, it is clear that they also have an influence on cell-to-cell signaling. For example, cleavage of the NC1 domain from type IV collagens is known to play a major role in the signaling of vascular development and remodeling. The strongest evidence for the involvement of MMPs at the neuromuscular junction is the fact that mice that lack MMP3 have increased junctional folds and have AChR receptors on their surface. This likely results from an accumulation of matrix molecules that would normally be removed by MMP3, particularly agrin. MMP2 and MMP9 have been shown to play an important role in the reinnervation of muscle following nerve damage. The fact that MMPs are present at the neuromuscular junction is not a surprise, since it is clear that the matrix must be remodeled to allow synaptic growth. The mechanisms that control MMP activation at the neuromuscular junction are still unknown.

Tissue Inhibitors of Metalloproteinases

Balancing the activity of the MMPs are the tissue inhibitors of metalloproteinases (TIMPs). There are four TIMPs, and one of these, TIMP2, has been shown to be present at the neuromuscular junction. Mice that lack TIMP2 have altered neuromuscular junctions. TIMPs inhibit MMPs, but are also required for the activation of some MMPs. Thus, there is a delicate balance between the activity of MMPs and TIMPs. The control of matrix structure and function is likely to be central to the mechanisms that control synaptic structure and function.

Conclusion

The synaptic basal lamina contains molecules that are common to all basal laminae throughout the body, and also a number of unique molecules. The synaptic basal lamina has both a structural role that links the cytoskeletons of the synaptic components, and a cell-to-cell signaling role. The organization of the synaptic basal lamina is extremely precise, and the maintenance of this structure is undoubtedly important in the organization of the synapse. The synaptic basal lamina is a dynamic structure, and proteases are constantly sculpting this complex matrix. The control of matrix structure and function is likely to be central to the mechanisms that control synaptic structure and function.

See also: Axonal Pathfinding: Extracellular Matrix Role; Neuromuscular Junction (NMJ): Mammalian Development.

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Neuromuscular Junction: Neuronal Regulation of Gene Transcription at the Vertebrate

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The Vertebrate Neuromuscular Junction: An Excellent System for Studying Neurotransmitter Receptor Accumulation in the Postsynaptic Membrane

Synapse formation in the nervous system is an example of highly coordinated subcellular differentiation driven by localized reciprocal interactions between the synapsing cells. These interactions result in the apposition of the molecules that mediate transmitter release in the nerve terminal and the ligand-gated ion channels that are opened by the transmitter in the target cell. To enable synaptic transmission to operate sufficiently rapidly, these two features of the synapse must be located within less than 100 nm of each other. This raises the question, what are the mechanisms that coordinate the appearance and spatial alignment of these crucial aspects of pre- and postsynaptic differentiation?

The vertebrate neuromuscular junction (NMJ) has served as a valuable model system for formulating and testing ideas regarding the cell–cell communication that governs synapse formation. Compared with synapses in the brain, which are small and are made by different neuronal types at different stages of development, the NMJs are large, have qualitatively similar properties, develop at a similar time, and are easily accessible for experimental manipulation. Moreover, new NMJs can be surgically induced to form in adult muscle. Furthermore, the electrical impulse activity which influences the expression of genes involved in synapse formation can be experimentally controlled independently in the pre- and postsynaptic cells forming these *de novo* synapses. Finally, combinations of transgenes can be readily transferred into the myofibers, allowing examination of their function for subsynaptic differentiation.

At NMJs, action potentials in the nerve cause acetylcholine (ACh) to be released from the nerve terminals. This ACh then binds to acetylcholine receptors (AChRs) in the postsynaptic muscle membrane. The muscle AChRs are ligand-gated ion channels which open on binding ACh. The flow of cations through the open channels causes a depolarization that induces an action potential which propagates along the muscle membrane, triggering muscle contraction. So that each action potential in the motor neuron is transmitted to the muscle fiber, AChRs must accumulate in the postsynaptic muscle membrane during development and be maintained there at high concentration throughout life.

Vertebrate skeletal muscle fibers are very large, multinucleated cells. The region of nerve contact at the NMJ occupies about 0.01% of the surface area of a typical muscle fiber. Two complementary processes account for the high density of AChRs in this region. First, in immature muscle, AChRs that are mobile in the plane of the cell membrane aggregate at sites of nerve–muscle contact. This aggregation is mediated by AGRIN, a protein which is released from the nerve terminal. Like all proteins, the clustered AChRs are constantly degraded and need to be continuously replaced. However, the electrical activity associated with muscle activation suppresses transcription of *AChR* subunit genes by muscle fiber nuclei. A second key process therefore exists to ensure that new AChRs continue to be synthesized near the NMJ in active muscles. Signals released by the motor neuron act to maintain a high level of *AChR* subunit gene expression by the small number of muscle nuclei in the immediate vicinity of the synapse. This article discusses the signals and molecular pathways by which the motor nerve maintains *AChR* gene transcription at the synapse despite its suppression in nonsynaptic regions. It should be noted, however, that in addition to *AChR* genes, nuclei at the synapse also express genes coding for other synaptic components at elevated levels (see **Table 1**).

Recent experiments have dramatically reshaped our understanding of the mechanisms regulating the accumulation of AChRs in the postsynaptic membrane of the vertebrate NMJ. Recent genetic studies *in vivo* have demonstrated convincingly that major conclusions drawn from earlier *in vitro* (muscle culture) studies were incorrect. The data from these *in vitro* studies (some of which are reviewed below) strongly suggested that neuregulin (NRG)/ARIA was the principal molecule stimulating synapse-specific synthesis of AChRs but had little effect on clustering AChRs whereas AGRIN, which clearly induced clustering, had no stimulating effect on synapse-specific synthesis of AChRs. However, the current evidence supports the conclusion that *in vivo*, AGRIN plays an essential role in both clustering of AChRs and their synapse-specific synthesis. In contrast, NRG/ARIA has only a minor role in postsynaptic AChR accumulation (but does

Table 1 Genes for which elevated expression levels by subsynaptic nuclei of the adult neuromuscular junction have been demonstrated

Gene	Function
AChRα	AChR subunit
AChRβ	AChR subunit
AChRδ	AChR subunit
AChRε	AChR subunit
Ache	Acetylcholinesterase, rapidly terminating ACh action by hydrolysis
Rapsyn	Membrane associated protein required for synaptic AChR clustering
Utrophin	Cytoskeletal protein involved in synaptic AChR clustering
Musk	Component of Agrin receptor complex, required for all aspects of postsynaptic differentiation
Dok-7	MuSK-interacting cytoplasmic protein, essential for MuSK activation
$Na_v1.4$	Voltage-activated sodium channel, expressed in adult muscle
Erbb2	NRG receptor
Erbb3	NRG receptor
Laminin–β2	Component of synaptic basal lamina, involved in presynaptic differentiation
Ncam	Neural cell adhesion molecule thought to be involved in neuromuscular adhesion

Data based on elevated transcriptional activity or on synaptic levels of respective mRNAs.

have other essential roles in the development of the neuromuscular system). The reader is asked to keep these points in mind while reading this article.

Expression of AChRs throughout Uninnervated Differentiating Myotubes Is Replaced by Synapse-Specific Expression in Innervated Fibers

The pattern of AChR expression along muscle fibers and cultured myotubes was initially resolved by electrophysiological mapping of ACh sensitivity, that is, changes in membrane potential in response to localized application of short pulses of ACh to the fiber surface. Later, application of labeled α-bungarotoxin (α-BTX), a snake venom protein that binds with very high affinity to AChRs, was used to visualize and quantify AChR distribution. Both methods revealed that AChRs are distributed along the full length of uninnervated and denervated muscle fibers, often grouped in small clusters, but that in mature innervated muscle, almost all surface AChRs are aggregated in the postsynaptic muscle membrane immediately opposite the nerve terminal. These findings indicated that there is a mechanism that tightly traps (clusters) AChRs at the synapse but left open the question as to whether in innervated muscle (1) *AChR* subunit genes are expressed by nuclei throughout the muscle, with the assembled receptors or unassembled subunit proteins or subunit mRNAs subsequently transported to the synapse, or if instead, (2) the *AChR* subunit genes are expressed primarily by subsynaptic nuclei. Molecular biological studies over the period ≈1985–93, applying techniques of increasing sophistication, definitively demonstrated synapse-specific transcription by subsynaptic nuclei.

Muscle AChRs are heteropentameric transmembrane proteins composed of *α, β, δ,* and either a *γ* or an *ε* subunit, each encoded by a separate gene. The AChRs present in immature muscle have the subunit composition $\alpha_2\beta\gamma\delta$ and are sometimes referred to as fetal while those in mature muscles have the composition $\alpha_2\beta\delta\varepsilon$ and are known as adult. The AChR *α, β, γ,* and *δ* subunits are expressed by differentiating myoblasts as part of their developmental program. Mammalian myoblasts fuse to form myotubes in two waves. In the first wave, myoblasts fuse to form a relatively small population of ‘primary’ myotubes that extend to both ends of the developing muscle. In a second wave, a few days after the first, other myoblasts that have undergone further proliferation line up along the primaries, generally in the central innervated region, and then fuse to form a much larger population of ‘secondary’ myotubes that make up the majority of fibers in adult muscle. During and after myoblast fusion, the expression of fetal AChR subunits along the entire length of the myotube (called global expression) is greatly increased relative to the levels expressed in unfused myoblasts.

It is at the developmental stage when primary myotubes are being formed that nerve processes, later followed by Schwann cells, appear in the developing muscle and begin to form neuromuscular contacts. These are characterized by clusters of fetal AChRs in the muscle membrane. Though the contacts at this stage lack many morphological and physiological characteristics of mature synapses, single quanta of ACh released from the nerve and activating the fetal AChRs are sufficient to generate action potentials in the myotubes, a process that is facilitated by their high input resistance and the long open time of fetal AChR channels. Thus, from the earliest stages of synapse formation, action potentials in the nerve induce muscle action potentials and contraction. Muscle action potentials repress *AChR* subunit gene expression by nuclei along the full length of the muscle fiber, and thus the consequence of innervation is that expression of *AChR* subunit genes by extrasynaptic nuclei becomes almost completely repressed as the muscle matures. An important exception is the

small group of muscle nuclei (termed 'synaptic' nuclei) that underlie the synaptic contact and continue to express high levels of *AChR* α-, β-, and δ-subunit genes in electrically active muscle. In the same nuclei, expression of the γ-subunit gene is replaced by that of the ε-subunit gene. This occurs during the early postnatal period in the rodent. The switch in subunit composition of the AChRs from $\alpha_2\beta\gamma\delta$ to $\alpha_2\beta\varepsilon\delta$ at the synapse results in channels with shorter open times but increased conductance for Na^+, K^+, and Ca^{2+} ions.

Do Motor Neurons Produce Signals That Induce Clustering of AChRs at the Synapse, Stimulate Synapse-Specific AChR Synthesis, or Both?

The central question addressed in this article is, what are the mechanisms that account for the production of AChR subunits at the synapse (synapse-specific expression) in the face of the global repression caused by muscle electrical activity? One possibility is that the synaptic nuclei differentiate independent of (and prior to) innervation to a state in which electrical activity does not suppress their expression of AChR subunits (discussed further in the section titled 'Myocentric model'). However, evidence from two experimental models of NMJ formation, nerve–muscle cocultures and the induction *in vivo* of ectopic synapses by surgical transplantation of foreign motor nerves onto nonsynaptic muscle regions, indicated that motor neurons produce signals that are capable of inducing activity-resistant accumulations of AChRs in the muscle plasma membrane opposed to the nerve terminal. Three hypotheses for the mechanism by which these nerve-derived signals cause AChR accumulation in the postsynaptic membrane were considered: that the signals induce clustering (aggregation) of AChRs, that they induce synapse-specific transcription of *AChR* subunit genes, or both. Using *in vitro* assays of AChR clustering and AChR synthesis rate, investigators set out to identify candidate signaling molecules.

A Nerve-Derived Factor Inducing AChR Aggregation: AGRIN

In a series of elegant experiments, McMahan and colleagues pursued the hypothesis that components of the basal lamina at the NMJ induce aggregation of AChRs. The basal lamina is the layer of extracellular matrix that ensheathes each skeletal muscle fiber and each myelinated axon. At the NMJ, a single layer of 'synaptic' basal lamina is present in the synaptic cleft between the nerve and the muscle. When adult muscles are damaged and allowed to regenerate within the persisting basal lamina sheath, AChRs aggregate at original synaptic sites (marked by the activity of acetylcholinesterase (AchE) that survives muscle fiber breakdown), even if the nerve is cut and its terminals degenerate. Material that could cause AChR aggregation in cultured chick muscle cells was found to be present in the extracellular matrix of the electric organ of the electric fish *Torpedo*, a modified NMJ of great size. This material was purified, found to be based on a protein, and named AGRIN. Antibodies against AGRIN label the synaptic basal lamina at frog and mammalian NMJs and prevent the AChR-aggregating activity of motor neurons. AGRIN is synthesized by motor neurons and released from motor nerve terminals (see also below). In mice in which expression of *Agrin* has been knocked out, nerve-induced AChR clustering fails to occur and nerves wander across the entire muscle, as they do in mice lacking the AGRIN receptor MUSK (**Figure 1**). There is thus very strong evidence that AGRIN released from the nerve mediates clustering of AChRs at developing NMJs. As pointed out above, AGRIN was not found to affect AChR synthesis rate in cultured myotubes. This is not the case *in vivo*, however, as discussed below.

Candidate Nerve-Derived Factors Inducing *AChR* Gene Transcription

Several molecules have been implicated in inducing the expression of *AChR* subunit genes by synaptic

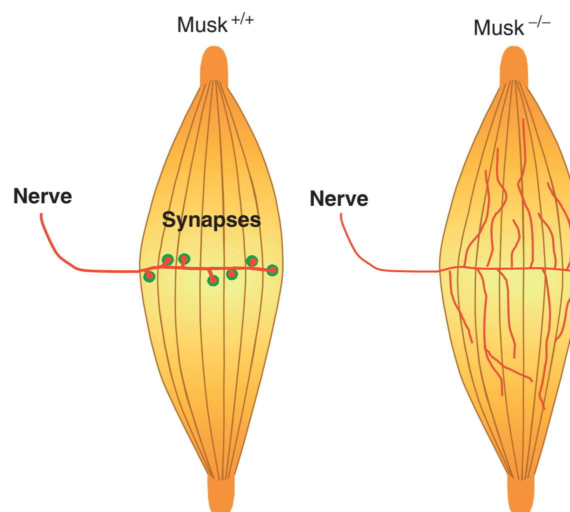

Figure 1 Deletion of AGRIN or of the AGRIN receptor MUSK prevents AChR clustering and neuromuscular synapse formation. Instead, nerve fibers grow along muscle fibers, suggesting that an AGRIN/MUSK-dependent stop signal is required for neuromuscular synapse formation.

nuclei. They include products of four genes, *Nrg1*, *Nrg2, Agrin*, *αCgrp*, as well as ATP. We very briefly discuss αCGRP, and ATP, and then turn to NRG1, NRG2, and AGRIN.

αCGRP and ATP

αCalcitonin gene-related peptide (αCGRP; acting via a G-protein-coupled receptor) and ATP (acting via P2YX receptors enriched in the synaptic membrane) are present in motor nerve terminals and enhance AChR subunit mRNA in cultured myotubes. ATP also affects the rate of AChR desensitization by high concentrations of cholinergic agonist. In contrast to AGRIN and NRG1, however, they have not been shown to be essential for NMJ formation *in vivo*. Specifically, ablation of the *αCgrp* gene in mice does not affect NMJ development, nor does abolishing presynaptic αCGRP and ATP delivery by sectioning the motor nerve abolish synapse-specific *AChR* transcription. However, this does not exclude a redundant role of these factors in this process.

Neuregulins

***In vitro* studies implicating NRG1 as a motor neuron-produced signal regulating postsynaptic *AChR* gene expression** Fischbach and colleagues reasoned that if motor neurons produce a signal that locally stimulates the synthesis of AChRs, an extract of spinal cord (where the cell bodies of motor neurons are located) would increase the rate at which receptors are inserted into the surface membrane of cultured muscle. They found that chick spinal cord and chicken brain extracts did accelerate the rate of new AChR insertion and that this 'acetylcholine receptor-inducing activity' (ARIA) had the biochemical characteristics of a protein. Peptide sequence of ARIA purified from chick brain revealed that this protein is encoded by the gene now designated *Nrg1*.

The NRG1 proteins are ligands for the receptor tyrosine kinases ERBB2, ERBB3, and ERBB4. Several features of NRG1 support the hypothesis that NRG1 is a signal used by motor neurons to induce the synthesis of the muscle postsynaptic specializations. These features include the following: (1) NRG1 is produced by motor neurons and transported along motor axons to the motor nerve terminals; (2) NRG1 receptors are expressed by muscle and become concentrated in the postsynaptic membrane; (3) NRG1 treatment of cultured muscle increases the amount of AChRα, AChRδ, and AChRε mRNAs; and (4) NRG1 activates signal transduction pathways and *cis*-acting DNA sequences implicated in regulating synapse-specific gene transcription (see discussion below of pathways involved in AGRIN-activated gene transcription). It is noteworthy that NRG1 has little effect on AChR clustering. Thus, a widely accepted view in the 1990s was that motor neurons induced postsynaptic differentiation via two signals: NRG1, which stimulated synapse-specific gene transcription, and AGRIN, which caused clustering and anchoring of AChRs and other synapse-specific proteins.

***In vivo* genetic studies have revealed relatively normal NMJ development in mice lacking neuromuscular NRG signaling** A role of NRG/ErbB was difficult to test *in vivo* by genetic approaches because mice homozygous for *Nrg1* or *Erbb* null alleles die due to defects in heart development at an embryonic stage before neuromuscular synapse formation begins. This difficulty has been circumvented through production of mice with tissue-specific mutations of the *Nrg1*, *Erbb2*, and *Erbb4* genes. Synapses begin to develop normally in mutants lacking *Nrg1* selectively in motor and sensory neurons. However, because neural *Nrg1* mutants die at birth (see below), that is, before the developmental time when nerve-induced electrical activity causes marked global suppression of AChR expression, the dispensability of *Nrg1* from neurons for synapse-specific *AChR* gene transcription cannot be tested in the adult in these genetic models. NMJ phenotypes in *Nrg2* mutants have not been analyzed, but mutants are viable, suggesting formation of functional NMJs.

The recent analysis of mice with muscle-specific deletion of both *Erbb2* and *Erbb4* genes has allowed clear assessment of the requirement for NRG-mediated nerve to muscle signal in neuromuscular synapse formation and maintenance. Ablation of both *Erbb2* and *Erbb4* rendered fibers insensitive to NRG, thus abolishing NRG signaling selectively to muscle (ERBB3 homodimers are inactive as they lack a kinase domain) while leaving ERBB2 and ERBB4 expression by Schwann cells and motor neurons unaffected. Synapses developed normally in these mutants (**Figure 2**). They were viable for months past birth, and synapse-specific *AChRε* and *AChRδ* transcription in the adult was only marginally affected. Therefore, NRG signaling from nerve to muscle is not essential for formation and maintenance of the subsynaptic apparatus. The function of muscle ERBB receptors in the postsynaptic membrane remains unclear.

Essential functions of NRG1 in neuromuscular development While the biological significance of muscle ERBB receptors, and possible *Nrg1*-mediated neuron-muscle, Schwann cell-muscle, and muscle autocrine signaling, remains unclear, there is no uncertainty that NRG1 signaling is essential for neuromuscular system development. First, mice homozygous for a targeted

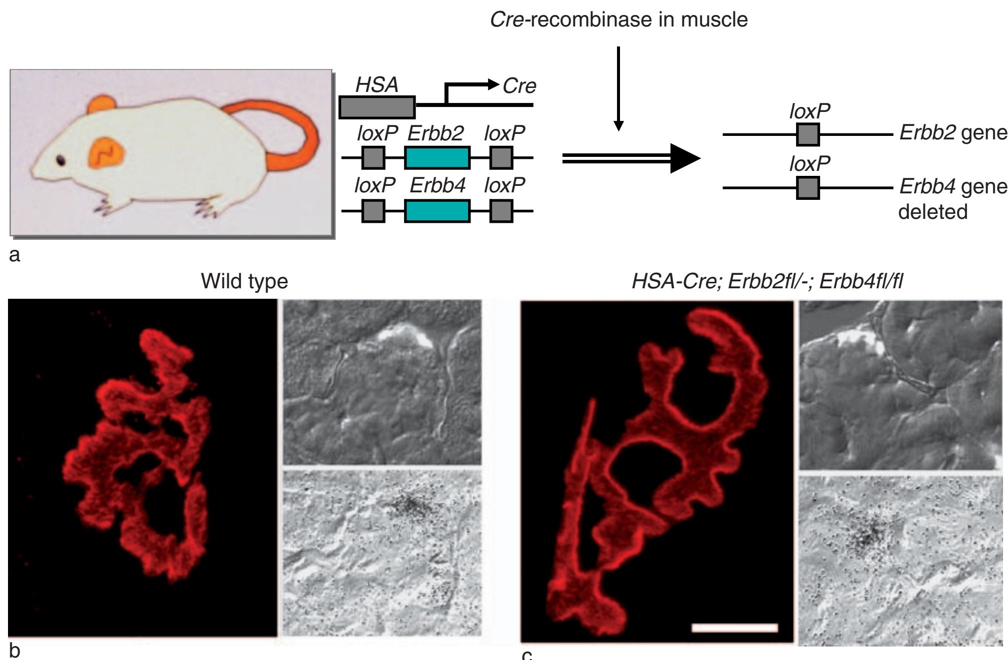

Figure 2 NRG signaling to muscle is not essential for neuromuscular synapse formation. Endplates develop normally in mouse mutants in which NRG/ErbB signaling to muscle is genetically abolished by Cre-induced recombination. (a) Muscle-specific *Erbb* deletion was induced by crossing a mouse line carrying a Cre transgene expressed under the control of a human skeletal actin promoter fragment (HSA-Cre) with a mouse line in which *Erbb* alleles were flanked by loxP sites. Endplates in (b) wild-type and (c) *Erbb*-deficient muscles appear similar (*en face* views (left), bar: 10 μm). Also shown are cross-sections after hybridization with a radiolabeled cRNA probe specific for the detection of *AChR* ε mRNA and development of photo emulsion. Note that accumulations of silver grains reflecting *AChR* ε mRNA co-localize with synaptic AChR clusters marked by fluorescent α-BTX (bar: 20 μm).

mutation that prevents production of Type III NRG1 protein die at birth because of respiratory paralysis and lack neuromuscular synapses because of degeneration of peripheral nerves. Analysis of these and other genetically altered mice, as well as cell culture studies, has demonstrated critical roles of NRG1 in proliferation, survival, migration, and myelination by Schwann cells. Second, targeted ablation of *Nrg1* in motor and sensory neurons (including γ-motor neurons and muscle spindle proprioceptive afferents) or of *Erbb2* in muscle prevents normal development of muscle spindles, causing abnormal gait.

AGRIN

AGRIN molecular variants and the AGRIN receptor MUSK AGRIN is secreted by muscle, nerve, and Schwann cells. As a consequence of differential mRNA splicing, multiple AGRIN proteins are produced by muscle, nerve, and Schwann cells (**Figure 3**). An N-terminal splice variant, NtA, binds to laminins, thus anchoring AGRIN to the synaptic portion of the muscle fiber's basal lamina. Another variant, TM, is a type II membrane protein with unknown function, expressed throughout the nervous system. NtA–AGRIN in turn is expressed in several C-terminal variants. The isoforms expressed by motor neurons include an 8, 11, or 19 amino acid insert (termed *B*- in chicken and *z*- in mouse) that is required for its function at the NMJ. While the three B/z variants differ somewhat in biological activity, they all act through a receptor tyrosine kinase (RTK) in muscle, MUSK, which is concentrated in the postsynaptic membrane. In contrast, AGRIN proteins lacking the B-/z-insert are expressed by terminal Schwann cells, along the entire length of muscle fibers, as well as in other tissues. The function of this AGRIN is poorly understood. In muscle, it does not activate MUSK, but it is involved in the organization of the muscle fiber's cytoskeleton.

***In vivo* genetic studies of AGRIN'S roles in synapse-specific gene expression** Consistent with the classical function of clustering AChRs, deletion of *Agrin* or of *Musk* genes abolishes synapse formation. It is interesting to note that in fetal muscle of mice lacking MUSK, no nerve-associated elevation of AChR subunit mRNA levels is seen, consistent with the idea that MUSK may be necessary for synapse-specific transcription (see also the section titled 'Models of neuromuscular synapse formation'). In these mice,

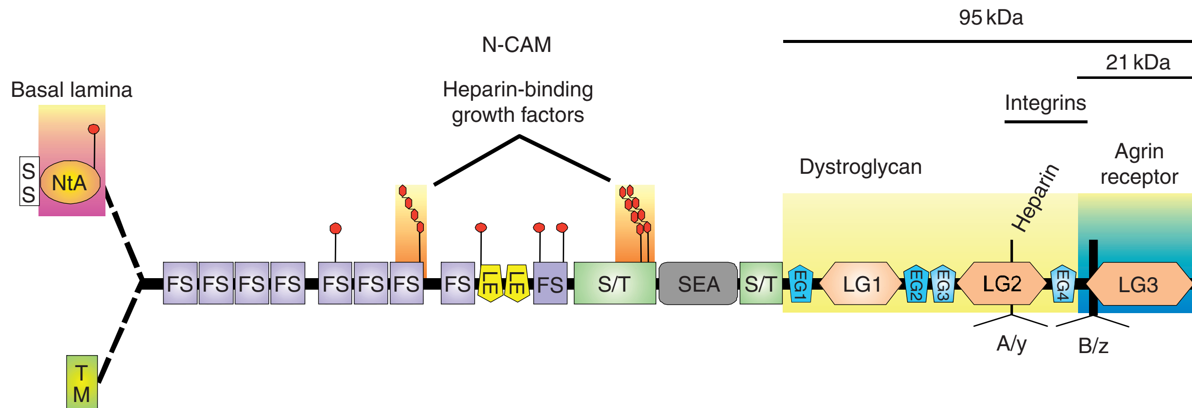

Figure 3 Structural domains of AGRIN and sites of alternative *Agrin* messenger RNA splicing (NtA vs. TM; A/y (0 vs. 4 amino acids); B/z (8 vs. 11 vs. 19 amino acids).) A miniAGRIN generated by fusion of an N-terminal fragment of Agrin (NtA), mediating binding to the basal lamina, to a C-terminal 21 kDa fragment comprising the B/Z-insert and LG3 is sufficient to activate MUSK and can induce ectopic nerve-free postsynaptic membranes, but with less potency than longer C-terminal fragments. SS: signal sequence required for secretion; LG: laminin globular domain. Binding regions to other partners (potentially) involved in NMJ formation are discussed in Bezakova G and Ruegg MA 2003 New insights into the roles of agrin. *Nature Reviews Molecular Cell Biology* 4: 295–308.

the growing motor neurons fail to make terminals but instead grow along the myotubes toward their ends. This suggests that a reciprocal signal from the muscle, in response to the AGRIN/MUSK interaction, is required for the induction of presynaptic terminals (**Figure 1**).

Positive evidence for a major physiological role of AGRIN in mediating synapse-specific *AChR* gene transcription comes from gain of function experiments in adult muscle. Recombinant neural AGRIN applied to nonsynaptic regions of normally innervated adult muscle fibers *in vivo* can induce the formation of functional ectopic, nerve-free, and Schwann cell-free postsynaptic membranes. These membranes contain accumulations of AChRs and all known molecules involved in AChR aggregation and anchoring and in *AChR* gene induction (muscle-derived NRG1, MUSK, and ERBBs) as well as the type of voltage-gated sodium channels concentrated at adult NMJs, $Na_v1.4$. The AChRs at these ectopic sites have the physiological properties of adult $\alpha_2\beta\varepsilon\delta$channels present at mature NMJs. Finally, the nerve-free ectopic membranes show synaptic folds characteristic of normal NMJs.

A striking feature of this inductive effect of AGRIN is that the ectopic postsynaptic membrane differentiation occurs in regions of the muscle where ongoing activity would normally suppress expression of *AChR* genes. Just as at normal NMJs, ectopically applied AGRIN induces the accumulation of a number of muscle nuclei. These nuclei, but not those elsewhere in the muscle fiber, express the genes *AChRε*, *Musk*, and, as implied from the activity-resistant AChR clusters, *AChRα*-, *β*-, and *δ*-subunits (**Figure 4**). In

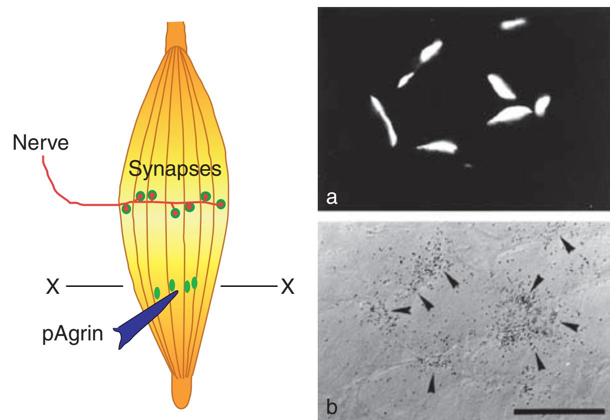

Figure 4 Ectopically applied AGRIN induces nerve-free, ectopic acetylcholine receptor (AChR) clusters and *AChRε*-subunit gene expression. Note that this occurs in spite of electrical impulse activity in the muscle fibers, which is maintained through the original synapses. Left: Ectopic expression of neural AGRIN by muscle fibers is induced by intracellular injection of AGRIN expression plasmid (pAgrin) into single muscle fibers. X- - - X denotes plane of cross-section shown on the right: (a) AGRIN-induced ectopic AChR clusters marked by fluorescent α-BTX; (b) same section after hybridization with a radiolabeled cRNA probe specific for the detection of *AChRε*-mRNA. Note that accumulations of *AChRε*-mRNA (arrowheads) co-localize with ectopic AChR clusters. Scale bar = 70 μm. (a, b) Reproduced from Jones G, Meier T, Lichtsteiner M, Witzemann V, Sakmann B, and Brenner HR (1997) Induction by agrin of ectopic and functional postsynaptic-like membrane in innerrated muscle. *Proceedings of the National Academy of Sciences of the United States of America* 94: 2654–2659, copyright (1997) National Academy of Sciences, USA.

addition, these nuclei express higher levels of the gene $Na_V1.4$ than do the nonsynaptic nuclei. Thus, the ectopic postsynaptic membranes induced by AGRIN are, at least qualitatively, similar to those

induced by transplanted motor neurons. It is interesting to note that recombinant AGRIN induces nerve-free ectopic AChR clusters and *AChRε* transcription in electrically active muscle, even in *Erbb*-deficient fibers. Therefore, AGRIN alone is sufficient for targeting *AChR* gene transcription to the synapse in electrically active muscle fibers.

Signaling Pathways by Which MuSK Regulates Postsynaptic Gene Expression

***Musk* expression in the postsynaptic membrane: A positive feedback loop?** AGRIN induces synapse-specific *AChR* gene transcription via MUSK. Whereas *Musk* is expressed throughout fetal muscle cells, it is downregulated later in development by electrical muscle activity. But if electrical activity suppresses *Musk* expression, how is MUSK maintained at synapses of active fibers? In adult fibers, forced expression of *Musk* mutants that are constitutively active activates endogenous *Musk*. This implies that MUSK protein is able to autoactivate the expression of the *Musk* gene. Thus synaptic *Musk* expression is stabilized in electrically active fibers by AGRIN/MUSK in a positive feedback signaling loop.

Signaling pathways AGRIN-induced *Musk* transcription is mediated, at least in part, by a regulatory sequence, CCAAGG, termed *N-box*, in the first intron of *Musk*. The same motif is also found in the promoters of *AChRε*- and *δ*-subunit genes, for which it mediates transcription induced by NRG1*β* in cultured myotubes as well as that induced by the nerve at synapses *in vivo*. Thus, the pathways activated by AGRIN, NRG1*β*, and the nerve converge. The AGRIN-dependent pathway has been examined by overexpression of constitutively active and inactive signaling components. AGRIN is known to activate the small GTPases Rac, Cdc42, and Rho. Forced expression of the active mutant RacV12 strongly activates *Musk* and *AChRε*, as does overexpression of active MKK7, an effector of Rac. In contrast, in muscle fibers *in vivo*, induction of *AChRε* and *Musk* by AGRIN is barely affected by overexpression of inactive ERBBs, consistent with the findings on *Erbb*-deficient muscles. However, overexpression of the inactive mutant RacN17, of the MAP kinase JNK, an effector of MKK7, and of inactive GAPB*β*, an effector of JNK, strongly inhibit transcription of *AChRε* and *Musk* both *in vivo* and *in vitro*. In combination, these data are consistent with a pathway at the synapse in which AGRIN/MUSK, activating in turn small GTPases RAC, MKK7, JNK, and GABP and ERM, members of the Ets family of transcription factors, converge on the same N-box in the *AChRε* and *Musk* genes that mediates their activation by the nerve and by NRG/ERBB (**Figure 5**).

It should be noted that the levels of AChRs and of *Musk* mRNA at ectopic sites induced by AGRIN or constitutively active MUSK are lower than at original synapses. Therefore, full transcriptional activation at the synapse may require synapse-specific signaling components acting downstream of MUSK and essential for its function (e.g., DOK-7), or neural signals in addition to AGRIN, such as NRGs or ATP, which may also target regulatory sequences other than the N-box. For example, NRG1*β* has been found *in vitro* to activate strongly *AChRε* via the transcription factor EGR1, and ATP via Elk-1. Moreover, *in vivo*, forced expression in muscle of a general Ets-dominant negative mutant competing for binding to N-box regulatory elements reduces *AChRδ*- and ε-without an obvious effect on *Musk* mRNA levels. Finally, the *AChRα* gene lacks an N-box in its promoter. Alternative pathways are further suggested for another synaptic component, UTROPHIN, which is part of the synaptic cytoskeleton anchoring the AChRs in the synaptic muscle membrane. Specifically, synaptic levels of *Utrophin* mRNA are not regulated by transcriptional activation alone but also by RNA stabilization.

MUSK and synaptic nuclei One factor thought to be important for synapse-specific transcription is the clustering of 5–8 nuclei underneath the synaptic muscle membrane, thus exposing them optimally to signals from the nerve terminal, such as AGRIN. The mechanisms governing this clustering process are not known. Hints come from studies of the control of cell migration, in which crucial roles of small GTPases of the Rho family, in particular of Cdc42, in defining cell polarity with respect to extracellular cues, are well established. Given that (1) recombinant AGRIN and constitutively active MUSK accumulate muscle nuclei in adult fibers, (2) subsynaptic nuclei are embedded in a rich and elaborate microtubular network, (3) efficient and persistent long-range migration requires stabilization of cell polarity, a process achieved through reorganization of the microtubule cytoskeleton, and (4) AGRIN activates Rho GTPases, it is plausible that nuclear translocation to the synapse is mediated through modifications of the cytoskeleton driven by AGRIN-activated Rho GTPases.

A number of proteins in addition to AChRs and MUSK display synapse-specific expression Although the role of neuromuscular NRG/ErbB signaling for synapse formation remains unclear, it is worth mentioning that synapse-specific expression of ERBBs is linked to the AGRIN/MUSK pathway. Both ERBB receptors and their mRNAs as well as *Nrg1β* precursors are observed at the synapse and at ectopic sites induced by AGRIN/MUSK. This indicates that the nerve, at

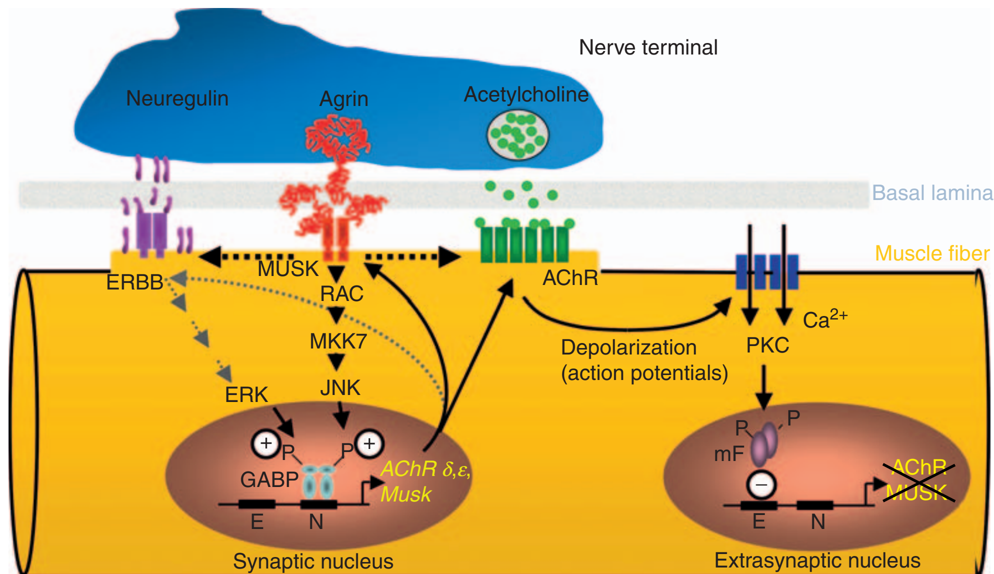

Figure 5 AGRIN from the nerve induces synaptic expression of acetylcholine receptors (AChRs) by targeting the expression of the *Musk* gene to the synaptic muscle region and aggregating AChRs in the synaptic muscle membrane. By activating MUSK in the muscle fiber, AGRIN induces (1) accumulation of muscle nuclei under the synapse via unknown mechanisms and (2) synapse-specific expression of (a) *Musk*, via a positive feedback loop (black solid arrows), as well as of other synaptic genes, including *AChRε*- and *δ*-subunits (indicated in yellow), all sharing the same regulatory sequence (N-box) in their promoters, and (b) *Erbb*s (broken gray arrows; pathways unknown). The activation of ErbB receptors by neuregulins (NRGs) from the nerve and muscle fiber can also stimulate transcription of genes with N-boxes and thus may strengthen the MUSK-induced expression level. Note that they may be enhanced further by other pathways (not shown). AGRIN also induces synapse-specific expression of (c) *AChRα*- and *β*-subunit genes (not shown, pathways unknown) and (3) subsynaptic accumulation of AChRs, MUSK, and ERBBs by anchoring them to the synaptic cortical cytoskeleton of the muscle fiber (broken black arrows). PKC is protein kinase C. ERK, RAC, MKK7, JNK are components of the respective signaling pathways; P denotes phosphorylation, mf is myogenic factor, E is promoter sequence binding mf.

least in part by secreting AGRIN, stimulates synaptic nuclei to express *Erbb* genes and to aggregate their products in the synaptic muscle membrane (see also **Table 1**).

Models of Neuromuscular Synapse Formation

Neurocentric Model

The simplest model to explain the induction and maintenance of synapse-specific gene transcription by the motor neuron in fetal muscle is that *Musk*, expressed constitutively in developing myotubes, is activated locally by AGRIN released from a motor neuron growth cone. This MUSK activation, in turn, stabilizes the expression of *Musk* in myonuclei closely adjacent to the site of contact (subsynaptic nuclei) against the downregulation of *Musk* expression throughout the muscle fiber caused by the electrical muscle activity which begins soon after the neuromuscular contact is established. Via a similar pathway, AChR expression is stabilized by AGRIN/MUSK, ensuring high synaptic ACh sensitivity in electrically active fibers. While NRG/ERBB signaling is not essential for postsynaptic membrane formation and maintenance, induction of genes by AGRIN/MUSK may be augmented by NRG from the nerve and/or the muscle converging on common regulatory elements, as well as by other hitherto unknown alternative pathways, perhaps mediated via NRG2, αCGRP, and/or ATP from motor neurons, muscle fibers, and/or Schwann cells, respectively. As the synapse further differentiates, the number and density of subsynaptic nuclei increase. The accumulation of extrasynaptic nuclei expressing *AChR* genes in response to ectopic AGRIN indicates that any nucleus can be reprogrammed by AGRIN to become a subsynaptic nucleus. Implicit in this model and the supporting data is the idea that during development, motor neuron terminals have the capability of inducing synapse formation anywhere along the length of the muscle fiber.

Myocentric Model

This alternative to the neurocentric model postulates that in fetal muscle, AChRs first cluster independently of neural influence and only later are stabilized by AGRIN from motor neurons contacting them. Conversely, AChR clusters not stabilized by AGRIN would be dispersed. The model is based on the observation that in fetal diaphragms of mouse

mutants lacking phrenic nerves, a central band of AChR clusters and of elevated level of *AChR* α-mRNA is seen in the area where synapses are normally made (although co-localization of nerve-free AChR clusters with focally elevated *AChR* mRNA has not been established at the subcellular level). As noted above, neither the central AChR clusters nor elevated AChR mRNA is seen in *Musk* mutant muscles, indicating an essential role of MUSK in this process. The myocentric model is supported by the observation that in certain muscles of the zebra fish, AChR clusters form prior to innervation and are then preferentially contacted by nerve processes. However, such 'prepatterning' is not generally required for fetal zebra fish neuromuscular synapse formation: AChR clusters follow, rather than precede, the appearance of motor neurons in other zebra fish muscles. A plausible explanation for the 'synaptic zone' in nerve-free mouse muscle is that (1) at high concentrations, MuSK auto-activates, and (2) high MuSK spatially coincides with developing myotubes elongating from the central muscle region and expressing elevated levels of MUSK and, as a consequence of auto-activated MuSK, high AChR levels.

A Unifying Model?

It is possible that the two models represent extreme states of an equilibrium between active and inactive MuSK. MuSK is activated not only by AGRIN but also by itself, depending on its abundance and that of other proteins (e.g., DOK-7) with which it interacts. The number of nerve-free AChR clusters in any particular situation is therefore likely to depend on the levels of expression of MUSK and these other proteins. For example, in myotubes expressing high MUSK levels, the number of nerve-free AChR clusters, and therefore the likelihood of a nerve process to encounter and stabilize a preformed AChR cluster, would be high. Conversely, in myotubes expressing low levels of MUSK, delivery of AGRIN by motor neurons would be required for initial MUSK activation and subsequent AChR cluster formation.

Conclusion

Among the candidates mediating the neural control of neuromuscular synapse formation, AGRIN and NRGs have for more than two decades commanded the widest attention. New genetic studies *in vivo* have dramatically redefined their roles in this process. The key pathways regulating the expression of synaptic AChRs, as well as their aggregation in the synaptic muscle membrane, are activated by AGRIN/MUSK. Synapse-specific transcription of several key synaptic genes appears to be regulated, at least in part, via a common pathway. In contrast, the neuregulins, which had been considered the primary candidates regulating *AChR* gene transcription, have turned out to be dispensable for this process. They are, however, essential for normal development of Schwann cells and muscle spindles.

See also: Neuromuscular Junction (NMJ): Mammalian Development; Neuromuscular Junction: Synapse Elimination.

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Neuromuscular Junction: Synapse Elimination

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Introduction

Motor neurons supply the last outposts of the motor system, where volition finally interfaces with and influences the material world. In higher vertebrates, the axon of an adult somatic motor neuron projects to a single anatomically defined muscle target. The motor axon then divides into several tens to hundreds of intramuscular collateral branches, each terminating on a muscle fiber at a neuromuscular junction (NMJ) to form its sole innervation. A high margin of safety for neuromuscular transmission ensures that every action potential generated in the motor neuron reliably triggers an action potential and contraction of all the muscle fibers it supplies. Thus, a motor neuron and its muscle fibers function together in a unitary fashion: The combined set is referred to as a motor unit. The number of muscle fibers in one unit is termed the motor unit size. Motor units are activated in an orderly fashion during voluntary movements, often sequentially by size. The variation in motor unit sizes (more than tenfold in some muscles and more than 1000-fold between other muscles) confers a remarkable dynamic range on the motor system as a whole.

All historic and contemporary images of adult NMJs reveal a remarkable and stereotyped pattern of the motor nerve supply to skeletal muscle fibers, found throughout the vertebrate subphylum but particularly well represented in mammals: Each well-defined motor junctional area, also called the 'endplate,' is supplied by the axon of one and only one motor neuron. However, this pattern is not formed *de novo*. Rather, the motor endplates of individual muscle fibers are initially supplied by terminals derived from several motor neurons. This state is called polyneuronal innervation. The transition from polyneuronal to mononeuronal innervation during development occurs by a process commonly known as synapse elimination, the subject of this article. The reduction in motor unit convergence (overlap) has another important consequence: Since in most cases the number of muscle fibers does not increase during synapse elimination, the degree of motor divergence also decreases; that is, synapse elimination also brings about a reduction in motor unit size, therefore ultimately limiting the maximum force a motor unit can produce.

Three main physiological factors regulate motor unit force production in vertebrate muscle. In addition to motor unit size, these are the frequency of activation and the functional 'type' of the muscle fiber. In rodents, where these factors have been studied in greatest detail, they all change during development. It is interesting to note that the three properties appear to be linked: Frequency of activation and intrinsic properties such as motor neuron and muscle fiber type play roles in steering the process of synapse elimination to its conclusion, thus determining the motor unit size.

Different rules govern the maturation of innervation patterns in smooth, cardiac, and some specialized types of voluntary striated muscle fibers that contract relatively slowly. Persistent, stable polyneuronal innervation is the norm in these muscle types, and they are therefore not discussed here. Synaptic remodeling also occurs during development of invertebrate muscles, and this plasticity is increasingly studied in the fruit fly, *Drosophila melanogaster*, mainly because of the power and pace of molecular genetic analysis in this species. However, although pruning of motor axon branches occurs during metamorphosis, neuromuscular synapse elimination comparable to that in mammalian muscles does not normally take place in *Drosophila*. The present discussion is therefore mainly restricted to mammalian skeletal muscle.

From π to μ

Neuromuscular synapses form about halfway through gestation in mice and rats. In some muscles, axonal growth cones appear to contact random points on the membrane of the immature muscle fibers (myotubes) and to induce specialized synaptic features in them. In other muscles, it appears that growth cones contact preformed postsynaptic sites. Either way, axons from other motor neurons then add to the first neuromuscular inputs. Thus, by birth, virtually all muscle fibers are innervated by terminals of several motor neurons, that is, polyneuronal innervation (π). The first evidence of polyneuronal innervation in mammalian muscle was obtained in 1916–17 in a study of fetal human tissue by Tello. Contemporaneously, Boeke reported polyneuronal innervation in reinnervated intercostal muscles after nerve injury in hedgehogs. Skeletal muscles of mice and rats are presently the tissues of choice for descriptive and mechanistic studies of neuromuscular synapse elimination both in neonates and in reinnervated muscles in adults.

More-recent interest in synapse elimination began with two electrophysiological exposés, one on neonatal

rat diaphragm muscle and the other on reinnervated adult rat hind limb muscles. Both studies showed that when the muscle nerves were stimulated with electric pulses of gradually increasing strength, discontinuous, stepwise increments occurred in the amplitude of the endplate potentials recorded with intracellular electrodes from individual muscle fibers. This is in contrast to the one-step response normally observed during graded nerve stimulation in adults. The simplest explanation of these observations is that multiple axons converging on the same endplate, and having distinct electrical excitation thresholds, are progressively recruited as the stimulating current is increased.

Polyneuronal innervation was also demonstrated using electrophysiological techniques at about the same time in other immature vertebrates, including tadpoles and chick embryos. These studies were complemented by physiological evidence of polyneuronal innervation in skeletal muscles of neonatal kittens by recording isometric tension. These studies showed that the force produced in response to the simultaneous stimulation of two immature motor axons is usually less than the arithmetic sum of the responses to stimulating both separately. This is also most simply explained by convergent innervation of muscle fibers.

More recently, direct histological evidence of polyneuronal innervation and synapse elimination has been obtained by confocal microscopy analysis of three- and four-dimensional image stacks (X,Y,Z +/− T), for example in preparations immunostained for neurofilament protein in axons (**Figure 1**) or expressing different fluorescent proteins. Such images have provided unequivocal confirmation of the convergence of several axon terminals on individual motor endplates at birth in rodents and synapse elimination during the first 3 postnatal weeks.

One early study has since achieved iconic status in the field: a comprehensive analysis of polyneuronal innervation and synapse elimination carried out by Jansen, Brown, and Van Essen. These investigators brought to bear a powerful combination of tension measurements, intracellular recordings, and histology. All contemporary studies and analysis of polyneuronal innervation and neuromuscular synapse elimination can be traced back to this study. Its main findings and conclusions were the following:

- Motor unit size is maximal at birth and declines roughly exponentially during the first 3 postnatal weeks, during which time there is no reduction in the total number of motor neurons. Therefore, synapse elimination is due to withdrawal of motor nerve terminals and pruning of axon collateral branches rather than to complete degeneration of individual motor neurons.

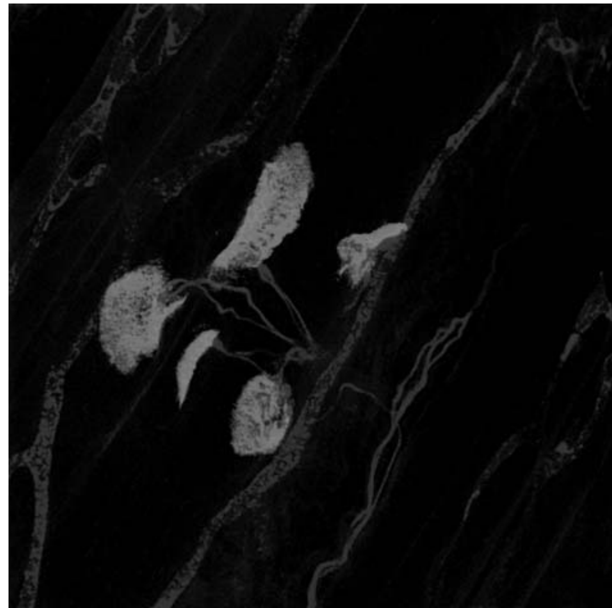

Figure 1 Polyneuronally innervated neuromuscular junctions in an 8-day-old mouse skeletal muscle, stained for neurofilament proteins in axons (green) and acetylcholine receptors on muscle fibers at motor endplates (red). Two axons can be seen projecting in the same motor endplate on several of these muscle fibers.

- The rate of synapse elimination is highest during the first postnatal week. However, virtually all muscle fibers remain innervated by at least two inputs during this period. Mononeuronally innervated muscle fibers emerge rapidly during the second to third postnatal weeks.
- Partial denervation at birth leads to reduced synapse loss in the surviving motor units but does not prevent synapse elimination completely. Mononeuronal innervation therefore arises partly by competition between the terminals of different motor neurons converging on the same endplate and partly by an intrinsic tendency of motor neurons to withdraw a proportion of their terminal branches.
- Implanting the cut end of a nerve that normally innervates one neonatal muscle into another, at some distance away, can result in stable polyneuronal innervation. The distance between converging synaptic inputs therefore mitigates elimination of polyneuronal connections: The further apart two inputs are, the more likely they are to persist and become stable.

A striking feature of the process of synapse elimination at the developing NMJ revealed by these studies is that the great majority of NMJs end up innervated by one and only one motor neuron μ. Although the response to partial denervation at birth suggests that there is a limit to the number of supernumerary branches that can be maintained, it is clear that the

usual result of synapse elimination is not consistent with a random loss of a fraction of the branches formed initially. This would leave some NMJs innervated by several axons and others by none. Rather, the observations point to some sort of local competition at each NMJ that results in a single 'winner.' (Whether synapse formation by motor axon branches is initially random is another unresolved matter.)

Comparable findings were subsequently reported by several groups using similar combinations of histological and electrophysiological methods in studies of reinnervated muscles in adults. Following certain forms of nerve injury, particularly crushing the nerve so that the surrounding connective tissue sheaths remain intact, the damaged motor axons grow back into the target muscle. Regeneration of damaged axons may restore some or all their original neuromuscular connections, in some cases leading to excellent functional recovery. When axons regenerate into partially denervated muscles, they often polyneuronally innervate endplates already occupied by intact motor nerve terminals. In such cases, a form of competition takes place between the original and the regenerated axons. The many similarities between synapse elimination in neonatal muscles and reinnervated adult muscles led to the working hypothesis that the underlying mechanisms are the same.

Synapse Elimination versus Synapse Degeneration

Ultrastructural analysis of NMJs established that the characteristics of synapses and axon collaterals undergoing elimination and withdrawal are quite unlike those of the orthograde (Wallerian) degeneration that occurs when axons are damaged. Wallerian degeneration is rapid and involves radical breakdown of cytoplasmic integrity and organelles within intramuscular axons and motor nerve terminals. By contrast, synapse elimination results in regression of a subset of the collateral branches in a motor neuron's axonal arbor. It is protracted over several days, and for the most part it involves gradual retraction of synapses and resorption of the affected branches into the parent axon trunk with no overt loss of integrity of the cytoplasm and organelles (e.g., mitochondria and synaptic vesicles appear to remain healthy).

Real-Time Visualization of Synapse Elimination *In Vivo*

An improvement in the basic description of the neonatal remodeling of motor units has followed the development of transgenic mice expressing fluorescent proteins in motor neurons. This enables all the connections made by individual motor neurons to be visualized at all stages of development. These transgenic mice have been made by inserting variants of the fluorescent jellyfish protein Green Fluorescent Protein into the mouse genome under the control of a chemically modified promoter, thy1.2, that has the effect of selectively driving protein expression mainly in neurons. Collectively, the members of this family of fluorescent transgenic mice are referred to below as XFP mice (X = R for red, Y for yellow, G for green, or C for cyan). Recently, via Cre-Lox floxed-stop tamoxifen induction, variants have been generated that express variable amounts of YFP. In all these cases, expression of the fluorescent proteins in neurons appears to be completely harmless: The transgenic mice are indistinguishable from their littermates until their brains, spinal cords, and peripheral nerves are viewed in a fluorescence microscope. Use of XFP mice has already revealed interesting new insights into the process of synapse elimination and is likely to continue to do so for some time.

Is competition local? Visualization of XFP-labeled NMJs during postnatal synapse elimination has confirmed and extended previous interpretations based on the more indirect methods applied for the first time by Jansen, Brown, and Van Essen. Studies of motor nerve terminal size, number, and disposition at different stages in the elimination process show that synapses and collateral branches retract into their parent nerve trunks such that during early stages, a dwindling fraction of each NMJ is occupied by the withdrawing synapses. The local competitions experienced by each of the numerous terminals of an immature motor neuron do not occur simultaneously but by an asynchronous process protracted over several days. At any given time, some of the terminals of a single neuron are 'victorious,' fully occupying the endplate, while others show variable fractional occupancies, giving way to or taking over the space occupied by the terminal synapses of other motor neurons. Takeover of endplates during synapse elimination has now been observed directly, by repeated visualization of polyneuronally innervated junctions whose axonal inputs are labeled by selective expression of YFP and CFP in different motor neurons. These direct observations appear to refute the hypothesis previously favored by some, that the main route to mononeuronal innervation was via elimination of acetylcholine receptors and their overlying synaptic boutons, with no takeover by the remainder. The routine takeover of endplates by one terminal, with simultaneous retraction of others, is now a generally accepted account of the normal process leading from polyneuronal to mononeuronal innervation. This view also accords with the progressive recovery

of synaptic area observed in partially denervated muscles following regeneration of injured axons, as suggested by static images of vital staining of regenerating and intact axons.

Expression of different fluorescent proteins (CFP and YFP) in different motor neurons has also been used to study unusual behavior at some NMJ. A few synapses show oscillating 'flip-flop,' in which first one of two inputs occupies most of the endplate at the expense of the other, but then the second gains the upper hand and the most fractional occupancy. It is unknown what determines this remarkable dynamic interaction or its ultimate outcome in favor of one of the two inputs, with complete elimination of the other.

At first glance, it is tempting to conclude that asynchronous retraction and flip-flop behavior suggest that synapse elimination is driven locally, entirely by competitive interactions between converging terminals. However, experiments show that asynchronous synapse loss also occurs when global properties such as axonal transport are interrupted. For example, axonal injury in adult mutant mice with the 'Wallerian degeneration, slow' mutation (*Wld*^{S}), in which orthograde degeneration is delayed, also results in protracted, asynchronous synapse retraction. Thus, observations of asynchronous synapse loss constitute insufficient evidence to conclude that local interactions drive synapse elimination (see below).

The fate of the losers Finally, detailed descriptions have been obtained by time-lapse imaging *in vivo* of the last stages of synaptic retraction. These have confirmed that removal of the losing synapse partly involves retraction of a collateral nerve branch into the parent axon trunk, identified by characteristic end bulbs (retraction bulbs) on the withdrawing axon branch. In addition, combined confocal and ultrastructural (electron microscopic) analysis has also shown that some retracting synapses and axon branches undergo fragmentation, with sequestration of the cellular residues, named axosomes, into Schwann cells and other phagocytic cells in the vicinity of the NMJ. Collectively, the retraction and axosomal fragmentation of synapses undergoing elimination appear remarkably similar to the loss of synapses following axotomy in some forms of neuromuscular pathology. This includes neuromuscular synaptic degeneration in *Wld*^{S} mutant mice after nerve injury; mouse models of some forms of motor neuron disease, such as in the SOD1G93A transgenic mouse model of amyotrophic lateral sclerosis (ALS); and in *smn* mutant models of spinal muscular atrophy (SMA; see below). However, it remains to be seen whether these remarkable morphologically similarities are caused by similar underlying molecular mechanisms.

Are There Intrinsic Hierarchies Among Motor Neurons?

A recent study by Kasthuri and Lichtman seems set to reignite debate over the forces that establish which synapses persist when two motor nerve terminals belonging to different motor neurons vie for exclusive occupancy of the same motor endplates. Transgenic mice expressing YFP in a small subset of motor neurons and CFP in a different subset were examined. Sometimes only two motor neurons supplying the same muscle were labeled, one yellow and the other cyan. Thus, the investigators were able to identify all the polyneuronally innervated motor endplates where both axons contributed a motor nerve terminal. They found that while both axons innervated variable fractions of the endplates they did not share, the same member of the pair nearly always occupied a greater fraction than the other at all endplates that they did share. This suggests that while competitive interactions take place locally at motor endplates, a pecking order or dominance hierarchy may operate within motor neuron pools, intrinsically biasing the outcomes of all their interactions with others during synapse elimination. An important prediction of this hypothesis is that all the branches of the motor neuron with the highest ranking should be 'winners.' By contrast, the lowest-ranked motor neurons should lose virtually all competitions and have either all or nearly all their terminals eliminated.

The suggestion of a fixed hierarchy among motor neurons needs to be viewed in the context of several previous studies. First, time-lapse observations of flip-flop (made by the same research group) made over several days *in vivo* suggest that if there is a hierarchy, then either it may be a dynamic one or the differences that determine the hierarchical rank could be very small and subtle. Second, indirect evidence based on isometric tension recordings in the watershed study by Jansen et al. suggests that some motor neurons withdraw their terminals from motor endplates in the absence of competitors, albeit this observation has been challenged by other studies and the issue remains unresolved. Third, axons regenerating into partially denervated muscles fail to displace sprouts from most of the terminals they previously innervated. Fourth, high levels of stable, persistent polyneuronal innervation are found following recovery from muscle paralysis. However, all but the first of these caveats are based on indirect evidence that predated the availability of transgenic XFP mice. It is to be hoped that the general availability of these mice will eventually allow clarification of the possibility of an intrinsic hierarchy among motor neurons.

The Role of Activity in Synapse Elimination

One of the questions examined in the immediate wake of the Jansen, Brown, and Van Essen study was whether activity plays an obligatory and decisive role in synapse elimination. In rats and mice, synapse elimination occurs in hind-limb muscles as the animals are first beginning to use those limbs to bear weight. By analogy in the visual system, use of the eye during a critical period when the precision of adult connectivity is being established plays a vital part in determining the outcome of that process. It therefore seemed plausible that something similar might happen at the NMJ. Several studies have explored the effect of changes in neuromuscular activity on both the timing and the outcome of synapse elimination, either in development or during reinnervation of adult muscle. The various studies have led to the following conclusions:

- Reducing activity delays elimination of polyneuronal innervation.
- Increasing activity makes synapse elimination occur sooner.
- Selectively altering activity levels in motor nerve terminals at NMJs supplied by convergent motor neurons frequently biases the outcome of synapse elimination in favor of the more active axons.
- Polyneuronal innervation is not an inherently unstable state: it persists indefinitely in active muscle under some experimental conditions.
- Activity is not strictly necessary for synapse elimination since it can occur at some NMJs even when they are experimentally paralyzed.
- Activity-dependent competitive vigor correlates with the synaptic strength of convergent inputs, that is, the amount of neurotransmitter release per unit area.

The overall conclusion from these studies is that activity is strongly influential but not strictly decisive in determining the outcome of synapse elimination. Other variables likely to mitigate competitiveness include intrinsic limits on the numbers of peripheral connections any one motor neuron can support, sensitivity to neurotrophic factors, and selective recognition or adhesion of motor neurons to specific muscle fibers based on either topographic or histochemical markers.

Muscle Fiber Type Specificity and Selective Synapse Elimination

Skeletal muscle fibers are metabolically heterogeneous. For example, some are specialized for brief, faster and others for prolonged, slower contractions. In addition, many are segmentally or compartmentally organized with axonal inputs constrained to arborize within the segmental or compartmental boundaries. Synapse elimination in development therefore occurs largely within rather than between segments or compartments. Occasionally, however, it appears that mistakes are made and editing of these occurs by axon branch pruning.

In rats and mice, some muscle fiber subtypes are already specified at birth: specifically, type I fibers expressing myosin isoforms characteristic of slow-twitch muscles. In contrast, fast-twitch type II muscle fibers develop mainly postnatally. In adults, these subtypes of fibers are selectively innervated by motor neurons whose activity patterns match the muscle fiber type characteristics. There is some evidence for nonselective innervation of muscle fiber types before synapse elimination occurs. Thus in mice in the first week after birth, individual motor units contain both fast and slow muscle fibers. By the time synapse elimination is complete, the homogeneity of the units has increased to nearly its adult level. This suggests that specific matching of motor neuron type to functionally appropriate muscle fiber types plays a role in synapse elimination. However, as yet there are no selective molecular markers for different motor neuron types, so this hypothesis awaits a stringent test.

Molecular Mechanisms of Synapse Elimination and Axonal Pruning

Progress toward understanding the molecular mechanisms that induce and execute synapse elimination has been exasperatingly slow. Neuromuscular synapses are virtually inaccessible to systematic biochemical analysis, because they comprise such a small fraction of the volume of skeletal muscles (unlike the brain, where synapses are the main constituent). However, several studies have used the incidence of polyneuronal innervation as a bioassay for the effects of pharmacological blockers, growth factors, and other treatments on synapse elimination. Virtually all these studies show only transient effects and have thus far provided no clear understanding at a molecular level of the causal events leading to mononeuronal innervation.

Neurotrophic Influences on the Rate of Synapse Elimination

Neurotrophic factors play a crucial role in maintaining the size of neuronal populations and the number and disposition of their connections. For instance, survival and growth of autonomic and sensory

neurons depend on maintenance of physiological levels of nerve growth factor. Some cortical neurons show similar requirements for the related neurotrophin, brain-derived neurotrophic factor (BDNF). Members of another class, the neurotrophic cytokines, have potent effects on motor neuron survival. The effects of different families of these cell-survival molecules have been tested on neonatal muscles to see whether they may play similar regulatory roles in synapse elimination. The results have been largely equivocal and/or negative. For example, administration of either BDNF or ciliary neuronotrophic factor delays synapse elimination in mice, but only by about a day. Remarkably, however, transgenic expression of glial cell line-derived neurotrophic factor (GDNF) in mouse muscle delays synapse elimination by about 1–2 weeks. Unfortunately, GDNF is unlikely to be a physiological regulator because many muscle fibers in normal mice do not express it at the right stage of development.

Role of Proteases and the Ubiquitin-Proteasome System

Some studies have focused on the possibility that regulated proteolysis of extracellular components of the NMJ might be involved in the elimination of supernumerary nerve terminals. There is some evidence that inhibition of either Ca-activated or serine proteases delays synapse elimination. Likewise, it has been reported that activation of protein kinase C delays synapse loss. At present, however, there is no detailed model of how these effects might operate during normal NMJ development.

Recently, there has been interest in the possible role of the ubiquitin-proteasome system (UPS), the mechanism within cells for targeted breakdown of proteins. Proteins tagged with a chain of ubiquitin monomers are recognized by chaperone proteins that convey them to proteasomes, organelles that execute protein degradation. Interest in this system stems partly from the role of the UPS in several forms of neurodegenerative disease, including ALS. There is evidence that the UPS plays a significant role in both Wallerian degeneration and axon pruning. For instance, synaptic degeneration is delayed in *Wld*^{S} mutant mice, in which there is expression of a chimeric protein, part of which contains the N-terminal 70 amino acids of a ubiquitination cofactor, Ube4b. Axotomy induces asynchronous synaptic retraction and fragmentation in these mice. Inhibitors of the UPS also delay axon pruning in *Drosophila* and axon degeneration in the SOD1G93A mouse model of ALS. However, there are as yet no published data on the role of the UPS in neonatal synapse elimination.

Role of Other Nonneural Cell Types at Immature and Adult NMJs

Parts of three cell types are thought to constitute the normal NMJ: muscle fiber, motor nerve terminal, and one or more perisynaptic (terminal) Schwann cells. These structures are bonded to one another by synaptic basal lamina. Nerve injury in adults triggers, after a latent period of about 3 days, proliferation and branching of terminal Schwann cells, which then form a scaffold of bridges between motor endplates. These appear to facilitate reformation of synapses by regenerating motor axons. The average number of Schwann cells per NMJ increases postnatally, but there is no compelling evidence that this process plays a role in selective elimination of inputs during synapse elimination. However, Schwann cells engulf degenerating nerve terminals after axotomy, during Wallerian degeneration, and appear to fulfill a similar function in synapse elimination, with formation of axosomal fragments. Recent availability of transgenic mice expressing fluorescent proteins in these terminal Schwann cells will permit longitudinal studies that should help establish whether they have a role in synapse elimination. NMJs also appear to be a favorable local environment for a population of so-called perisynaptic fibroblasts. These cells have been observed at most NMJs in frogs, chickens, rodents, and human muscle. They lie outside the basal lamina, but although they react by spreading and expression of specific cell adhesion molecules, their precise functions at NMJs remain intriguing but unknown.

Synapse Elimination and Neurodegenerative Disease

Most neurodegenerative diseases show an increased likelihood of onset with age. This applies to most adult forms of motor neuron disease (MND), such as ALS. Most adult forms of MND (more than 90%) are sporadic, the remainder inherited. About 20% of the inherited forms (i.e., less than 2% overall) are due to mutations in genes coding for superoxide dismutase (SOD). However, the availability of good animal models of SOD-dependent ALS has led to this form's being the most widely studied. Overexpression of SOD genes in transgenic mice leads to defective axonal transport and motor neuron degeneration, with clinical signs resembling the human disease. Other forms of MND are neonatal and juvenile. These include SMAs of various types, the most aggressive and earliest onset being type I (Werdnig-Hoffman disease), which is lethal within the first few months of life. In contrast to ALS, most forms of SMA are inherited, and in more

than 95% of cases, the mutations responsible have been pinpointed to the *smn* (survival of motor neurons) gene. There are good animal models of these forms of motor neuron disease as well.

Recent studies of neuromuscular pathology in mouse models of ALS and SMA, as well as other mutants, such as *pmn* and *wasted*, have revealed some remarkable common features. Specifically, neuromuscular synapses within some motor units in all these models degenerate or retract into their parent axons at early stages in the disease process, in advance of axonal or motor neuron cell body degeneration. This has led to three views: first, that these motor neuron disease variants may represent a form of dying-back neuropathy, beginning with primary pathology at neuromuscular synapses; second, that neurodegenerative processes in general may be compartmentalized, with independent mechanisms regulating the degeneration of synapses, axons, and cell bodies; and third, that degeneration of synapses in circumstances like MND may share some common molecular mechanisms with normal forms of synaptic plasticity, including synapse elimination in postnatal development.

These views are not universally held and remain open to stringent experimental validation. For instance, although there may be strong morphological and physiological similarities between developmental synapse elimination and MND pathology in SOD-dependent ALS, recent data suggest that synaptic degeneration in SOD1G93A mouse variants shows loss of synaptic vesicle proteins in advance of degeneration, which synapses undergoing elimination in development do not. Moreover, synapses in SOD1G93A mice degenerate synchronously in some muscles, suggesting a primary axonopathy (consistent with the disruptive effect of the SOD-1 mutation on axonal transport).

Synapses are known to be especially vulnerable to defects in axonal integrity, perhaps partly due to the high metabolic and energetic demands placed on them by the exigencies of synaptic transmission. Thus, it is perhaps not surprising that early loss of synapses could lead to the erroneous conclusion that these foci are the primary loci of disease. On the other hand, primary synaptic pathology has not been conclusively ruled out, either for SOD1-related ALS or for any other form of MND. Synapses contain abundant mitochondria, so these foci of oxidative metabolism may be especially vulnerable to direct, primary effects of oxidative stress. Either way, genetic or pharmacological strategies aimed directly at inhibiting synaptic degeneration could prove highly effective in mitigating disease progression. For instance, *pmn* mutant mice cross-bred with Wld^S mice showed strong evidence of mitigation of disease progression. However, transferring the Wld^S gene to SOD1G93A mice has, at best, only a weak neuroprotective effect. This may be partly explained by the weakening of the protective effect of the Wld^S gene on synapses as mice age. Screens of mutations induced by ethylnitrosourea are currently under way in a search for novel mutations that may protect synapses as effectively as Wld^S protects axons. Such mutations could ultimately be exploited so as to mitigate disease progression in several forms of motor neuron disease.

Summary

We still do not know precisely what genetic, environmental, or even stochastic factors determine whether a synaptic bouton should persist or be removed from a π-junction or indeed whether there is a single deciding factor. Activity levels and sensitivity to neurotrophic factors, selective recognition between motor neurons and muscle fibers, and other intrinsic properties of motor neurons and the muscle fibers they innervate may all help to establish the size of motor units, shaping their organization and orderly recruitment, as we accumulate repertoires of behavior during development that we carry with us into adulthood. Ultimately, all these potential regulators may individually play influential rather than decisive roles. For example, perhaps inactivity induces and sustains polyneuronal innervation merely by stimulating and maintaining nerve branching, enhancing the opportunities for competitive interactions to occur between synaptic terminals. Intrinsic determinants of synaptic strength, differential sensitivity to neurotrophic factors, and differential adhesion may then bias the outcome of the subsequent interplay as motor terminals advance or retreat over endplate territory defined by the boundaries of postsynaptic receptor clusters they require to mediate their primary function. But the final outcome could also be generated by chance, through some fundamentally stochastic process. By analogy, reliable chemical synaptic transmission is produced by an underlying stochastic mechanism in which action potentials elevate the probability of exocytosis rather than determining precisely how many vesicles fuse with release sites in presynaptic active zones. Perhaps chance and necessity, an apt epithet for the forces regulating molecular evolution, may also prove to be a fitting summary of the interplay between environment andgene expression leading to development of functionally appropriate innervation patterns, not only at NMJs but throughout the developing nervous system, thereby individualizing all voluntary behavior.

See also: Neuromuscular Junction (NMJ): Mammalian Development; Neuromuscular Junction: Neuronal Regulation of Gene Transcription at the Vertebrate.

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Schwann Cells and Plasticity of the Neuromuscular Junction

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Introduction

The neuromuscular junction (NMJ) has served as a prototype synapse due to its relatively large size, simple organization, and easy accessibility. Similar to the chemical synapse in the central nervous system, the vertebrate NMJ is composed of three adjacent cellular components: the presynaptic nerve terminal, the postsynaptic specialization, and the synapse-associated glial cells. These synapse-associated cells are called perisynaptic Schwann cells (PSCs) or terminal Schwann cells at the NMJ (**Figure 1**). Previous studies on the NMJ have primarily focused on the roles of nerve terminals and postsynaptic components, while the role of PSCs has been largely overlooked. However, from the 1990s, a barrage of studies have revealed multiple roles of glial cells in synaptic function, formation, and plasticity at NMJs, as well as at central synapses. Emerging from these studies on synapse–glia interactions is the concept of the 'tripartite' synapse, which asserts that, along with the presynaptic nerve terminal and the postsynaptic specialization, synapse-associated glial cells should be considered as an active and integral partner of the vertebrate chemical synapse as well. In this section, we describe the key findings of synapse–glia interactions and the involvement of PSCs in the plasticity of the vertebrate NMJ and focus on the multiple roles PSCs play in synaptogenesis, maintenance, remodeling, and regeneration of the NMJ.

The General Characteristics of Perisynaptic Schwann Cells at the Neuromuscular Junction

In the vertebrate neuromuscular system, motor axons are wrapped around by myelinating Schwann cells, which are responsible for the saltatory conduction of nerve impulses; however, motor nerve terminals are capped by nonmyelinating PSCs instead. The existence of Schwann cells at the vertebrate NMJ was suspected long before the days of electron microscopy. Soon after the initial descriptions of vertebrate NMJs, Louis-Antoine Ranvier was the first to report clusters of 'arborization nuclei' in 1878. These nuclei accompany nerve terminal arbors at NMJs, and are distinct from muscle fiber nuclei. Subsequent histological studies identified these arborization nuclei as nuclei of teloglia, or terminal Schwann cells. The survival of these teloglia after nerve degeneration further suggests that they are non-neuronal cells. Electron microscopy studies in the 1950s and 1960s provided much detailed information about NMJ morphology, confirming the intimate association between terminal Schwann cells, or PSCs, and nerve-muscle contacts.

At the mammalian NMJ, PSCs do not invade the synaptic cleft between the nerve terminal and the muscle surface, and the exclusion may be attributed to laminin 11 in the synaptic cleft. At the frog NMJ, however, PSCs do extend finger-like processes looping around the nerve terminal in between active zones, and are colocalized with F-actin concentrated at the nonrelease domains of the frog nerve terminal. PSC fingers contain L-type calcium channels, but the function of PSC fingers, if any, is unknown. There are approximately three to four PSC somata per NMJ in both mammalian and amphibian muscles, and the PSC number correlates with the junctional size. PSCs are capped with basal lamina, which contain some molecules that are distinct from those in the synaptic cleft and in the extrasynaptic basal lamina of the muscle fiber. It has been suggested that the extracellular matrix associated with PSCs may play a role in guiding nerve terminal sprouts at the frog NMJ, although the molecular mechanisms remain to be studied (see below).

The advance in understanding the role of PSCs has been aided by the availability of several probes that can label them, although most of these probes are not specifically for PSCs as they can also label axonal Schwann cells. The most commonly used probe to label mammalian PSCs is an antibody to S-100, a calcium-binding protein. Recently, transgenic mice expressing green fluorescent protein (GFP) under the control of human S100B gene have been generated to reveal GFP expression in axonal Schwann cells and PSCs at living NMJs. Other probes – such as antibodies to glial fibrillary acidic protein (GFAP), neural cell adhesion molecule (NCAM), L1, LNX-1, growth-associated protein-43 (GAP-43), and the low-affinity nerve growth factor (NGF) receptor p75 – can also be used to label PSCs, although the latter two probes label PSCs only after nerve injury or blockade of nerve activity. Surprisingly, antibodies to myelinating glial markers – such as protein zero (P_0), myelin-associated glycoprotein (MAG), galactocerebroside, and 2′,3′-cyclic nucleotide 3′-phosphodiesterase – can also label PSCs even though they are nonmyelinating. The role, if any, of these myelinating markers in the differentiation of PSCs is not known.

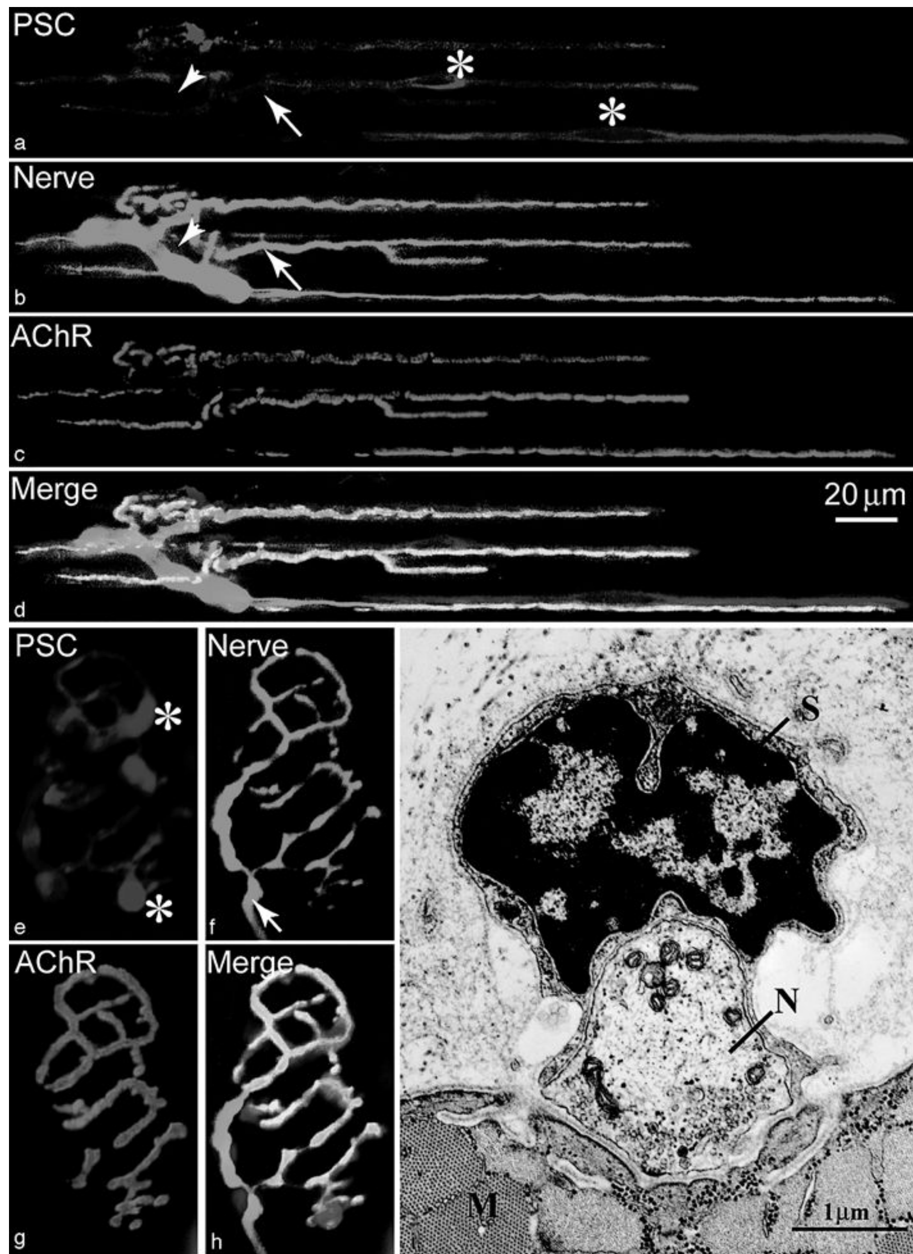

Figure 1 The tripartite organization of vertebrate neuromuscular junctions (NMJs). (a–d) A frog NMJ fluorescently labeled with a monoclonal antibody, mAb 2A12, for perisynaptic Schwann cells (PSCs) (a, green); antineurofilament and antisynapsin I antibodies for axons and presynaptic nerve terminals (b, blue); and α-bungarotoxin (α-BTX) for postsynaptic acetylcholine receptors (AChRs) (c, red). Note that mAb 2A12 labels PSC somata (asterisk in (a)) and processes (arrow in (a)), but not Schwann cells along the axon (arrowheads in (a) and (b)). As seen in the merged image (d), the three components of the NMJ are closely aligned with one another. Scale bar in (d) applies to (a)–(c). (e–h) An NMJ in a transgenic mouse that expresses green fluorescence protein (GFP) in Schwann cells (e, green) and cyan fluorescence protein (CFP) in nerve terminal (f, blue). The NMJ is also labeled with α-BTX for AChRs (g, red). Note that GFP is expressed not only in PSC somata (asterisks in (e)) and processes, but also in Schwann cells along the preterminal axon (arrow in (f)). The tripartite arrangement of the NMJ is further demonstrated in the merged image (h). (i) Electron micrograph of a frog NMJ in cross section shows the PSC (S) with its electro-dense nucleus capping the nerve terminal (N), which is apposed to postjunctional folds on the muscle fiber (M). Scale bar = 1 μm. (a–h) Reproduced from *Journal of Neurocytology*, vol. 32, 2003, 423–1037, Special issue – the neuromuscular junction, Ko CP and Thompson W, Cover picture, copyright Spring with kind permission of Springer Science and Business Media. (i) Reprinted from Reddy LV, Koirala S, Sugiura Y, Herrera AA, and Ko CP (2003) Glial cells maintain synaptic structure and function and promote development of the neuromuscular junction *in vivo*. *Neuron* 40: 563–580, with permission from Elsevier.

In addition to the above probes, there are two probes available for selectively labeling frog PSCs: peanut agglutinin (PNA), which recognizes the extracellular matrix molecules associated with PSCs; and a monoclonal antibody (mAb) 2A12, which labels the external surface membrane of PSCs. One major advantage of these two probes is that they do not interfere with synaptic function and can thus be used as vital probes for PSCs in living frog muscles. Furthermore, mAb 2A12 combined with a complement treatment can be used to selectively ablate PSCs from living frog NMJs without destroying axonal Schwann cells. The application of this complement-mediated lysis provides a unique approach to test the necessary role of PSCs *in vivo* (see below). Unfortunately, neither PNA nor mAb 2A12 labels mammalian PSCs.

With the availability of these probes and the development of new tools such as calcium imaging, it is now possible to investigate synapse–glia interactions at the NMJ. For example, it has been shown that PSCs not only can sense synaptic activity by increasing their intracellular calcium levels but also are capable of modulating transmitter release when G-proteins or intracellular calcium levels are manipulated pharmacologically. Studies on the reciprocal interactions between PSCs and nerve terminals have also unraveled the multiple roles of PSCs in synaptic development, maintenance, and repair at the NMJ, as detailed below.

Role of PSCs in Synaptogenesis

The intimate association between PSCs and nerve terminals seen at adult NMJs has begged a question as to whether synapse–glia interactions also play an essential role in the development of NMJs. It has been shown that, during development, neuronal supply of neuregulin-1 (NRG1) is required for the survival of axonal Schwann cells as well as PSCs. Disruption of the neuregulin pathway by knocking out NRG1, or its receptor ErbB2 or ErbB3, in transgenic mice results in the absence of Schwann cells both in the peripheral nerves and NMJs. Despite the lack of Schwann cells in these transgenic mice, motor axons still project to their target muscles and form transient nerve-muscle contacts. These studies suggest that, during development, while axons are required for the survival of Schwann cells, Schwann cells are required neither for axonal navigation to target muscles nor for the initial formation of nerve-muscle contacts. Consistent with this suggestion is the finding that functional nerve-muscle contacts can be formed in *Xenopus* nerve-muscle cultures in the absence of Schwann cells. However, several lines of evidence suggest that Schwann cells are essential for the subsequent growth, maturation, and maintenance of developing NMJs. First, Schwann cells are present at normal NMJs soon after initial nerve-muscle contacts are formed. Second, the number of PSCs at the NMJ increases and correlates with the size of end plates during the period of rapid synaptogenesis. Third, in the transgenic mice that are deprived of Schwann cells, although initial nerve-muscle contacts are formed, the newly formed synaptic contacts cannot be maintained without the support of Schwann cells. This suggests a possible maintenance role of Schwann cells (see below). However, the specific role of PSCs at developing mammalian NMJs cannot be confirmed since axonal Schwann cells too are absent in these transgenic mice.

To examine the direct role of PSCs in synaptogenesis, repeated *in vivo* observations of tadpole NMJs have been conducted. They revealed that PSCs extend processes beyond nerve terminals, and the subsequent growth of nerve terminals, as well as the addition of synapses, occurs preferably along the preceding PSC sprouts. The necessity of PSCs at developing frog NMJs is confirmed using the PSC ablation technique. Repeated *in vivo* observations demonstrate that less synaptic growth and more synaptic retraction, including a complete loss of synapses in some cases, occur in tadpole muscles several days after PSC ablation. Therefore, PSCs and their sprouts play an important role in guiding extending nerve terminals and promoting synaptic growth, as well as maintaining newly formed NMJs in tadpole muscles.

The necessary role of PSCs in synaptic growth has also been implicated at mammalian NMJs. For example, although partial denervation in adult muscles results in sprouting of both nerve terminals and PSCs (see below), partial denervation in neonatal rat muscles does not induce nerve terminal sprouting. This result could be attributed to the apoptosis of PSCs following nerve injury in neonatal muscles. The withdrawal of nerve terminals seen in mutant mice lacking ErbB2 and ErbB3 is also consistent with a potential role of PSCs in the maintenance of developing synapses (although the involvement of axonal Schwann cells cannot be excluded). Furthermore, both exogenous application of NRG1 to developing rat muscles and induction of constitutively active ErbB2 in neonatal mouse Schwann cells activate the sprouting of nerve terminals and PSCs. Thus, NRG1-ErbB signaling provides an essential mechanism of how axons influence the development of PSCs.

However, the molecular mechanisms of how PSCs promote the growth and maintenance of nerve terminals at developing NMJs are not understood. It has been shown that Schwann cell-conditioned medium

promotes synaptogenesis in *Xenopus* nerve-muscle cultures. One candidate for mediating Schwann cell-promoted synaptogenesis in tissue cultures may be transforming growth factor (TGF)-β1. Whether TGF-β1 and other not-yet-identified factors also promote synaptogenesis *in vivo* remains to be explored. In addition to synaptogenesis, Schwann cell-derived molecules can enhance synaptic transmission in *Xenopus* nerve-muscle cultures. Application of Schwann cell-conditioned medium causes an acute increase in the frequency, but not the amplitude, of spontaneous synaptic currents by more than 100-fold. The identity of the Schwann cell-derived molecules responsible for enhancing transmitter release is currently unknown. Whether Schwann cells also enhance transmitter release at developing NMJs *in vivo* remains to be investigated.

Role of PSCs in Synaptic Maintenance at the Adult Neuromuscular Junction

Do PSCs also play a pivotal role in maintaining adult NMJs similar to developing NMJs? This question has been examined at frog NMJs using complement-mediated cell lysis, as described above for tadpole NMJs, to selectively ablate adult PSCs *in vivo*. Shortly after PSC ablation (within 5 h), neither the ultrastructure of nerve-muscle contacts nor synaptic transmission is affected. Thus, PSCs do not acutely modulate synaptic function and are dispensable for the short-term maintenance of frog NMJs, although PSCs are capable of modulating transmitter release by pharmacological treatments. In contrast to the lack of acute effect, partial or total retraction of nerve terminals is observed at frog NMJs 1 week after PSC ablation. In addition, transmitter release is decreased approximately by half. The overall muscle function as seen in the nerve-evoked muscle twitch tension is also reduced. These observations demonstrate that PSCs are essential for the long-term maintenance of NMJs. The mechanisms by which PSCs maintain adult NMJs are not known. The retraction of nerve terminals does not seem to be attributed to nerve degeneration as there are no signs of Wallerian degeneration – such as electron dense cytoplasm and swollen mitochondria – observed in retracting nerve terminals. The absence of global nerve terminal detachment after PSC ablation suggests that PSCs do not simply serve as mechanical 'glue' to attach nerve terminals with muscle fibers. Schwann cells have been shown to express a variety of trophic factors, including TGF-β1, and extracellular matrix (ECM) molecules. The potential involvement of these trophic factors and ECM molecules in maintaining nerve terminal structure and function remains to be determined.

Recent studies suggest that PSCs also play a similar maintenance role at adult mammalian NMJs. In a transgenic mice model, in which constitutively active ErbB2 is conditionally expressed in Schwann cells, PSCs sprout massively and nerve terminals also extend processes following PSC sprouts in adult muscles. Furthermore, when the expression of active ErbB2 is turned off, both PSC sprouts and nerve terminal sprouts retract. These findings suggest that PSCs can support nerve terminal growth and that activation of the neuregulin signaling pathway in Schwann cells is sufficient to induce nerve-terminal sprouting. However, it is important to investigate the necessary role of PSCs at adult mammalian NMJs using loss-of-function approaches, such as blocking the neuregulin-signaling pathway in PSCs or deleting PSCs *in vivo*. Unfortunately, the fact that the transgenic mice lacking Schwann cells die at birth precludes the study of PSCs' role in synaptic maintenance at adult mammalian NMJs. Since mAb 2A12 does not recognize mammalian PSCs, it cannot be used to ablate PSCs with the complement-mediated cell lysis approach in mammalian muscles. Recently, it has been shown that a subtype of anti-disialosyl antibodies that is found in Miller Fisher syndrome (MFS) preferentially binds and kills PSCs via membrane attack complexes at mouse NMJs. Despite the damage of PSCs following this antibody and complement treatment, both NMJ morphology and synaptic transmission in mammalian muscles are not acutely affected. This result is similar to the finding from frog NMJs following acute PSC ablation. However, no study on the long-term effect after PSC ablation has been performed in adult mammalian muscles. It would also be interesting to examine whether defects in PSCs may contribute to MFS or other neuropathies.

Role of PSCs in Synaptic Remodeling, Degeneration, and Regeneration

Remodeling

Mature NMJs are not static, but undergo synaptic remodeling throughout adult life. For example, extension and retraction of nerve terminals can be seen with repeated *in vivo* observations at frog NMJs in normal intact muscles. Similar to nerve terminals, PSCs are also very dynamic in frog muscles. Using PNA as a vital probe for PSC-associated extracellular matrix, it has been shown that PSCs and associated ECM often extend processes beyond nerve terminals, which grow along the preceding PSC sprouts. Similar dynamic behavior of PSCs and nerve terminals can occur even within minutes, as observed at adult toad NMJs *in vivo*. These results suggest that PSCs

guide nerve terminal growth during synaptic remodeling at adult amphibian NMJs. Although mammalian NMJs are relatively less plastic than amphibian NMJs, extension of short processes of PSCs and minor nerve terminal filopodia and lamellipodia has been seen at adult mammalian NMJs as well. The remodeling plasticity of mammalian NMJs is much more pronounced in androgen-sensitive muscles, and PSC number correlates with end plate size, which can enlarge or shrink in response to androgen treatment or castration, respectively.

Degeneration and Regeneration

After nerve injury, PSCs become phagocytic as they engulf the debris of degenerating nerve terminals and occupy the denervated end plate. Surprisingly, miniature end plate potentials can be recorded even at denervated end plates, albeit at a much lower frequency than those recorded from intact end plates. These spontaneous potentials at denervated end plates are thought to be caused by acetylcholine released from PSCs, but the functional significance of these Schwann cell miniature potentials remains unknown.

Besides being phagocytic, PSCs become very dynamic after nerve degeneration as PSCs sprout their processes profusely beyond the original end plate sites. PSC sprouting can also be induced by blockade of nerve activity. Regenerating axons not only grow along axonal Schwann cells but also extend nerve terminal processes along PSC sprouts. This indicates the importance of Schwann cells in promoting synaptic restoration. Furthermore, regenerating nerve terminals can follow PSC 'bridges,' which are formed by PSC processes between neighboring end plates, to innervate adjacent denervated junctional sites. Using mice that express GFP in Schwann cells and cyan fluorescent protein (CFP) in axons, it has been demonstrated that PSC sprouts induced by denervation guide regenerating nerve terminals. Similar results using vital probes to label PSCs and nerve terminals have also been found in frog muscles after nerve injury and regeneration. However, in contrast to mice, frog PSC sprouting is not induced immediately following nerve injury, but is triggered only upon the arrival of regenerating axons. Taken together, these findings from mammalian and frog muscles strongly suggest that PSC sprouts guide nerve terminal extension and play a central role in the reinnervation of the original junctional sites after nerve injury.

The importance of PSC sprouting in synaptic repair has also been shown in muscles after partial denervation. PSC bridges are formed as PSC sprouts extend from denervated end plates to contact innervated end plates following partial denervation. Consequently, nerve terminals from the innervated end plates grow along these PSC bridges to innervate the neighboring denervated end plates. Thus, similar to PSC sprouts induced by the total axotomy, PSC bridges can guide regenerating nerve terminal sprouts and help synaptic repair following partial damage of axons in adult muscles. The necessity of PSC sprouts in inducing nerve terminal sprouting has also been implicated. As discussed above on the role of PSCs in synaptogenesis, partial denervation of neonatal muscles does not trigger nerve terminal sprouting, which could be attributed to the apoptosis of developing PSCs following nerve injury. Furthermore, poor reinnervation has been reported in aging muscle as well as in *mdx* mice (a model for Duchenne muscular dystrophy), in which PSC bridge formation is impaired. Thus, PSC bridges likely are required for inducing nerve terminal sprouting.

The mechanisms by which PSC sprouting is induced following nerve injury are not fully understood. Activating neuregulin signaling pathway by expressing constitutively active ErbB2 receptors in PSCs can induce PSC sprouting, suggesting that the neuregulin-ErbB signaling pathway may play a role in PSC sprouting and nerve terminal sprouting at adult NMJs, similar to developing NMJs. It has been shown that nerve terminal sprouting can be induced by ciliary neurotrophic factor (CNTF) and insulin-like growth factor 1 (IGF-1). Whether these trophic factors, and other molecules such as GFAP, GAP-43, and p75, which are upregulated in PSCs after nerve injury or inactivity, mediate PSC-induced nerve sprouting is unknown. Nerve activity has also been shown to influence PSC sprouting. For example, although blocking synaptic activity by botulinum toxin or α-bungarotoxin induces sprouting of both nerve terminals and PSC processes, inactivity prevents the formation of PSC bridges in partially denervated muscles. On the other hand, direct stimulation or exercise of partially denervated muscles also impairs PSC bridge formation and nerve terminal sprouting. It is not clear how activity and inactivity can both inhibit PSC bridge formation following partial denervation.

In addition to their guidance role for regenerating presynaptic nerve terminals after nerve injury, PSCs may also modulate postsynaptic specializations by expressing neuronal isoforms of agrin. PSC-derived agrin may explain the appearance of AChR clusters underneath PSC sprouts in frog muscles following nerve injury and regeneration. In addition to agrin, PSCs also express neuregulin-2, which may promote AChR synthesis and synaptic differentiation at mammalian NMJs. Whether and how the postsynaptic role of PSCs may contribute to synaptic repair remains to be further examined.

Conclusions

Despite the traditional view of glial cells as a passive supporting player at chemical synapses, growing evidence has demonstrated that glial cells are an integral and essential component of synapses in both central and peripheral nervous systems. In this section, we have described the roles of PSCs during the formation, maintenance, and regeneration of the NMJ. Axonal navigation to target muscles and the formation of initial nerve-muscle contacts do not require the presence of Schwann cells; however, PSCs are essential for guiding extending nerve terminals and promoting synaptic growth, as well as maintaining developing NMJs. In adult muscles, while PSCs are dispensable for the short-term maintenance of NMJs, long-term absence of PSCs causes retraction of nerve terminals and reduction in synaptic transmission. This is possibly due to the lack of trophic support provided by PSCs. PSC sprouts guide nerve terminal sprouts during synaptic remodeling in intact adult muscles, as well as guide regenerating nerve terminals after nerve injury. NRG1-ErbB signaling is thought to play an essential role in the survival and sprouting of developing PSCs, as well as in inducing sprouting of PSCs and nerve terminals in adult denervated muscles. Similar to PSCs, astrocytes also play multiple roles at CNS synapses; therefore, synapses should be viewed as a tripartite structure, taking into account the contribution of glial cells in the formation, function, and maintenance of synapses. The molecular mechanisms by which synapse-associated glial cells contribute to multiple aspects of the synapse are not well understood. Identification of these mechanisms in the future will lead to better understanding of how synapses form, function, maintain, and repair.

See also: Neuromuscular Junction (NMJ): Mammalian Development; Schwann Cells and Axon Relationship.

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Glia and Synapse Formation: An Overview

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Introduction

The majority of mature synapses are not just composed of a pre- and postsynaptic neuronal element, but also an astrocytic process that envelops the synapse (**Figure 1**). This close spatial relationship has led to the term 'tripartite synapse' to acknowledge the glial contribution. Their synaptic localization means that glial cells are ideally placed to monitor and respond to synaptic activity. Indeed, one glial cell can contact and ensheathe thousands of synapses formed between many different neurons. This morphological specialization suggests important roles for glia in synaptic development and function, which are reviewed here.

Glial Induction of Synaptogenesis

Glial cells are in the right place at the right time to play an active role in neuronal synaptogenesis. For example, there is a temporal correlation between when retinal ganglion cell (RGC) axons reach their target structure, the superior colliculus, and when they form synapses. There is a delay of 1 week between target innervation and synaptogenesis, during which time glia are generated, which suggests that neurons require glial-derived signals to enable them to form synapses. The mechanism by which glial signals act is still unclear. Are they permissive in allowing synapses to form, with the location of synapses determined by neurons, or do they instruct neurons where to form synapses?

The first demonstration of a role for glial cells in neuronal synaptogenesis was by Pfrieger and Barres in 1997. They showed that when RGCs were cultured in the presence of astrocytes they possessed significantly more synaptic activity than when cultured alone in serum-free media. This was later shown to be due to astrocytes inducing the formation of new synapses in addition to enhancing the efficacy of existing synapses. This effect was not due to astrocytes enhancing the survival of RGCs, as the same effect was seen when astrocytes were added after most of the neuronal death had occurred and RGC survival factors were present in the media. Contact between astrocytes and neurons was not required, as the same effect was seen if the astrocytes were grown in contact with the neurons or placed in a feeding layer above them, thus demonstrating that a soluble signal released from astrocytes increased the number of synapses. Interestingly, astrocytes in culture did not require a neuronal signal to stimulate secretion of synaptogenic factors – they were constitutively released. Addition of media conditioned by astrocytes was equally effective in inducing synapses as a feeding layer of astrocytes.

There are a number of lines of evidence demonstrating that the synapses induced by astrocytes are functional (**Figure 2**). First, electrophysiological recordings from RGCs showed an increase in the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) following exposure to astrocyte signals. Second, immunostaining for pre- and postsynaptic markers showed a sevenfold increase in co-localized pre- and postsynaptic puncta, defined as a synapse, following astrocyte addition. Third, FM dye-uptake studies showed that presynaptic vesicle recycling was enhanced by astrocytes. Fourth, electron microscopy analysis of the synapses induced between RGCs by astrocyte exposure showed them to be ultrastructurally normal, exhibiting presynaptic vesicles and an electron-dense postsynaptic density. Taken together these observations show that the synapses induced to form between neurons by glial cells are fully functional.

Glia Induce Synapses in Multiple Neuron Classes

Since this initial study, numerous researchers have demonstrated a role for glial cells in inducing synaptogenesis in multiple classes of neurons. In addition to glutamatergic RGCs, spinal motor neurons, γ-aminobutyric acid (GABA)ergic hippocampal neurons, and glycinergic neurons all show enhanced synapse formation in the presence of glial cells. Also, synaptogenic effects are not restricted to astrocytes. Oligodendrocytes and Schwann cells, whose primary function is to myelinate and ensheathe axons, have also been shown to induce neuronal synaptogenesis.

Perisynaptic Schwann cells, specialized Schwann cells that are present at the neuromuscular junction synapse, are involved in guiding nerve terminals to the muscle during development. These specialized Schwann cells have been shown to enhance synaptogenesis between cultured motor neurons from rodents and between motor neurons and target muscle in *Xenopus*. Perisynaptic Schwann cell processes make contact with the muscle first and the nerve terminal uses the processes as a guide to locate and innervate the muscle. Selective ablation of perisynaptic Schwann

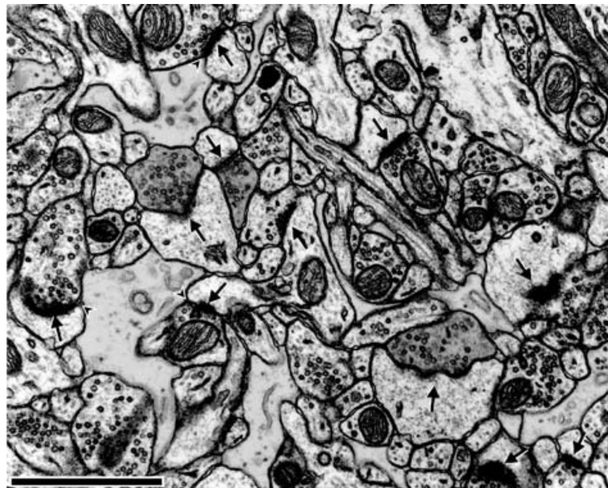

Figure 1 Electron micrograph illustrating the close relationship of astrocytic processes to synapses in the hippocampus. Astrocytic profiles are illustrated (blue) in the vicinity of 11 synapses (arrows). On this section, three synapses have astrocytic profiles at their perimeters (arrowheads). Scale bar = 1 μm. Reproduced from Ventura R and Harris KM (1999) Three dimensional relationships between hippocampal synapses and antrocytes. *Journal of Neuroscience* 19(16): 6897–6906, with permission.

cells in *Xenopus* tadpoles resulted in a decrease in the number of synapses that formed, the retraction of nerve terminals that had already formed synapses, and the loss of postsynaptic acetylcholine receptors.

GABAergic hippocampal neurons undergo enhanced synaptogenesis in the presence of astrocytes, and as for RGCs, the astrocytic effect is via a soluble factor. Astrocytes induce an increase in pre- and postsynaptic clusters, increased surface levels of $GABA_A$ receptors, and increased frequency of miniature inhibitory postsynaptic currents (mIPSCs). One of the downstream effects of astrocyte signals is to regulate brain-derived neurotropic factor (BDNF) and tyrosine receptor kinase B (TrkB) signaling between neurons to enhance synaptogenesis, specifically the maturation of the postsynaptic site and insertion of $GABA_A$ receptors, but the identity of the astrocytic signal is currently unknown.

Glia Induce Synaptogenesis via Secreted Factors

Much work has focused on identifying the soluble signals secreted by astrocytes that induce formation of functional synapses. To date two signals have been identified, thrombospondin (TSP) and cholesterol, but there are likely to be many more.

TSPs are a family of large extracellular matrix proteins that can mediate both cell–cell and cell–matrix interactions. Addition of TSP to cultured RGCs increased the number of structural synapses that formed, to the same extent as that induced by astrocytes. TSP-1 and -2 are expressed in the developing brain during the peak period of synaptogenesis but decrease by adulthood. Mice in which both TSP-1 and -2 had been knocked out formed 30% fewer synapses in their brains. These observations have led to the hypothesis that immature astrocytes provide a developmental window during which synaptogenesis can occur, by producing a permissive environment via the secretion of TSP.

Interestingly, the synapses induced by TSP are postsynaptically silent – they lack the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid subtype of glutamate receptor (AMPAR) but contain extrasynaptic *N*-methyl-D-aspartate receptors (NMDARs). This suggests that a second astrocyte-derived signal is required to trigger insertion of AMPARs into the postsynaptic membrane and make the synapse fully functional.

Another astrocyte-secreted factor suggested to be involved in synaptogenesis is cholesterol bound to apolipoprotein (ApoE). Cholesterol is required for normal neuronal synaptogenesis and can be provided by glia when neuronal cholesterol is lacking. Cholesterol enhances presynaptic function and transmitter release, and contributes to dendrite development, although these effects are strongest in clonal-density cultures where neuronal cholesterol may be lacking. Indeed, it has been demonstrated that if cholesterol is depleted from neurons in culture, then surface AMPARs are reduced due to disruption of lipid rafts.

Therefore, multiple astrocyte-derived signals promote both synapse formation and function. There are many signals yet to be identified, and it remains to be seen whether different signals induce the formation of different synapse types or between different classes of neurons.

Contact-Mediated Synaptogenesis

Glia can also enhance synaptogenesis by contact-mediated signaling. It has been shown that integrin-mediated astrocyte–neuron contact was required to initiate synaptogenesis between embryonic hippocampal neurons in culture. In these experiments neurons were encircled by noncontacting astrocytes to provide trophic support, but very few synapses were formed even in the presence of soluble astrocyte factors. However, when an astrocyte was added directly to a neuron and physically contacted it, multiple synapses were formed. In contrast, direct contact by astrocytes was not needed to induce postnatal RGCs to form synapses. This raises the question of whether astrocytic contact during embryonic development can render neurons receptive to secreted factors that can induce synaptogenesis.

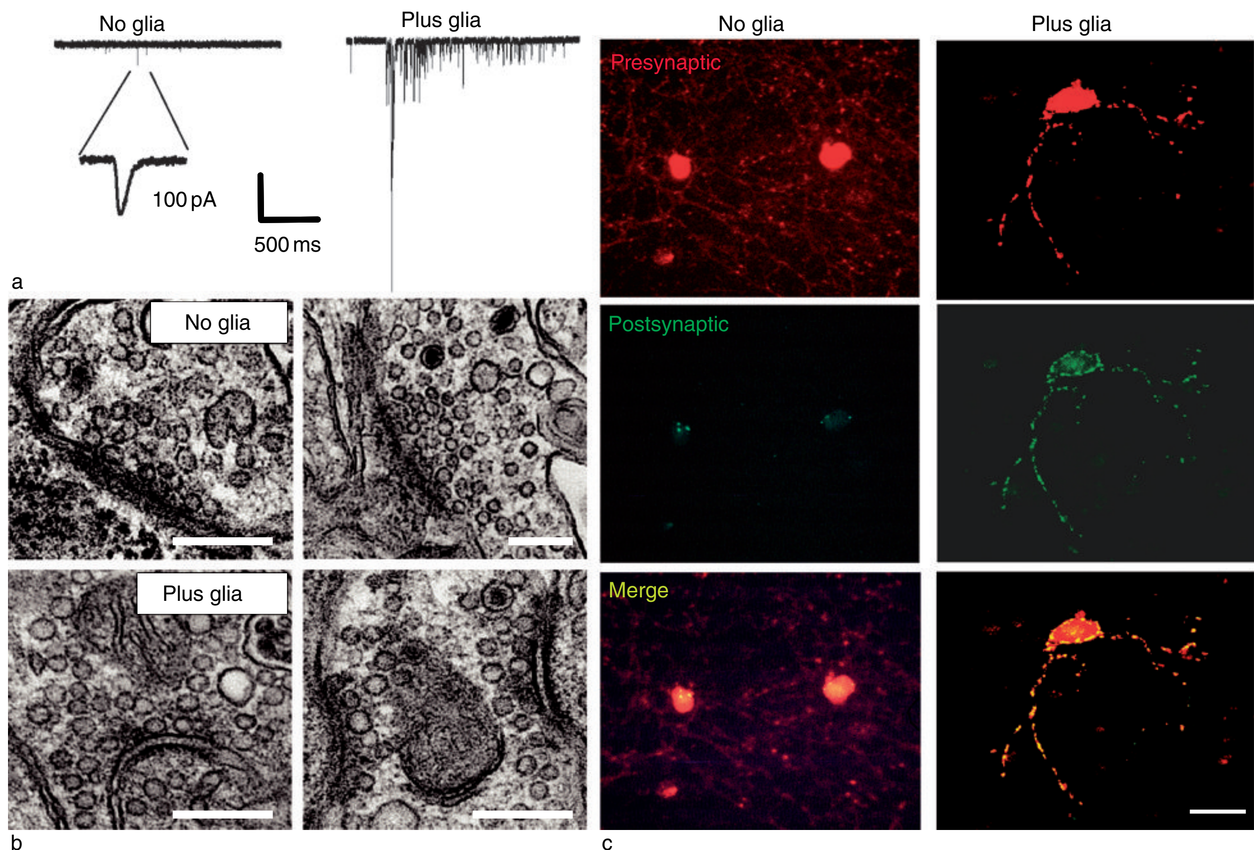

Figure 2 Glia induce functional synapses to form between retinal ganglion cells in culture. (a) Electrophysiological recordings from cultured retinal ganglion cells. In the absence of glia, only a low frequency of small spontaneous synaptic currents is observed, whereas in the presence of glia large spontaneous synaptic currents are frequently observed. (b) Electron micrographs of synapses between retinal ganglion cell neurons cultured in the absence of glia and presence of glia. There is no apparent difference in synaptic ultrastructure between the two conditions. Scale bar = 200 nm. (c) Glia increase the clustering of pre- and postsynaptic proteins and their co-localization. Staining of retinal ganglion cells with an antibody against the presynaptic marker synaptotagmin shows that in the absence of glia presynaptic markers are diffuse (left), whereas in the presence of glia presynaptic markers are discretely clustered (right). Staining of retinal ganglion cells with an antibody to the postsynaptic marker PSD-95 reveals that in the absence of glia there are relatively few PSD-95 puncta, whereas in the presence of glia numerous puncta are apparent. In the absence of glia there is little overlap between pre- and postsynaptic markers, whereas in the presence of glia there is a high degree of overlap. Scale bar = 50 μm. Reproduced from Ullian EM, Sapperstein SK, Christophernon KS, and Barres BA (2001) Control of synapse number by glia. *Science* 291(5504): 657–661, with permission.

Glia Influence Synapse Elimination

During development an excess of synapses are formed, but there is a reduction to the final adult number by a process of synapse elimination. It is hypothesized that elimination can be due to retraction of presynaptic terminals and axons or by degeneration of axons and subsequent phagocytosis of the debris by surrounding glial cells. It has now been suggested that glia are not just scavenging axonal debris, but are actively involved in the pruning process. In the *Drosophila* nervous system, glial cells were shown to engulf axonal varicosities prior to their degeneration. When the ability of glia to phagocytose was perturbed, axon pruning was significantly inhibited. A separate electron microscopy (EM) study showed peroxidase from labeled neurons inside neighboring glial cells in lysosomal compartments, suggesting phagocytosis. These findings are not direct evidence, but strongly suggest that glia actively contribute to pruning in *Drosophila*.

A similar process of axon removal by Schwann cells, termed axosome shedding, occurs during synapse elimination at the mammalian neuromuscular junction. This raises the possibility that glia actively contribute to synapse elimination in mammals. A combination of time-lapse imaging and serial EM showed that as axons disappeared they shed small membrane-bound particles termed axosomes, and that these axosomes appeared inside neighboring Schwann cells. It was suggested that this is a novel

means of transferring information between the cytoplasmic compartments of different cell types.

Glia Modulate Synaptic Strength

In addition to dictating the formation of synapses during development, glial cells are also actively involved in modulating the strength of mature synaptic connections. They do this by releasing factors that alter the number of neurotransmitter receptors present at synapses, and via factors that modulate the release of neurotransmitter and act as cofactors for receptor activation.

Glia, particularly microglia, release cytokines, including tumor necrosis factor-α (TNF-α), which has been shown to modulate synaptic strength via effects on the trafficking of AMPA and $GABA_A$ receptors. Addition of TNF-α to cultured hippocampal neurons increased the surface levels of AMPA receptors while simultaneously decreasing surface $GABA_A$ receptors, thus leading to an overall strengthening of the synapse, a phenomenon known as homeostatic synaptic scaling. Blockade of the actions of endogenous TNF-α led to a decrease in surface AMPA receptors. A glial source for TNF-α was shown by experiments in which TNF-α knockout (KO) glia were cultured with wild-type (WT) neurons and synaptic scaling due to activity blockade was no longer present. There were no deficits in long-term potentiation (LTP) in hippocampal slices from TNF-α KO mice, showing that glial TNF-α is involved in controlling overall synaptic strength but has no role in plasticity.

Glial Release of Transmitters and Neuromodulators

What are the effects of astrocytic transmitter release on synaptic transmission? Astrocytes have been shown to release ATP, which acts to inhibit overall neuronal excitability, and glutamate receptor agonists, which enhance overall activity levels.

Astrocytic ATP release has been implicated in heterosynaptic depression, a process whereby synapses neighboring those that have undergone LTP are depressed. ATP can exert its effects either as itself or by being converted to adenosine in the extracellular space by ectonucleotidases. In the hippocampus glial-derived adenosine has been shown to act on presynaptic A1 receptors, leading to inhibition of calcium channels, a decreased probability of vesicular release, and reduced frequency of sEPSCs. These effects were long-lasting and occurred over a large area – many synapses were inhibited. It is not clear if this inhibition was due to the diffusion of adenosine through the extracellular space or to the release of ATP from multiple regions on the astrocyte or multiple astrocytes within a network.

It has been suggested that glutamate can be released from astrocytes in an activity-dependent manner. Astrocytic glutamate has been shown to activate extrasynaptic NMDAR on neighboring neurons, which could contribute to excitation of the cell. As there is little evidence that glutamate can be accumulated in glia, it remains uncertain whether these effects are attributable to glial glutamate release or release of some other neuroactive substance.

In addition to the release of transmitters that directly activate neurotransmitter receptors, a role for astrocytes in releasing receptor co-agonists has been proposed. D-Serine, which has been suggested to be the endogenous ligand of the glycine binding site of the NMDAR, is expressed in glial cells but not neurons, and is necessary for NMDAR to be activated. There is circumstantial evidence that D-serine can be released from astrocytes in response to neuronal activity and that this release is necessary for hippocampal LTP. When astrocytes were cultured in contact with hippocampal neurons, the neurons were capable of undergoing LTP, whereas neurons grown in media conditioned by astrocytes (but without contact) were not. The addition of exogenous D-serine restored the ability of neurons grown in astrocyte-conditioned medium to undergo LTP. These data, and further studies carried out in hippocampal slices, suggest that astrocytes are actively involved in synaptic plasticity by regulating the availability of D-serine.

Glia Influence Synaptic Structure, Stability and Location

Studies on cultured neurons have shown that if synapses are induced by astrocytes and then the astrocytes are removed, these initial synaptic connections are lost. While the identity of this maintenance factor is unknown, this demonstrates that a constant astrocytic signal is needed in order to maintain synaptic connections.

Studies have shown transient physical coupling between astrocytic processes and synapses via ephrin/EphR interactions in the hippocampus. Dendritic spines express EphA4, which interacts with ephrin-A3 on astrocytic processes. Activation of EphA4 via ephrin-A3 caused a reduction in the length of dendritic spines by 30% and the collapse of 20% of spines, leading to an overall reduction in spine density. Conversely, inhibition of EphA4 caused an increase in spine length and a more disorganized pattern of spines appeared. EphA4 expression levels decrease during

development, and it is present in an inactive form in the adult brain. This raises the question of whether developmental contact between astrocytic processes and dendritic spines plays a role in the elimination and localization of synapses.

Live confocal imaging studies have produced new information about the motility of astrocytic processes at synapses. Imaging of green fluorescent protein (GFP)-labeled astrocytes in brain slices revealed that astrocytes frequently extend and retract fillipodial processes, on a timescale of minutes. Interestingly, this motility only occurred at synaptic sites, not at regions where astrocytes were contacting neuronal somata or blood vessels, and occurred in numerous brain regions. These observations add to the evidence that astrocytes are actively monitoring the synaptic environment.

In the hypothalamo-neurohypophysial system (HNS) astrocytes are capable of fully retracting from synapses, in a reversible manner, under the control of hormonal signals. When oxytocin is released it causes astrocytes to retract from neighboring neurons, which then receive more synaptic inputs. Astrocytes were previously blocking these sites and preventing neuronal innervation. In this way astrocytes can control the location and number of synaptic inputs that a neuron receives, thus influencing the overall activity and output of the neuron. This is a normal physiological process, and a similar phenomenon has been demonstrated in the cerebellum when astrocytic transmitter receptors were altered. Climbing fibers release ectopic vesicles directly onto Bergmann glia (BG) in the cerebellum. Released glutamate activates AMPA receptors on the BG; glutamate spilling over from neighboring synapses would not be at a high enough concentration to do this. BG express calcium-permeable AMPA receptors lacking the GluR2 subunit, which usually confers calcium impermeability to the channel. Expression of GluR2 in BG led to retraction of glial processes from the Purkinje cells (PCs) that they are normally in close association with and caused the PCs to be aberrantly innervated by multiple climbing fibers. Therefore, ectopic vesicular release of glutamate directly onto BG AMPA receptors signals the Bergmann glial cell in a calcium-dependent manner to remain in close apposition with the PC it is surrounding.

In the central nervous system it has been hard to interpret the effects of removal of astrocytes on synaptic function in mature animals. Experiments in which astrocytes have been ablated have had effects mainly in the cerebellum, with the loss of BG. This led to widespread death of granule neurons, presumably due to excitotoxicity from glutamate that accumulated, as the BG were no longer removing it from the extracellular space. The more global effects of astrocyte ablation on neuronal survival make it hard to infer anything about effects on synaptic function.

More subtle perturbations in astrocyte function do lead to alterations in synaptic physiology. Mice in which the astrocyte-specific intermediate filament protein glial fibrillary acidic protein (GFAP) has been deleted show altered synaptic plasticity, with enhanced levels of LTP. Deletion of GFAP will probably affect the structure of astrocytic processes and presumably their synaptic apposition, although whether this is responsible for the enhanced LTP remains to be determined.

An elegant study using the frog neuromuscular junction as a model system investigated the effects of removing synaptic glia from a mature synaptic contact. Perisynaptic Schwann cells in an intact adult frog were selectively labeled with a monoclonal antibody, exposed to complement, and lysed via the complement cascade. This approach left the presynaptic motor neuron terminal and the postsynaptic muscle cell intact, but removed their synaptic partner, the perisynaptic Schwann cell. Interestingly, this had no effect on the structure or function of the synapse until 1 week after the ablation. At this time presynaptic function decreased by half and there was a tenfold increase in the retraction of presynaptic terminals from the muscle. Therefore, glial cells play a role in stabilizing mature synaptic contacts, both in the central and peripheral nervous systems.

The studies discussed here demonstrate that glia are constantly monitoring the synaptic environment and can alter their structure and synaptic association in response to neuronal activity. This is yet another way in which they can control synaptic activity. Glial cells are ideally placed to respond to alterations in neuronal activity and to integrate information from many sources.

Conclusions

Glial cells are intimately associated with synapses at all stages of development and adult life, both in the central and peripheral nervous system. Glia induce the formation of synapses via the secretion of synaptogenic substances, and secrete additional signals that regulate both pre- and postsynaptic function. Glia contribute to the maintenance of synaptic structure and arrangement, ensuring that neurons receive the correct pattern of innervation.

See also: Schwann Cells and Plasticity of the Neuromuscular Junction.

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$GABA_A$ Receptors: Developmental Roles

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Introduction

In adult neurons, GABAergic synapses provide most of the transmitter-gated inhibitory drive, whereas glutamatergic synapses are responsible for the ongoing excitatory drive that impinges on neurons. $GABA_A$ receptor-mediated signaling also plays a key role in the control of neuronal excitability and the generation of behaviorally relevant patterns and oscillations. Activation of $GABA_A$ receptors synchronizes the activity of principal neurons, leading to the generation of gamma and other network-driven oscillations. Blockade of GABAergic receptors generates seizures and other insults, reflecting the importance of an equilibrated activity of GABAergic and glutamatergic signaling. In cortical structures, GABAergic interneurons represent around 10–15% of the total neuronal population and constitute a very heterogeneous population of neurons with unique features that facilitate the control of all aspects of the activity of principal neurons. One key question in developmental neurobiology is how GABA signaling is installed during maturation and how the balance between excitation and inhibition is preserved throughout. This is an important issue considering the fact that any imbalance would lead to either excitotoxic consequences, if the excitatory glutamatergic drive prevails at any time, or inadequate construction of cortical networks and migration disorders, if inhibitory GABA signaling prevails. An equally important question is the maturation of network-driven patterns that shift from silence to adult networks that generate behaviorally relevant patterns. How does the developmental sequence of intrinsic voltage- and transmitter-gated currents provide a substrate for the development of networks? This is of course central in our understanding of how activity modulates genetic programs to entrain the construction of cortical networks.

Studies initiated primarily in Paris almost 20 years ago have unraveled a developmental program in the establishment of GABAergic signaling that appears to have been conserved throughout evolution. This cascade of events includes several key rules that appear also to provide a progressive shift of developing neurons that will first generate a universal primitive pattern that runs on the engine before the network has a sufficient density of synapses and is capable of generating adult patterns and the integrative functions associated with them. These events and how they shed light on the maturation of neurons and networks are discussed in the following sections.

The Excitatory/Inhibitory Shift of GABA Actions during Development

The activation of GABA receptors leads to the opening of a channel primarily permeable to chloride. In adult neurons, the intracellular concentration of chloride is quite low (a few millimoles per liter) and GABA will induce an influx of chloride and a hyperpolarization associated with a shunt of membrane potential that prevents the generation of action potentials. In developing neurons, studies using a wide range of recording techniques, including single GABA channels and perforated patch recordings that are impermeable to chloride, show that the intracellular chloride concentration ($[Cl^-]_i$) is higher (in the range of tens of millimoles per liter) than in adult neurons (**Figure 1**). As a consequence, the activation of $GABA_A$ receptors will lead to quite different actions, including a depolarization, the activation of sodium spikes, and voltage-dependent calcium currents leading to an excitation and an increase of $[Ca^{2+}]_i$. Perhaps even more strikingly, the activation of $GABA_A$ receptors leads to a removal of the voltage-dependent Mg^{2+} block of *N*-methyl-D-aspartate (NMDA) channels and thus to an additional increase of $[Ca^{2+}]_i$. This 'synergistic' action of GABA receptors and NMDA receptors that is quite opposite to the actions of GABA in adult neurons has important consequences in terms, notably, of plasticity. Several studies indicate that coactivations of GABA and NMDA receptors or calcium channels lead to alterations of synaptic efficacy of GABAergic synapses, including a long-term depression or potentiation of GABAergic synapses according to the source of calcium influx activated. They also modulate the formation of novel synapses in neurons that are quiescent. Thus, repetitive electrical stimulation of neurons that are silent – with no functional synapses – triggers the expression of GABAergic synapses, as reflected by the presence of spontaneous GABAergic postsynaptic currents (PSCs).

The developmentally regulated reduction of $[Cl^-]_i$ and the excitatory (E) to inhibitory (I) shift of the actions of GABA (E to I) have now been confirmed in all developing brain structures and animal species studied, suggesting that they have been preserved throughout evolution. It is suggested that excitatory actions of GABA provide a source for calcium influx

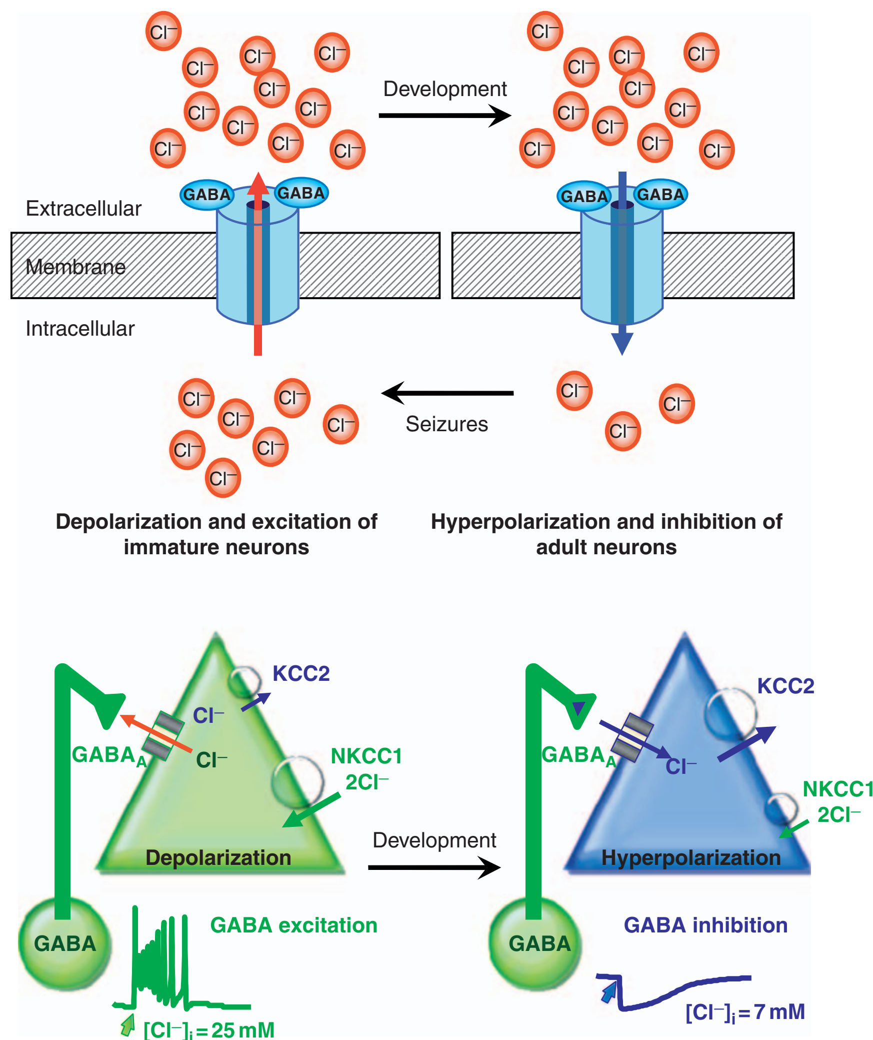

Figure 1 The developmental shift of γ-aminobutyric acid (GABA) actions. In immature neurons, there is a high intracellular concentration of chloride (Cl^-). This is reduced during maturation, leading to an excitatory-to-inhibitory shift. The mechanisms include an early expression of a chloride importer (NKCC1) and a late expression of a chloride transport extruder (KCC2). From Ben-Ari Y (2002) Excitatory actions of GABA during development: The nature of the nurture. *Nature Reviews Neuroscience* 3(9): 728–739.

needed for growth while avoiding the possible excitotoxic actions of unbalanced glutamatergic drive, because of the small magnitude of GABA excitation and the shunting actions intrinsic to its operation. In this model, the E-to-I shift occurs when the density of glutamatergic synapses reaches a level that requires an efficient compensatory inhibition.

How Does the E-to-I Shift Occur?

The concentration of $[Cl^-]_i$ is regulated by several cotransporters, and two of them appear to play an important role (**Figure 1**):

1. KCC2 is the principal transporter for Cl^- extrusion from neurons. KCC2 extrudes K^+ and Cl^- using an electrochemical gradient for K^+. Developmental studies suggest that KCC2 is exclusively expressed in mature neurons: the KCC2 that extrudes chloride follows a developmental expression curve that corresponds well with the higher $[Cl^-]_i$ at an early stage. An early expression by gene transfection of the transporter leads to the expected E-to-I shift. Interestingly, in neuronal cultures, an early expression of the transporter at a time when immature neurons have high $[Cl^-]_i$ leads, in addition to the expected shift, to a selective increase of the density of GABAergic – but not glutamatergic – synapses in the transfected neuron. Therefore, the intracellular concentration of chloride plays an important role in synapse formation and by a yet unknown mechanism may regulate the establishment of GABAergic synapses.

2. The NKCC1 is a membrane transport protein that mediates chloride uptake across the plasma membrane, internalizing one Na^+, one K^+, and two Cl^- ions in an electroneutrally coupled fashion. NKCC1 also plays a housekeeping role in cell volume homeostasis. The early expression of this chloride importer plays an important role in maintaining high intracellular $[Cl^-]_i$. Studies using selective antagonists of this transporter suggest that it also plays an important role in early epileptogenesis.

The mechanisms responsible for the reduction of $[Cl^-]_i$ and the excitatory-to-inhibitory shift of GABA actions are not fully understood. Some observations suggest that GABA itself may do the job – that is, it is the activation of immature GABAergic synapses providing the signal that activates KCC2 and the removal of chloride. This is based on neurons in cultures that do not exhibit the shift when GABA$_A$ receptors are blocked. Other studies, however, cannot be reconciled with such a mechanism and suggest that the shift is programmed and occurs even when GABA signaling is blocked.

Therefore, the E-to-I shift adheres clearly to a basic mechanism that includes the expression of mechanisms that remove excess chloride. However, it remains to determine quantitatively the alterations of $[Cl^-]_i$ that occur dynamically during ongoing activity in developing neurons. The issue is to understand how chloride is dynamically regulated when an accumulation occurs and how that process is modified by activity. Other observations suggest that in a variety of pathological conditions, KCC2 is downregulated, leading to a chronic accumulation of chloride occurring on a persistent basis. This suggests that pathological insults in adults recapitulate the developing brain situation, but also that $[Cl^-]_i$ is an important signal in neurological disorders.

When Does the Shift Occur?

The E-to-I shift occurs in a species- and structure-dependent manner. In the rodent hippocampus, the shift occurs during the second postnatal week, although there are important differences between neurons according to their developmental stage: the shift will occur earlier in interneurons or CA3 pyramidal neurons that mature before CA1 pyramidal neurons. In other structures, the E-to-I shift will occur earlier or later according to developmental programs. This is particularly important in humans and subhuman primates, which have an extended developmental period, because recently born cortical neurons will have excitatory GABA whereas adjacent neurons that belong to the same neuronal population but that became postmitotic at an earlier stage will already operate with thousands of inhibitory-operated GABAergic synapses. This heterogeneity must be taken into account in models of network development. In addition, in a clinical perspective, drugs that augment the efficacy of GABA actions (e.g., benzodiazepines) will affect GABA signaling differently in the mother's brain and that of the embryo at a given developmental stage.

GABAergic Synapses Are Formed before Glutamatergic Synapses

In adult neurons, applications of GABA receptor antagonists generate seizures because of the removal of a tonic and phasic inhibitory drive, whereas similar applications of glutamate receptor antagonists produce a full blockade of ongoing excitatory activity. Studies performed in immature slices indicate, in contrast, that similar applications of GABA receptor antagonists block ongoing activity instead of generating seizures, suggesting that the excitatory drive is provided by GABA. However, the effects of antagonists can be misleading, particularly in developing networks, because of their heterogeneous features and expression of different receptor subunits. Direct demonstration that GABA signaling does in fact mature before glutamate signaling matures came from studies performed in primate hippocampal neurons *in utero* and rodent embryos and early postnatal neurons; in these studies neurons were first recorded, their physiological properties were determined, and morphological *post hoc* reconstruction of neurons was performed to determine their developmental stage (**Figure 2**). This showed that silent neurons with no active PSCs have a small axon and little or no dendrites, neurons with a small apical dendrite have only GABAergic PSCs, and neurons with both GABA and glutamate PSCs have longer and more developed apical and basal dendrites. Neurons with glutamate but no GABAergic PSCs were not encountered, suggesting that GABAergic synapses are formed first on apical dendrites and glutamatergic synapses are formed only once the postsynaptic neuron has reached a certain degree of maturity (**Figure 2**). Since afferent glutamatergic axons are already present at an early stage, this sequence is not due to a late arrival of inputs but rather to different requirements for the formation of GABA and glutamatergic synapses: glutamatergic synapses require a more developed postsynaptic target to form synapses than GABAergic axons. Therefore, once neurons have a primitive apical dendrite, they will form GABAergic but not

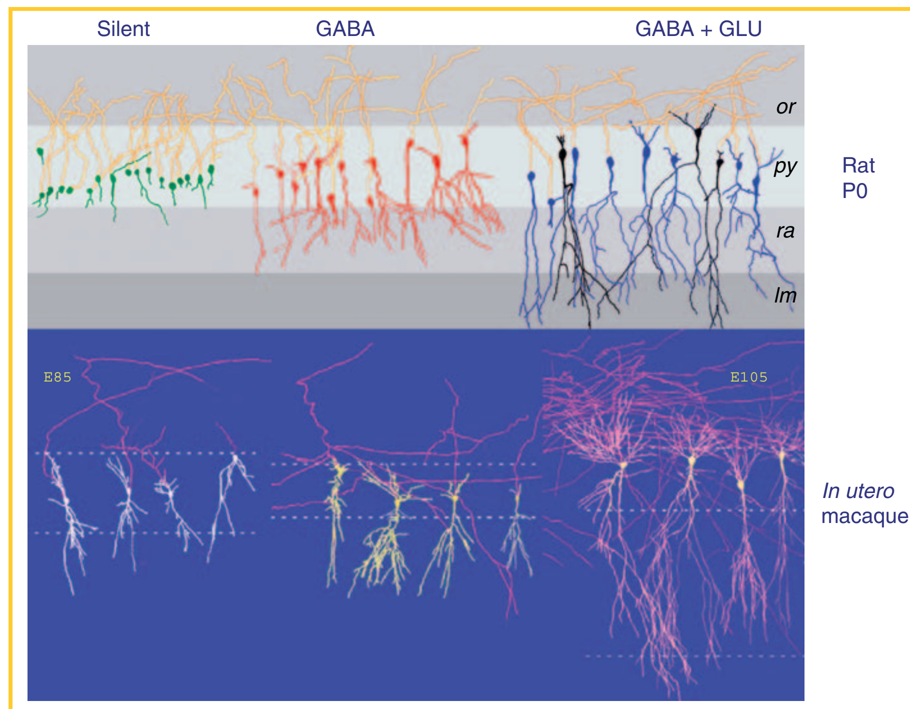

Figure 2 Early formation of γ-aminobutyric acid (GABA) synapses and the GABA–glutamate (GLU) sequence. CA1 pyramidal neurons from rodent slices at birth (top) and from primate hippocampus at various gestational stages (E85 to E105; bottom) were recorded in slices and reconstructed after identification of their synaptic responses. In both cases, silent neurons with no functional postsynaptic currents are small, with an axon but no developed dendrites; neurons with GABA but no GLU postsynaptic currents have a small apical dendrite, and neurons with both GABA and GLU postsynaptic currents have an elaborated apical and basal dendrite. (Top) Data from Tyzio R, Represa A, Ben-Ari Y, et al. (1999) The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. *Journal of Neuroscience* 19(23): 10372–10382. (Bottom) Data from Khazipov R, Esclapez M, Caillard O, et al. (2001) Early development of neuronal activity in the primate hippocampus *in utero*. *Journal of Neuroscience* 21(24): 9770–9781.

glutamatergic synapses, and only once they have well-developed apical and basal dendrites will they also have functional glutamatergic synapses. A similar developmental gradient is observed in other structures. Interestingly, studies in proliferating neurons in the adult fascia dentata show a similar gradient: newly proliferating neurons have initially only excitatory GABAergic synapses and only when they have a more mature dendritic arbor and morphology do they also have functional glutamatergic synapses. Therefore, this sequence is somehow obligatory; neurons must express the E-to-I shift during their maturation. One consequence of this second developmental rule is that at an early stage, GABA provides most if not all ongoing activity.

If GABAergic synapses mature before there is maturation of glutamatergic synapses, then the neurons that synthesize and release GABA must mature before glutamatergic neurons do. This indeed was found to be the case. Patch clamp recordings from immature GABAergic interneurons and reconstruction revealed that they follow the same sequence as the principal neurons do, but earlier: *in utero*, in rodents, interneurons first have GABAergic synapses, and when they are more developed also have glutamatergic synapses, at a stage when most pyramidal neurons are silent. Thus, at E18, in rodents, most pyramidal neurons have no functional synapse at a time when most interneurons already operate with functional GABA and glutamate synapses. In fact, the earliest patterns recorded *in utero* are generated by a network of interneurons, indicating that these neurons, which control the generation of many fundamental adult patterns, also drive early patterns. Therefore, in spite of their long journey along the tangential migration pathway, GABAergic neurons, at least in the hippocampus, become postmitotic first, then establish synapses and extend a dendritic and axonal arbor to organize the first network pattern. The first functional synapses in the developing hippocampus are formed between GABAergic interneurons.

Giant Depolarizing Potentials: A Primitive Network Oscillation, Present in All Developing Systems, That Disappears When the E-to-I Shift Is Completed

Studies performed initially in the developing hippocampus *in vivo* and *in vitro* have unraveled a unique primitive pattern that disappears around the second postnatal week when the E-to-I shift has been completed. This pattern was named 'giant depolarizing potentials' (GDPs; **Figure 3**), since it was identified in intracellular recordings and was characterized by its long-lasting duration and high amplitude at resting membrane potential. GDPs are characterized by the presence of synchronized synaptic currents mediated by excitatory GABA and glutamatergic PSCs and, particularly, by NMDA receptor-mediated PSCs, suggesting that the GABA and NMDA synergistic actions play a central role in GDP generation. GDPs disappear in the hippocampus by the second week of postnatal life, when more elaborated behaviorally relevant patterns, including gamma oscillations, are first recorded. This pattern is universal in that similar oscillations have been observed in a wide range of developing structures and preparations, including the spinal cord and neocortex. The key signal is the completion of the E-to-I shift that is associated with the loss of GDPs, suggesting that the excitatory actions of GABA are instrumental in the generation of GDPs. GDPs can be generated in small slices in all subregions of the hippocampus, suggesting that a small network of GABAergic interneurons and principal cells can generate them.

To study the propagation of GDPs, an interesting preparation was developed: the intact hippocampi from both hemispheres, along with their dissected connecting commissure, are placed in a triple chamber with three independent compartments, allowing a perfusion of the three components with different liquids. With this preparation, GDPs were found to propagate from one hemisphere to the other, and within the same hippocampus to propagate following developmental gradients: higher-frequency GDPs generated in more developed subregions propagate to less developed regions that generate less frequent GDPs. The entire hippocampus behaves like a syncytium, with all regions generating GDPs but more developed regions having a higher density of glutamatergic synapses generating GDPs with a higher probability of propagating to less developed regions. GDPs have been recorded *in vivo* in rodents, including in their neocortex. These patterns may correspond to the 'delta brush' pattern observed in preterm babies.

GDPs thus provide a signal that entrains large networks together at an early developmental stage by means of the excitatory actions of GABA and the activation of NMDA receptor-driven PSCs that are very long-lasting in immature neurons because of the expression of subunits with longer kinetics. The long-lasting kinetics of GDPs are highly suitable

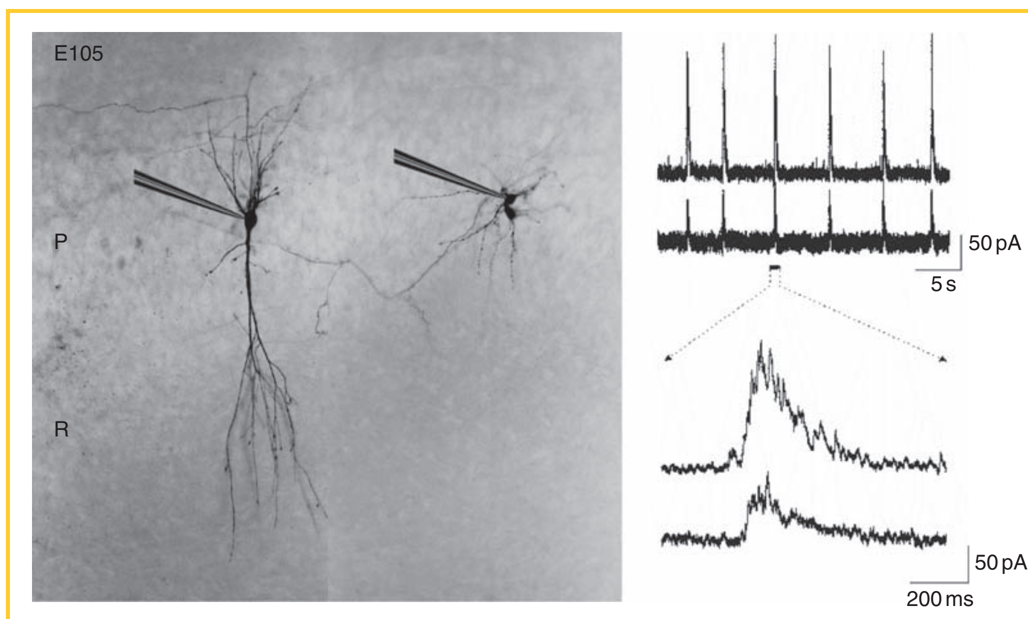

Figure 3 Giant depolarizing potentials in primate hippocampal neurons *in utero* (E105; P, pyramidal layer; R, stratum radiatum). Two neurons were patch clamp recorded and reconstructed *post hoc*: one was a pyramidal neuron and the other was an interneuron. Both had synchronized giant depolarizing potentials. From Khazipov R, Esclapez M, Caillard O, et al. (2001) Early development of neuronal activity in the primate hippocampus *in utero*. *Journal of Neuroscience* 21(24): 9770–9781.

to activate a very heterogeneous population of neurons, some of which have very few active synapses and others of which already have large numbers of synapses. The long time constant of GABaergic PSCs, in comparison with AMPA receptor-driven PSCs, is useful: with a short kinetic event it would have been quite difficult to activate neurons endowed with a few active synapses. This is an instrumental property that will enable neurons that fire together to 'wire' together, and most likely facilitates the formation of functional units. It remains to determine the mechanisms that eliminate GDPs and the relation between the density of active synapses and this shift. Also, the role of activity in that elimination will have to be better understood.

Developmental Curve of Primate Neurons

Are the principal elements of this cascade present in primate neurons *in utero*? This is quite important, considering the relevance to humans, but also because of the ontogenetic implications of better determining the developmental curves of neuronal maturation in rodents and primates. A pioneer study recorded central primate neurons during fetal development: a cesarean operation was performed in pregnant macaques, the fetal brain was removed, and the entire developmental sequence was determined using a morphofunctional characterization of the properties of neurons in relation to their developmental stage (**Figures 2** and **4**). At midgestation, pyramidal neurons of CA1 are immature with little or no dendrites and no functional synapses (i.e., no PSCs). Next, neurons have a small apical dendrite and only GABAergic synapses, and only later do they also form glutamatergic synapses. A quantitative analysis revealed that axons develop before dendrites do; also, whereas at midgestation neurons have no spines, a few weeks before full term they have as many as 7000 spines; these data allow quantitation of the speed of formation of synapses in primate central neurons. GABA excites immature neurons, as reflected by the fact that a GABA receptor antagonist has no effect at midgestation but generates seizures a few weeks later. This also reflects the importance of seizures *in utero*, in that neurons are able to generate synchronized patterns *in utero* and are highly excitable (see later).

The curve depicting this maturational sequence in primates, which in fact is more completed than that in rodents, is shown in **Figure 4**. Note that GDPs are present until a few weeks before delivery; this suggests that the loss of GDP signals is an important maturational stage, when networks can generate behaviorally relevant patterns. Again, the E-to-I

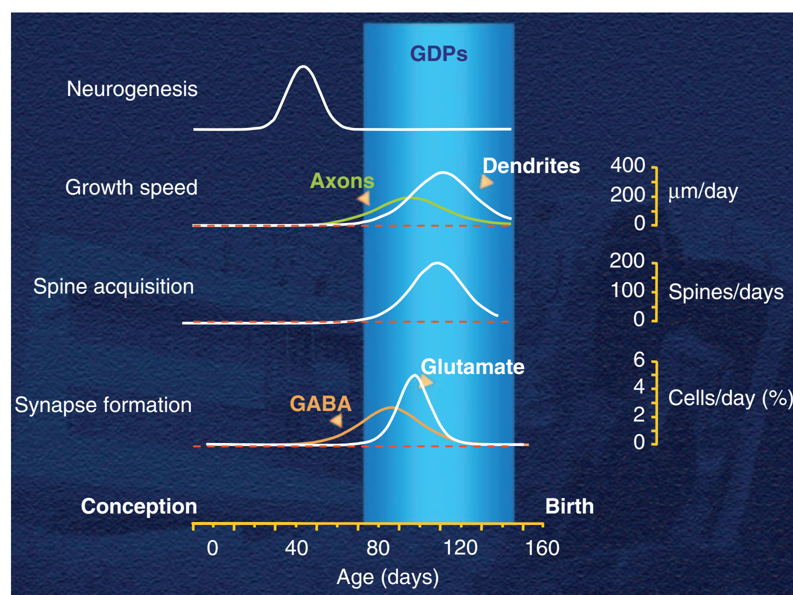

Figure 4 Boltzmann equation used to depict various functions during maturation of primate neurons *in utero*, from neurogenesis in the hippocampus to speed of growth of axons, spine acquisition, and synapse formation. All values are derived from intracellular recordings and reconstruction of primate neurons in slices *in utero* (removed by cesarean surgery), with values calculated. Note the early formation of axons (prior to dendrite formation), the early maturation of GABA signals, and the rapid formation of spines, between midgestation and delivery, shifting from 0 to over 7000 synapses a few weeks before delivery. Note also the domination of giant depolarizing potentials (GDPs) until a few weeks before delivery, indicating that GDPs in primates are an *in utero* pattern, in contrast to that in rodents. From Khazipov R, Esclapez M, Caillard O, et al. (2001) Early development of neuronal activity in the primate hippocampus *in utero*. *Journal of Neuroscience* 21(24): 9770–9781.

shift is an important signal, since the disappearance of GDPs and the completion of the E-to-I shift indicate that at this stage there is a sufficient density of glutamatergic synapses and a network capable of integrative activity. This curve is therefore an important source of information on network development in primates.

Paracrinic Action of GABA Prior to Synapse Formation

In all developing peripheral (i.e., the neuromuscular junction) or central cells, receptors are established before synapses are. Does this contribute to the process that established cortical networks? Do the excitatory actions of GABA also play a role at that earlier stage? Studies performed in immature hippocampal neurons confirm the presence of functional receptors at an early stage and indicate that stimulation of immature neurons that bear no functional synapse generates a long-lasting current present only at an early stage. This early current is mediated primarily by GABAergic receptors, as the current is almost entirely blocked by GABA receptor antagonists. The remaining current is NMDA, but not AMPA, receptor driven. Therefore, these early expressed receptors somehow sense a release of GABA and generate long-lasting currents in neurons with no functional synapses.

How are the transmitters released? Blocking both sodium and calcium currents and vesicular release with toxins fails to block that response. This response persists even in recordings made in a knockout in which vesicular release had been obliterated by inactivating Munc18, a protein required for vesicular release. Therefore, a nonvesicular release of GABA occurs before the conventional vesicular release, and this diffuses to distal neurons to generate long-lasting currents. Again, GABA appears to operate earlier than glutamate does and to exert an important control on early activity.

Are these early actions of GABA – and to a lesser extent, glutamate acting on NMDA receptors – functional? Studies using slice cultures indicate that blocking GABA receptors at a time when neurons have no synapses retards considerably neuronal migration. Therefore, a nonvesicular release of GABA acts on neurons that have receptors, but no functional synapses, to modulate neuronal migration.

The diffusion of GABA to distal sites depends on the efficacy of transporters. Using GABA and glutamate transporter blockers, it was found that glutamate, but not GABA, transporters operate at an early stage. Therefore, GABA will diffuse to distal sites and act in a paracrinic manner whereas the concentration of extracellular glutamate is tightly controlled from the start to prevent toxic actions. In keeping with this finding, *in vivo* and *in vitro* injections of a glutamate transport blocker generate long-lasting oscillations and seizures with a clinical and electrographic pattern reminiscent of encephalopathies.

Trophic Actions of Excitatory GABA

Early studies suggest that GABA exerts a plethora of trophic actions in several systems. GABA stimulates neuronal growth and dendritic arborization, synapse formation, and neuronal differentiation. GABA also exerts an important action on neuronal proliferation by means of action on DNA in neuroblasts. Therefore, at all early stages, GABA can modulate important functions by means of its excitatory action.

I-to-E Shift after Insults: Pathogenesis Recapitulates Ontogenesis

It has long been known that high-frequency electrical activity induces an accumulation of chloride – most likely because the capacity to remove chloride is limited. This is most conspicuously shown in seizures and other insults associated with high-frequency electrical activity. Such activity leads to excitatory actions of GABA in a transient or even quasi-permanent manner; excitatory actions of GABA have been observed in human epileptic tissue months after the last seizure, and various insults, including trauma and lesions, also lead to a long-lasting I-to-E shift.

Recent studies using a convenient novel *in vitro* preparation have enabled determination of the mechanism of induction and expression of this shift and of the electrographic events required for its occurrence. A triple chamber was constructed to accommodate the two intact hippocampi and the commissure connection in three independent chambers that can be perfused with different liquids. One hippocampus was perfused with a convulsive agent (kainate) and the other with an artificial cerebrospinal fluid (ACSF). Seizures generated in one hippocampus propagated to the other hippocampus and led to the formation of a chronic mirror focus, since after a few seizures, the naive hippocampus (the one that did not receive kainate but was subjected to recurrent propagated seizures) became epileptic and generated seizures when disconnected from the other hippocampus. This situation persisted for up to 2–3 days (i.e., as long as the preparation could be kept alive *in vitro*).

This preparation was then used to determine the conditions required for the shift. Applications of an NMDA or GABA receptor antagonist to the naive side – while kainate was applied only to

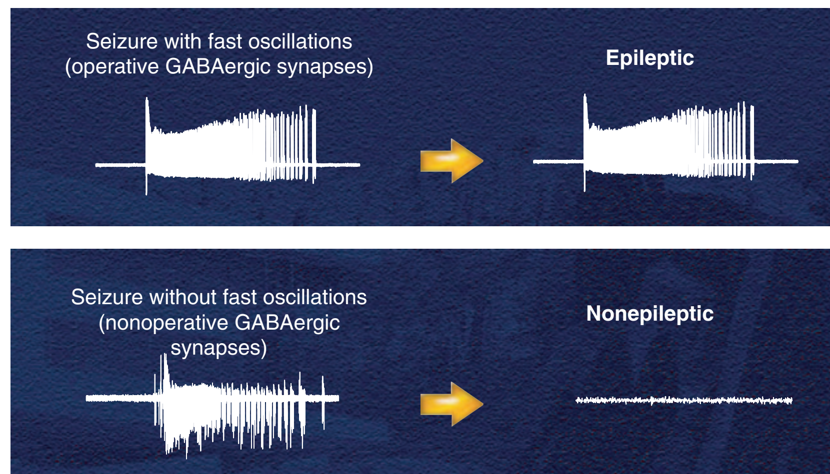

Figure 5 Seizures beget seizures in the triple chamber *in vitro*. When a seizure is generated in one intact hippocampus, it travels to the other hippocampus and leads to the formation of a secondary epileptogenic mirror focus, capable of spontaneously generating seizures. However, this transformation of a naive network to one that seizes requires high-frequency oscillations in the propagated paroxysmal events. If the electrographic event does not include high-frequency oscillations, it will not lead to long-lasting consequences. Since high-frequency oscillations require functional γ-aminobutyric acid (GABA; and *N*-methyl-D-aspartate) signaling for their generation, blocking GABA receptors does not block the seizures but rather prevents their pathogenic consequences: GABA receptor antagonists are anticonvulsive but not antiepileptogenic. From Khalilov I, Holmes GL, Ben-Ari Y (2003) *In vitro* formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures. *Nature Neuroscience* 6(10): 1079–1085.

the stimulated hippocampus – prevented the formation of a mirror focus, although this did not block the propagation of seizures. Therefore, functional GABA and NMDA receptors are required for seizures to beget seizures, although they are not required for the propagation of seizures. What are the elements in the seizure needed for this transformation to take place? When time frequency curves were constructed to evaluate the components of the seizure, it was found that high-frequency oscillations (60–90 Hz) are present in the ictal events that led to the formation of a mirror focus: seizures that included lower frequencies failed to generate a mirror focus, and blocking GABA or NMDA receptors also blocked the high-frequency components of the seizures (**Figure 5**). Therefore, the GABA–NMDA synergy is needed for seizures to activate a persistent transformation of a network from that of control to an epileptic one. Finally, using this preparation, GABA was found to excite neurons in the chronically epileptic tissue, suggesting that epileptogenesis has induced a shift to an immature state: epileptogenesis recapitulates ontogenesis. This illustrates the dynamic properties of the shift and its contribution to neurological disorders.

Implications of These Basic Rules and a General Model of the Establishment of Activity in Developing Cortical Networks

These observations suggest that the unique features of GABA receptors that are not shared by other transmitters (notably, glutamate) – that is, the capacity to shift from excitation to inhibition in an activity-dependent manner – play an important role in early brain development. The high $[Cl^-]_i$ that appears to be a basic feature of developing neurons, most likely an evolutionarily conserved feature, is used by developing neurons to provide a small excitatory drive and is needed to augment intracellular calcium concentrations with little danger, when compared to the potentially toxic actions of glutamate. The long duration of GABA PSCs – when compared to AMPA receptor-driven PSCs – will also facilitate the generation of synchronized events in neurons that have a few functional synapses. The synergistic actions of GABA and NMDA receptors will further stimulate these effects, in particular when the long-lasting actions of the NMDA receptor subunits, present at an early developmental stage, are considered. The synergistic actions of GABA and NMDA will generate the first network-driven pattern capable of synchronizing a wide range of neurons at very different developmental stages. This primitive pattern, which has little informative content, is present to activate neurons synchronistically; it will disappear when a sufficient density of glutamatergic synapses is present and when the E-to-I shift has taken place in the vast majority of GABA synapses. Only at that stage is the network capable of generating more complex, behaviorally relevant patterns. This suggests that this sequence provides a solution to the issue of how to develop excitatory and inhibitory drives while preventing a

transient imbalance between excitation and inhibition that would have potentially excitotoxic actions.

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NEUROGENESIS, NEUROTROPHISM, AND REGENERATION

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Cerebral Cortex: Symmetric vs. Asymmetric Cell Division

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Introduction

The human cerebral cortex contains approximately 10^{10} cells that exhibit a wide array of physiological properties; these cells are wired together by synaptic connections that show remarkable specificity. This highly ordered structure is organized along two axes. Vertically, the cerebral cortex can be divided into six discrete laminae, each of which displays distinct afferent and efferent connections. Horizontally, cortical columns within each region of the cortex subserve specific aspects of cortical function, such as processing somatosensory or visual information or coordinating motor outputs. Despite this impressive architecture, cortical neurons can be grossly subdivided into two major classes, the excitatory pyramidal or principal neurons (80%) that are glutamatergic and provide the major structural inputs and outputs of the cortex, and the inhibitory GABAergic interneurons (~20%) that regulate cortical function through local connections. Pyramidal neurons with characteristic axonal connections, dendritic morphologies, and physiological properties are segregated in each of the different cortical layers. For instance, morphologically, deep-layer (V/VI) neurons have a bigger soma compared to the superficial-layer (II/III) neurons, which typically contain smaller cell bodies. Moreover, a majority of layer V/VI pyramidal neurons project to subcortical structures, including the thalamus, basal ganglia, and spinal cord, whereas layer II/III neurons project intracortically to other cortical areas. Similarly, cortical interneurons at least in some cases are enriched within specific cortical laminae. While the cell bodies of the Martinotti interneurons are found predominantly in the deep layers of the cortex, the neurogliofor interneurons are preferentially found in the superficial cortex. A similar subdivision of both of these populations occurs within the horizontal plane, as best shown by the expansion of input cortical layers (layers I and IV) in sensory regions such as visual cortex and enlargement of output layers of the cortex (layers V and VI) in motor regions. Understanding how such exquisite organization is established during development is one of the major challenges in developmental neurobiology.

Efforts to understand how cortical cellular diversity arises dates back to the late ninteenth century. The first systematic attempts to explore the organization of the developing brain came from the study of early neurogenesis, a time when epithelial cells span their fibers across the entire cortical wall. These epithelial cells, which appear to be equivalent to what we today call 'radial glial cells,' were referred to by His as 'spongioblasts' based on their elongated spongelike appearance and were thought to give rise solely to glial cells. The other 'cell type' observed by His was the rounded 'germinal cells' that reside at the apical surface of the neural tube, which he proposed were the mitotically active neuronal progenitors. This view was challenged by Magini, who observed dramatic morphological variations within the spongioblasts, and suggested that the spongioblasts and germs cells were simply the same population at different points in the cell cycle, an idea that was later confirmed by Sauer. Sauer reached this conclusion through the observation that cell nuclei at the basal side of the neural tube contain an increased complement of DNA (i.e., S phase), while those adjacent to the ventricle were tetraploid (i.e., M phase). This suggested that neural progenitors undergo 'interkinetic nuclear migration' as they progress through the cell cycle and concurrently undergo dynamic changes in their cellular morphology and nuclear position. That this occurred was definitively shown in 1959 where the movements of dividing cells were followed using *post hoc* analysis of cells previously labeled with [^{3}H]thymidine. This autoradiographic method revealed that cortical progenitor cells indeed undergo DNA replication at a distance from the ventricular surface, and then subsequently translocate to the apical surface, where the cells round up and undergo mitosis. From these divisions arise daughter cells that either terminally differentiate into neurons and migrate to the pia or undergo a second cycle of proliferation. Although nuclei prior to neurogenesis translocate across the entire width of the neural tube, subsequent to the onset of neurogenesis, interkinetic migration is confined to a region adjacent to the lateral ventricle. The geographical restriction of cells undergoing interkinetic nuclear migration led to this region being designated as the 'ventricular zone.'

Within the cortex, postmitotic cells exit the ventricular zone and migrate along radial glia toward the pial surface. Surprisingly, the structural role of radial glial cells in guiding migration in the cortex was established long before it was appreciated that the radial glia were themselves the progenitor population that gave rise to newborn neurons. Indeed, for the three decades after radial glial cells were known to support neuronal migration, the radial glia and cortical neuronal progenitors were considered to be two distinct cell lineages, with the former solely

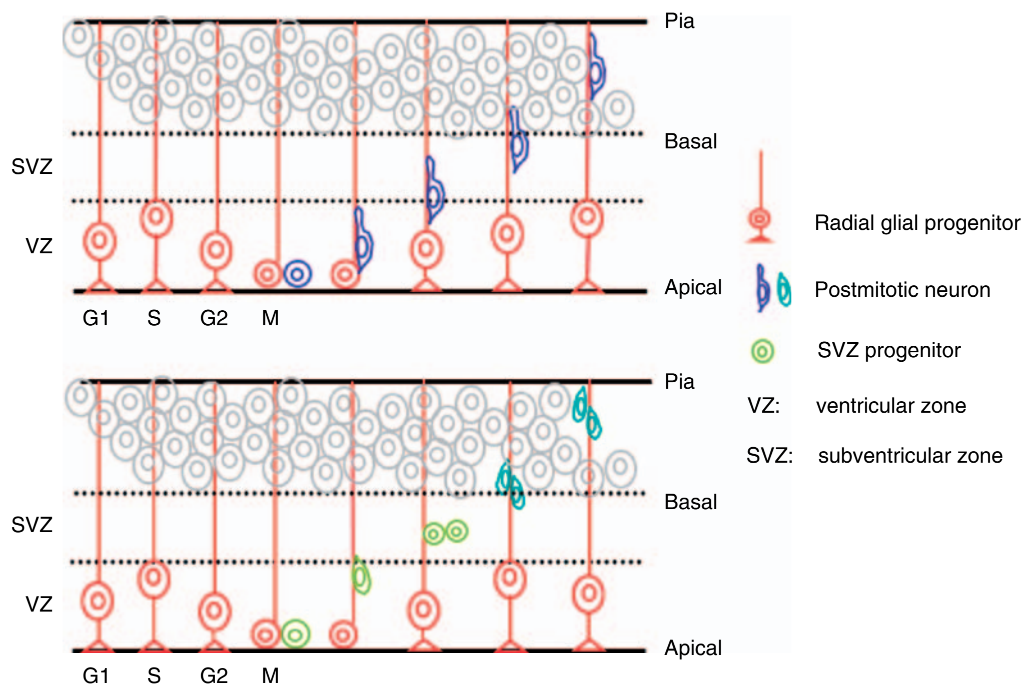

Figure 1 Asymmetric and symmetric cell division in the cerebral cortex. Top panel: Radial glial progenitors undergo interkinetic nuclear migration during the cell cycle. One asymmetric cell division results in the production of two daughter cells, one of which remains in the VZ and inherits the radial fiber and the other of which translocates outward toward the pia along the radial fiber to become a postmitotic neuron. Bottom panel: Alternatively, SVZ progenitors arise from the VZ and subsequently undergo symmetric divisions to produce two postmitotic neurons. Adapted from Noctor SC, Martinez-Cerdeno V, Ivic L, et al. (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nature Neuroscience* 7: 136–144.

producing astrocytes. However, the seminal paper by Noctor and Kriegstein used time-lapse imaging of retrovirally infected cortical progenitors to demonstrate that radial glial cells, in addition to supporting the migration of nascent neurons, gave rise to them. These studies revealed that neurons assembled in close radial units are not only clonally related, but also that radial glial cells can simultaneously give rise to both differentiated neurons and progenitor cells (**Figure 1**). This once and for all established that radial glial cells are the major and perhaps sole progenitor for cortical pyramidal neurons. Moreover, although previous analysis suggested that asymmetric cell divisions occurred in the cerebral cortex, this analysis provided the first definitive evidence establishing the existence of this phenomenon.

Cell-Type Specification in the Neocortex

Temporal Determinants: Birthdate and Laminar Fate

How is the diversity of distinct layer neuronal subtypes created from a restricted progenitor pool within the VZ? The VZ appears to utilize temporal cues to sequentially produce different subclasses of neurons, rather than simultaneously generating multiple progenitor pools, each of which gives rise to particular cell type. Studies examining the origin of distinct neuronal subtypes indicate specific neuronal populations arise at precise times during development, presumably in response to changing temporal determinants. The first evidence that birthdate was a strong predictor of neuronal fate in the cerebral cortex came from [^{3}H] thymidine studies in rodents; it was revealed that the administration of a tritiated thymidine injection at distinct times during neurogenesis resulted in the labeling of neurons within particular cortical laminae. Moreover, this occurred in an inside-out fashion, where early-born neurons occupy the deepest position within the cerebral cortex, while later-born cells migrate past their ascendants, to take up more superficial positions. Hence, in the mature cortex, layer VI neurons are born first and layer II neurons are born last. The only exception to this rule is the earliest-born neurons, the Cajal–Retzius (RC) cells that transiently reside in layer I. The inside-out relation of laminar position to birthdate, although evident in other mammals, is more precise in higher species, such as primates. This has been interpreted as suggesting that the progenitors within the ventricular zone undergo multiple cell cycle divisions, resulting in the production of neurons destined for sequentially more superficial positions.

Direct evidence for the sequential production of cortical neurons came from both static and live-imaging clonal analysis of ventricular progenitors using retroviruses. These studies revealed that individual cortical progenitors produced multiple subtypes over a prolonged period during cortical development. These clonal and birthdating studies raised the question as to how neural progenitors in the ventricular zone change their distinct laminar fate according to temporal cues. One possibility is that extrinsic cues surrounding the ventricular zone change over time to influence the fate of the progenitor cells. Alternatively, it was proposed that progenitors cells might be intrinsically programmed to sequentially produce distinct neuron types at particular developmental timepoints. To test the relative contribution of extrinsic and intrinsic factors to temporal cortical cell-fate determination, classical transplantation experiments in ferrets were carried out to heterochronically challenge ventricular zone progenitors by transplanting them reciprocally to earlier or later developmental timepoints. Specifically, in these experiments early-born progenitors fated for deep layers of cortex (layer VI) were transplanted to periods of development when late-born neurons within layers II/III were being generated. Conversely, cortical progenitors that would normally produce layers II/III were transplanted into host cortical ventricular zone at a time when layer V was being generated. The results revealed a forward ratcheting in the potential of progenitors as development progressed. While early progenitors were found to be competent to adopt later fates when transplanted to older hosts (i.e., progenitors normally fated to produce layer V neurons upon transplantation to later stages of development could produce layer II/III neurons), the converse was not true (i.e., progenitors from later timepoints in development did not produce earlier-born populations of neurons even when transplanted to host animals where earlier populations of neurons are being produced). These results highlight that the cell fate in the cerebral cortex is largely established by intrinsic determinants and that progenitor potential becomes progressively restricted as development progresses. Interestingly, while the molecular mechanism governing these fate restrictions is not well understood, timed transplants of early-born cells to late donors revealed that the fate of neurons is set within 6 h of their final S phase.

Asymmetric and Symmetric Division of Cortical Progenitors Function in the Generation of Cortical Cell Diversity

What is the mechanism by which a common pool of cortical progenitors within the germinal zone gives rise to a broad diversity of neurons over a prolonged period of time? It has been postulated that the mode of cellular division utilized by the progenitor is central to this process. During early periods of neurogenesis, a majority of cortical progenitors undergo symmetric divisions that produce two progenitor cells, in order to expand the cortical progenitor pool. However, that a common progenitor produces distinct types of neurons over multiple cell divisions also implies that later there is a second process by which two daughter cells can assume distinct cell fates. Given that postmitotic daughter cells are produced sequentially, it suggests the existence of asymmetric divisions where one daughter cell reenters the cell cycle, while the other exits to differentiate into a mature neuron. That this can occur hints that during an asymmetric division the two daughter cells adopt fundamentally distinct intrinsic cell-fate programs, where asymmetric divisions unequally bestow cell-fate determinants into only one of the two daughter cells. Considerable efforts have been made to identify these 'determinants.' *Drosophila* neurogenesis has provided a perfect context to study this question, as neuroblasts in the central nervous system (CNS) as well as sensory organ precursor cells in the PNS undergo asymmetric divisions, depending on the precise orientation of their cleavage plane. Notably, the protein Numb, together with Prospero and the adaptor proteins Miranda and Pon, are asymmetrically localized to the basal surface of neuroblasts during metaphase. Therefore, when the orientation of the cleavage plane is horizontal to the apical surface, the daughter cell on the basal side inherits the majority of determinants, such as Numb protein. Thus, asymmetrical cell division requires two events, establishing the cell polarity by regulating the orientation of the mitotic spindle along the apical–basal axis, and targeting Numb and associated proteins to the basal side. Identification of asymmetric cell-fate determinants has come from analysis of the aberrations in the neural lineages of mutant flies, where one of the critical determinants, such as Numb or Prospero, is lost. The loss of these results in a randomized spindle orientation and a corresponding perturbed cell fate. Although the precise function of asymmetric localization of Numb is not clear, it is likely that Numb functions as an inhibitor of Notch signaling, which is required for maintaining neuroblasts in a progenitor state.

In the cerebral cortex, it has been suggested that the plane of cell cleavage may also determine the fate of the resulting daughter cells. Examination of the ferret cortex has shown that around 15% of the mitosis in the ventricular zone has a horizontally oriented cleavage plane, where, following division, one daughter cell retains the apical contact and remains in the ventricular zone, while the basal daughter cell migrates away from the ventricular

zone and becomes a postmitotic neuron. Interestingly, the mouse homolog of *Drosophila* Numb, m-Numb, is asymmetrically localized to the apical membrane of the dividing progenitors in the ventricular zone, suggesting a conserved function of Numb in determining progenitor outcome in mammals. However, a majority of the cell divisions exhibit a vertical or randomly oriented cleavage plane. Thus, the cleavage orientation is not clearly bifurcated into distinct division modes as is the case in flies. Hence, as the 15% of the horizontal cleavage cannot account for all neuronal production during peak neurogenesis period, the relationship between the cleavage plane and asymmetric division, and its contribution to developmental fate in the cerebral cortex, need to be clarified.

What, then, is the function of Numb and Notch proteins in the cerebral cortex? In mammals, the interpretation of loss-of-function of either of the genes is not straightforward, as there are multiple homologs of both genes that appear to functionally compensate for each other. In mouse two *numb* homologs exist, mouse *numb* (*m-numb*) and *numblike* (*nbl*). Mice that lack *m-numb* have highly perturbed development and die prior to E11.5. Although the $nbl^{-/-}$ mutant alone does not exhibit an apparent neural developmental phenotype, this is likely a result of functional compensation between these two genes, as double mutants demonstrate a more severe phenotype and die around E8.5. To circumvent this early lethality, a conditional allele of *m-numb* was generated to test the requirement of this gene during the neurogenic period. The outcome of these experiments suggested that Numb has pleiotropic roles during the corticogenesis period, as distinct phenotypes were observed, depending on when the gene was removed. Conditionally ablating *m-numb* and *nbl* with Cre-recombinase driven under the Nestin promoter, which specifically removes genes starting around E8.5, resulted in an early depletion of the cortical progenitor pool. Similarly, compound removal of *m-numb* and *nbl* during later corticogenesis using a late Cre driver, D6-Cre (which becomes active only after E10.5), also suggested that both genes are required to maintain the progenitor cells in an undifferentiated state. However, removal of *m-numb/nbl* gene function using an Emx1-Cre (which becomes active at E9.5 and results in complete recombination within the cerebral cortex by E12.5) resulted in hyperproliferation of the cortical progenitors as well as inhibition of neuronal differentiation, suggesting that in this context Numb acts to direct progenitors to a neuronal fate. Taken together it is apparent that further work is needed to assess why the function of Numb is diametrically opposite, depending on the context of its removal.

More clear-cut is the function of Notch signaling during neurogenesis. Loss of Notch signaling results in the precocious differentiation of neural progenitors, while overexpressing a constitutively active form of Notch (caNotch) results in inhibition of neurogenesis and promotion of a radial glia cell fate. It is notable that the transient ectopic activation of Notch signaling early in neurogenesis during the period when deep-layer neurons are produced does not disrupt the normal temporal progression of neurogenesis. Upon removal of the ectopic Notch activation, upper-layer corticogenesis resumes normally. Hence even though the transient activation of Notch prevents progenitors from differentiating, on removal of Notch signaling the normal temporal progression of neurogenesis resumes. This implies that the primary function of Notch in cortical development is to maintain the progenitors in an undifferentiated state, but it does so without disrupting the developmental clock that controls the competence of progenitors. In the future, better understanding of the precise roles of Numb and Notch in cortical development, as well as their genetic and biochemical interactions, should prove informative. In addition, whether asymmetrical cell division determines cell fate or merely controls the length of corticogenesis as a means of expanding the number of neural progenitors needs to be clarified.

Does the Segregation of Progenitors to the Ventricular Zone and Subventricular Zone Represent a Bifurcation of Cell Fates?

The types of neurons produced in the cerebral cortex are clearly restricted by temporal cues within the ventricular zone; however, the mechanisms that determine layer neuron specification may not solely depend on temporal changes in progenitor competence. Recent studies have suggested that the restriction in neuronal class production can occur by a spatial segregation of cortical progenitors. In this regard, the emergence of the secondary germinal zone in the neocortex, the subventricular zone (SVZ), may play an important role in determining the identity of upper-layer neurons in the cerebral cortex. The SVZ is a transient zone that is juxtapositioned between the ventricular zone and the intermediate zone. It was traditionally defined by those progenitors that do not undergo interkinetic nuclear migration as they transit through the cell cycle. The SVZ is also distinguishable from the ventricular zone by its lack of pseudostratified epithelial morphology.

It has been postulated that since SVZ develops later than the VZ, SVZ progenitors predominantly give rise to the glial cells at the end of corticogenesis. However, since the peak neurogenesis period of upper-layer (layer II–IV) neurons overlaps with the

expansion of SVZ progenitors, the alternative view is that the spatial segregation between SVZ and VZ may directly restrict the fate of distinct layer neurons. This hypothesis is further supported by the strong correlation in genes expressed in both SVZ and upper-layer neurons. Two genes, *Svet1* and *Cux2*, specifically label mitotic cells in the SVZ. Notably, the level of expression in the SVZ population declines as the progenitors in this pool are depleted. In turn, *Svet1* and *Cux2* are expressed in postmitotic neurons that comprise neurons in layers II–IV. Beyond this simple correlation in gene expression, mutants that have abnormal development in SVZ also show abnormalities in the differentiation of upper layers of cortex. In *Pax6* mutants, the expression of *Svet1* and *Cux2* is downregulated in SVZ progenitors, and the upper-layer neurons fail to be properly established. Furthermore, expression of both *Svet1* and *Cux2* is inverted in *reeler* mutants, suggesting that these genes define the intrinsic properties of upper-layer neurons rather than their laminar position or migratory patterns. It is notable that progenitors change their mode of cell division as they transit from the VZ to the SVZ. During corticogenesis, a majority of VZ progenitors undergo asymmetric divisions, in which the two daughter cells adopt distinct fates to remain a progenitor or become a postmitotic neuron (**Figure 1**, top panel). However, SVZ progenitors are more likely to follow a symmetric pattern of division, where two daughter cells either become a pair of postmitotic neurons or together remain as progenitors (**Figure 1**, bottom panel). It is worth mentioning that not all the progenitors undergo mitosis in the SVZ, even during the peak neurogenesis of upper-layer neurons, and it remains possible that within the VZ and the SVZ there exists greater heterogeneity of progenitors than has yet to be appreciated. In this regard it is notable that in the primate neocortex studies of neurogenesis have subdivided the SVZ into inner and outer zones, the latter of which has been described to be another major source of cortical neurons. The emergence of SVZ during evolution may be the consequence of an evolutional strategy of expanding the diversity or numbers of cortical neurons that can be produced at a given time. Hence during evolution, in addition to the size and complexity of the architecture of the cerebral cortex, the cellular diversity may also have increased.

Intrinsic Factors of Cortical Cell-Fate Specification

Fundamental to cortical neurogenesis is the prolonged production of different siblings from a common pool of progenitors within the VZ and SVZ. This almost certainly depends on intrinsic cues to direct distinct neuronal fates. This process allows the progenitor pool to expand and simultaneously produce different cell types at precise times during neural development. What, then, is the identity of temporal determinants that alter the fate of the neuron types produced? As previously mentioned, cortical progenitors appear to utilize a ratcheting mechanism by which the neuronal types produced over time become progressively restricted. Moreover, later-born progenitors retain a limited ability to differentiate into neuron types prior to their own birthdate. In rodents, the molecular identity of this progressive restriction is best understood in the switch from generating the earliest-born CR cells to deep-layer projection neurons. This process is regulated by the transcriptional repressor *Foxg1*. In the cerebral cortex, *Foxg1* is expressed in the majority of the cortical projection neurons, with the exception of layer I CR neurons. Moreover, in the absence of *Foxg1*, cortical progenitors fail to generate later-born neurons and instead continue to produce the earliest-born CR neurons. In order to determine the cell-autonomous function of *Foxg1* in cell-fate specification, *Foxg1* was conditionally inactivated in deep-layer progenitors. Interestingly, removal of *Foxg1* at E13 (the birthdate of deep-layer neurons) results in the resumption of CR cell production in the cerebral cortex. By labeling the progenitors that were born subsequent to the removal of *Foxg1* with BrdU, it was confirmed that the cells that were normally destined to become deep-layer neurons instead adopted an earliest-cell fate. This work has recently been replicated *in vitro*, where the fate of individual neuroblasts can be directly followed. These studies suggest the early cell-fate specification in the mammalian cerebral cortex is determined by an active mechanism whereby a transcriptional repressor prevents the later-born neurons from adopting an earlier fate.

How is the cortical cell fate established during the subsequent corticogenesis period? It has been reported that a zinc-finger-containing transcription factor, *Fezl*, which is specifically expressed in corticospinal motor neurons in layer V, directs both the specification and identity of these cell types. Loss of *Fezl* results in both the loss of corticospinal neurons and the failure of subcortical projections. Furthermore, overexpression of *Fezl* near the end of layer V genesis can partially override the transition of cortical progenitors to produce neurons that migrate to layer IV, which instead send a projection through the internal capsule. It remains to be explored whether the competence window of cortical progenitors responding to either *Foxg1* or *Fezl* is limited. Beyond this, it seems clear that a great deal

more remains to be learned concerning the intrinsic mechanisms by which temporal competence is controlled within the cerebral cortex.

A Cascade of Intrinsic Determinants Controls the Production of Neurons in *Drosophila*

The progressive restriction of progenitors appears to be central for the specification of distinct cell types in a variety of tissues, such as in the retina and hematopoietic systems. This mechanism appears to be evolutionarily conserved, as similar rules appear to underlie the specification of cells in the nerve cord of invertebrates. In particular, work in *Drosophila* has begun to elucidate the molecular determinants controlling the precisely orchestrated production of different neuronal subtypes during development. In flies, the cell-lineage relationships of distinct neuron types have been well characterized and transcription factors that are specifically expressed in neuroblasts at distinct times have been shown to be required for the acquisition of specific neuronal identities. For instance, the well-characterized NB7–1 lineage consists of 30+ neurons, including 5 Eve+ neurons (U1–U5) and their siblings, and subsequently 20+ interneurons. Analogous to the mammalian cortical neurons, the earliest-born U1 neurons reside in the deepest position and project a longest distance to their target muscle, whereas the latest-born U5 neurons reside superficially and send their processes only proximally. The neuroblasts that produce the 5 Eve+ distinct types express Hunchback (Hb), Hb/Kruppel (Kr), Kr, Pdm, and Pdm/Castor (Cas). It has been demonstrated by removing the function or overexpressing these genes that both Hb and Kr are necessary and sufficient to determine the first-born and second-born cell fates, respectively. Furthermore, overexpression of Hb induces Kr, and similarly expression of Kr induces Pdm, showing that sequential expression of these transcription cascades is tightly regulated to produce these distinct neuron types in an invariant order. It has also been demonstrated that the sequential expression of fate-determining genes is largely cell intrinsic, as the expression occurs in isolated neuroblasts in culture and even can partially progress in the absence of cell cycle progression (Kr-Pdm-Cas). Although corresponding mammalian homologs of Hb (Ikaros family genes), Pdm (SCIP/Oct-6), and Cas (Casz1) exist, the contribution of these factors to temporal lineage progression is not equivalent (SCIP) or has not been addressed (Ikaros, Casz1). It is also noteworthy that in *Drosophila*, the temporal progression in neuroblast identity is dependent on transcriptional factors that positively promote neuroblast identity. This is in contrast with the model in the mammalian cerebral cortex, where at least during the earliest corticogenesis, cell fate is regulated by a repressive mechanism by which a transcriptional repressor, *Foxg1*, prevents deep-layer neurons from adopting an earliest-born CR cell fate. The extent to which future analysis will reveal further similarities between mammalian cortical neurogenesis and that seen in the *Drosophila* nerve cord is presently unclear. Regardless of the precise details, it seems likely that the study of *Drosophila* neuroblasts will prove informative in helping us understand how cortical progenitors generate the diversity seen in the mature cortex. Furthermore, it seems inevitable that considerable progress will be forthcoming concerning the specific transcription cascades involved in generating cerebral cortex neuron diversity, as well as with regard to the mechanisms that ensure symmetric and asymmetric cell divisions that allow the production of the appropriate numbers of neurons at the appropriate times.

See also: Neurogenesis in the Intact Adult Brain.

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Differentiation: The Cell Cycle Instead

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Introduction

Generation of the central nervous system (CNS) requires a fine balance between the processes of cell proliferation, cell fate determination, and differentiation, but the coordination between these processes has only relatively recently begun to be understood. One can envisage models in which cell cycle status influences cell differentiation or, conversely, in which the process of differentiation has effects on the cell cycle. However, it is becoming increasingly clear that there are complex multidirectional links between processes at many stages (**Figure 1**). To understand these links, we must first examine what is known about cell division in the CNS.

Cell Division in the Nervous System

The entire nervous system must be generated from division of a relatively small number of initial precursors during development, and, not surprisingly, a strategy of both symmetrical and asymmetrical cell divisions is adopted. First, neuroepithelial (NE) cells, the stem cells of the mammalian nervous system, can divide symmetrically to generate two identical daughters in what are known as proliferative divisions. Alternatively, they can divide asymmetrically to produce one NE cell and one non-stem cell progenitor or differentiating neuron. Non-stem cell precursors may also divide again to form neurons and/or glia, generating enough cells to populate the nervous system. Neuroepithelial cell progeny can adopt any neural or glial cell fate. However, as development progresses, NE cells divide to produce stem cells with a more restricted range of fate known as radial glial (RG) cells, and most brain neurons are derived from RG cell divisions. Finally, cell proliferation is coordinated with regulated cell death to generate the nervous system.

The progeny of neurogenic divisions (i.e., divisions that generate differentiating neurons) must adopt the correct fate in response to both internal and external cues, and it is becoming increasingly apparent that this process is both coordinated with and influenced by the cell cycle. Indeed, this is obviously essential if the correct number and distribution of neurons and glia are to form in the animal, although the complexity of the relationships involved has meant that, until recently, relatively little has been known about the links between cell cycle and differentiation in the nervous system.

Interestingly, the transition from NE to RG cells and their subsequent progression from proliferative to neurogenic divisions may be influenced by cell cycle length; in particular, G_1 phase elongates as this process proceeds. Experimentally, when a chemical cyclin-dependent kinase (cdk) inhibitor olomucine was used in whole mouse embryo cultures at a dose sufficient to lengthen but not block the cell cycle, this resulted in premature neurogenesis. The 'cell cycle length hypothesis' has been proposed to explain these and other results, where it is suggested that a longer G_1 phase allows factors that promote progression from NEs to RGs to neurons greater time to act. Thus, a lengthened cell cycle will promote these transitions.

Cell Cycle and Cell Fate in the *Drosophila* Nervous System

Important information about the molecular links between cell cycle and cell fate has emerged from studying less complex organisms, which are amenable to genetic manipulation. This is perhaps most vividly illustrated by the differentiation of *Drosophila* neuroblasts, which divide and differentiate in a stereotypical manner that makes them particularly amenable to experimental study. Neuroblasts, the stem cells of the *Drosophila* nervous system, can self-renew but also generate a variety of different neurons and glia; a neuroblast division results in generation of another neuroblast and a smaller ganglion mother cell, which will itself divide again to generate two postmitotic neural or glial daughters. The number of times a neuroblast will divide depends on the expression of a sequential series of transcription factors, which are intimately involved in determining the identity of their progeny. If the cell cycle is blocked prior to neuroblast cell division using a mutant of the cell cycle activator String (cdc25), cells maintain expression of the first transcription factor in the series, hunchback. Therefore, it is passage of the cell cycle and not just passage of time that is needed for the changing pattern of transcription factor expression. However, when both String and Hunchback are knocked out, the cell cycle-arrested neuroblasts do go on to express the normal sequence of transcription factors, so this later phase must be cell cycle independent, indicating the complexity of the situation.

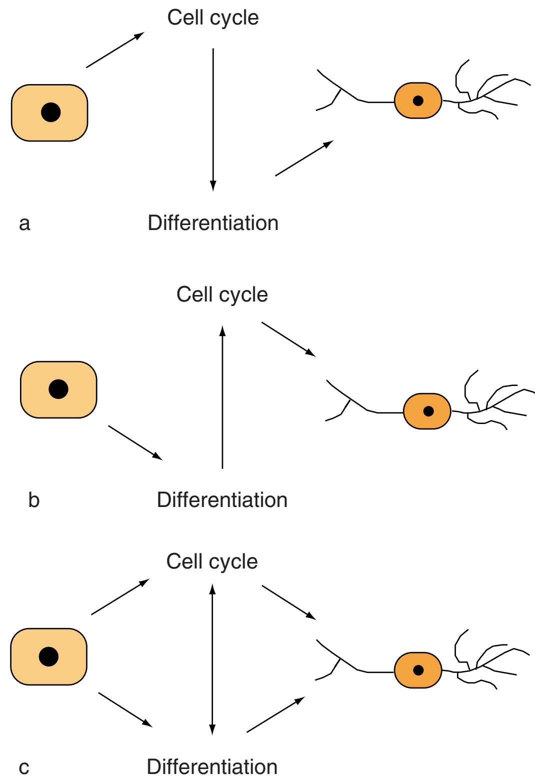

Figure 1 Models linking cell cycle and differentiation processes on the transition from neural precursor (left) to differentiated neuron (right).

Cell Cycle Stage and Cell Fate

The fate of the neurons produced from ganglion mother cell (GMC) divisions is generally determined by expression of transcription factors during the last cell cycle. The same phenomenon may be observed in *Xenopus* retinal progenitors, when transcription factors which determine specific cell fates are upregulated concomitant with a downregulation of cell cycle activator molecules.

Cortical development in mammals shows an unusual 'inside-out' morphology in which the older neurons are found nearer to the ventricular zone, whereas younger-born neurons migrate through to the upper cortical layers, and cells in different layers ultimately adopt specific fates. McConnell and Kaznowski investigated the influence of cell cycle phase on laminar identity in mice. Neuronal precursors from young animals were labeled with tritiated thymidine and then transplanted to an older host. If the cells were transplanted in S phase of the final cell cycle, they were found in layer 3, indicative of an earlier than expected fate, whereas if they were transplanted in mitosis, the cells were ultimately found in layer 6, their expected site. Therefore, it appears that laminar fate is determined in S phase of the final cell cycle and that cell cycle status influences differentiation. There are several ways in which cell cycle stage could influence cell fate, and these may be dependent on events specific to that stage.

Exit from the cell cycle, transiting from G_1 into a G_0 phase, is required for neuronal differentiation. Therefore, it is intuitive that factors influencing the cell cycle will influence differentiation; indeed, cell cycle regulators can directly influence the activity of determination factors. Moreover, there could be cell cycle-dependent expression of transcription factors that influence determination. This is clearly seen, for example, with the homeobox transcription factor prox1, required for determination of horizontal cells in the retina, which is maximally expressed at G_2 phase.

Other characteristics of specific cell cycle phases can also be exploited to coordinate cell fate determination and differentiation. For instance, as described previously, cortical neuronal cell fate seems to be largely determined in S phase. This may be a result of chromatin remodeling which can occur most efficiently at this phase, when complex chromatin structures are opened up to allow DNA replication. This suggestion is supported by evidence that chromatin remodeling complexes play a crucial role in neuronal differentiation. For instance, mice lacking Srg3, a core subunit of the SWI/SNF chromatin remodeling complex, have severe defects in neuronal proliferation and differentiation. Moreover, neurogenin is a central basic helix–loop–helix (bHLH) transcription factor that influences cell fate in many areas of the nervous system, at least in part, by influencing chromatin structure via recruitment of remodeling complexes.

Cell fate can also be determined in mitosis by asymmetrical distribution of determining factors, which is affected by orientation of the mitotic machinery. For instance, in *Drosophila*, Prospero is a transcription factor that is preferentially passed to GMCs and inhibits neuroblast (NB)-specific gene expression. Failure to divide results in repression of NB identity. In addition, blocking the function of the mitotic kinases cdc2 and Aurora A leads to impairment of cell fate determinant localization. Moreover, cyclin E expression may influence symmetry of neuroblast division in the *Drosophila* abdomen, independent of its effects on the cell cycle.

Cell Cycle Genes Regulate Cell Fate and Differentiation in the Nervous System

Differentiation of neurons is likely to be influenced by the proliferation status of the cells. In addition, many instances are now emerging of cell cycle regulators playing a direct role in influencing cell fate and differentiation in the nervous system and even playing a role in some postmitotic aspects of neuronal

function. Although our understanding is far from complete, some interesting themes are emerging, particularly the occurrence of two independent but complementary functions residing within the same cell cycle regulator. Such an approach allows precise coordination between cell cycle exit and differentiation processes and, as such, makes good developmental sense.

The retina is a good system in which to study whether cell cycle influences cell fate because a wide range of different cell types are produced from a single neurogenic precursor type. Normally, early born cell types arise from precursors that exit the cell cycle at an early stage, whereas the converse is true for late-born cell types. Cycling, *per se*, is not essential for producing cell diversity; blocking DNA replication early in retinogenesis still results in production of cells of all the expected histogenic cell types. However, the proportions of these cells are influenced by the cell cycle. For example, atonal-type bHLH transcription factors can influence histogenesis; overexpression of Xath 5 promotes early fates such as the production of retinal ganglion cells. Early cell cycle exit, brought about by co-overexpression of the *Xenopus* cdki Xic1, potentiates this effect, whereas inhibiting the cell cycle by overexpression of cyclin E does the opposite – inhibiting early and biasing toward late cell fates.

Cell Cycle Progression

The cell cycle is driven forward by the action of cdks (**Figure 2**) that phosphorylate key targets such as initiators of DNA replication, proteins that regulate nuclear envelope breakdown and spindle formation, and so on to bring about cell cycle transitions. Different cdks act at different phases of the cell cycle, and their activity depends on their binding to specific cyclins. Cyclins, in turn, are regulated not only by transcription but also at the level of protein stability.

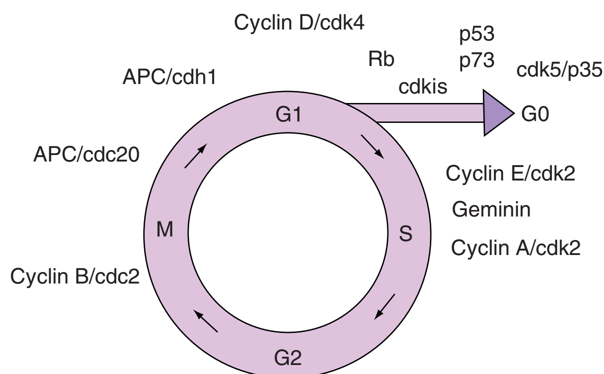

Figure 2 The cell cycle. Cell cycle regulators act at different phases of the cell cycle and also influence cell fate and differentiation in the nervous system.

For instance, the transition from G_2 to M phase is brought about primarily by the activity of cylin B/cdc2. To exit mitosis, cells must degrade cyclin B, and this is effected by ubiquitin-mediated proteolysis controlled by the anaphase-promoting complex (APC), bound to cdc20. Cyclin B levels are kept low in G_1 phase by the continuing activity of the APC, this time bound to cdh1.

Another key transition point in the cell cycle occurs in G_1 and is known as the restriction point (R). After cells have passed R, they can traverse the rest of the cell cycle without any further external stimulus, and passage through R corresponds to the phosphorylation and inactivation of the retinoblastoma protein. Alternatively, cells can exit the cell cycle before R and enter G_0, a process that is facilitated by the activity of cyclin-dependent kinase inhibitors (cdkis). After cell cycle exit into G_0, cells undergo differentiation (**Figure 2**).

Cyclin-Dependent Kinase Inhibitors

The role of cdkis in neuronal differentiation of mammals has been reasonably well studied, particularly using an oligodendrocyte model system. O-2A cells are bipotential progenitors that have the ability to differentiate into type 2 astrocytes or oligodendrocytes. Differentiation in the presence of mitogens is regulated by an internal clock that measures time rather than cell divisions, and the cdki p27Kip1 (Kip1) is a component of this clock; Kip1 levels rise progressively through O-2A cell divisions and then reach a plateau at the time of cell cycle exit and differentiation. Experimentally, the speed of the clock can be altered by growing cells at different temperatures. This works by changing the levels of Kip1, a protein whose stability is controlled by ubiquitin-mediated proteolysis. Interestingly, the cdki p21Cip (Cip1) is also involved in O-2A differentiation; Kip1 is needed for cell cycle arrest, but Cip1 may have a role in differentiation independent of its ability to control cell cycle progression. In mice, it has been shown that Kip1 can independently promote neural differentiation and migration in the cerebral cortex and at least some of these functions are independent of its ability to inhibit cdks. A third mammalian cdki, p57 Kip2 (Kip2), can regulate differentiation of neurons where it has been shown to play two roles in amacrine cell differentiation – promoting cell cycle exit and then postmitotically influencing amacrine subpopulation development.

Indeed, although the most well-established function of cdkis is to inhibit the kinase activity of cyclin-dependent kinases, many studies have demonstrated that cdk activity can directly affect neuronal

differentiation as well as affect postmitotic neuronal functions. Nerve growth factor (NGF)-induced differentiation of PC12 cells into neurons is associated with a decrease in cdc2 and cdk2 activity, and strikingly, inhibition of cdc2 and cdk2 activity alone is sufficient to trigger PC12 cell differentiation. Normally, Cip1 levels rise dramatically on PC12 cell differentiation, and indeed, ectopic expression of Cip1 alone is enough to mimic NGF-induced differentiation and trigger the formation of neurons.

The Cdki *Xenopus* Xic1 has complementary but independent roles in regulating other functions independent of its ability to arrest the cell cycle. Müller glial cells are the last cells to be born in the *Xenopus* embryonic retina, and this coincides with accumulation of the highest levels of Xic1. Overexpression of Xic1 in the retina strongly promotes formation of Müller glial cells at the expense of neurons. Mutational analysis of Xic1 has demonstrated that this activity resides near its N-terminus and is independent of its ability to arrest the cell cycle or inhibit overall cdk kinase activity. Similarly, Xic1 is required for differentiation of primary neurons in *Xenopus*, the first neurons to differentiate out of the neural plate. Again, this activity is independent of cell cycle effects and found near the N-terminus. Interestingly, in this case Xic1 has been shown to act at an early stage of neurogenesis, in parallel with the bHLH transcription factor neurogenin, where it may influence neurogenin protein stability. Such cell cycle-independent functions of cdkis in the mammalian nervous system have also recently emerged.

Cyclins and Cyclin-Dependent Kinases

Different levels of cyclins have interesting influences on neuronal cell fate and differentiation. Increasing the level of cyclin E in the developing *Xenopus* retina promotes late-born cell fate types. In mammals, cyclins D1 is found at high levels in proliferating cells of the retina and cerebellum, whereas cyclin D3 is found specifically in differentiating retinal Müller glial cells. Cyclin D2 knockout mice have a decrease in granule cell number and have no stellate interneurons. However, basket and Golgi interneurons are unaffected in the mice even though they come from the same precursor, suggesting that cyclin D2 influences cell type as well as cell cycle. Cyclin D1 null mice have reduced thickness of all retinal cell layers, possibly the result of a reduction in proliferation and specific apoptosis of photoreceptors. Surprisingly, cyclin D1 levels rise on differentiation of PC12 cells, but because there is apparently no increase in associated cdk4 kinase activity, the role of this is unclear.

Cdk5 is unusual among the cdks in that it does not seem to play a direct role in cell cycle progression. Instead, it has important functions in postmitotic neurons, in which it complexes with the p35 and p39 proteins to regulate differentiation and neurite outgrowth. However, because cdk5 can also complex with cyclin D and Kip1, it may also have influences on the cell cycle, although these have been poorly investigated. However, one study has shown that cdk5 can phosphorylate and stabilize Kip1 protein in postmitotic neurons, in which it is required to regulate neuronal migration.

The Anaphase Promoting Complex

Cell cycle progression is driven forward by the action of cdks, which are activated by binding to cyclins. To drive the cell cycle in one direction, cyclins must be cyclically destroyed and this is brought about via their ubiquitin (Ub)-mediated proteolysis. The APC is an E3 ligase required to facilitate conjugation of Ub to a variety of substrates, including cyclin B. In mitosis, APC is activated in part by the presence of a cdc20 subunit to target mitotic substrates, whereas this is replaced by cdh1 in G_1, in which the APC still has E3 Ub ligase activity. Remarkably, APC/cdh1 is highly expressed in postmitotic neurons, and a cdh1 knockdown has been shown to promote neurite outgrowth. Moreover, APC/cdh1 controls synaptic size in *Drosophila* via its ability to control stability of the liprin protein. Again, we see a protein complex with dual roles in controlling the cell cycle and other aspects of neuronal differentiation/function.

The Retinoblastoma Protein

One of the key targets for phosphorylation by cdks that allows them to control cell cycle progression is the retinoblastoma protein (Rb). Rb is required to regulate the passage through G_1 phase. At its most simple level, underphosphorylated Rb protein, found in early G_1, binds to and represses the transcriptional activity of the E2F family of transcription factors, whose targets include many genes that are essential for progression into S phase. As G_1 proceeds, Rb becomes progressively phosphorylated first by cyclin D/cdk4 and then by cyclin E/cdk2. Phosphorylated Rb is no longer able to bind to E2Fs, so it releases them to allow transcription of E2F-dependent genes and transition into S phase. However, it should be emphasized that this is a very simplified view of Rb function; Rb has been shown to bind numerous proteins besides E2F, where the function of these interactions is often unknown, and there are several members of the Rb family with at least partially overlapping functions.

Rb was first identified as a gene inherited in families with children who develop multifocal retinoblastoma

tumors of the eye, indicating an important role in cell proliferation and/or differentiation of the retina. Because Rb is thought to be a crucial cell cycle regulator in most, if not all, cell types, it is perhaps surprising that we still do not understand why the greatest tumor predisposition is for cancers of the nervous system. Mutations of Rb family members have also been found in astrocytomas, glioblastomas, and gliosarcomas, but it is also clear that Rb heterozygous individuals are susceptible to other nonneurological tumors later in life, particularly osteosarcomas. Indeed, some component of the Rb pathway, although not necessarily Rb, is thought to be mutated in the vast majority of human tumors.

An early function for Rb in the development of the nervous system was inferred from the severe neurological phenotype seen in Rb null mice, which die by embryonic day 14. However, it has been shown that many of these problems arise as a result of placental insufficiency; if Rb null embryos are rescued with wild-type placentas, then they have a relatively normal nervous system. However, Rb is strongly expressed in the CNS and, in common with Kip1, its overexpression alone is enough to induce differentiation of neuroblastoma cells, indicating specific roles in neural tissue.

It seems likely that Rb may influence neuronal differentiation independently of its ability to control cell cycle, and indeed, in some cultured cell systems, regulation of cell cycle and differentiation by Rb can be separated. At a molecular level, our best understanding of how Rb can directly influence differentiation comes from the study of myogenesis. Interestingly, studies mainly of cultured cells have shown that Rb not only facilitates cell cycle exit but also cooperates with the bHLH myogenic factor MyoD to activate the MEF2 transcription factor. It has also been reported that Rb may directly influence bHLH factor function by binding Id2, an inhibitor of bHLHs with which it can dimerize but these resultant dimers can no longer bind to DNA and activate transcription. Moreover, a study has shown that Rb may be a coactivator for the neurogenic bHLH NeuroD1.

The p53 Family

p53 is a transcription factor whose activity is disrupted in a wide range of tumors. p53 can transactivate both Cip1 and genes involved in apoptosis, and it has been implicated in neurogenesis, although its precise function has been difficult to identify. p53 does seem to accumulate in the nucleus of neuronal cells at an early stage of differentiation. Moreover, a dominant-negative form of p53 inhibits the cell cycle exit and differentiation of O-2A cells on treatment with thyroid hormone. p73, another p53 family member, can also induce the expression of Cip1. p73 null mice are missing the earliest sort of neurons, Cajal–Retzius cells. Moreover, retinoic acid treatment induces the differentiation of a retinoblastoma cell line and this results in upregulation of p73. Overexpression of p73 alone will promote differentiation of these cells, whereas a dominant negative form of p73 blocks retinoic acid-induced differentiation; therefore, it seems that p73 can also regulate both cell cycle and differentiation.

Geminin

Geminin is an excellent example of a protein with dual functions in cell cycle and differentiation. The gene was initially identified in two independent screens, one for proteins that could perturb early *Xenopus* neural development and the other searching for proteins that are specifically degraded during mitosis. It has subsequently become apparent that geminin has at least two functions: (1) to regulate the loading of the mini-chromosome maintenance proteins onto prereplicative DNA prior to S phase and (2) to maintain neuronal progenitors in a proliferative precursor-like state. It performs this second function by apparently binding to and blocking the function of Brg1, a component of the SWI/SNF chromatin remodeling complex, which is required to act with the bHLH neurogenic transcription factors Neurogenin and NeuroD to promote neural differentiation. Geminin levels decrease in differentiating neurons and this may be required to trigger neuronal differentiation. Moreover, other studies have shown that geminin can also interact with Six- and Hox-related transcription factors, so its ability to modulate transcription both within and outside the nervous system may be widespread.

Differentiation and Patterning Factors Regulate the Cell Cycle

Based on the evidence described previously, it is clear that both cell cycle status and cell cycle regulators influence many aspects of cell fate choice and differentiation in the nervous system. Conversely, much evidence is emerging that molecules controlling cell fate choice and differentiation also have direct effects on the cell cycle.

A paradigm for what we might expect to occur in nerves is seen during the differentiation of skeletal muscle: the bHLH transcription factor MyoD not only drives expression of muscle structural genes but also upregulates cdkis to promote cell cycle exit. This is complemented by interactions with Rb and

cyclinD/cdk4 that regulate MyoD function. Similar interactions may occur in differentiating neurons. Mouse P19 embryonal carcinoma cells can be differentiated into neurons on overexpression of several neurogenic bHLH proteins, including neurogenin 1 and neuroD2, and this is accompanied by upregulation of Kip1 and cell cycle exit. Moreover, achaete-scute, another bHLH protein, transcriptionally regulates the mitotic phosphatase and CDC25 homolog string. Also in *Drosophila*, Prospero, an asymmetrically distributed homeodomain protein, controls the expression of cyclin E, cyclin A, and string. In addition, it can work indirectly via the bHLH protein deadpan to control levels of dacapo, a *Drosophila* cdki.

Expression of other tissue-specific transcription factors in the nervous system influences cell proliferation but only in the regions where they are usually expressed, indicating a requirement for cofactors; overexpression of Optx2, Six3, and Rx1 results in very large eyes due to extra cell proliferation, whereas loss of Optx2 results in the upregulation of two cdkis. Interestingly, the winged helix transcription factor XBF1 has dual roles, both recruiting cells to a neural fate and controlling their proliferation via regulation of cdki transcription, having different effects at high and low doses.

The internal clock that drives the sequential expression of a series of transcription factors in *Drosophila* neuroblasts, leading their progeny to adopt different fates, has been described previously. This transcription factor series may also be used as a way for NB cells to 'count' their number of cell divisions; prolonged expression of early genes such as Hunchback and Kruppel results in the generation of extra ganglion mother cells of an early type. A similar counting mechanism may exist for NBs in postembryonic flies, in which loss of a coiled coil protein, Mushroom body defect, results in generation of extra progeny.

Signal transduction pathways responsive to extracellular ligands are also known to regulate both cell fate and differentiation and cell cycle coordinately, although the molecular details of these interactions have only recently come to light. The two pathways about which probably the most is known are those of Notch and Hedgehog (Hh) signaling.

The Notch signaling pathway is used widely throughout the nervous system, and its role in allowing scattered neurons to differentiate out from a field of equivalent neural precursors has been particularly well studied. In general, active Notch signaling keeps neural cells in an undifferentiated state and, in several systems, also favors gliogenesis at the expense of neurogenesis. In some contexts, such as the mouse nervous system, Notch signaling keeps cells in cycle, and this has been proposed as a mechanism whereby cells remain undifferentiated. However, in other systems, Notch activation promotes cell cycle exit, such as in the *Xenopus* retina and neural plate. How does Notch signaling influence the cell cycle? Again, this seems to be context dependent. Hes1, a component of the Notch signaling pathway, can repress Cip1 expression in PC12 cells and inhibit NGF-induced differentiation, whereas in the skin, Notch induces Cip1 and promotes differentiation. Confusingly, in the *Xenopus* neural plate, Notch signaling inhibits cdki expression but nevertheless promotes cell cycle exit, possibly by downregulation of cyclin A and cdk2 mRNAs. This may reflect an effect of Notch required to inhibit the differentiation function of the cdki, rather than to influence its cell cycle function. Moreover, Notch may control levels of cdkis posttranslationally via effects on the level of E3 ubiquitin ligase SCF Skp2, which can target Kip1 and Cip1 for proteolysis.

The Hh pathway regulates cell proliferation in several areas of the vertebrate CNS, including the hippocampus, cerebellum, forebrain, spinal cord, and retina. Again, the effects of Hh signaling on proliferation seem to be context and species dependent. For instance, upregulation of Hh signaling in the mouse retina results in proliferative cells persisting inappropriately, whereas in the zebrafish loss of Sonic Hh results in inhibition of Kip2 and increased proliferation.

Other signaling pathways influence cell cycle in the CNS. Wnt signaling is also likely to play an important role in precursor cell proliferation by regulating levels of D-type cyclins and c-myc. If the downstream Wnt effector β-catenin is put under a neuron-specific enhancer, these mice develop enlarged brains. Moreover, transforming growth factor-α has a dose-dependent effect on proliferation in the retina. Other pathways, such as fibroblast growth factor and bone morphogenetic protein signaling, influence cell fate and differentiation in the neuroectoderm, and it seems likely that in the future we will obtain a better understanding of their influence over cell cycle events in the CNS.

Conclusions

Neural precursors must proliferate, adopt the correct fate, and then differentiate at the appropriate time. Thus, it seems obvious that there must be constant feedback between these processes for development of the exquisitely patterned CNS, yet our understanding of these links has only recently begun to emerge. One key point has been the realization that several cell cycle regulators have complementary but separable roles in the processes of fate determination, differentiation, and cell cycle control, providing important insight into the

elegant ways that have evolved to coordinate these events. From this base, our understanding is likely to grow considerably in the near future.

See also: Cerebral Cortex: Symmetric vs. Asymmetric Cell Division; Helix–Loop–Helix (bHLH) Proteins: Hes Family; Helix–Loop–Helix (bHLH) Proteins: Proneural; Neurogenesis in the Intact Adult Brain; Terminal Differentiation: REST.

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Programmed Cell Death

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Introduction

During the embryonic, fetal, larval, and early postnatal stages of development, there is a massive loss of undifferentiated and differentiating cells in most tissues and organs, including the central and peripheral nervous systems. This cell loss is a normal part of development, and it occurs by a highly regulated process known as programmed cell death (PCD). Developmental PCD is defined as the spatially and temporally reproducible, tissue and species-specific loss of cells whose occurrence serves diverse functions and is a process required for normal development. Perturbation of this normal process can be maladaptive, resulting in pathology.

History

Although the occurrence of developmental PCD was first reported in the middle of the nineteenth century, it was not until the middle of the twentieth century that the occurrence and significance of PCD in the nervous system began to be appreciated by embryologists and developmental biologists. In a series of seminal papers by Viktor Hamburger and Rita Levi-Montelcini in the 1930s and 1940s, it was shown that the sensory and motor neurons in the spinal cord of the chick embryo are generated in excess during neurogenesis, followed by the PCD of approximately one-half of the original population. The period of cell loss was found to occur as sensory and motor neurons were establishing synaptic connections with their peripheral targets (e.g., muscle in the case of motor neurons). In a conceptual *tour de force*, Hamburger and Levi-Montalcini proposed that developing neurons compete for limiting amounts of target-derived survival-promoting signals (the winners survive and the losers undergo PCD) and in this way neurons are thought to optimize their innervation of targets by a process known as systems matching. It was this conceptual framework that led to the discovery of target-derived neurotrophic molecules and to the formulation of the neurotrophic theory that has been a major factor in fostering progress in this field for over 50 years. According to the neurotrophic theory, neurons that compete successfully for neurotrophic molecules avoid PCD, owing to the expression of survival promoting intracellular molecular genetic programs. The discovery of molecular genetic programs for both survival and death has revolutionized the study of PCD, resulting in the publication of thousands of articles each year since the early 1990s.

Evolution

The occurrence of massive cell death during development is, on the one hand, counterintuitive, whereas, on the other hand, the fact of its existence in many vertebrate and invertebrate species argues for its significance as an adaptively significant and fundamental part of embryogenesis. The death of occasional cells during development is to be expected in biological systems in which accidental deleterious events may be lethal to individual cells. However, because the stereotyped death of large numbers of developing cells in all members of a species cannot be explained in this way, it raises two basic questions regarding the evolution of PCD: (1) because PCD occurs by a metabolically active, genetically regulated process (see the sections titled 'Molecular regulation of cell death and survival by neurotrophic factors' and 'Intracellular regulation of cell death'), how and why did the molecular mechanisms involved arise during evolution; and (2) what are the adaptive reasons for massive developmental cell death in the nervous system (see the section titled 'The functions of PCD in the nervous system')? A plausible answer to the first question is that PCD arose as a defense mechanism for eliminating abnormal cells that threatened the survival of the organism. According to this argument, the death-promoting intracellular machinery arose in host cells to defend against the spread of viral infection and, in response, viruses evolved survival-promoting genetic mechanisms to block or counter the host cell death-promoting defenses. Because these death- and survival-promoting mechanisms have been found to mediate PCD in a diversity of organisms (see the section titled 'Intracellular regulation of cell death'), they are considered to represent an evolutionarily conserved core molecular genetic program that has been coopted by the nervous system for regulating cell numbers.

The Functions of PCD in the Nervous System

PCD in the nervous system involves both neurons and glia, and it occurs in the central and peripheral nervous system, the brain, and spinal cord and in virtually all neuronal and glial cell types. Accordingly, it is

Table 1 Some possible functions of developmental PCD in the nervous system

Differential removal of cells in males and females (sexual dimorphisms)
Deletion of some of the progeny of a specific sublineage that are not needed
Negative selection of cells of an inappropriate phenotype
Pattern formation and morphogenesis
Deletion of cells that act as transient targets or that provide transient guidance cues for axon projections
Removal of cells and tissues that serve a transient physiological or behavioral function
Systems matching by creating optimal quantitative innervation between interconnected groups of neurons and between neurons and their targets (e.g., muscles, sensory receptors)
Systems matching between neurons and their glial partners by regulated glial PCD (e.g., Schwann cells and axons)
Error correction by the removal of ectopically positioned neurons or of neurons with misguided axons or inappropriate synaptic connections
Removal of damaged or harmful cells
Regulation of the size of mitotically active progenitor populations
Production of excess neurons may serve as an ontogenetic buffer for accommodating mutations that require changes in neuronal numbers in order to be evolutionary adaptive (e.g., increases or decreases in limb size may require less or more motor neuron death for optimal innervation)
Regulated survival of subpopulations of adult-generated neurons as a means of experience-dependent plasticity

not surprising that the diversity of adaptive roles for PCD differs according to the animal species, cell type, nervous system region, and stage of development. A list of some of the most common reasons for PCD in the nervous system is provided in **Table 1**. Of these, the one that has received the most attention because of its central role in establishing optimal functional connectivity in neuronal circuits is systems matching (**Figure 1**). In birds and mammals, the evidence is compelling that for postmitotic neurons that are establishing interconnections with targets and afferents, systems matching, occurring in the framework of the neurotrophic hypothesis (see the section entitled 'Introduction'), is the primary reason for why a significant proportion of neurons (20–80%) undergo PCD. An analogous kind of systems matching adjusts myelinating glial cell numbers to the number of available axons.

PCD by Autonomous versus Conditional Specification

PCD occurring during systems matching represents a paradigm example of the conditional specification of cell fate during development. Developmental biologists have identified two kinds of pathways that cells use for specifying their differentiated fate or phenotype. One pathway, autonomous specification, involves the differential segregation of cytoplasmic signals into daughter cells following mitosis. In this way, cells become different from one another by the presence or absence of these cytoplasmic signals with little if any contribution from signals from neighboring cells. The other pathway, conditional specification, requires signals from other cells (cell–cell interactions) to progressively restrict differentiation and determine whether a cell lives or dies. These cell–cell interactions can be of four types: (1) juxtacrine (direct cell–cell or cell–matrix contact); (2) autocrine (a secreted signal acts back on the same cell type from which the signal arose); (3) paracrine (a secreted diffusible signal from one cell that acts locally on a different cell type); and (4) endocrine (signaling via the bloodstream). In the developing vertebrate nervous system, neuronal and glial survival is largely dependent on conditional specification involving paracrine interactions that use neurotrophic factors (NTFs). For neurons, the most commonly used NTFs are members of three major families: (1) neurotrophins, (2) the glial cell line-derived family ligands (GFLs), and (3) ciliary NTFs. Each of these contains several distinct members that act preferentially on specific types of neurons via membrane-bound receptors. By contrast, the survival of glial cells depends on different families of trophic factors such as the neuregulins and insulin-like growth factors. Neurons and glial cells use paracrine signaling to promote survival by ligand–receptor interactions. However, there are some situations in which paracrine interactions can signal death versus survival.

Molecular Regulation of Cell Death and Survival by NTFs

The dependence of neurons on NTFs for survival intuitively leads to the conclusion that the inactivation of NTF receptors, and the signal transduction associated with them, leads to the activation of cell death events (**Figure 2**). This has been best studied with neurotrophins and their receptors, notably the

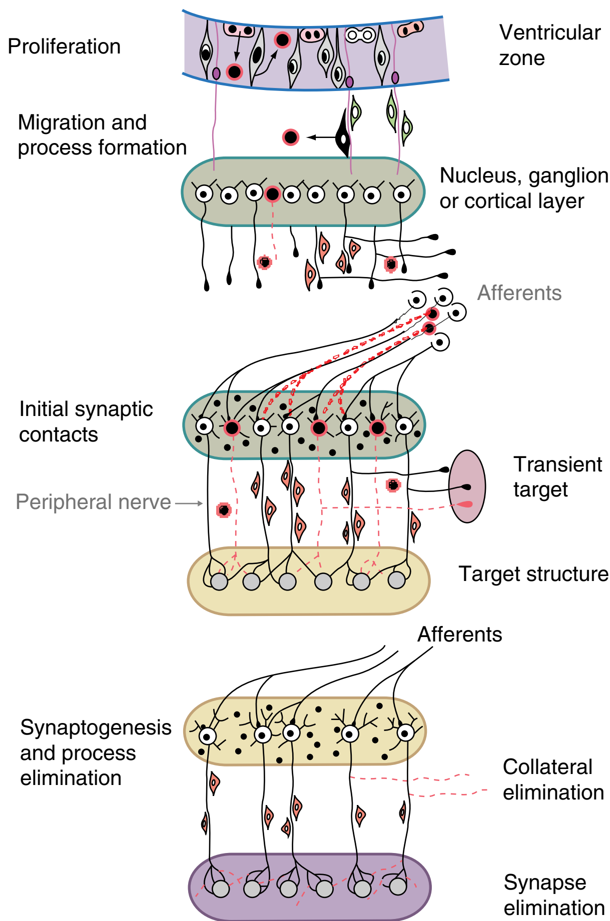

Figure 1 Schematic illustration of some key steps in neuronal development. Neurons undergoing PCD (●) are observed during neurogenesis in the ventricular zone, during migration and while establishing synaptic contacts. Schwann cells in developing nerves also undergo PCD. ◊ represents peripheral glial (Schwann) cells; ● represents CNS glia (astrocytes and oligodendrocytes); ⊙ represents surviving, differentiating neurons (motor neurons) whose targets are skeletal muscle.

Trk receptors and p75 (**Table 2**). Activation of the trk receptors results in the activation of the phosphatidylinositol-3 kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways. Akt, a substrate of PI3-K, has been reported to phosphorylate Bad, promoting its association with 14-3-3 and preventing the inactivation of Bcl-2 and Bcl-x. Activation of PI3-K/Akt, extracellular signal-regulated kinase-1/2 (ERK1/2), protein kinase (PK)C, or PKA promotes the serine phosphorylation of Bad and Bcl-2, and Gsk-3B phosphorylates and inactivates Bax – events that are associated with cell survival. The survival-promoting activity of phosphorylated Bcl-2, however, is controversial, being associated with motor neuron survival-induced trophic support, whereas in neurons treated with microtubule destabilizing agents it appears to promote death. The c-jun N-terminal kinase (JNK) pathway is reported to exhibit increased activity in neurons triggered to die. The activation of one of its substrates, c-jun, is also thought to play a role in mediating neuronal death. For example, the JNK activation of the BH3 proteins Bim and DP5 has been associated with neuronal death, and a JNK–p53–Bax pathway appears to be critical for death in specific cell types. Nonetheless, the JNK pathway has also been reported to be a critical mediator of survival-promoting events such as neurite outgrowth and may serve as a double-edged sword in neurons through changes in intracellular localization or specific activation or inactivation of individual isoforms.

The role of the p75 nerve growth factor (NGF) receptor in promoting neuronal survival or death is complicated. For example, complexes of p75 and the trk receptors bind neurotrophins and promote survival, while complexes of p75 and sortilin appear to promote binding of the proneurotrophins resulting in the death of neurons. Activation of p75 in the absence of Trk activation has most notably been associated with the death of neurons, but this appears to be developmentally regulated. The precise mechanism by which p75 promotes death is unclear. However, p75 is a member of the tumor necrosis factor receptor family, and another member of this family better known for its death promoting activity is Fas/CD95. Engagement of Fas by Fas ligand leads to the activation of caspase 8. This event is dependent on the formation of a death-induced signaling complex (DISC). Homotrimerization is the first step in this process. Engagement of Fas by its ligand can only occur when it is homotrimerized. This family of receptors is characterized by the presence of a death domain (DD) on the cytoplasmic region of the receptors. The Fas-associated death domain-containing protein (FADD) can then bind to Fas. In addition to the DD, FADD also contains a death effector domain (DED). Procaspase 8 binds the DED. The autolytic nature of caspases leads to active capsase 8 that can then go on to directly activate caspase 3 (in type 1 cells, e.g., thymocytes) or cleave Bid leading to changes at the mitochondria (in type 2 cells, e.g., hepatocytes and neurons) and cell death.

Intracellular Regulation of Cell Death

Many of the intracellular mechanisms mediating neuronal cell death were investigated following initial work that suggested that new gene expression was required for the process. Horvitz and coworkers provided some of the first evidence that there was indeed a genetic component of PCD from their work in the 1980s with the free-living nematode *Caenorhabditis elegans*, although it was two decades later

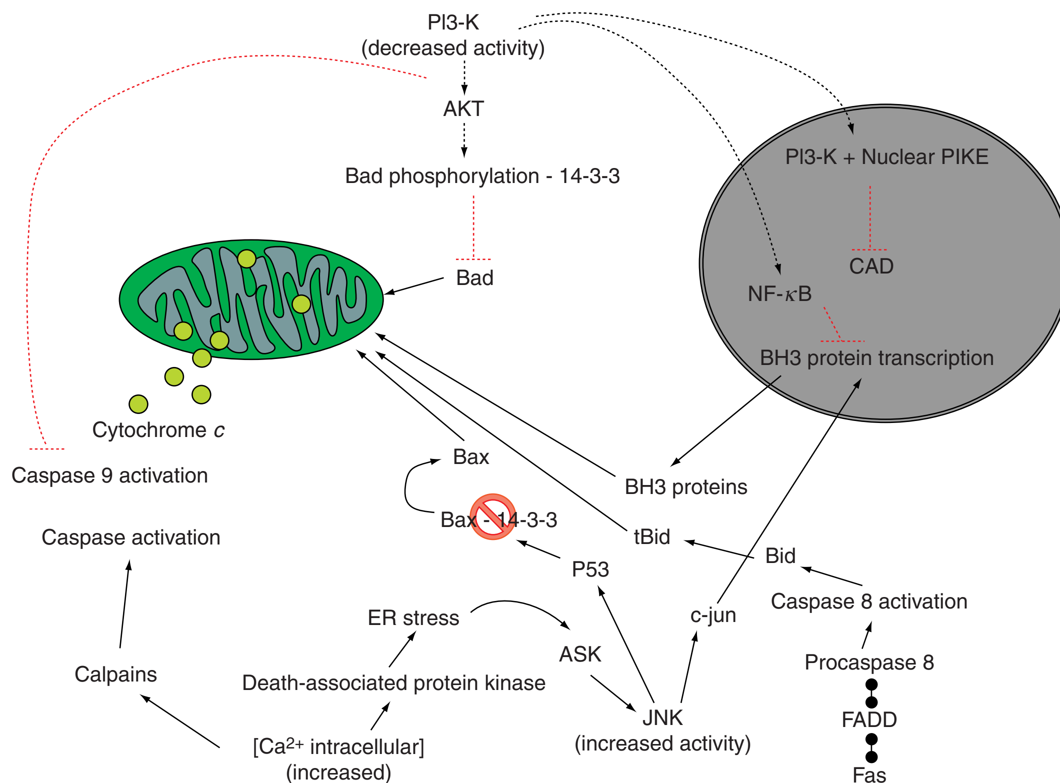

Figure 2 Signals to die. Changes in signal transduction pathways, activation of receptors containing a DD, or changes in intracellular calcium concentrations have been identified as events that can lead to the activation of cell death-specific events. Loss of trophic support results in decreased activation of the phosphatidylinositol-3 kinase (PI3-K), thereby releasing inhibitory factors linked to this pathway. Alternatively, increases in intracellular calcium concentrations, activation of the FAS receptor, and increased c-jun terminal kinase activation are associated with the mitochondrial changes and caspase activation that occurs in cell death. ASK, apoptosis signal-regulating kinase; ER, endoplasmic reticulum; FADD, Fas-associated death domain-containing protein; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor κB; PIKE, phosphatidylinositol-3 kinase enhancer.

that the significance of this work was fully appreciated (**Figure 3**). As these genes were identified, the sequence of events leading to the death of cells was pieced together.

The presence of Bcl-2 family proteins appears critical for mediating the survival or death of nervous system cells. During development, Bcl-2 expression is correlated with neuronal survival. With continued maturation and development, this expression declines while Bcl-x expression increases. The specific mechanisms responsible for this change in expression are currently not known. On the other hand, the expression of pro-death Bcl-2 family proteins such as Bid or Bax appears to be consistent throughout development, whereas the intracellular localization of these proteins appears to change in cells undergoing death. In healthy cells, Bax is localized more in the cytoplasm, whereas in dying cells the majority of Bax localizes to organelle membranes, including the mitochondria. The localization of Bax to the mitochondria corresponds to the release of cytochrome *c* into the cytoplasm. Once in the cytoplasm, cytochrome *c* binds Apaf-1, causing a conformational change in Apaf-1 to reveal a caspase recruitment (CARD) domain. In the presence of ATP, a heptamer of the cytochrome *c*–Apaf-1 complex is formed. Procaspase 9 has a high affinity for the CARD domain and localizes to the heptamer. This complex is referred to as the apoptosome. With the increased local concentration of procaspase 9, the autolytic property of caspases leads to the generation of active caspase 9. As an initiator caspase, active caspase 9 can cleave procaspase 3, resulting in the active form of this protease (**Figure 4**). The activation, inactivation, or destruction of specific substrates significantly contributes to the rapid degeneration of the cell. Caspase activity also leads to the activation of endonucleases that may play a role in the changes in nuclear morphologies that are observed in may cell types.

The removal of the dying cell is most likely just as critical an event in the cell death process as any of those already discussed. The event is accomplished by adjacent cells that can be nonprofessional phagocytes (e.g., Schwann cells) but more often involves

Table 2 Specific molecules associated with cell death in neurons[a]

Receptors	Fas
	P75
	Trk receptors
Signal transduction pathways	PI3K–Akt
	ERK1,2
	ERK 5
	JNK
	PLC
	PKC
	PKA
Bcl-2 proteins	Bcl-2
	Bcl-x
	Bid
	Bik
	Bag
	Bax
	Bad
	Dp5
	Bim
	Puma
Mitochondrial proteins	Cytochrome *c*
	SMAC/Diablo
	XIAP
	AIF
	Endonuclease G Omi/HtrA2
Caspases	
Initiator caspases	Caspase 8
	Caspase 9
Effector caspases	Caspase 2
	Caspase 3
Inflammatory caspases	Caspase 1
Other proteins	Apaf-1
	Amyloid precursor protein/ β-amyloid
	PARP
	p53
	p35
	Ubiquitin
	E2F
	Cyclin D1
	Cyclin E
	Cyclin B
	Cdk4/6
	Cdk2
	Cdk1
	Rb
	p130
	HIF1-α
	CAD
	iCAD

[a]AIF, apoptosis-inducing factor; CAD, caspase-activated DNase; HIF1-α, hypoxia-inducing factor; iCAD, inhibitor of CAD; JNK, c-jun N-terminal kinase; PARP, poly(ADP-ribose) polymerase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; XIAP, x-linked inhibitor of apoptosis protein.

dedicated phagocytes such as tissue macrophage (microglia) or circulating monocytes. During central nervous system (CNS) development, resident microglia are not present in large numbers among dying cell populations. Intuitively, some signal must be sent by the dying cells to recruit the phagocytes into the area. One such chemotactic factor has been identified in nonneuronal cells, and interestingly, this factor is the phospholipid lysophosphatidylcholine. Caspase 3 activation appears to be necessary for the release of this factor. Once the phagocytic cells are in the region, they must be able to distinguish dying from healthy cells. Changes on the dying cell's surface include revealing of thrombospondin 1 binding sites, exposure of phosphatidyl serine, ATP-binding cassette (ABC-1) molecules, and carbohydrate changes. The phagocyte, in turn, expresses the phosphatidylserine receptor, as well as thrombospondin receptors that recognize the thrombospondin bound to the binding site on the dying cell, lectins that bind to the carbohydrate changes, and ABC-1 molecules that bind to like molecules. Phagocytosis occurs only when multiple changes are recognized, and this phagocytosis is limited and does not result in macrophage secretion of cytokines that would normally induce an inflammatory or immune response if foreign antigens were phagocytosed.

Different Types of Neuronal Death

Many individuals studying cell death often rely on the critical papers of Currie, Kerr, and Wyllie to define and characterize the different types of cell death. These investigators described in detail two distinct morphological changes associated with cell death. An active process, apoptosis, was characterized by specific morphological changes that included condensation of nuclear chromatin, shrinking of the cytoplasm, and a breaking up of the cell into membrane-bound particles that were phagocytosed. Necrosis, on the other hand, was characterized by a swelling of the cytoplasm with eventual bursting of the cell (**Figure 5**). In this case, intracellular components are spilled into the extracellular space where they could initiate inflammatory and immune responses. However, electron microscopy studies of the developing nervous system indicated that neuronal death could not be so easily defined by only these two modes of death. During the naturally occurring death of some neurons, initial changes in dying cells are observed in the cytoplasm where there is an increase in the diameter of the cisternae of the rough endoplasmic reticulum (RER). Mitochondrial swelling was also observed, although it was not clear if these changes occur in the same cell. Nonetheless, during normal development initial changes are observed in the cytoplasm with little nuclear alterations in dying cells. The cell then appears to round up and break into pieces that are phagocytosed. Although cytoplasmic cell death appears to be more prominent

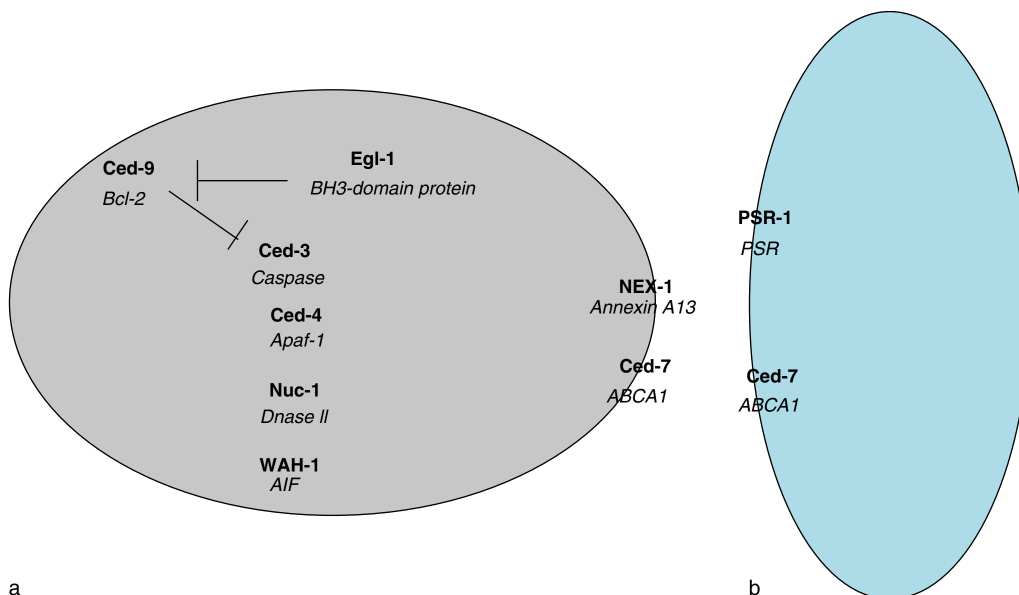

Figure 3 Genes for the key regulators of cell death: (a) dying cell; (b) engulfing cell. Many of the key regulators of cell death are evolutionarily conserved. Many of the genes for these proteins were initially identified through genetic mutations in the free living nematode (bold type), with homologs later being identified in *Drosophila* and mammals (italics).

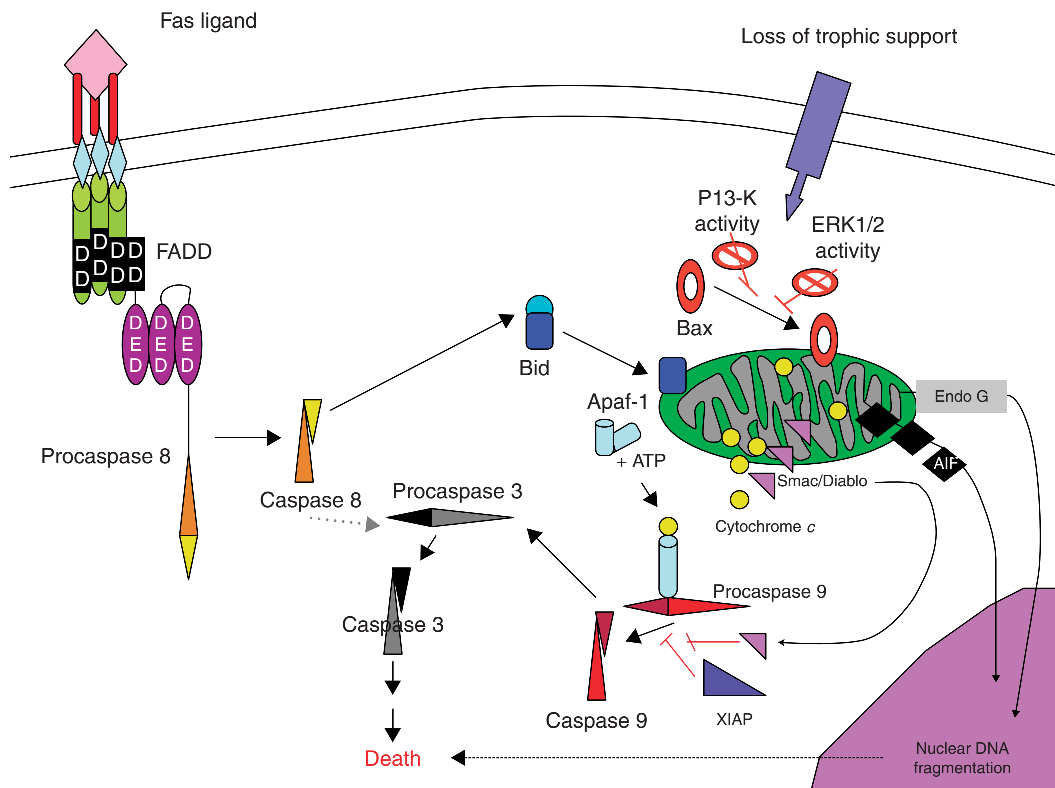

Figure 4 Many of the critical components of neuronal death and their apparent sequence of activation during development. The mitochondria release many regulators that can lead to death with or without caspase activation. Neurons are considered type 2 cells where FAS engagement results in minimal caspase 8 activation, resulting in the cleavage of Bid and its subsequent movement to the mitochondria. The localization of Bax to the mitochondria appears to be a critical event in the death of neurons. AIF, apoptosis-inducing factor; ATP, adenosine triphosphate; DD, death domain; DED, death effector domain; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain-containing protein; PI3-K, phosphatidylinositol-3 kinase; XIAP, x-linked inhibitor of apoptosis protein.

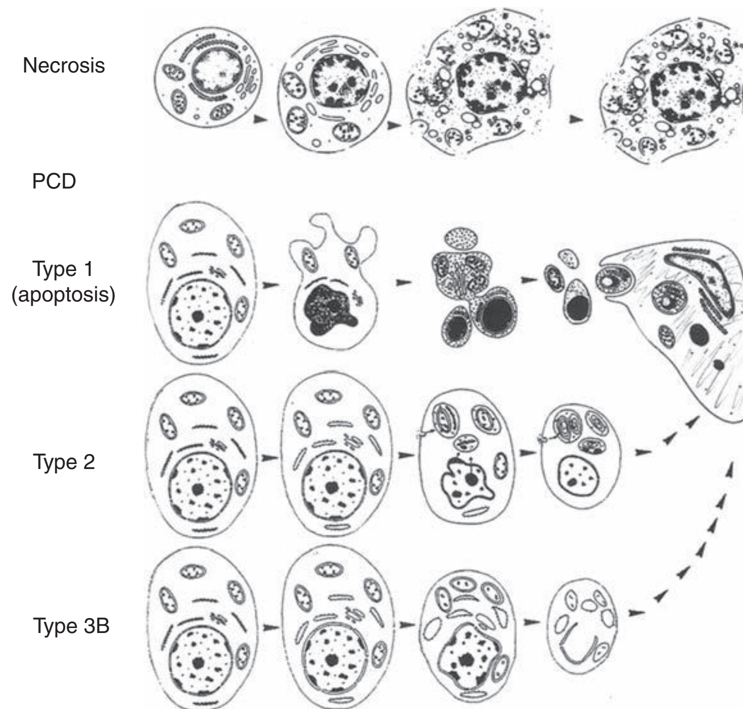

Figure 5 Necrotic death and the three most common types of programmed cell death (PCD) observed in the nervous system. Type 1 meets the criteria for apoptosis. In PCD, phagocytes remove the resulting corpses, whereas necrosis in many cases results in inflammation.

during development, nuclear or apoptotic death also occurs. A third type of death is autophagy, characterized by the formation of numerous autophagic vacuoles. Nuclear changes may also occur in this type of death.

It is important to note that all three types of neuronal death appear to reflect a metabolically active process. Accordingly, it appears that a homogeneous mode of suicide does not occur but, rather, that processes leading to death appear to be context and developmentally dependent. One example is a neuronal population undergoing apoptosis when nuclear condensation is a prominent feature. On the other hand, in animals in which caspases have been inhibited or deleted, the same neuronal population undergoes a delayed death with major changes occurring in the cytoplasm.

Pathological Neuronal Death

Many of the mechanisms that have been identified as playing a role in neuronal death during development are reported to be reactivated in the mature pathological nervous system. Alterations in the expression of Bcl-2 proteins are observed in animal models and in postmortem human tissue in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's chorea (HC), amyotrophic lateral sclerosis (ALS), and stroke. Caspase activation has also been reported in these and other disorders. Interestingly, although reports of apoptotic cells are present in these conditions, the majority of degenerating cells exhibit morphological changes reminiscent of cytoplasmic or autophagic death. This is most notable in the mutant SOD1 mouse model of ALS. During development, dying motor neurons exhibit many features of apoptosis, including possible Fas activation, Bax translocation, mitochondrial dysfunction, and caspase activation and condensation of nuclear chromatin. By contrast, in the adult spinal cord of SOD1 mice, degenerating motor neurons exhibit cytoplasmic vacuolization, mitochondrial dilation, and protein aggregation. Nonetheless, some of the cell death-associated events that occur during development are also observed. It is becoming increasingly recognized that neuronal dysfunction is most likely the event responsible for clinical symptoms in ALS and other neurodegenerative diseases. In experiments in which motor neuron death is inhibited in the SOD1 mouse, disease progression and survival of the animal are only very modestly affected; muscle denervation still occurs, and the animals die prematurely. Results such as this call into question the practical application of inhibiting cell death as a therapeutic approach for these disorders.

See also: Autonomic and Enteric Nervous System: Apoptosis and Trophic Support During Development; Autophagy and Neuronal Death.

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Autonomic and Enteric Nervous System: Apoptosis and Trophic Support During Development

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Introduction

Programmed cell death (PCD) is an evolutionarily conserved process in multicellular organisms that is important for morphogenesis during development and for the maintenance of tissue homeostasis in organs with ongoing cell proliferation. PCD also occurs in many tissues in response to injury. Apoptotic cell death is one form of PCD that provides a mechanism to remove individual cells from tissues without damage to adjacent cells. Defects in molecular mechanisms that regulate cell death cause both cancer and abnormal development. Because many aspects of apoptosis have been reviewed over the past decade, this article focuses on the developmental role of apoptosis in the sympathetic, parasympathetic, and enteric nervous system and provides only a brief overview of apoptotic mechanisms.

Apoptotic Cells and Molecular Mechanisms

Apoptosis is a form of cell death characterized by cell shrinkage, plasma membrane blebs, nuclear chromatin condensation, and DNA fragmentation. Phosphatidylserine, usually restricted to the inner face of the plasma membrane in live cells, also partially redistributes to the extracellular face of the plasma membrane. These changes largely result from activation of a set of cysteine-aspartyl-specific proteases called caspases. Caspases can be activated via either an extrinsic pathway or an intrinsic pathway. The extrinsic pathway is activated by binding of extracellular ligands to cell surface death receptors like Fas (Apo-1 or CD95), tumor necrosis factor receptor-1 (TNFR-1/p55/CD120a), interferon receptor, and TRAIL (TNF-related apoptosis-inducing ligand or Apo2-L) receptor. This binding leads to the recruitment of a variety of proteins that activate procaspase-8 or procaspase-10. In contrast, the intrinsic pathway is characterized by cytochrome c release from mitochondria. Cytochrome c in the cytoplasm induces heptamerization of Apaf-1, that then binds to and activates procaspase-9. These initiator caspases (-8, -9, and -10) activate the effector caspases (caspase-3 and caspase-7). The effector caspases have many substrates (estimated to include 0.5–5% of cellular proteins), induce DNA cleavage and mitochondrial permeabilization, and activate additional proteolytic cascades that eventually result in cell death.

Apoptosis in the Nervous System and the Discovery of Neurotrophic Factors

PCD is common in most parts of the developing nervous system, where 20–80% of all neurons produced during embryogenesis die before adulthood. Typically, these cells are eliminated by PCD at the time that they would normally innervate their targets. In this setting, PCD is thought to occur to allow matching of the neuronal population to the size of the innervation target via target-derived, trophic factor-dependent cell survival. This mechanism also efficiently eliminates cells with abnormal migration or abnormal axon targeting because cells that fail to innervate appropriate targets do not receive adequate trophic factor. The observation that developing sympathetic and sensory neurons were dependent on exogenous trophic factor for survival led to the discovery of nerve growth factor (NGF) by Rita Levi-Montalcini and Stanley Cohen. This discovery prompted work that in turn resulted in the discovery of the related proteins brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. An *in vitro* sympathetic neuron survival assay was also critical for discovery of neurturin (NRTN) and for the identification and characterization of the related proteins glial cell line-derived neurotrophic factor (GDNF), artemin (ARTN), and persephin (PSPN). Furthermore, molecular mechanisms of neonatal sympathetic neuron survival *in vitro* have been studied extensively and form the basis of much of what we know about neuronal apoptosis. In the rat, for example, superior cervical ganglion (SCG) sympathetic neurons are NGF dependent from embryonic day (E)16 to postnatal day (P) 7. NGF deprivation during this time results in PCD within 48 h. With this assay, investigators demonstrated that apoptosis is triggered after NGF withdrawal by increased c-Jun protein levels and c-Jun phosphorylation, increased translocation of the proapoptotic protein Bax from the cytosol to mitochondria, and Bax-induced cytochrome c release that causes apoptosis via the intrinsic pathway. Release of Smac/DIABLO and HtrA2/Omi from mitochondria is also important to relieve caspase inhibition by inhibitor of apoptosis proteins so that caspases can become activated.

Lessons from Mutant Mice

The neurotrophic factors NGF, BDNF, NT-3, GDNF, NRTN, and ARTN are critical for the normal development and maintenance of the peripheral nervous system. Depending on the specific cell type and developmental time point, these factors determine nervous system structure and function via a number of distinct effects including preventing or in some cases promoting cell apoptosis. These proteins have other important roles as well:

- Promotion of cell survival
- Promotion of cell proliferation
- Support for neuronal differentiation
- Promotion of directed cell migration
- Promotion of neurite extension
- Axon guidance

Each of these actions is mediated by binding of trophic factors to specific cell surface receptors. There is also some ability for specific trophic factors to activate multiple receptors at least *in vitro*. Preferred receptor–ligand interactions, alternate receptor–ligand interactions, and a summary of interactions that are important *in vivo* are presented in **Figure 1**. Briefly, NGF, BDNF, and NT-3 bind to and activate the transmembrane tropomyosin-related kinase (Trk) receptors TrkA, TrkB, and TrkC, respectively. In addition, NT-4 activates TrkB, whereas NT-3 can directly activate TrkA and TrkB, but with lower efficiency than it activates TrkC. All these NTs also bind to the low-affinity receptor p75NTR. The interaction with p75NTR inhibits the activation of Trk receptors by their nonpreferred ligands and in some cases improves intracellular signaling. However, in the absence of specific Trk receptor activation, binding of proneurotrophins to p75NTR promotes cell death. For example, pro-BDNF binding to p75NTR on sympathetic neurons, which express TrkA but not TrkB, promotes cell death, but NGF promotes cell survival by activating both TrkA and p75NTR. This dual role for p75NTR in sympathetic neurons is thought to improve the specificity of axon targeting by eliminating cells whose axons innervate the wrong target and arrive at a source of BDNF but not of NGF. The proteins GDNF, NRTN, ARTN, and PSPN form a separate family of trophic factors called the GDNF-related ligands (GFLs). GFLs all activate the transmembrane tyrosine kinase Ret. Instead of binding directly to Ret, however, GFLs activate Ret by binding to a glycosylphosphatidylinositol-linked cell surface receptor (GFRα1–4). Each GFL preferentially binds to a specific GFRα protein, with GDNF, NRTN, ARTN, and PSPN interacting best with GFRα1, GFRα2, GFRα3, and GFRα4, respectively. There is some *in vitro* cross-talk between GFLs and nonpreferred GFRα proteins, as indicated in **Figure 1**, but these interactions generally require higher trophic factor concentrations and do not appear to be physiologically important *in vivo*. However, receptor

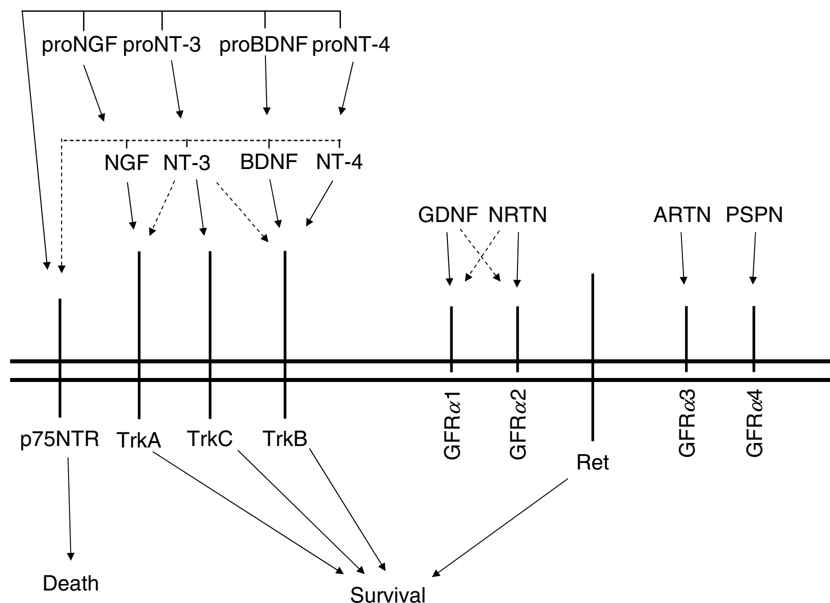

Figure 1 Summary of neurotrophic factors and their receptors. Solid arrows show preferred receptor–ligand interactions. Dashed arrows show nonpreferred interactions between ligands and receptors. For neurturin (NRTN) and glial cell line-derived neurotrophic factor (GDNF), the nonpreferred interactions do not appear to be important *in vivo*. For neurturin (NT)-3, nonpreferred interactions are important *in vivo*. Interactions of neurotrophins with p75NTR are also likely to be physiologically relevant. ARTN, artemin; BDNF, brain-derived neurotrophic factor; GFR, growth factor receptor; NGF, nerve growth factor; PSPN, persephin.

cross-talk appears important in the pharmacological response to exogenously administered factors, presumably acting at supraphysiological levels.

Given the complexity of these systems, determining the relevance of specific trophic factors and their receptors for neuronal development and function has required a combination of *in vitro* studies and the analysis of mutant animals. While many conclusions can be drawn from this work, several important themes have emerged:

1. Patterns of trophic factor receptor and ligand expression correlate well with trophic factor dependence *in vivo* but incompletely predict function.
2. For many developing neurons, trophic factor dependence changes during development such that cells initially dependent on one factor become dependent on a different trophic factor at a later developmental stage.
3. Trophic factors may promote both survival and proliferation early in development or may direct precursor migration. At later stages, these factors may support survival (e.g., at the time of target innervation) or provide trophic support without being essential for survival.
4. Trophic factor dependence *in vitro* does not imply developmental stage-specific programmed cell death *in vivo*. Cell death *in vivo* requires both trophic factor dependence and trophic factor deficiency.
5. Trophic factor receptor expression also does not imply trophic factor-dependent survival since specific cell populations may respond to multiple trophic factors simultaneously.

A few examples will clarify the strategies employed to determine cell number in the autonomic and enteric nervous system (ENS). Effects of specific receptor and ligand mutations on sympathetic, parasympathetic, and enteric neurons are summarized in **Table 1**.

Trk Receptors and Ligands Regulate Cell Death *In Vivo* in the Sympathetic Nervous System

The sympathetic chain in the mouse embryo arises at E11.5, and at that time, TrkC is expressed, but TrkA is not detectable. By E13.5, when the SCG forms, both TrkC and TrkA are detected in the SCG. TrkA expression becomes more robust by E15.5 and continues to be expressed at high levels throughout postnatal development. TrkC levels in the SCG fall significantly by P0, when only a few cells have detectable TrkC expression. In contrast, TrkB is not detected in sympathetic neurons at any developmental stage.

The pattern of TrkA receptor gene expression correlates well with the normal role of TrkA and NGF in development of the mouse sympathetic nervous system. In TrkA−/− mice, for example, neuron number is normal in the sympathetic chain at E11.5 and in the SCG and sympathetic chain at E13.5. By E15.5, there are 15% fewer SCG neurons in TrkA-deficient mice than wild-type (WT). By E17.5, TrkA−/− mice had 35% fewer SCG neurons than WT mice did, and almost all SCG neurons are lost in TrkA−/− mice by P9. At each of the periods investigated, the reduction in SCG neuron number in TrkA−/− mice was attributable to increased cell death. Similarly, in NGF-deficient mice, all SCG neurons are absent by P14, and increased cell death was observed at P3. These changes in neuron number demonstrate the critical role for NGF/TrkA signaling in sympathetic neuron survival and are consistent with results obtained in the 1960s with exposure to neutralizing antibodies to NGF. They also define a period of trophic factor-dependent cell death that correlates well with the timing of trophic factor dependence of SCG neurons in primary culture. Moreover, the striking similarity of the SCG phenotype for NGF−/− and TrkA−/− mice highlights the importance of this preferred ligand–receptor interaction.

In contrast to these results with TrkA−/− and NGF−/−mice, however, the effect of TrkC or NT-3 deficiency is less easily predicted based on gene expression patterns. For example, although TrkC is expressed in the sympathetic nervous system as early as E11.5, the SCG is normal at all developmental stages in TrkC−/− mice. This contrasts with the 50% reduction in SCG neurons found in NT-3-deficient mice. The time during development when NT-3 is required for sympathetic neuron survival, however, has been controversial, with an early report suggesting increased SCG precursor cell death between E11 and E17 and a later study using more animals failing to reproduce this observation and demonstrating increased programmed cell death after birth. Furthermore, injection of anti-NT-3 antibodies into neonatal rats caused a 60–80% loss of SCG neurons, suggesting a role for NT-3 in postnatal SCG survival. Together these observations demonstrate the complexity of determining how specific trophic factors regulate neuronal apoptosis, especially in cells with multiple trophic factor receptors. They further suggest that although NT-3 critically regulates SCG apoptosis, it may perform this function primarily via TrkA receptor activation.

The role TrkB and BDNF in SCG neuron survival is even more remarkable. Since TrkB is not expressed in the sympathetic nervous system, it might initially have been predicted that the SCG would be normal in

Table 1 Neuron number in autonomic and enteric ganglia of mutant mice

	Sympathetic		*Parasympathetic*				*Enteric*
	SCG	*Chain ganglia*	*Ciliary*	*Submandibutar*	*Sphenpalatine*	*Otic*	
TrkA KO	100% loss by P9						
NGF KO	100% loss by P7						
TrkB KO	Normal						
BDNF KO	36% increase						
TrkC KO	Normal						Selective neuron loss
NT-3 KO	50% loss						Selective neuron loss
p75NTR KO	Increased						
Ret KO	Abnormal, but variable	Abnormal	48% loss	30–47% loss	99–100% loss	99–100% loss	99% loss
GFRα1 KO	Normal			33% loss	99–100% loss	99–100% loss	99% loss
GDNF KO	30% loss		40% loss	36% loss	99% loss	86% loss	99% loss
GFRα2 KO	Normal			42% loss	Normal	40% loss	
NRTN KO	Normal		50% loss	45% loss	Normal	Normal	Normal
GFRα3 KO	Abnormal, but variable	Abnormal					
ARTN KO	Abnormal, but variable	Abnormal					Normal

BDNF−/− mice. Remarkably, SCG neuron number is increased by 36% in BDNF−/−compared to WT animals at P15. This was hypothesized to occur because pro-BDNF promotes sympathetic neuron cell death by binding to p75NTR. Indeed, WT SCG neuron number decreases by 42% because of PCD between P0 and P23, but SCG neuron number increases between P0 and P23 in P75NTR−/− mice. These results suggest that both BDNF and p75NTR are required for naturally occurring cell death in the SCG of WT mice. Further support for this hypothesis is the observation that mice deficient in both p75NTR and TrkA have markedly reduced sympathetic neuron apoptosis compared to TrkA−/− animals. This mechanism allows for efficient elimination of cells with misguided axons that encounter pro-BDNF and reinforces the importance of TrkA activation for SCG survival.

Ret Signaling Promotes Sympathetic Neuron Precursor Migration and Supports Axon Extension Required for Sympathetic Neurons to Innervate Targets and Encounter Trk Ligands

Ret activation via GFRα3 and ARTN is essential for normal sympathetic nervous system development. In this case, however, Ret signaling appears to be important for neuronal precursor migration and axon extension, but not directly for cell survival. This conclusion is based on the following observations. Ret−/− mice have smaller-than-normal SCG ganglia that appear in a more caudal position than in WT animals. Furthermore, sympathetic fiber innervation of targets in the nasal mucosa, eye, and skin of Ret−/− mice is almost completely absent, while sympathetic innervation density of the submandibular salivary gland is significantly reduced. In addition, there are defects all along the sympathetic chain in Ret−/− mice, including smaller-than-normal ganglia, abnormally located ganglia, and reduced target innervation. Detailed evaluation of the mechanism of these defects demonstrated that Ret−/− sympathetic neuron precursor lineage commitment appears normal, but differentiation of these cells is delayed, and neuronal cell death increases between E16.5 and P0 compared with WT mice. This increased cell death was particularly interesting since Ret is expressed in all sympathetic precursors at E11.5, but most of these cells lose Ret expression by E15.5. Furthermore, although increased cell death occurred in Ret−/− SCG, it did not occur preferentially in Ret-expressing cells.

Additional analysis demonstrated that the Ret ligand ARTN is expressed in blood vessels along the normal route of sympathetic axons where ARTN potently stimulates SCG axon growth and is a chemoattractant that directs axon pathfinding. It is interesting that ARTN- and GFRα3-deficient mice have variable defects in the SCG. Specifically, SCG size was near normal in ARTN−/− and GFRα3−/− mice if the ganglia were normal and in position and peripheral targets were innervated, but SCG size was markedly diminished and apoptosis was increased in mice with abnormally located SCG and reduced peripheral target innervation. Defects in ARTN−/− and GFRα3−/− mice were detected as early as E10.5, when sympathetic neuron precursors emerge from the neural crest, demonstrating an important role for Ret/ARTN/GFRα3 signaling in precursor migration and neurite extension. Overall, these data suggest that increased cell death in the sympathetic nervous system of Ret−/−, ARTN−/−, and GFRα3−/− mice, compared with WT mice, occurs because of failure to properly innervate targets and subsequent deficiency in target-derived trophic factor (i.e., NGF deficiency). The Ret ligand GDNF also appears to be important for SCG development; since GDNF−/− mice have been reported to have a 30% loss of SCG neurons. Although GDNF supports the survival of cultured SCG neurons, the precise role of GDNF for SCG neuron survival *in vivo* has not been defined. Furthermore, mice missing the preferred GDNF receptor GFRα1 have a normal SCG at P0, which suggests that GDNF effects on the SCG are mediated via an alternate signaling pathway. These analyses demonstrate the complexity of interpreting mouse phenotypes and primary culture data and the importance of careful mechanistic time-course studies to define the role of specific trophic factors and their receptors in neuron survival. They also demonstrate robustly that programmed cell death in response to trophic factor deficiency at the time of target innervation provides a powerful mechanism to ensure survival of only the sympathetic neurons that correctly innervate their targets.

Programmed Cell Death in the Parasympathetic Nervous System

Cell death in parasympathetic neurons is much less well studied than in sympathetic neurons, but these cells do not appear to rely on neurotrophins (NGF, BDNF, or NT-3) for survival. Furthermore, although ciliary ganglion cell survival is supported by ciliary neurotrophic factor (CNTF) *in vitro*, CNTF does not appear to perform this function *in vivo* during normal development. Instead, Ret activation by GDNF and NRTN appears to be the most important determinant of parasympathetic neuron number. Unfortunately, a detailed analysis of cell apoptosis and proliferation

within the parasympathetic nervous system of mutant mice has not been performed for most cell populations. Nonetheless, several important conclusions can be drawn about the role of GDNF and NRTN in parasympathetic neurons:

1. Different parasympathetic neuron populations respond differently to the loss of Ret signaling. For example, otic and sphenopalatine ganglia are essentially completely absent from Ret−/− and GDNF−/− mice, but the reduced cell number in the ciliary and submandibular ganglia of these mice is much less dramatic (**Table 1**).
2. Reduced number of ganglion cells does not imply increased apoptosis. This is demonstrated by analysis of the sphenopalatine ganglion in GDNF and GFRα1−/− mice. In these animals, the sphenopalatine ganglia are absent as early as E12.5. Furthermore, bromodeoxyuridine labeling in GDNF−/− mice demonstrated reduced proliferation of sphenopalatine ganglion cell precursors, but terminal transferase deoxyuridine triphosphate nick end labeling (TUNEL) analysis failed to demonstrate any increase in cell death. Thus, at least in the sphenopalatine ganglion, GDNF/Ret signaling is required for precursor proliferation and not for survival of mature neurons.
3. Reduced parasympathetic ganglion cell survival may result from abnormal ganglion cell precursor migration. This is illustrated by the otic ganglion in GDNF−/− mice. Like the sphenopalatine ganglion, most otic ganglion cells are absent in GDNF−/− animals. In this case, however, otic ganglion cell precursors migrate abnormally, and TUNEL staining demonstrated increased cell death in the abnormally migrating precursors.

Thus, while programmed cell death may be important for parasympathetic nervous system development, it is much less well understood than in the sympathetic nervous system. In particular, mechanisms for eliminating cells with abnormal axon targeting have not been well documented in the parasympathetic nervous system. Indeed, target innervation by parasympathetic sphenopalatine neurons is dramatically reduced in NRTN−/− and GFRα2−/− mice, but sphenopalatine ganglion numbers are normal, which suggests that these cells are not dependent on target-derived trophic factors for survival.

Mechanisms Governing Neuron Number in the ENS

The ENS is a complex network of neurons and glia within the bowel wall that controls intestinal motility, responds to sensory stimuli, and regulates intestinal secretion and blood flow. To perform these functions, there are roughly as many neurons in the ENS as in the spinal cord, and the ENS comprises many distinct neuron subtypes that differ in function, transmitter phenotype, and pathways of axon pathfinding. Mechanisms of axon targeting in the ENS, however, and mechanisms to ensure that enteric neurons have correctly innervated their targets, are not yet understood. Furthermore, the ENS presents challenges that do not occur in other regions of the nervous system. This is especially true within the myenteric plexus since specific subtypes of myenteric neurons must extend their axons either orally (toward the mouth) or aborally (toward the end of the bowel) for the gut to function normally. Although there must be axon guidance cues present during development to direct these axons, adjacent regions of the bowel wall appear remarkably similar in the mature organism. Thus, unlike the sympathetic nervous system, where targets of innervation are far from the neuronal cell bodies and target-derived trophic factor dependence is an excellent mechanism for ensuring that only correctly targeted neurons survive, it is difficult to imagine how apoptosis could be used in the ENS to eliminate neurons whose axons project in the wrong direction. It is easier to imagine that apoptotic pathways could be important for enteric neurons projecting outside the muscular gut wall (e.g., to villi), but this has not yet been investigated.

With these ideas in mind, it is perhaps not surprising that apoptosis does not appear to occur within the developing or adult ENS of WT mice. This is not to suggest that enteric neurons are trophic factor-independent. Indeed, TrkA, TrkB, TrkC, p75NTR, Ret, GFRα1, and GFRα2 are all expressed within the ENS. Furthermore, enteric neurons undergo apoptotic cell death in primary culture when they are deprived of trophic factors and clearly respond to a variety of neuronal survival factors, including GDNF, NRTN, and NT-3, *in vitro*. In addition, Ret−/−, GFRα1−/−, and GDNF−/− mice miss essentially all enteric neurons from small bowel and colon. In fact, defective Ret signaling is the most commonly identified etiology of distal colon aganglionosis in humans (i.e., Hirschsprung disease). NT-3- and TrkC-deficient animals also have abnormal ENS development, with striking reduction in some subpopulations of enteric neurons. In contrast to these results, NRTN−/− and GFRα2−/− mice have a normal density of enteric neurons, but as in the parasympathetic nervous system, enteric neurons of NRTN−/− and GFRα2−/− mice are smaller than normal, with reduced neuronal projections, at least in some subtypes of neurons. Finally, apoptosis commonly occurs in ENS precursors of mice with a variety of mutations that cause intestinal aganglionosis, including in Ret−/−,

$Sox10^{Dom}/Sox10^{Dom}$, and Phox2b−/− animals. Thus, since ENS precursors depend on trophic factors for survival, but apoptosis does not appear to occur during normal ENS development, these factors must be produced in an adequate supply in WT animals to prevent programmed cell death. This further implies that ENS precursor proliferation must be carefully regulated to avoid producing more neurons than can be supported by available trophic factor. One way this occurs is that the availability of GDNF directly determines the rate of ENS precursor proliferation. Both increases and decreases in GDNF availability alter enteric neuron number via changes in precursor mitotic rates.

Apoptosis in Neuronal Injury

In addition to the physiologic role of apoptosis during normal development, cellular injury may also cause cell death via apoptotic pathways. In the ENS, for example, apoptosis has been reported in age-related myenteric neuron loss, anti-HuD-associated paraneoplastic syndrome, colitis-induced neuronal injury, and diabetes-associated ENS injury. Furthermore, at least in mouse models of diabetes, apoptosis can be reduced by providing additional trophic factor (GDNF) *in vivo*. It is interesting that in contrast to the effect of diabetes on the ENS, sympathetic neuron cell death does not appear to occur in diabetic rats. Thus, once again, the importance of apoptotic pathways in the autonomic nervous system and the ENS is specific to the age of the animal, the apoptotic trigger, and the neuronal subtypes evaluated. Presumably, these differences in the extent of apoptosis in different regions of the nervous system and at different times during life reflect both the availability of trophic factors to support survival and the abundance of intracellular pro- and anti-apoptotic proteins.

Summary

Programmed cell death is important for tissue morphogenesis during development and for the maintenance of tissue homeostasis during adult life. In part, apoptosis is valuable because it provides a way for the organism to specifically eliminate single cells that are no longer needed. This is important in the nervous system, where correct axon targeting and matching of the target size to the number of innervating nerve fibers is critical for function. Because both too much and too little cell death could be detrimental, carefully regulated intracellular and extracellular control mechanisms have been established to control PCD. Many of these mechanisms have been studied in detail in the sympathetic nervous system, where target-derived trophic factors are required for neuron survival during a defined developmental period when neurons are innervating their targets. Because trophic factors are active at the axon tip and are produced in the axon target this strategy effectively eliminates neurons that fail to innervate an appropriate target. Similar strategies are employed in most regions of the nervous system.

Mechanisms of PCD in the autonomic nervous system and the ENS highlight several important themes. First, for PCD to be useful for tissue morphogenesis, the axon tip and neuron cell body must be in different environments. For this reason, PCD tends to occur as neurons are innervating their final targets instead of when they first begin to extend axons. Because many parasympathetic neuron cell bodies are embedded in their targets and many enteric neurons project axons to targets in an environment similar to that around the cell body, it is more difficult to see how PCD could be used in the parasympathetic nervous systen and the ENS to ensure proper axon targeting. Indeed, it is difficult to find evidence that PCD occurs as a part of normal development in either the ENS or the parasympathetic nervous system. Second, for PCD to be effective, different targets need to produce different trophic factors, and subsets of neurons must respond to only a limited array of trophic factors. Even more effective would be a strategy to actively eliminate cells exposed to the 'wrong' trophic factor. This explains the variety of trophic factors and receptors present in the nervous system and the role of p75NTR to induce cell death in the absence of Trk receptor activation. Indeed, given the wide array of neuron subtypes and targets, it is remarkable that the nervous system can be established with so few trophic factors and receptors. It is probably for this reason that developing neurons may respond to a combination of trophic factors or change trophic factor dependence during development. Finally, most postmitotic neurons are needed throughout the life of the organism. For this reason, resistance to apoptosis in neurons that have correctly innervated their targets is important for longevity. In the nervous system, this is accomplished by limiting trophic factor-dependent cell survival to a small developmental period. It is important to note that this requires changes in the intracellular machinery for apoptosis once targets are innervated.

Tremendous advances have been made over the past decade in understanding the role of apoptosis in development and disease, but many challenges remain. In particular, it would be valuable to develop additional strategies to prevent neuronal cell death after injury. Ideally, these strategies should target specific cell populations since global inhibition of apoptosis is likely to be carcinogenic. Continued detailed analysis of the molecular mechanisms of apoptosis

and survival in different defined cell populations is therefore critical to allow targeted therapy and reduce disease-related morbidity and mortality.

See also: Enteric Nervous System: Neurotrophic Factors; Programmed Cell Death.

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Autophagy and Neuronal Death

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Autophagy and Its Role in the Mediation of Autophagic Cell Death

Autophagy

Although this article is primarily concerned with neurons, and the implication of autophagy in their death, it will be necessary to draw general principles from studies on other cell types, because autophagy is a general phenomenon occurring in virtually all types of cell, and the most convincing molecular analyses of its role in cell death have been done in nonneuronal cell lines.

Autophagy is the mechanism by which cells degrade parts of their own cytoplasm using the lysosomal machinery. There are several types of autophagy, including microautophagy, the direct capture of tiny portions of cytosol by invagination of lysosomal membranes; chaperone-mediated autophagy, a specific mechanism for degrading cytosolic proteins containing a particular pentapeptide consensus motif; pexophagy, the specific autophagocytosis of peroxisomes; and macroautophagy, which involves the engulfment of sizeable regions of cytoplasm, including organelles, in double-membrane vesicles called autophagosomes. Macroautophagy is the best-studied type of autophagy, and the only type that has been studied in detail in relation to cell death. This article will therefore deal primarily with macroautophagy.

Macroautophagy (**Figure 1**) is initiated by the formation of autophagosomes (about 400–800 nm in diameter) from cup-shaped double-membranous structures called isolation membranes or phagophores, which engulf cytosolic components, including organelles. The isolation membrane then closes to form the autophagosome. The origin of the isolation membrane is still a matter of debate. There is evidence that it may arise from various sources including smooth endoplasmic reticulum and the *trans*-Golgi network, but recent research on yeast indicates that a major source of its membrane is an independent punctate structure called the 'pre-autophagosomal structure.' The autophagosome fuses with a lysosome to form an autolysosome, where the enclosed material is broken down. The term 'autophagic vacuole' includes both autophagosomes and autolysosomes.

Autophagy (including macroautophagy) is involved in the normal turnover of cell contents and is enhanced by cellular stresses, against which it provides protection, for example, by replenishing the pool of free amino acids in the case of amino acid depletion, or by eliminating damaged proteins. Also, by reducing the size of stressed cells, autophagy reduces their metabolic burden. Thus, in many situations, autophagy promotes the health and survival of cells.

Autophagic Cell Death: Origins of the Concept

Despite the life-promoting roles of autophagy, macroautophagy has also been associated with cell death, and the term 'autophagic (or type 2) cell death' is used as a morphological classification for dying cells containing numerous autolysosomes. This occurs frequently during embryonic development and is probably the most common type of cell death in insect metamorphosis, but (macro)autophagic features are also associated with many cases of pathological cell death including heart failure, excitotoxicity, and neurodegenerative diseases.

Historically, the development of electron microscopy permitted the discovery of (macro)autophagy in the early 1960s, and this was soon followed by numerous ultrastructural studies from the mid-1960s onward, showing an abundance of autolysosomes in dying cells in many situations, including most cases of metamorphosis. Nevertheless, even as late as the 1990s, only a few authors considered that the autophagy was instrumental in the cell death.

The reasons for this reluctance were multiple. One was that autophagy had from the moment of its discovery been understood to play physiological roles in healthy cells, for example, the provision of breakdown products for reuse and the elimination of abnormal proteins, and several authors interpreted its presence in the dying neurons to reflect an unsuccessful survival-promoting mechanism for eliminating damaged regions of cytoplasm. Many other authors were influenced by the (then) widely accepted 'suicide bag hypothesis' of De Duve, discoverer of the lysosome, according to which cell death is achieved by the release of hydrolases from the lysosomes; the status of this hypothesis is still controversial. Then, as the suicide bag hypothesis gradually fell out of favor in the 1970s and 1980s, the simultaneous rise in popularity of a somewhat rigid dichotomy according to which all cell death had to be apoptosis or necrosis did not encourage openness to alternative mechanisms of cell death. Indeed, proponents of the apoptosis–necrosis dichotomy maintained that

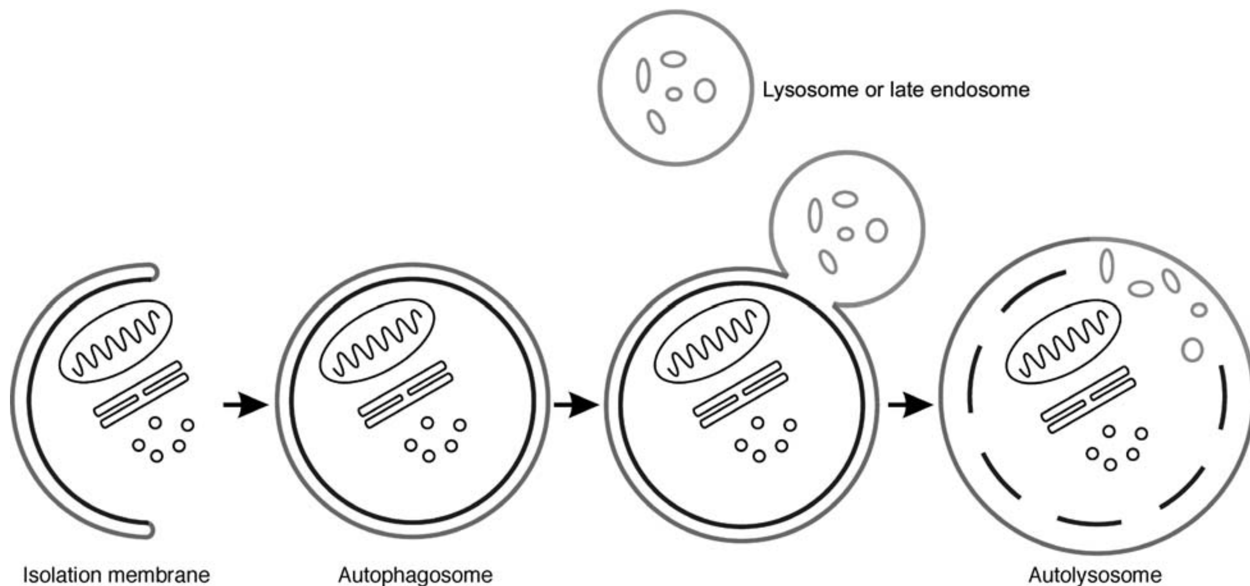

Figure 1 Macroautophagy. An isolation membrane (formed from smooth endoplasmic reticulum, the *trans*-Golgi network, or the 'pre-autophagosomal structure') enwraps cytosolic components and closes to form the two-membraned autophagosome, which then fuses with a lysosome or late endosome to give an autolysosome, in which the engulfed contents and the inner autophagosomal membrane (shown in green) are degraded.

autophagic dying cells were in fact undergoing apoptosis and that the autolysosomes were either a protective reaction or an irrelevant epiphenomenon. And, finally, it has to be admitted that a death-mediating role for the autophagy had not been proved, and in several cases very strong autophagy can occur without neuronal death.

The idea of autophagy-mediated cell death was, however, supported in the 1980s by experiments on neuronal death in the target-deprived isthmo-optic nucleus in chick embryos. (The isthmo-optic nucleus is the source of efferents from the brain to the retina in birds.) This neuronal death was characterized by abundant autolysosomes that ultimately filled most of the cytoplasm, and also by the loss of DNA from the nucleus to neighboring lysosomes. The fact that a cell's own DNA was being degraded by autophagy went against the view that the autophagy was a survival-promoting reaction to cellular stress.

Prevention of Autophagic Cell Death by Pharmacological Inhibitors of Autophagy

Nevertheless, a death-promoting role for autophagy gained only limited acceptance until it could be proved that inhibiting it prevented cell death. Initial evidence for this was provided in the 1990s by the death-preventing effects of 3-methyladenine (3-MA), an inhibitor of the formation of autophagic vacuoles that has been described as 'specific' but only in the limited sense that it does not alter the overall level of protein synthesis. Sandvig and van Beurs first showed, in 1992, that cell death, in this case toxin induced, could be prevented by 10 mM 3-MA. Subsequently, similar doses of 3-MA were shown to prevent (partially or completely) or delay cell death with autophagic characteristics in many situations including sympathetic neurons deprived of nerve growth factor, telencephalic neurons exposed to chloroquine, and cerebellar granule neurons deprived of serum and potassium. In all cases, the dying cells were shown to contain numerous autophagic vacuoles, and their rescue by 3-MA was accompanied by a reduction in their content of autophagic vacuoles. The suppression by 3-MA of autophagy is probably due to its inhibition of class III phosphatidylinositol 3-kinase (PI3-K), but it was uncertain whether this is also the basis of its protection against autophagic cell death, because its pharmacological profile is poorly characterized and it probably affects other enzymes. It was therefore important to test whether better-characterized inhibitors of PI3-K (LY294002 and wortmannin) could have similar protective effects. In many situations, these inhibitors are proapoptotic, because they inhibit the powerfully protective class I PI3-K pathway, so a protective effect due to inhibition of class III PI3-K (and hence autophagy) can easily be masked; but in serum-deprived PC12 cells, LY294002, wortmannin, and 3-MA have all been shown to be protective, apparently through the blockade of autophagy.

Prevention of Autophagic Cell Death by Interference with Autophagy Genes

However, even the better-characterized PI3-K inhibitors affect other cellular processes as well as autophagy, and definitive evidence for the death-mediating role of autophagy was provided only recently, by studies involving RNA interference of specific autophagy genes.

Our understanding of the control mechanisms of autophagy (particularly macroautophagy) depends to a great extent on intensive studies on autophagy in yeast, where about 30 genes controlling the initiation and execution of autophagy have been identified during the last decade. Until recently, these were grouped into three main gene families (apg, aut, and cvt), according to the genetic screens in which they were detected, but the functional distinctions between these families do not appear to be very clear-cut, and in the current terminology all the genes are grouped into the single 'atg' (autophagy gene) family (atg1–atg29 at the time of writing, but new members continue to be discovered). A detailed description of how these genes control autophagy would be beyond the scope of this chapter, but it is highly relevant to our present concerns that many of the yeast genes have vertebrate (including mammalian) homologs, and that certain of them, including atg5, atg6 (beclin 1), and atg7, are essential for the formation of autophagosomes.

This fact was used in two key papers in 2004 in which macroautophagy was blocked by RNA interference of atg5, atg6 (beclin 1), and atg7 (as well as by pharmacological inhibitors of autophagy) in cell lines whose apoptotic machinery had been deactivated genetically or pharmacologically. In both papers, pure autophagic cell death occurred and both the autophagy and the accompanying cell death were prevented by the RNA interference (and by the pharmacological inhibitors). Although a role for the autophagy genes in processes other than autophagy cannot be entirely ruled out, the fact that silencing each of the three genes prevented the autophagic cell death is strong evidence that the (macro)autophagy is not merely an epiphenomenon, or a defensive reaction, but is actually involved in mediating the cell death.

The Importance of Autophagy-Mediated Cell Death in Relation to Apoptosis

Autophagic cell death, as judged morphologically, seems to be the commonest type of cell death in physiological situations of massive cell death leading to the destruction of a tissue, as in many cases of metamorphosis and in some radical cases of mammalian embryonic tissue remodeling, whereas apoptosis appears to be the usual mechanism where sporadic dying cells occur in a tissue destined to survive. Thus, if autophagy could be assumed to mediate cell death in all cases of morphologically identified autophagic cell death, one could conclude that the autophagic death mechanism was of almost equal importance to the apoptotic mechanism.

Unfortunately, this is currently uncertain. While the reliability of 3-MA in protecting against many different cases of autophagic cell death does suggest that the autophagic death mechanism is of widespread importance, the more specific studies with RNA interference (or antisense) are still few in number, and situations have been reported in which massive autophagy can occur in cells without them dying. Moreover, there is evidence that a lysosomal, presumably autophagic, mechanism can initiate caspase activation and apoptosis. This is clearly different from autophagic cell death, which in many cases has been shown to be caspase independent, but does mean that morphological evidence for autophagy cannot be taken as proof of autophagy-mediated cell death. Thus, although the existence of an autophagic death mechanism is now difficult to deny, its generality and importance are still matters of debate.

Indeed, it has recently been argued that autophagy may mediate cell death only in very artificial situations where apoptosis has been deactivated (as in the two RNA interference studies mentioned above). Even if this were true, it would not detract from the importance of autophagic cell death in many pathological situations, where apoptosis may indeed have been deactivated either genetically (as in cancers that have become resistant to apoptosis-promoting chemotherapy) or pharmacologically (as in anticaspase neuroprotective protocols). But it has recently been shown that downregulation of atg5 by antisense technology protected against interferon-γ-induced autophagic cell death in HeLa cells whose apoptotic machinery had not been inhibited. Moreover, pharmacological blockade of autophagy by inhibition of PI3-kinase actually enhances the apoptotic machinery by increasing caspase-3 activation, but it can still prevent or delay cell death.

Thus, the autophagic death mechanism can be effective without the artificial deactivation of apoptosis, but its generality and importance are still not entirely clear.

Autophagy in Neuronal Death and Neurodegeneration

Autophagy in Neuronal Death

Although our mechanistic understanding of autophagic cell death has come largely from studies of

Table 1 Reports of neuroprotection by 3-MA

Situation	*Autophagic morphology?*	*Protection by 3-MA?*	*Caspase-dependent?*	*Reference*
Cerebellar granule neurons deprived of serum and potassium	Yes	Partial protection	Partially	Kaasik et al. (2005)
Cerebellar granule neurons in low potassium	Yes	Yes	Yes	Canu et al. (2005)
Telencephalic neuronal cultures exposed to chloroquine	Yes	Yes	No	Zaidi et al. (2001)
Sympathetic neurons treated with cytosine arabinoside	Yes	Cell death delayed	No	Xue et al. (1999)
Sympathetic neurons deprived of NGF	Yes	Cell death delayed	No	Xue et al. (1999)

nonneuronal cells, there is considerable morphological evidence for autophagic 'neuronal' death in all the main situations where neurons die: in natural development, in various pathological situations, and in experimental models, as is discussed below. In addition, there are a few studies showing the prevention of autophagic neuronal death by 3-MA (see **Table 1**).

Autophagic Neuronal Death during Development

Reports of autophagic neuronal death occurring naturally during development are relatively few, and most concerned anuran metamorphosis, including the death of the Rohon–Beard neurons, a transient population of sensory neurons that undergoes 100% cell death. In mammals, one is able to find only one relevant report; it concerned autophagic neuronal death in the developing cerebral cortex. This paucity of reports suggests that autophagic cell death plays only a relatively minor role in naturally occurring neuronal death in mammals (and other higher vertebrates). This fits with the generalization made above, that autophagic cell death occurs most commonly in physiological situations of massive cell death leading to the destruction of a tissue. However, caution is required, because in many studies isolated autophagic dying cells may have been mistaken for phagocytes, which they resemble morphologically and in their expression of autophagic markers.

Failure in competition for retrograde neurotrophic support is believed to be a major cause of naturally occurring neuronal death, and numerous studies of neuronal death in development have involved axotomy or other means of depriving neurons of retrograde support. In some cases, the resulting neuronal death was autophagic, but in many others it was clearly not. The reasons for the differences are unclear, but one factor may be the developmental stage. This was first indicated by an elegant study by Decker in 1978 on motor neuronal death in larval frogs. He found that very early axotomy caused a pyknotic (apoptosis-like) morphology, whereas very late axotomy caused classic chromatolysis. But axotomy at an intermediate stage caused the 'genesis of numerous secondary lysosomes in degenerating cells' – in other words, cell death with an autophagic morphology.

Studies on the isthmo-optic nucleus of chick embryos showed an age dependence that was similar to the above but not quite so clear cut. Early deprivation of retrograde support by blocking axonal transport in the isthmo-optic axons led to isthmo-optic neuronal death with a mixed morphology that was both pyknotic and autophagic, whereas later transport blockade caused a purer form of autophagic cell death with only minimal pyknosis (**Figure 2**). This neuronal death was also characterized by strong endocytic activity, a phenomenon that has since been observed in several subsequent studies of stressed, but not necessarily dying, neurons. Isthmo-optic neuron death could also be provoked by de-afferentation, but this caused no signs of autophagy, and when combined with blockade of retrograde support it decreased the autophagic characteristics of the dying neurons.

Neuronal Autophagy in Acute Neurological Conditions

The neuronal cell death in virtually all acute neurological conditions (e.g., stroke, traumatic brain injury, and neonatal asphyxia) shares a common mechanism: excitotoxicity, excessive depolarization that is usually due to the excessive activation of glutamate receptors, especially the *N*-methyl-D-aspartate (NMDA) subtype. Excitotoxic neuronal death is generally considered to be necrosis (most commonly) or apoptosis or a combination of the two, and, until recently, the presence of enhanced autophagy in these conditions was largely ignored. However, over the last few years, morphological evidence for intense autophagy and an increase in the autophagosomal marker LC3-II have been reported in several experimental models of cerebral hypoxia–ischemia, and an increase in the autophagy gene beclin 1 has been reported in a model of traumatic brain injury. NMDA receptor activation has likewise

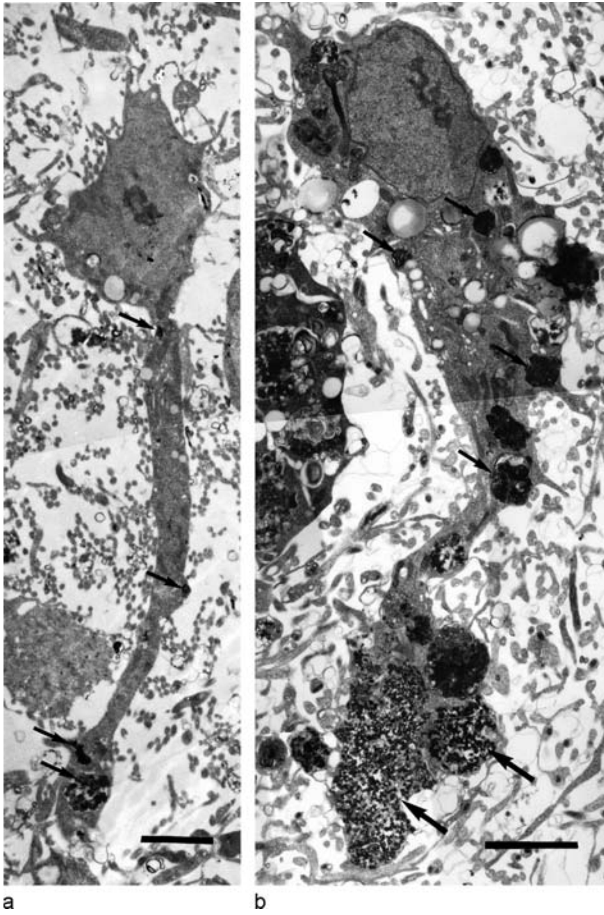

Figure 2 Electron micrographs of two autophagic dying neurons in the isthmo-optic nucleus of 14-day-old chick embryos. The death of both neurons was provoked by an injection of colchicine in their axonal target territory, which blocked axoplasmic transport, depriving them of retrograde support. (a) Early stage of neuronal death. Several vacuoles are found in the cytoplasm of the soma and main dendrite. The nucleoplasm and cytoplasm are somewhat denser than in a healthy neuron. (b) Advanced stage of neuronal death. The vacuoles are larger and more numerous, many containing membranous whorls. The arrows indicate vacuoles labeled with intravascularly injected horseradish peroxidase (in this and several other cases autophagic dying neurons have been shown to be strongly endocytic). Scale bar = 2 μm. From Hornung JP, Koppel H, and Clarke PGH (1989) Endocytosis and autophagy in dying neurons: An ultrastructural study in chick embryos. *Journal of Comparative Neurology* 283: 425–437.

been shown to induce autophagic neuronal death, in organotypic hippocampal cultures. This neuronal death was also characterized by strong endocytosis of exogenous horseradish peroxidase (**Figure 3**). However, it is currently unknown whether the autophagy in acute neurological conditions and excitotoxicity mediates cell death.

Autophagy in Neurodegenerative Diseases

In contrast to acute neurological conditions, neurodegenerative diseases involve progressive neuronal degeneration over periods of many months or years. Changes in the endosomal–lysosomal system, including increased macroautophagy, have been reported in virtually all neurodegenerative diseases including Alzheimer's, Huntington's, and Parkinson's diseases, prion diseases, and amyotrophic lateral sclerosis. The causes and roles of the increased macroautophagy are difficult to establish in human diseases, but additional information from experimental models provides some preliminary hypotheses. From models of Alzheimer's, Huntington's, and Parkinson's diseases, there is evidence that the macroautophagy may in many cases be involved in clearing protein aggregates from affected neurons, and hence be protective, but may also lead to autophagic neuronal death.

In Huntington's disease, the autophagy seems to be primarily protective. This disease involves massive neuronal death in the striatum as a result of the presence of an expanded polyglutamine repeat in the Huntington gene product. The dying neurons have a strongly autophagic morphology, and the autophagy appears to be a defense mechanism because the experimental enhancement of autophagy in fly and mouse models of Huntington's disease reduces the accumulation of polyglutamines as well as the neuronal death, whereas inhibition of autophagy has the opposite effect on both.

In Parkinson's disease, the situation is more ambiguous. The best-known neuropathological characteristics of this disease are the degeneration of dopaminergic neurons of the substantia nigra, and the presence of cytoplasmic inclusions called Lewy bodies in these neurons before they die. Lewy bodies contain ubiquitinated aggregates of α-synuclein and other proteins. There are reports that this neuronal death can have an autophagic morphology. Some cases of early-onset Parkinson's disease involve a mutation in the α-synuclein gene. In cultured PC12 cells, overexpression of mutant but not wild-type α-synuclein causes an impairment in the ubiquitin–proteasome system and the presence of ubiquitinated protein aggregates, an accumulation of autophagic vacuoles, and increased nonapoptotic autophagic cell death. Thus, although the increased autophagy may be an attempt to protect the cells by clearing the protein aggregates, it may also be involved in mediating the neuronal death.

Alzheimer's disease is characterized by the presence of (extracellular) β-amyloid plaques and filamentous tangles, primarily in the hippocampus and cerebral cortex. Both are currently believed to be involved in the degenerative changes in these brain regions. Pronounced macroautophagy has been demonstrated in the affected neurons, and β-amyloid has been shown to be generated by the proteolytic cleavage of β-amyloid precursor protein. In a mouse model of the

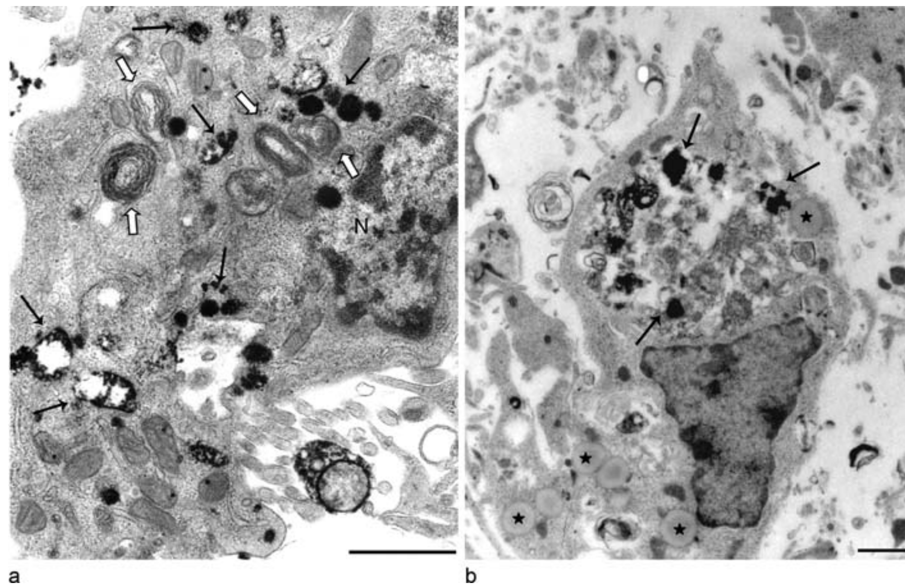

Figure 3 Autophagic degenerating neurons in the CA1 region of organotypic hippocampal cultures after 2 h (a) or 8 h (b) of exposure to 100 μM NMDA. (a) Thick white arrows, membranous whorls (autolysosomes); black arrows, endosomes labeled with horseradish peroxidase; N, nucleus. (b) Clumps of peroxide labeling within a large vacuole; stars, putative unlabeled endosomes. Scale bar = 1 μM. Reproduced from Borsello T, Croquelois K, Hornung JP, and Clarke PGH (2003) *N*-methyl-D-aspartate-triggered neuronal death in organotypic hippocampal cultures is endocytic, autophagic and mediated by the c-Jun N-terminal kinase pathway. *European Journal of Neuroscience* 18: 473–485, with permission from Blackwell Publishing.

disease, a similar neuronal macroautophagy occurs, and this happens rather early, before the extracellular β-amyloid deposits, but the maturation of autophagosomes to autolysosomes appears to be impaired. At later stages, there is a further accumulation of autophagosomes, and these are rich in β-amyloid. Inducing or inhibiting macroautophagy elicits parallel changes in macroautophagy and β-amyloid production, suggesting that in this case the macrophagy may contribute to the disease process, but not necessarily through autophagic cell death.

Neuronal Autophagy in Lysosomal Storage Diseases

Lysosomal storage diseases are caused by mutations in the genes encoding various lysosomal hydrolases, leading to the accumulation (or 'storage') of partially digested substances in lysosomes. Different lysosomal storage diseases cause degenerative and other changes in different organs of the body, including in some cases the brain (e.g., in Tay–Sachs disease and Niemann–Pick C disease). Whereas most neurodegenerative diseases involve increased lysosomal digestion, lysosomal storage diseases are caused by a 'decrease' in one particular component of lysosomal digestion, but this can lead to complex changes in many different cellular signaling pathways. Since the genetic mutation directly affects the lysosomal system, autophagic digestion must presumably be affected. There have been few studies of autophagy in neuronal death in these diseases, but in a mouse model of Niemann–Pick C disease there was massive degeneration of cerebellar Purkinje cells, which had features consistent with autophagic cell death.

See also: Programmed Cell Death.

Further Reading

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Neurotrophins: Physiology and Pharmacology

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Introduction

Nerve growth factor (NGF) was the first neurotrophin family member and the first nervous system growth factor to be identified. NGF was originally isolated from a mouse sarcoma based upon its ability to promote the hypertrophy of, and fiber outgrowth from, peripheral sensory neurons. Subsequently, other neurotrophin family members were identified, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). Although originally described as a potentially novel growth factor, neutrotrophin-5 was found to be identical to NT4. Two other neurotrophin members, neurotrophin-6 and-7, were identified in fish but do not appear to have corresponding homologs in mammals.

Neurotrophin Production and Release

Neurotrophin proteins are synthesized as precursors of approximately 270 amino acids (30–35 kDa) and are subsequently cleaved to yield mature proteins of ~120 amino acids (13 kDa) in length. While most physiological actions of neurotrophins have been ascribed to the mature proteins that generally function as soluble, noncovalently linked homodimers, recent studies have identified potentially critical roles for the unprocessed neurotrophin precursors (see later). Elements within the precursor sequence of newly generated neurotrophins presumably direct the intracellular sorting of neurotrophin molecules into one of two distinct pathways, resulting in either constitutive or regulated secretion. This process of intracellular sorting plays a critical role in determining the possible spectrum of physiological actions mediated by a given neurotrophin molecule by determining when and where neurotrophin secretion will take place. While neurotrophins were originally believed to function exclusively as target-derived factors that mediate signaling in a retrograde manner, recent studies have provided substantial evidence that some neurotrophins function partially, or exclusively, as anterograde molecular signals.

Neurotrophin Actions Are Mediated by Distinct Cell Surface Receptors

Once neurotrophins are released from the cell of origin, their actions are mediated by binding to distinct cell surface receptors. The specificity of neurotrophin action is controlled primarily by the pattern of expression of receptors in distinct populations of neurons located at the site of neurotrophin release.

Two distinct classes of neurotrophin receptors have been identified. The p75 neurotrophin receptor (p75NTR) is a member of the tumor necrosis receptor family that binds all of the mature neurotrophins with a similar, nanomolar affinity. Recent studies have indicated that the p75NTR can also bind unprocessed neurotrophin precursors with a high affinity. The second class of neurotrophin receptors is made up of three distinct members of the tropomyosin-related kinase (Trk) receptor family. The various neurotrophins show some degree of specificity with respect to their binding of distinct Trk receptors as shown in **Figure 1**. Binding of neurotrophins to their respective Trk receptors occurs with an affinity 100-fold greater than binding of mature neurotrophins to the p75NTR. Moreover, the various Trk family members have highly homologous intracellular domains, suggesting they use similar signaling pathways to mediate their effects. Differential splicing of TrkB and TrkC genes can also produce neurotrophin receptors that lack the intracellular kinase domains. The functions of these truncated receptors are unknown, but studies have suggested that they may play a role in regulating the availability or presentation of secreted neurotrophins to full-length and active receptors.

The physiological role of the p75NTR has been a subject of considerable study. The p75NTR was initially thought to act as a coreceptor, conferring high-affinity binding of neurotrophins to their respective Trk receptors and promoting retrograde transport of neurotrophins from their targets. However, in the absence of Trk receptor expression, the p75NTR may promote apoptosis via multiple pathways, including an increase in nuclear factor-kappa B (NF-κB) activity and by inducing sphingomyelin hydrolysis into ceramide (**Figure 2**). Neurotrophin binding to the p75NTR also leads to increased activation of c-Jun N-terminal kinase (JNK), a stress-activated protein kinase that has also been implicated in cell death. Binding of the precursor for NGF to the p75NTR has recently been demonstrated to increase apoptotic death signaling; the consequence of binding other neurotrophin precursor molecules to this receptor remains to be determined. A key factor in determining the fate of neurotrophin signaling through the p75NTR appears to be the extent to which Trk receptors are co-expressed; in the presence of Trk receptors, neurotrophins primarily support cell survival, whereas

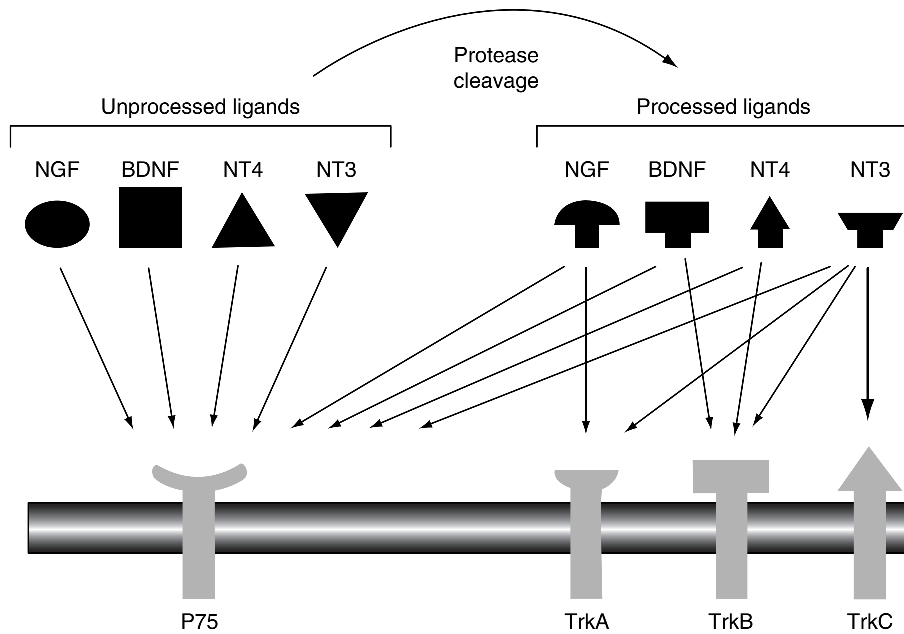

Figure 1 Neurotrophins binding to their receptors. All neurotrophin precursors associate with the p75NTR with similar high-affinity binding. Processed (mature) neurotrophin proteins also bind to the p75NTR with a 100-fold lower affinity. Mature neurotrophins bind with high affinity to members of the tropomyosin-related kinase (Trk) family. Interactions with Trk receptors show a high degree of ligand specificity, with nerve growth factor (NGF) binding selectively to TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4) binding to TrkB, and NT3 binding primarily to TrkC. From Segal RA (2003) Selectivity in neurotrophin signaling: Theme and variations. *Annual Review of Neuroscience* 26: 299–330.

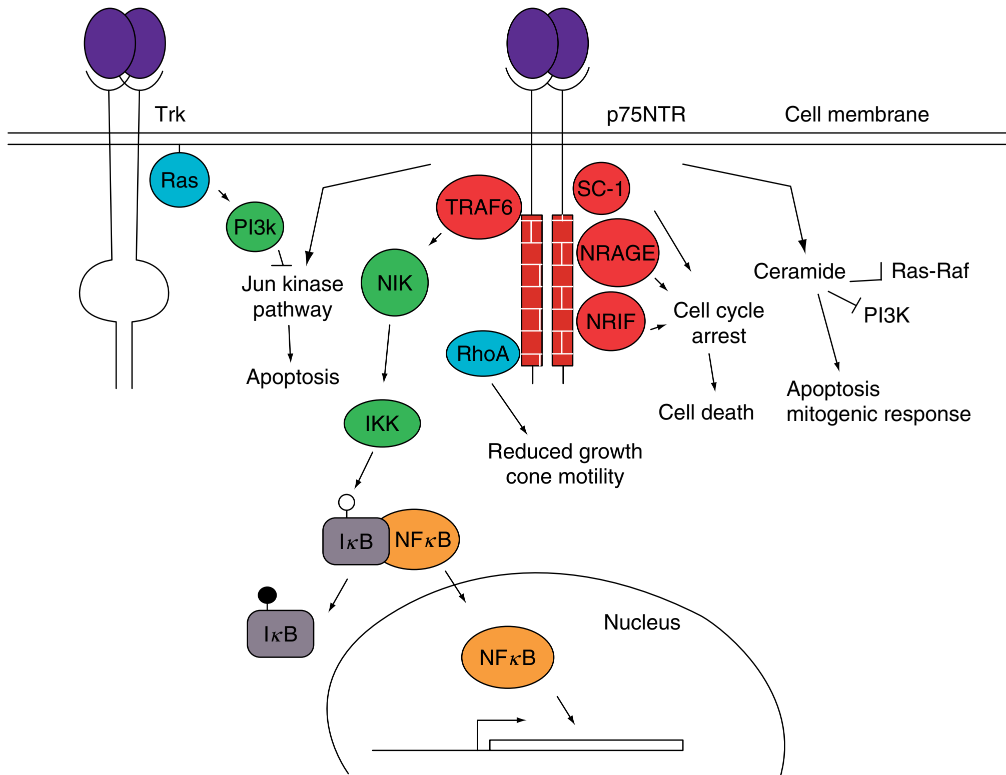

Figure 2 Neurotrophin signaling through the p75 neurotrophin receptor (p75NTR). Binding of neurotrophins to the p75NTR leads to alterations in the function of many associated proteins, leading in turn to changes in gene expression and apoptosis. Adapter proteins are shown in red, kinase proteins in green, small G-proteins in blue, and transcription factors in brown. Trk, tropomyosin-related kinase; PI3K, phosphatidylinositol 3-kinase; NIK, NF-κB-inducing kinase; IKK, inhibitor of κB kinase; IκB, inhibitor of κB; NF-κB, nuclear factor-kappa B; TRAF6, tumor necrosis factor receptor-associated kinase-6; SC-1, Schwann cell factor-1; NRAGE, neurotrophin receptor-interacting MAGE (from melanoma antigen); NRIF, neurotrophin receptor-interacting factor. From Huang EJ and Reichardt LF (2001) Neurotrophins: Roles in neuronal development and function. *Annual Review of Neuroscience* 24: 677–736.

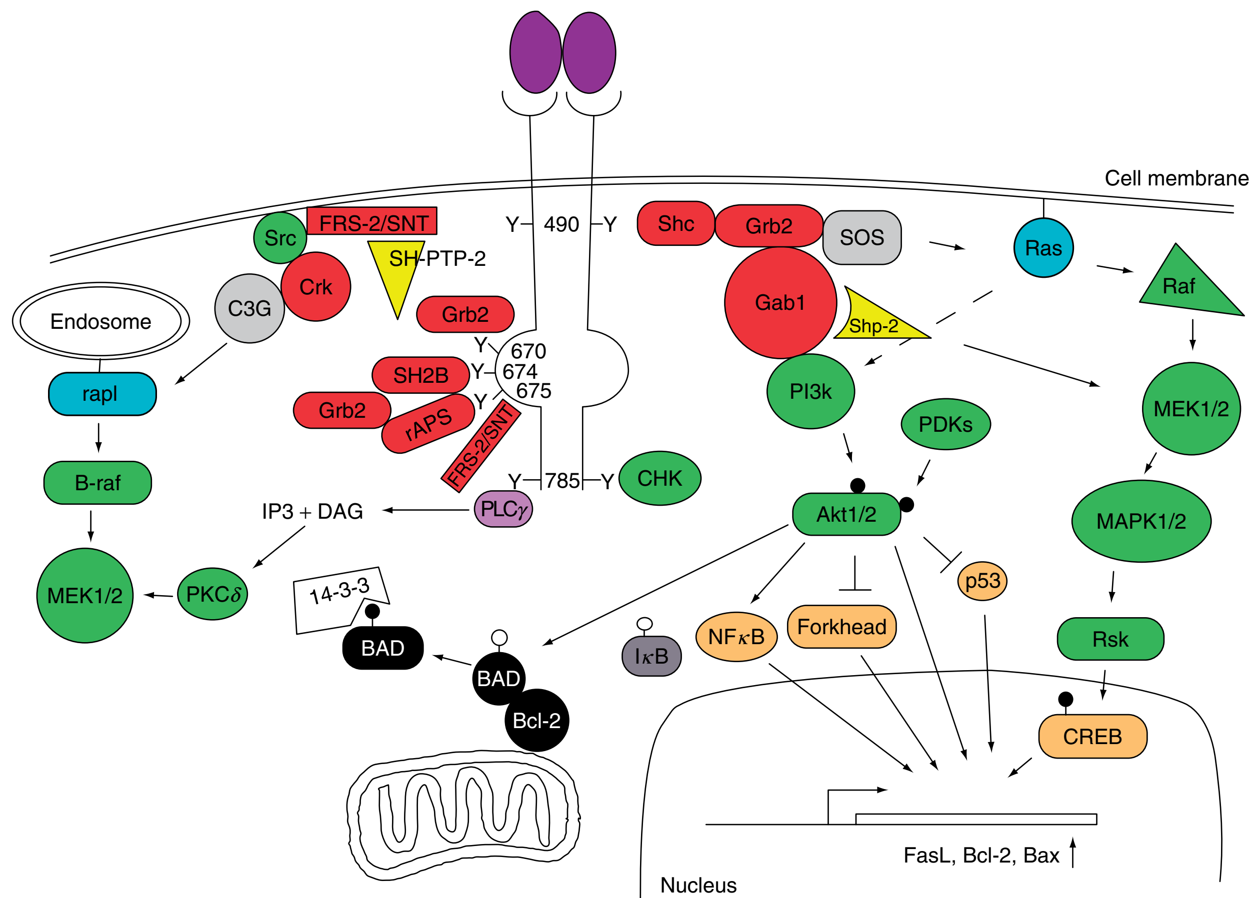

Figure 3 Neurotrophin signaling through Trk receptors. Binding of mature neurotrophins to Trk receptors leads to the phosphorylation of a wide variety of associated proteins, which ultimately induce alterations in gene expression. Neurotrophin actions through Trk receptors affect neuronal survival, neurite outgrowth, cell morphology, and synaptic efficacy. Adapter proteins are shown in red, kinase proteins in green, small G-proteins in blue, and transcription factors in brown. From Huang EJ and Reichardt LF (2001) Neurotrophins: Roles in neuronal development and function. *Annual Review of Neuroscience* 24: 677–736.

in the absence of Trk receptors, neurotrophin signaling through p75NTR primarily mediates cell death.

Binding of mature neurotrophins to their respective Trk receptors induces dimerization of the receptors and the activation of kinase activity. Increased phosphorylation activity mediated through activated Trk receptors drives cellular responses through three distinct molecular pathways as indicated in **Figure 3**, resulting ultimately in a variety of transcriptional changes. Neurotrophin signaling through Trk receptors can influence many aspects of cell function, including cell survival, neurite outgrowth, and cellular differentiation.

Neurotrophin Physiology

Cell Survival

One of the classic physiological functions attributed to neurotrophins is the regulation of cell survival, specifically within neuronal populations expressing a particular type of neurotrophin receptor. Within the peripheral nervous system, tightly regulated neurotrophin expression is thought to be responsible for controlling neuron number through the process of programmed cell death, whereby a precisely timed and limited expression of target-derived neurotrophins limits the survival of responsive neurons projecting to a given peripheral target. Data from genetically mutant mice with targeted deletions of specific neurotrophins have provided evidence supporting a role for neurotrophins as target-derived regulators of neuronal survival in the periphery. Consistent with known patterns of neurotrophin and receptor expression, targeted deletion of either specific neurotrophins or their receptors results in profound, yet specific, losses of distinct populations of peripheral neurons during development. For instance, targeted deletion of NGF results in a nearly complete loss of peripheral sympathetic and nociceptive sensory neurons that express TrkA receptors. Targeted NGF deletion, however, does not significantly reduce the number of TrkC-expressing peripheral sensory neurons.

Based upon observations made within the peripheral nervous system during development, it was initially postulated that neurons within the central nervous system (CNS) may also rely upon neurotrophin support for their survival, not only during development but also in the adult. In spite of this prediction, there is strikingly little evidence suggesting that neurotrophins play a significant role in promoting cell survival within the CNS, either during development or in the adult. For instance, basal forebrain cholinergic neurons, which possess both p75NTR and TrkA receptors throughout life and show numerous physiological responses to NGF, show minimal deficits in developmental cell survival following targeted deletions of either NGF or its corresponding TrkA receptor. Moreover, deletion of the cellular source of neurotrophins in the adult animal also fails to cause cell death. However, neurotrophins appear to have many other effects upon neurons in the CNS, especially pertaining to cellular differentiation, target innervation, and synaptic plasticity.

Cell Differentiation and Morphology

In addition to their well-defined actions on cell survival, neurotrophins have been implicated in numerous aspects of cellular differentiation. Some of the earliest studies with NGF demonstrated that this neurotrophin induced dramatic outgrowth of neurites from developing peripheral neurons. Subsequently, the various members of the neurotrophin family have been implicated in mediating a variety of morphological changes, in a multitude of distinct neuronal populations, including the extension and guidance of axons, hypertrophy of the cell body, and changes in dendritic complexity and dendritic spine density. For example, respective expression of BDNF or NT3 in cortical target regions of extending axons during brain development appears to define zones of axon growth termination in strikingly laminar patterns, helping to establish the detailed topography of mature innervation. In addition to controlling the initial outgrowth of neuronal processes, neurotrophins have been strongly implicated in the process of maintaining target innervation in both central and peripheral targets. One of the most striking effects observed in NGF knockout animals is the failure of NGF-sensitive cholinergic neurons to retain appropriate innervation of their central targets.

Neurotrophins also play an important role in other aspects of cellular differentiation, helping to determine the ultimate fate and function of certain cells. For example, NGF promotes the differentiation of sympathoadrenal precursors into sympathetic neurons, as opposed to adrenal chromaffin cells. Neurotrophins also play more subtle roles in neuronal function by regulating the expression of neurotransmitters, ion channels, and receptors, both during development and, in many cases, throughout adulthood.

Synaptic Function and Synaptic Plasticity

In addition to regulating neuronal function, neurotrophins have direct actions on synaptic function and synaptic plasticity. Application of neurotrophins at developing and mature synapses can stimulate or modulate neurotransmitter release under many circumstances. The induction of long-term potentiation (LTP), a possible electrophysiological substrate for long-term storage of information in the nervous system, is potently regulated by BDNF. *In vitro* and *in vivo* applications of BDNF to the hippocampal formation are capable of inducing long-lasting synaptic potentiation in the absence of any additional excitatory stimulus. Moreover, the ability to induce LTP in the hippocampus with an electrical stimulus is greatly attenuated when BDNF signaling is disrupted. Together, these results suggest that BDNF may play a critical and necessary role in regulating synaptic plasticity in certain populations of neurons. Whether or not the regulation of synaptic plasticity is a selective property of BDNF is unknown at present, but NGF and NT3 have not been demonstrated to influence synaptic function and LTP in a similar manner.

Neurotrophin Pharmacology

The potent survival effects of neurotrophins on neurons of the peripheral nervous system originally discovered 50 years ago fostered speculation for many years that neurotrophins may also promote the survival of CNS neurons, thereby representing pharmacological candidates for treating neurodegenerative diseases. The degeneration of specific neuronal populations is a hallmark of the most common neurodegenerative disorders, including losses of midbrain dopaminergic neurons in Parkinson's disease and the loss of basal forebrain cholinergic neurons as one component of multisystem cell loss in Alzheimer's disease. The hypothesis that neurotrophins might represent a new class of therapeutic molecules was substantiated by studies published in the mid-1980s, demonstrating that NGF prevented the injury-induced death of basal forebrain cholinergic neurons in adult rats. NGF neuroprotection of adult cholinergic neurons was also reported after lesions in the brains of adult primates. Other studies reported that spontaneous atrophy and dysfunction of basal forebrain cholinergic neurons in aged rats and primates could also be reversed by NGF administration. Further, NGF treatment significantly ameliorated

Table 1 Potential clinical uses for neurotrophins

Neurotrophin	*Targeted cell population*	*Disease*
Nerve growth factor	Basal forebrain cholinergic	Alzheimer's disease
	Sensory nociceptive	Peripheral neuropathy
Brain-derived neurotrophic factor	Cortical neurons	Alzheimer's disease
	Spinal motor neurons	Amyotrophic lateral sclerosis
	Midbrain dopaminergic	Parkinson's disease
	Cortical neurons	Acute brain injury/stroke
Neurotrophin-3	Sensory proprioceptive	Peripheral neuropathy

age-related memory impairments. Collectively, these findings established a theoretical basis for pursuing clinical trials targeting NGF therapy for the treatment of Alzheimer's disease, whereby a significant loss of basal forebrain cholinergic neurons is postulated to contribute to ongoing cognitive decline.

Analogous experimental paradigms explored the possibility that the neurotrophins could protect cell populations affected in a diverse spectrum of neurodegenerative diseases, including nigral neurons, motor neurons of the spinal cord, and dorsal root ganglion sensory neurons (see **Table 1**).

Clinical Trials with Neurotrophins

The potent actions of neurotrophins in preventing neuronal degeneration and augmenting cell function made them intriguing candidates for clinical testing in human neurological disorders. To date, neurotrophins and other growth factors have been tested in clinical trials in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke, and peripheral neuropathy. Trials are also under consideration in treating Huntington's disease and spinal cord injury.

In spite of the extensive animal data supporting the potential use of neurotrophins in treating a variety of neurological diseases, no studies to date have yielded clear evidence of efficacy. One of the most important limiting factors in testing the clinical potential of neurotrophins has been the availability of an effective, safe, and sustained method of delivering growth factors to the brain. Because neurotrophins are large and charged proteins, they do not cross the blood–brain barrier after peripheral administration. When infused into the cerebrospinal fluid space of the brain (intraventricular or intrathecal infusions), growth factors usually fail to diffuse to neuronal targets. In the case of NGF, intraventricular or intrathecal infusions broadly disseminate the neurotrophin throughout the subarachnoid space of the brain and spinal cord, inducing the migration of Schwann cells into the CNS, stimulating nociceptive axons of the dorsal root ganglia (DRG) (resulting in pain), eliciting sympathetic axon sprouting around the cerebral vasculature, and causing weight loss. Thus, clinical testing of neurotrophins requires a delivery method that both achieves adequate growth factor concentrations at target neurons that may lie deep within the brain, and restriction of growth factor delivery to target regions in order to avoid adverse effects of broad growth factor dissemination in the cerebrospinal fluid space. Chronic intraparenchymal growth factor infusion into the brain, or growth factor gene delivery into the brain, may be a means of achieving these goals.

Indeed, recent signs of possible growth factor activity in Alzheimer's disease and Parkinson's disease have been reported using targeted intraparenchymal delivery methods. In Alzheimer's disease, a recent phase I clinical trial of NGF gene delivery to cholinergic neurons was reported to increase glucose utilization in the cortex by positron emission scanning, and to elicit new growth of axons toward the NGF source. Potential effects on cognition are being explored in phase II clinical trials of NGF gene delivery in Alzheimer's disease. A clinical trial of growth factor gene delivery in Parkinson's disease is also under way (using neurturin, a glial-derived neuronal growth factor). Three recent clinical trials using intraparenchymal infusion of glial-derived neuronal factor (GDNF) have also been conducted in Parkinson's disease. The development of these targeted, restricted, and sustained growth factor delivery methods raises the possibility that the next few years will definitively establish whether growth factors will prove useful for the treatment of CNS disorders.

Neurotrophins have also undergone clinical testing for peripheral neuropathy: NGF is a tropic factor for a subpopulation of nociceptive sensory neurons with cell bodies in the DRG, and NT3 is a tropic factor for large-diameter proprioceptive neurons of the DRG. NGF was tested in patients with diabetes using intermittent peripheral subcutaneous injections, but failed to show efficacy. Once again, problems with potentially subtherapeutic doses, inadequate targeting, and rapid peripheral degradation of NGF limited the

interpretability of the study. NT3 is also being tested in clinical trials for drug-induced peripheral neuropathy. Solutions to the challenge of targeted, sustained, and safe growth factor delivery for treatment of peripheral nervous system disorders await identification, to determine whether early potential signals of growth factor activity in the CNS may also be achievable in the peripheral nervous system.

See also: Enteric Nervous System: Neurotrophic Factors.

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Enteric Nervous System: Neurotrophic Factors

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Introduction

Our understanding of the roles of neurotrophic factors in the enteric nervous system (ENS) has lagged behind that in other parts of the peripheral nervous system, largely because of the complexity of enteric neuronal circuitry. Neurons of the ENS are located within the gut wall, in numerous small, irregular-sized ganglia located along the entire length of the gastrointestinal tract. The circuitry of the ENS is difficult to analyze experimentally, because, unlike many parts of the nervous system, where well-identified neurons make clearly defined connections with their target tissues, enteric ganglia, particularly the myenteric ganglia, are composed of a mixture of different neuronal types in varying proportions, with diverse projections to a range of target cell types.

When the prototypic neurotrophic factor, nerve growth factor (NGF), was first identified and its effects on sensory and sympathetic neurons characterized, its possible effects on the ENS were not studied. In the early 1990s, however, when other members of the neurotrophin family, notably brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) and their receptors, were identified and their properties and actions began to be elucidated, it was found that several of these proteins were expressed in the gut. At that stage, their localization within the gut wall had not been fully characterized, but analysis of the effects of NT-3 on neural precursors isolated from the embryonic gut indicated that NT-3 promoted differentiation of both enteric neurons and enteric glial cells. These studies provided the first indication that neurotrophins may play a role in the ENS. More recently, supporting evidence for this hypothesis has come from transgenic mice in which NT-3 expression is disrupted; these mice exhibit abnormalities in the ENS (see later).

Soon after identification of the neurotrophins, two major discoveries provided a breakthrough in the study of neurotrophic factors and the ENS. First, in the early 1990s, was the discovery that a mutation in the c-ret gene was associated with Hirshsprung disease, a congenital condition in which varying lengths of the terminal colon are aganglionic, and have no enteric neurons. At this time, although the c-ret gene was known to encode a tyrosine kinase receptor, its ligand was unknown. c-ret −/− knockout mice were found to die shortly after birth, and among other abnormalities were found to lack enteric neurons (and glia) from all regions of the gut except for the esophagus. Soon after this finding, in 1996, transgenic animals lacking the newly discovered neurotrophic factor, glial cell line-derived neurotrophic factor (GDNF), were found to display an identical phenotype to the c-ret −/− mice, and it was discovered that c-ret was the signaling receptor for GDNF. c-ret forms part of a receptor complex, together with a glycosyl phosphatidylinositol (GPI)-linked GDNF binding receptor, now known as GFRα1. GFRα1 knockout mice also display the aganglionic phenotype. Since the discovery of GDNF, three related proteins were discovered, at least one of which, neurturin (NTN), is also active in the ENS.

Since these first seminal studies suggesting roles for GDNF and NT-3 in the developing ENS, much more work has been carried out on the expression and actions of neurotrophic factors in the gut. Most detailed studies have focused on establishing the roles of neurotrophic factors in the development of enteric neurons, a process which is not described in detail here. Increasing efforts, however, are now being put into investigating the patterns of expression and actions of neurotrophic factors in the gut after birth and in maturity, with the aim of determining if these factors are involved in neural plasticity in the adult ENS and if disruption of neurotrophic factor support may be involved in some pathological changes that take place in the ENS, or in ENS aging.

Neurotrophic Factors and Their Receptors in the Gut

The main neurotrophic factors and/or their receptors that have been shown to be present in the gut, or active on enteric neurons, and hence implicated in the development and/or the maintenance of the ENS, are shown in **Table 1**. It is important to note, however, that in addition to factors classified as neurotrophic factors, other secreted proteins, peptides, and molecules play important roles in the survival, differentiation, and maintenance of the ENS. Examples of such proteins that act on the developing ENS before birth include the hedgehog family members, Indian and Sonic hedgehog, which may be involved in the patterning of the ENS; the bone morphogenetic proteins, BMP-2 and -4, which are involved in early specification of enteric neurons; and endothelins, which inhibit neuronal differentiation and may thus prevent precocious neuronal differentiation in the

Table 1 Neurotrophic factors and neurotrophic factor receptors involved in ENS development and/or maintenance[a]

Neurotrophic factor family	*Receptors*	*Comments*
Neurotrophins	Common low-affinity p75 receptor; high-affinity tyrosine kinase receptors (Trks), with preferential specificity:	NGF, NT-3, and BDNF all expressed in the gut; varied reports of receptor expression by enteric neurons (see text)
Nerve growth factor (NGF)	TrkA	
Brain-derived neurotrophic factor (BDNF)	TrkB	
Neurotrophin-3 (NT-3)	TrkC	Evidence for roles of NT-3 in ENS both pre- and postnatally; some evidence for roles of other factors (see text); no evidence for NT-4/5 expression in the gut, but extrinsic sensory innervation of intestine severely affected in NT-4 −/− mice
Neurotrophin-4/5 (NT-4/5)		
Glial cell line-derived neurotrophic factor (GDNF) family	Common signaling tyrosine kinase receptor c-ret; glycosyl phosphatidylinositol (GPI)-linked binding GFRα family, with preferential specificity:	
GDNF	GFRα1	GDNF and NTN and their receptors expressed in the gut; established roles for GDNF in early ENS development; NTN has later actions
NTN	GFRα2	
Artemin	GFRα3	Some evidence for artemin expression in gut and action on enteric neurons (see text)
Persephin	GFRα4	No evidence for expression or actions of persephin in the gut
Neuropoietic cytokines	Tripartite receptor, comprising:	Evidence that an as yet unknown factor, probably related to CNTF or LIF, acts at a neuropoietic cytokine receptor complex in the ENS; activation of receptor complex implicated in prenatal development, but roles uncertain; some actions postnatally
Ciliary neurotrophic factor (CNTF)	CNTFRα	
Leukemia inhibitory factor (LIF)	Gp130	
Oncostatin M	LIFRβ	
Cardiotrophin-1		

[a]Note: this is not an exhaustive list of all factors/receptors in these families.

developing gut. In several cases these proteins act by influencing the responsiveness of neural precursors to neurotrophic factors such as GDNF.

Other growth factors, proteins, peptides, and even molecules traditionally associated with processes such as neurotransmission may also have neurotrophic actions in the ENS. Examples include fibroblast growth factor 2, which may be expressed by enteric neurons and which promotes neurite outgrowth from postnatal myenteric neurons *in vitro*; adenosine; serotonin, which promotes differentiation of fetal neuronal precursors in culture; and also vasoactive intestinal peptide (VIP) and nitric oxide, which have been found to promote survival of adult myenteric neurons in culture. These molecules are not further considered here.

Roles of Neurotrophic Factors in Early (Prenatal) ENS Development

The most detailed understanding of neurotrophic factor action in the gut is that of GDNF, in particular its roles during early development of the cells of the ENS, which arise from specific regions of the neural crest. Since this topic is covered in detail elsewhere, only an outline is provided here.

Glial Cell Line-Derived Neurotrophic Factor

GDNF is essential for development of the ENS. It is expressed in the early intestinal mesenchyme, just after the arrival of migrating neural crest-derived progenitors in the developing gut; these progenitors express the signaling receptor for GDNF, c-ret, as or just before they arrive in the gut. In cell culture models, GDNF has been found to promote survival, proliferation, and differentiation of ENS progenitor cells as well as their migration along the growing gut. As already mentioned, transgenic mice in which the GDNF gene is disrupted have an aganglionic gut distal to the esophagus and are not viable. Targeted disruption of either the c-ret or the GFRα1 genes produces animals with a similar ENS phenotype.

Neurturin

NTN is also expressed in the developing gut mesenchyme, and GFRα2 is expressed by developing enteric ganglion cells. Both NTN and GFRα2, however, are first detected slightly later than GDNF and GFRα1 in

the developing mouse gut. *In vitro*, NTN promotes the survival and proliferation of enteric neural progenitors; *in vivo*, targeted disruption of the NTN or GFRα2 genes results in mice which exhibit abnormalities of the ENS. The enteric abnormality in NTN and GFRα2 knockout animals results in a reduction in neuronal perikaryon size, a small but consistent reduction in myenteric neuronal numbers, and a reduction in the density of cholinergic nerve fibers in adult animals. GFRα2 knockout mice also display a reduction in the density of substance P-immunopositive fibers. Both NTN –/– and GFRα2 –/– transgenic animals also exhibit abnormalities in gut function, including reduced smooth muscle contractility and reduced responses to electrical stimulation and carbachol. These changes may contribute to the poor growth of NTN –/– and GFRα2 –/– mice seen after birth.

Artemin

A recent study has shown that overexpression of artemin in mice results in hyperplasia of myenteric ganglion cells, and that artemin has a small effect on enteric neurons *in vitro*. Clear evidence for expression of GFRα3 by enteric neuronal precursors or differentiated enteric neurons, however, is lacking, and it is possible that the effects observed in this study were due to an action of artemin binding to GFRα1 or GFRα2.

Neurotrophin-3

NT-3 is expressed in the developing intestinal mesenchyme, and TrkC receptors are expressed by enteric neural progenitors. *In vitro*, NT-3 promotes survival of progenitors and differentiation of enteric neurons and glia. *In vivo*, NT-3 –/– and TrkC –/– transgenic mice have enteric ganglia, although recent evidence has shown that NT-3 null mice have reduced numbers of myenteric and submucosal neurons. Conversely, transgenic mice in which overexpression of NT-3 is targeted to the myenteric plexus have increased numbers of myenteric neurons, of a larger size than those in wild-type mice. Evidence also indicates that NT-3 acts together with other factors, and may be important in later stages of fetal ENS development.

Nerve Growth Factor and Brain-Derived Neurotrophic Factor

Although NGF, BDNF, TrkA, and TrkB transcripts have all been detected in the developing gut, there is no evidence that they have a role in early ENS development.

Neuropoietic Cytokines

The neuropoietic cytokines ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) are expressed in the gut; CNTF has been detected in the fetal mouse gut, and LIF is expressed in postnatal rat gut. Neural crest-derived neural precursors immunoselected from embryonic day 14 (E14) fetal rat gut express the receptor, CNTFRα, as do developing neurons in the E16 and E18 rat ENS. Although CNTF and LIF knockout mice appear to have a normal ENS, transgenic mice in which either the CNTF receptor α or LIF receptor β are disrupted die at birth and have reduced numbers of enteric neurons; in particular, they exhibit a loss of substance P-immunoreactive and nitric oxide synthase (NOS)-immunoreactive neurons that innervate smooth muscle.

Although the actions of all these different types of neurotrophic factors have been summarized separately here, evidence suggest that they are likely to interact; ENS development is dependent upon a range of neurotrophic and other factors.

Developmental Disorders of the ENS That Involve Neurotrophic Factors

The main developmental disorder of the ENS, Hirschprung disease, has been much studied, and disruption of the GDNF/c-ret signaling pathway as a major cause of Hirschprung disease is well established. The involvement of disruption to the endothelin signaling pathway in Hirschprung disease has also been well described. The possible involvement of other neurotrophic factors in developmental disorders of the ENS, however, has been little studied. Deficiency in neurotrophin expression has been described in the transitional zone of some Hirschprung disease patients. Dysregulation of neurotrophic signaling may also be involved in the etiology of infantile pyloric stenosis, a condition in which infants have hypertrophic pyloric muscle associated with abnormal innervation.

Neurotrophic Factors and Postnatal Changes in the ENS

The mammalian ENS undergoes a number of changes after birth, during the periods of gut growth and dietary change. Although most enteric neurons differentiate during fetal development and enteric ganglia are formed before birth, neural crest-derived stem cells have been identified in the postnatal gut and a small population of neural precursors has been shown to withdraw from the cell cycle postnatally. The relative proportions of different types of enteric neuron in newborn animals have been found to be different from those in adults. The cholinergic population, in particular, is not fully established at birth, and in rodents, the numbers of neurons expressing VIP, pituitary adenylyl cyclase-activating peptide (PACAP),

and neuronal NOS (nNOS) in the myenteric plexus have been shown to increase during the first few weeks of life. The morphological properties of enteric neurons also change; in addition to an increase in cell soma size, neuronal processes must grow appropriately in parallel with their target tissues and it is likely that 'fine tuning' takes place, to fully establish enteric circuitry at a structural level and also at the neurochemical level – for example, in the expression of neurotransmitter receptors. Neurotrophic factors are likely to play a role in these changes. Evidence in support of a postnatal role for neurotrophic factors and their receptors comes from studies of their expression and actions in the postnatal gut.

Expression of Neurotrophic Factors and Their Receptors in the Postnatal Gut

The neurotrophic factors that are active in the fetal ENS – GDNF, NTN, and NT-3 – continue to be expressed in the postnatal rodent gut. Evidence from immunohistochemical studies of human tissues indicates that BDNF and LIF are also expressed in the gut during the postnatal period. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) of mRNA extracted from whole gut has been used to analyze possible changes in expression levels of GDNF and NTN and their specific GPI-linked receptors postnatally. This work indicates that there may be a decline in the level of expression of GDNF in the first few postnatal weeks, while that of NTN may increase, reaching a maximum at postnatal day 14 in mice. These changes in expression are mirrored by possible decreases in the levels of GFRα1 and increase in GFRα2 during the same time period. Interpretation of such analysis of mRNA levels in whole gut, which contains a mixture of cell types, however, does not determine if local expression levels of proteins change, and is further complicated by the rapid postnatal growth of the different gut layers. In this context it is worth noting that other studies have reported continued high levels of expression of both GDNF and NTN into adult life. Possible changes in expression levels are nevertheless potentially of importance, since data from both NTN and GFRα2 null transgenic mice indicate that NTN may play a role in the later development of the ENS, particularly of cholinergic neurons, some of which have been reported in other studies to differentiate postnatally.

The expression of other neurotrophic factors and their receptors during postnatal development has been studied using immunohistochemical techniques. In human infants, TrkA and TrkB receptors have been found to be expressed by both enteric neurons and glia, while TrkC receptors are expressed only by enteric neurons. BDNF and NT-3 are also expressed by enteric ganglion cells. Detailed analysis to localize neurotrophic factor and receptor expression to particular neuronal subpopulations, however, has not been performed during the postnatal period. It should also be noted that a different pattern of expression of TrkB has been described in adult rodent gut (see later). Nevertheless, expression of neurotrophic factors by enteric neurons in postnatal animals suggests that these factors could be involved in establishing interneuronal connections within the developing ENS circuitry.

Actions of Neurotrophic Factors on Postnatal Enteric Neurons and Glia

In vitro work supports the hypothesis that neurotrophic factors play a role in the postnatal development of the ENS. Studies of isolated myenteric ganglion cells in dissociated cell culture show that myenteric neurons from postnatal rats respond to exogenously applied GDNF, NGF, NT-3, and CNTF. GDNF and NT-3 promote neuronal survival and neurite extension, NGF promotes neuritogenesis, and CNTF has been reported to also promote neurite sprouting *in vitro*. There is also evidence that GDNF and NT-3 may have effects on the neurochemical properties of cultured myenteric neurons, possibly promoting expression of VIP. Study of the effects of GDNF on neurons at different postnatal ages indicates that older (postnatal day 14) myenteric neurons may be less responsive to GDNF than are those from younger (postnatal day 7) animals, an observation that is consistent with the molecular data on GFRα1 and GFRα2 mRNA levels in postnatal mice.

Neurotrophic Factors and the Adult ENS: Roles in Intestinal Neurophysiology and Neuroprotection?

Expression of Neurotrophic Factors and Their Receptors in the Adult Gut

Expression of neurotrophic factors and their receptors in the gut continues into adult life. GDNF and NTN mRNA expression in the adult rat gut has been confirmed by molecular techniques, and GDNF protein has also been shown by immunoassay to be present at high levels in the adult gut. Analysis of mRNA indicates that GDNF and NTN expression continues even in aged (24 months old) rats. Although relatively few studies have been performed, immunohistochemical evidence indicates that some, but not all, enteric neurons in adult humans, rats, mice, and other vertebrates express NGF, BDNF, or NT-3. Co-expression of the three neurotrophins by enteric neurons has not been examined to date. In rat intestine, NT-3-immunoreactive neurons were found to be abundant in the

submucous plexus, but sparse in the myenteric plexus, and most NT-3-immunopositive neurons also expressed the peptide VIP.

Analysis of receptor expression indicates that adult enteric neurons express c-ret, GFRα1, GFRα2, TrkA, and TrkC, while TrkB expression seems to be confined to glial cells. Recent immunohistochemical analysis in adult rat colon showed that 71% of myenteric neurons are immunopositive for TrkA, and 78% express the low-affinity neurotrophic receptor, p75. More than 60% of myenteric neurons express both receptors. The functional significance of this widespread expression of NGF receptors was demonstrated by application of exogenous NGF, which resulted in TrkA phosphorylation. Double labeling demonstrated that TrkA was expressed by different types of enteric neurons, including choline acetyltransferase-immunopositive and nNOS-immunopositive neurons. Whether or not the level or pattern of receptor expression by individual neurons changes during the life span remains to be determined, although there is evidence that responsiveness of myenteric neurons to NGF, NT-3, and GDNF is maintained throughout life.

Roles of Neurotrophic Factors in the Adult and Aging Gut

What is the role of neurotrophic factors in the mature and aging ENS? Recent evidence shows that there may be both a neuromodulatory action, affecting gut function, and a neuroprotective role, promoting neuronal survival.

Effects of neurotrophic factors on gastrointestinal motility The acute effects of BDNF in the central nervous system have been well documented. The observation that BDNF and other neurotrophins are expressed by enteric neurons therefore led to the suggestion that they may also have rapid and short-lived actions in the gut, as well as, or instead of, longer term neurotrophic effects.

Evidence that neurotrophins may have effects on intestinal physiology came indirectly, when it was noted that patients in clinical trials involving treatment with recombinant BDNF or NT-3 for neurological disorders had an increased frequency of bowel movements and diarrhea. Subsequent trials of the possible therapeutic use of NT-3, administered three times a week subcutaneously, for treatment of patients with idiopathic constipation also resulted in a dose-dependent enhancement of colonic transit and improved passage of stools. Gastric emptying and both intestinal and colonic transit were accelerated in these patients. Studies of the effects of NGF, recombinant BDNF, and recombinant NT-3 infused into the tail vein of rats showed that these factors had similar effects on colonic motility: an increase in the spike frequency, amplitude, and duration. Recombinant NT-3 (but not the other factors) also promoted myoelectrical activity in the stomach and small intestine. The mechanisms by which the neurotrophins exert these effects, however, are unclear. The effect in humans began 24–48 h after onset of treatment, so could possibly be due to a relatively rapid trophic effect, perhaps on the efficacy of particular nerve types or neurotransmitter systems. To date, analysis of the effects of these factors *in vitro* has not provided clear evidence on their mode of action *in vivo*.

Protective roles of neurotrophic factors in the ENS Recent evidence indicates that neurotrophic factors may have protective roles in the ENS during maturity and in aging. Effects of neurotrophic factors in model systems in which enteric neuronal damage was induced either by hyperglycemia or by reactive oxygen species (ROS) have been investigated.

Enteric neuropathy is a well-documented complication of diabetes, and loss of enteric neurons has been demonstrated in humans and in animal models. The causes of enteric nerve damage in diabetes have not been established, although hyperglycemia is likely to be involved. In recent work, the effects of elevated glucose levels on cultured fetal enteric neurons were examined. Glucose produced a dose-dependent increase in apoptosis in these cultures, an effect that was significantly reduced in the presence of GDNF. Importantly, *in vivo*, overexpression of GDNF also protected against apoptosis, specific neuronal loss, and motility changes seen in streptozotocin-induced diabetic transgenic mice.

GDNF and NT-3 have also been shown to reduce generation of ROS and protect against ROS-induced apoptosis *in vitro*. When isolated segments of the muscularis externa containing the myenteric plexus are incubated in the presence of dihydrorhodamine, which fluoresces when exposed to ROS, addition of GDNF and NT-3, but not NGF, reduces the fluorescent signal. Furthermore, when myenteric ganglia are incubated in the presence of the ROS generator menadione, some myenteric neurons undergo apoptosis. More do so in samples from aged animals, fed an *ad libitum* diet. Incubation in the presence of GDNF or NT-3, but not NGF, reduced the number of apoptotic neurons detected in this model. Interestingly, this protective effect was lost in the aged *ad libitum*-fed animals, but not in rats fed a calorically restricted diet.

Are Neurotrophic Factors Involved in Clinical Conditions Affecting the ENS?

The continued expression of neurotrophic factors in the adult gut and their receptors by enteric neurons

also leads to the question of whether disruption of neurotrophic factor signaling is involved in clinical conditions that affect the ENS. Enteric neurons innervate all cell types in the gut, so neurotrophic factors may directly or indirectly influence all types of intestinal pathophysiology. One area in which neurotrophins have been implicated is that of the inflammatory bowel diseases, where, in addition to the inflammatory damage to nonneuronal gut tissues, abnormalities of the ENS have been described.

In experimental colitis, pretreatment with neutralizing antibodies to NGF or NT-3 resulted in exacerbation of the inflammatory response, implying that these factors normally exert a protective effect in this model of inflammatory disease. In humans with either Crohn's disease or ulcerative colitis, increased expression of NGF and NT-3 was seen, at both the mRNA and the protein levels. Selective losses of NGF-sensitive enteric neurons in experimental colitis, however, have also been described, and intestinal mast cells have also been shown to express both NGF and TrkA. Enteric neurons have also been shown to cause mast cell degranulation. Neuroimmune interactions in the gut are therefore complex, and although neurotrophic factors are likely to play a role in inflammatory conditions, much remains to be learned about this and other aspects of their actions in the gut.

See also: Autonomic and Enteric Nervous System: Apoptosis and Trophic Support During Development; Enteric Nervous System Development.

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Glial Growth Factors

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Glial cells are a major source of growth factors in the healthy and injured central and peripheral nervous systems. Glia-derived growth factors regulate differentiation and function of glial cells in an autocrine fashion, and they modulate neuronal process formation, migration, and survival during development. An important function of glial growth factors is the regulation of cellular repair processes after injury, degeneration, and demyelination in the peripheral and central nervous systems. Changes in growth factor synthesis, release, and action are also associated with the formation of glia-derived brain tumors.

Expression of Growth Factors by Different Glial Subpopulations

Satellite Cells

Satellite cells are specialized glial cells in the sensory ganglia ensheathing cell bodies of pseudounipolar sensory ganglion cells. During development, satellite cell-derived nerve growth factor (NGF) is an important determinant for neurite outgrowth from sensory neurons since, in the rat, neurite outgrowth from neonatal sensory neurons after removal of satellite cells is induced by NGF.

After peripheral nerve injury in the rat, NGF and neurotrophin NT-3 induce sprouting of noradrenergic nerve terminals in the dorsal root ganglia. Satellite cell-derived NGF also affects expression of the nicotinic acetylcholine receptor in rat sensory neurons. NGF also seems to affect satellite cells since NGF depletion reduces reactivity of rat trigeminal satellite cells after inferior alveolar nerve injury.

Other growth factors released from satellite cells are vascular endothelial growth factor (VEGF), the transforming growth factor (TGF)-βs, and TGF-α. Thus, VEGF plays an important role in the establishment and maintenance of blood vessels in the dorsal root ganglia during development, whereas TGF-β signaling is altered after peripheral nerve transection as indicated by changes in the distribution of TGF-β and the TGF-β type I and type II receptors in peripheral nerves and mechanoreceptors after traumatic injury. TGF-α and its receptor reveal strong upregulation in satellite cells in response to peripheral nerve lesioning.

Schwann Cells

Schwann cells are the myelinating cells of the peripheral nervous system and thus ensheathe with their processes axons in peripheral nerves. Perturbed Schwann cell functioning is a key feature of several demyelinating disorders, the most prominent of which is multiple sclerosis (MS). Schwann cells synthesize and release a wide variety of growth factors, such as neurotrophins, neuregulatory cytokines, TGF-βs, glial cell line-derived neurotrophic factor (GDNF), epidermal growth factors (EGFs), and platelet-derived growth factor (PDGF).

High levels of NGF are found in Schwann cells *in vivo* and in primary cell cultures *in vitro*. NT-3 mRNA is expressed at low concentrations in cultured Schwann cells but is upregulated upon immortalization. In response to nerve injury, neurotrophins are differentially up- or downregulated in Schwann cells. Thus, low levels of NGF in normal Schwann cells are greatly increased after injury. Also, transection of the sciatic nerve leads to an increase in the mRNAs for brain-derived neurotrophic factor (BDNF) and NT-4 but to a decrease of that for NT-3.

Schwann cells of the sciatic nerve also express ciliary neurotrophic factor (CNTF) beginning on postnatal day 8 and reaching adult levels on day 21. CNTF expression in Schwann cells is regulated by axonal factors since after axotomy, CNTF expression recovers only after regeneration of the axonal processes. Whereas in cultured Schwann cells mRNAs for CNTF and leukemia inhibiting factor (LIF), another neuregulatory cytokine, are high, myelinating Schwann cells *in vivo* show only CNTF expression and levels of LIF mRNA are low. However, retrograde transport of LIF and CNTF is increased after nerve lesioning.

In Schwann cells and their precursors *in vivo*, both TGF-β2 and -β3 are expressed, whereas TGF-β1 is upregulated only after injury. Thus, in the transected sciatic nerve, TGF-β1 mRNA is induced in the distal nerve stump, whereas that for TGF-β3 is repressed. Also during axon regeneration, TGF-β1 mRNA is transiently increased. In contrast to the *in vivo* situation, in cultured Schwann cells expression of TGF-β1 mRNA is high but decreases when axonal contact is mimicked by the application of forskolin. A striking feature of the effects of TGF-β on Schwann cells is that application of TGF-β together with tumor necrosis factor-α (TNF-α) can induce Schwann cell death, whereas either cytokine alone is ineffective.

Whereas in normal circumstances GDNF expression in Schwann cells and satellite cells is low, it shows transient upregulation after injury. Thus, GDNF is

upregulated in the distal nerve stump of the transected sciatic nerve, as well as in the satellite cells of the trigeminal ganglion.

Schwann cells are also known to express TGF-α and other neuregulins, as well as the neuregulin receptor ErbB1, on their surface. In accordance with this, neuregulins act as autocrine stimulators of Schwann cell proliferation during development. Similar observations have been made for PDGF, the expression of which in rat Schwann cells is high at birth, followed by a continuous postnatal decrease in nonmyelinating but persisting high levels in myelinating Schwann cells.

Oligodendrocytes

Oligodendrocytes are the myelinating cells of the central nervous system (CNS) and thus, like Schwann cells in the peripheral nervous system (PNS), ensheathe the axonal processes by their processes. Oligodendrocytes and their precursor cells also provide an important source of growth factors such as the neurotrophins and several TGF-β superfamily members. Thus, they express NGF, NT-3, and BDNF, probably mediating the autocrine control of proliferation and survival of immature oligodendrocytes and of O-2A precursor cells. In O-2A precursor cells, different TGF-β isoforms, such as TGF-β1 and -β3, are expressed, whereas mature oligodendrocytes express TGF-β2 and-β3. In cultured oligodendrocytes, all three TGF-β isoforms are present.

Astrocytes

Astrocytes in the CNS mediate the transport of nutrients and waste products between blood vessels and neurons, and they also modulate neuronal activity and synaptic transmission. Due to their important role during gliosis and neuronal repair, growth factor synthesis by astrocytes has been intensely studied. Thus, astrocytes in the healthy CNS release NGF, NT-3, and NT-4, whereas reactive astrocytes after gliosis start to express BDNF together with NGF, a process which at least *in vitro* seems to depend on interleukin (IL)-1β and interferon-γ (IFN-γ).

Astrocytes also release high amounts of fibroblast growth factor-2 (FGF-2), mRNA of which can be found in cortical, hippocampal, and spinal cord astrocytes with a strong postnatal increase. FGF-2 seems to act in an autocrine manner on astrocyte differentiation since in FGF-2$^{-/-}$ mice astroglial gap junction coupling and neurotransmitter sensitivity are changed. Astroglial FGF-2 is also responsible for the induction of endothelial tight junctions and thus for the formation of the blood–brain barrier in the CNS.

Astrocytes express neuregulatory cytokines such as CNTF, which can be found in type 1 astrocytes of the optic nerve and the olfactory bulb. Also, primary cultures of astrocytes contain CNTF mRNA and protein. Astroglial CNTF expression is upregulated during brain lesions such as entorhinal cortex lesions, hippocampal deafferentiation, optic nerve transection, and ischemia. Also, LIF is expressed in cultured astrocytes of variable origins and is upregulated after trauma and during inflammation.

However, astrocytes do not express EGF itself but synthesize other members of this growth factor family, such as TGF-α and the neuregulins. TGF-α has been found in astrocytes of the corpus callosum, striatum, and globus pallidum. Astrocytes also express ErbB1, the receptor for TGF-α, and therefore it is thought to act in an autocrine manner. In reactive astrocytes, TGF-α and its receptor are transiently reduced. Other neuregulins which can be found in cultured astrocytes are highly upregulated during reactive gliosis. Thus, GGF-2 and NDF have been detected in human white matter astrocytes of the spinal cord and in the cortex.

Of the insulin-like growth factors (IGFs), only IGF-1 is expressed in astrocytes, which *in vitro* has been shown to promote astroglial proliferation in an autocrine manner. During remyelination, IGF-1 is upregulated in astrocytes, together with its receptor, and is also increased after ischemia, probably acting in a protective manner.

With regard to the TGF-β superfamily members, in astrocytes a highly heterogeneous expression pattern can be found. Thus, astrocytes in the uninjured brain express only TGF-β2 and -β3, whereas after lesioning TGF-β1 is also upregulated. In contrast, GDNF is not expressed in astrocytes of the adult brain but can be found during early postnatal development. However, GDNF is released from cultured fetal human astrocytes and in astrocytes *in vivo* after brain damage. GDNF is also highly upregulated in gliomas *in situ* and in glioma cell lines.

Ependymoglia

Ependymoglia line the ventricular wall and the choroid plexus and, thus, are important for substance transport and for establishing the blood–liquor barrier. FGF-2 is expressed in the choroid plexus epithelia of both lateral and third ventricles, in ependymal cells of the third ventricle, and along the lateral sides of the lateral ventricles. All positive cells reveal a similar distribution with apical labeling, as well as some cytoplasmic staining. This suggests a transepithelial transport of growth factors such as FGF-2 via ependymal cells into the underlying brain parenchyma. However, FGF-2 also seems to play a role in the autocrine regulation of ependymal cells since both EGF and FGF-2 have been shown to cause proliferation of

ependymal precursor cells in the adult rat spinal cord. Another growth factor which is expressed in ependymal cells, at least during development, is GDNF, which has been demonstrated in human prenatal cortical plate ependyme from the age of 10 weeks onward.

Müller and Bergmann Glial Cells

Müller glial cells are persisting radial glial cells in the retina and express a large variety of cytokines and their receptors. Thus, expression of NGF, BDNF, and NT-3, along with neurotrophin receptors trkB and trkC, but not of trkA, has been reported. Also, glial maturation factor-β, another cytokine, is synthesized in these cells. Human Müller cells *in vivo* express high amounts of FGF-2. In addition, FGF-2 has been shown to downregulate IGF-1 in Müller cells. *In vitro*, Müller cells release TNF-α and nitric oxide upon stimulation with lipopolysaccharide and IFN-γ. TGF-β2 and -β3 are also expressed in Müller cells. Glucose and pH influence expression of VEGF in Müller cells. VEGF183 has been reported as a Müller cell-specific splice variant of this growth factor. Müller cells also express CNTF and FGF-2.

Bergmann glia, the persisting radial glia in the cerebellar cortex, express some growth factors and growth factor-related proteins. Thus, IGFBP2 is expressed in these cells during postnatal cerebellar maturation in parallel to the expression of IGF-1 in cerebellar Purkinje neurons. Bergmann glia cells also express IL-6.

Role of Glial Growth Factors in Glial Cell Differentiation and Function and the Formation of Glial Brain Tumors

An important function of glial growth factors is para- and autocrine regulation of the differentiation and maturation of glial cells, a feature which is of utmost importance for pathological changes of the nervous system (**Figure 1**).

Autocrine Regulation of Astrocyte Differentiation

With regard to autocrine regulation of astrocyte differentiation, studies from the 1980s reported that astrocyte-conditioned medium is able to inhibit astrocyte proliferation compared to fresh medium – a feature that has been attributed to the secretion of growth inhibitory factors by these cells.

The complex network of para- and autocrine effects of glial cells during astrocyte development is highlighted by the fact that differentiation of O-2A progenitor cells into either oligodendrocytes or type 2 astrocytes seems to depend on growth factors released from type 1 astrocytes. Type 1 astrocytes have been shown to secrete PDGF, thus stimulating O-2A progenitor cell proliferation and oligodendrocyte differentiation. Later, type 1 astrocytes secrete CNTF, thus initiating differentiation of type 2 astrocytes.

The LIF-receptor and its ligands play an important role in astrocyte differentiation. Thus, neural precursor cells isolated from LIFR$^{-/-}$mice fail to generate glial fibrillary acidic protein (GFAP)-positive astrocytes, whereas precursors from heterozygous mice differentiate normally. Also, *in vivo* LIFR$^{-/-}$ mice show low levels of GFAP. In contrast, animals deficient in LIF show reduced GFAP levels only in some brain regions, suggesting that LIF is not the predominant endogenous ligand for the LIF receptor.

Region-specific effects of glia-derived factors on astrocyte differentiation can also be observed in the cerebellar cortex, in which granule cell precursors are differentiated into astroglial cells by sonic hedgehog and bone morphogenic peptides. Growth factor-stimulated cells initially express both GFAP and neuronal markers and later switch to S100-beta, a marker of differentiated astrocytes.

Despite their important role in neuronal stem cell proliferation and differentiation, with regard to astrocytes, FGF-1 and FGF-2 are primarily involved in the regulation of astrocyte proliferation. Nevertheless, GFAP immunoreactivity is reduced in mice with a genetic defect in FGF-2. Also, Fgf-8b, a splice variant of this growth factor, has been shown to promote astroglial differentiation of a subpopulation of E15 cortical precursor cells in culture.

Regulation of Oligodendrocyte and Schwann Cell Differentiation by Glial Growth Factors: Importance for Etiology and Treatment of MS

A central role in paracrine and autocrine regulation of oligodendrocyte differentiation is played by neuregulins, which in the normal human CNS are produced by astrocytes and neurons. Thus, NRG-1α and -1β and their receptors can be found in cultured oligodendrocytes from neonatal rat pups. Under these conditions, less differentiated oligodendrocytes contain both NRG isoforms in the cell bodies but not in the processes, whereas only NRG-1β was found in the nucleus. In contrast, differentiated oligodendrocytes contained both isoforms only in cytoplasm and cell processes. Similar effects could be observed for embryonic striatal oligodendrocyte precursor cells, which express Nrg-1 as well as its specific receptors, ErbB2 and ErbB4, but not ErbB3. Likewise, inhibition of Nrg-1 activity by the addition of soluble ErbB3 decreases the mitotic activity of Nrg-1 on oligodendrocyte precursor cells.

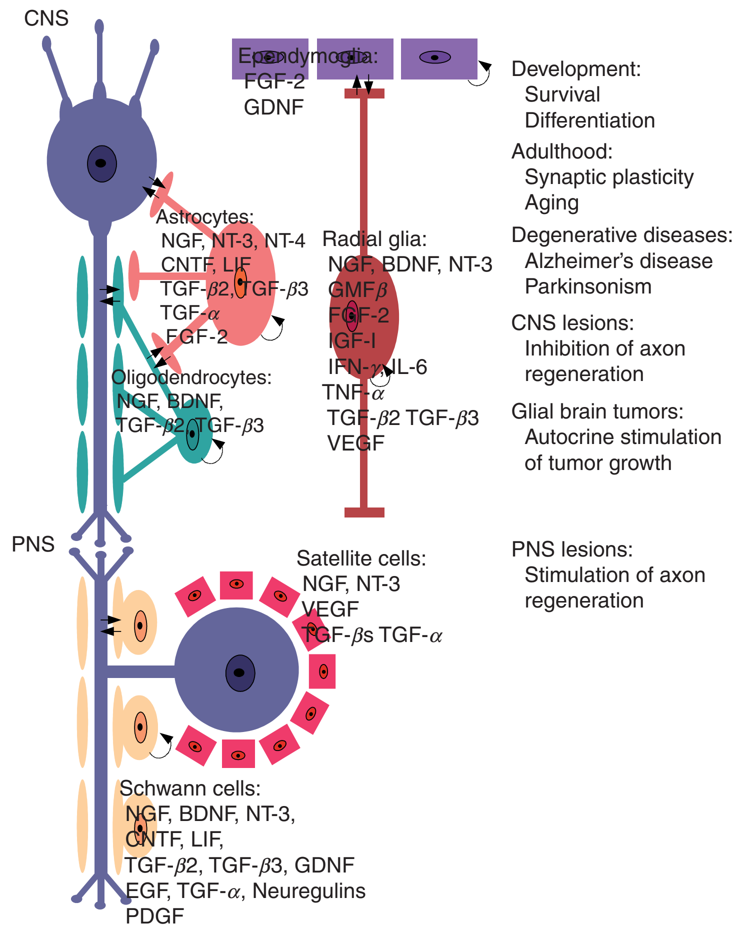

Figure 1 Graphical summary of expression and effects of glial growth factors in the central and peripheral nervous systems. A sophisticated network of glia–neuronal, interglial, and autocrine actions of glial growth factors regulate normal development and function of the nervous system, degeneration and regeneration after nerve injury, as well as brain tumor growth.

Despite these *in vitro* findings, neuregulins seem to also be important for myelination *in vivo* since mice with a null mutation in the nrg-1 gene show defects in oligodendrocyte differentiation. This also seems to be important for the pathology of MS since in active and chronically active MS lesions, expression of astrocyte-derived neuregulin is reduced and thus may contribute to impaired remyelination in MS patients.

Other glial growth factors influencing oligodendrocyte differentiation and myelination are PDGF and IGF-1. Thus, in spinal cord explants, NRG-1 and PDGF, but not LIF, enhance myelination. PDGF has also been shown to be a potent regulator of oligodendrocyte progenitor migration and proliferation in oligodendrocyte cultures. IGF-1 acts on myelin-forming cells to promote normal myelination and remyelination after injury. Thus, in experimental MS models, the neuregulin isoform GGF-2, IGF-1, and several neurotrophins promote remyelination following inflammatory demyelination.

Differentiation-stimulating effects of IGF-1 on oligodendrocyte precursors have also been detected *in vitro*, in which the addition of IGF-1 induced a high proportion of precursor cells to differentiate into galactocerebroside-positive oligodendrocytes, whereas the proportion of type 2 astrocytes was unaffected. In addition, IGF-1 promotes proliferation of O-2A precursor cells.

Also in myelinating Schwann cells, growth factors released from glial cells are involved in remyelination after damage. Thus, in neuron–Schwann cell cocultures, GGF-1, an NRG-1 isoform, inhibits myelination by preventing axonal segregation and ensheathment. In addition, treatment of established myelinated cultures with GGF-1 results in demyelination that frequently begins at the paranodes and progresses to the internodes. In contrast, FGF-2 and TGF-β1 inhibited myelination but did not cause demyelination, suggesting that this effect is specific to the NRGs. The NRG receptor proteins erbB2 and erbB3 are expressed on ensheathing and myelinating Schwann cells and rapidly phosphorylate upon GGF-1 treatment.

In Schwann cells, both NRG-1β and -1α are colocalized in cytoplasm and its processes. The Schwann

cell nucleus has weak immunoreactivity for both NRG-1 isoforms, although NRG-1β is predominant. ErbB2 and ErbB3 receptors, transducing the NRG-1 signal in Schwann cells, are found throughout cytoplasm and processes and are also localized in the nucleus. Stimulation of Schwann cells with mitotic agents induces nuclear translocation of NRG-1β.

Regulation of Radial Glia Differentiation

Little information is available on the role of glial growth factors in the differentiation of radial glia. Thus, in the mammalian retina proliferation of Muller glia is stimulated by EGF in a dose-dependent manner, whereas astrocyte proliferation is stimulated by FGF-1 and FGF-2. In contrast, proliferation of glial precursor cells is stimulated by FGF-1, FGF-2, and PDGF, but not by EGF. The inhibitory role of FGF signaling for Muller cell differentiation is further supported by the fact that inhibition of the endogenous FGF receptor by cotransfecting a dominant-negative form results in an increased number of Muller cells, suggesting a balance between FGF signaling and other signaling cascades to modulate retinal precursor cell fate.

Growth Factors in Glial Brain Tumors

Genetic changes in glial cells and their precursors lead to some of the most devastating human brain tumors, such as glioma, glioblastoma, and astrocytoma. During the process of tumor transformation, para- and autocrine stimulation of tumor cell proliferation is a key mechanism for tumor growth providing at the same time a starting point for pharmacological intervention of this process.

Glioma cells are among the oldest known sources for a number of different growth factors, such as FGF-2 and GDNF, which have also been shown to affect glial cell proliferation in an autocrine manner. Thus, glioma cells express high levels of FGF-2 protein and high-affinity FGF receptors and therefore are mitogenically responsive to FGF-2. Likewise, a knockdown of GDNF or its receptor, GFR-α1, by antisense RNA results in reduced proliferation of rat C6 glioma cells, suggesting a role in autocrine growth stimulation.

A growth factor that has been implicated in the regulation of glioblastoma growth is PDGF, which is coexpressed with its receptor in this tumor type and has been shown to regulate tumor cell proliferation. Because it is a secreted factor, PDGF not only has autocrine effects on producing cells but also paracrine actions on other tumor cells and on the tumor microenvironment. Thus, PDGF is involved in the regulation of tumor cell migration and tumor angiogenesis. In addition, human glioblastoma cells show a general increase in the expression of autocrine growth regulators such as TGF-α, TGF-β, and FGF-2 compared to normal human brain tissue.

Also in astrocytomas, activity and regulation of a number of mitogenic signaling pathways is aberrant. Thus, upregulation of growth factor receptors such as EGFR, PDGFR, and c-Met, as well as signaling intermediates such as Ras, protein kinase C, and others which have been shown to positively regulate tumor proliferation and cell cycle progression, has been demonstrated.

Effects of Glial Growth Factors on Differentiation and Function of Neurons in Both Healthy and Injured Nervous Systems

Glial growth factors are important regulators of normal neuronal differentiation and of neuron survival in the developing and adult brain. Disturbances of glial growth factor release are therefore supposed to play a role during CNS and PNS lesioning and in the etiology of various neurological disorders (**Figure 1**).

Regulation of Neuronal Differentiation during Development

Glial growth factors are important for the migration of neuronal progenitors during development. Thus, intraventricular injection of NT-4 or overexpression of BDNF lead to defects in cortical layering. Cortical layering defects can also be observed in FGF-2 knockout mice. GGF-1 promotes the migration of neuronal progenitors along radial glial fibers.

Glial growth factors can also influence axonal pathfinding, as shown for netrin-1 in the internal capsule. Netrin-1 is released from oligodendrocytes but not astrocytes. Together with netrin-1, engrailed-1 is directing axons of association neurons, projecting ipsilaterally to motor neurons in the spinal cord. Consistent with this, axonal pathways are altered in netrin-1-deficient mice. A number of glia-derived growth factors, such as TGF-α, have been shown to induce neuritogenesis.

An important role for glial growth factors is the regulation of neuron survival during periods of ontogenetic neuron death. Thus, FGF-2 can prevent neuron loss in the chick ciliary ganglion. Ciliary neurotrophic factor is also able to rescue motor neurons in the embryonic chick lumbar spinal cord during ontogenetic cell death. Moreover, TGF-βs enhance survival of chick ciliary ganglionic neurons *in vitro* synergistically with different neurotrophins and CNTF. Surprisingly, immunoneutralization of TGF-βs during chick development *in vivo* enhances neuronal survival.

Glia-Derived Growth Factors during Puberty and Aging

A late developmental event is puberty, during which gonadal steroids induce plastic changes in certain brain regions. In hypothalamic astrocytes, this is accompanied by the production of TGF-α and neuregulins, which elicit astroglial secretion of prostaglandin E2, stimulating the release of neuronal luteinizing hormone-releasing hormone. Hence, overexpression of TGF-α in the hypothalamus accelerates puberty, whereas blockade of TGF-α or neuregulin delays this process.

Growth factor synthesis by glial cells is also changed during aging, in which, together with astrogliosis, a continuous upregulation of TGF-β1 can be observed. Also, a decrease in NGF has been observed in a senescence accelerated mouse strain. Likewise, in aging mice an increase in IL-6 and a decrease in IL-10 release from glial cells can be observed.

Glia-Derived Growth Factors during Neurodegenerative Diseases

Neuronal degeneration during Parkinson's disease (PD) and Alzheimer's disease (AD) is accompanied by astrogliosis and thus upregulation of several glia-derived growth factors. On the other hand, some glial growth factors are downregulated in these diseases, such as FGF-2 expression, which is reduced in the substantia nigra of PD patients, and NGF expression, which is reduced in the hippocampus of both PD and AD patients. *In vitro* studies on growth factors such as FGF-2, GDNF, CNTF, and BDNF suggest that they are important for survival of dopaminergic midbrain neurons.

According to this, altered levels of glial growth factors are common in postmortem samples of the brains of AD patients. Thus, β-amyloid of senile plaques induces IL-1β and FGF-2, probably stabilizing the disease conditions. Whereas FGF-2 is expressed in astrocytes in the center of senile plaques, FGF-1 can be found in astrocytes surrounding the plaque. Also, other growth factors are upregulated in astrocytes of the brains of AD patients, such as endothelin-1, TGF-β2, IGF-1, and hepatocyte growth factor. In contrast, expression of BDNF is reduced in astrocytes surrounding senile plaques.

Role of Glia-Derived Growth Factors in Neural Regeneration in the Central Nervous System

It has long been known that neuron regeneration after lesioning is enhanced by soluble proteins released from glial cells (**Table 1**). Thus, NGF prevents death and promotes fiber growth of transected cholinergic neurons. Similarly, the release of IGF-1 from astrocytes is involved in neuron regeneration after cuprizone-elicited demyelination of the CNS. Notably, IGFs are also potent regulators of remyelination following CNS damage.

Glia-derived factors such as Nogo are also involved in the inhibition of axonal regeneration after spinal cord lesioning. Inhibition of the Nogo receptor could provide a means to overcome this obstacle to neuronal regeneration after spinal cord transection. However, Nogo is not the only axon outgrowth inhibitor

Table 1 Growth factor expression in different glial subpopulations

	SC		*Oligodendrocytes*		*Astrocytes*		*MG*	*BG*
	Normal	*Lesioned*	*Normal*	*Lesioned*	*Normal*	*Reactive*		
NGF	++	++++	+++	nd	+++	++++	++	nd
BDNF	+	+++	+++	nd	−	+++	++	nd
NT-3	++	−	nd	nd	++	nd	++	nd
NT-4	+	++	nd	nd	++	nd	nd	nd
CNTF	+++	+	−	−	+	+++	++	nd
LIF	+	++	nd	nd	+	+++	nd	nd
TGF-β1	−	+++	+	+++	−	+++	−	nd
TGF-β2	++	+	nd	nd	++	nd	++	nd
TGF-β3	+++	+	nd	nd	++	nd	++	nd
GDNF	+	+++	nd	nd	−	+++	nd	nd
TGF-α	++	++++	nd	nd	+	nd	nd	nd
Neuregulins	++	nd	nd	nd	++	nd	nd	nd
PDGF	++	nd	nd	nd	nd	nd	nd	nd
FGF-2	−	nd	nd	nd	+	+++	++	nd
IGF-1	nd	nd	nd	nd	nd	nd	++	nd

BDNF, brain-derived neurotrophic factor; BG, Bergann glia; CNTF, ciliary neurotrophic factor; FGF-2, fibroblast growth factor-2; GDNF, glial cell line-derived neurotrophic factor; IGF-1, insulin like growth factor 1; LIF, leukemia inhibitory factor; MG, Müller glia; nd, not determined; NGF, nerve growth factor; NT, neurotrophin; PDGF, platelet-derived growth factor; SC, Schwann cells; TGF, transforming growth factor.

since other astroglial molecules, such as collagen IV, are similarly active.

Also in brain regions in which axonal regeneration occurs throughout life, astrocyte-derived growth factors such as NGF, BDNF, GDNF, and neurturin seem to be involved since transplantation of astrocytes from the olfactory bulb to the spinal cord promotes axon regrowth after transection.

PDGF upregulation in astrocytes of the facial nucleus after transection of the facial nerve is important for neuronal regeneration. Similarly, astroglial CNTF expression is increased in fields of axonal sprouting in the deafferented hippocampus after entorhinal cortex lesioning. Likewise, BDNF, NT-3, and GDNF are induced in astrocytes of the spinal cord adjacent to a lesion.

Role of Glia-Derived Growth Factors in Regeneration of Peripheral Nerves

Transection of a peripheral nerve leads to apoptotic cell death of dorsal root ganglion neurons. This is probably due to a reduction in the expression of neurotrophins and neurotrophin receptors, and as a consequence, exogenously applied growth factors are able to counteract these effects. An important endogenous source for growth factor release in the distal nerve stump after peripheral nerve transection is Schwann cells, which release NGF, BDNF, NT-4/5, and p75. In addition to these factors, CNTF and LIF are needed for axonal regeneration, which act synergistically on nerve regeneration. Also, GDNF is upregulated in Schwann cells of the distal nerve stump after nerve injury and remains so for more than 5 months.

See also: Macroglial Lineages; Schwann Cells and Axon Relationship; Schwann Cell Development.

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Neural Stem Cells: Adult Neurogenesis*

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Introduction

Adult neural stem cells (NSCs) are cells in the adult nervous system that can self-renew and differentiate into all types of neural cells, namely neurons, astrocytes, and oligodendrocytes. Free-floating neurosphere and adherent monolayer cultures are the methods that are commonly used to expand and characterize NSCs. Both of these types of cultures contain heterogeneous populations of cells, including the putative NSCs and their progeny. The self-renewal of adult NSCs is determined by serial passage and clonal analysis. The multipotency of adult NSCs is characterized by their ability to differentiate into all types of neural cells from single neurospheres or single cell clones. The existence and characteristics of adult NSCs have been mostly analyzed *in vitro* and have yet to be corroborated *in vivo*. Therefore, the term progenitor has often been used to loosely describe all proliferating precursor cells. Progenitor is a generic term for any dividing cell with the capacity to differentiate. This includes putative stem cells in which the self-renewal and/or multipotency have not yet been demonstrated. Precursors are cells from which more mature cells can be formed.

Location of Adult NSCs in the Mammalian Brain

The phenomenon that new neurons are generated in the postnatal brain was first described by Altman and colleagues in the 1960s. It is now known that neurogenesis continues in the adult central nervous system at two locations: the subventricular zone (SVZ; also known as the subependymal layer) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. In rodents, new neurons generated in the SVZ migrate through the rostral migratory stream (RMS) and incorporate into the olfactory bulb, whereas new neurons generated in the SGZ migrate a short distance into the granular cell layer and become granule neurons (**Figures 1(a)–1(c)**).

*For recent up to date reviews please also refer to:
Zhao C, Deng W, and Gage FH (2008) Mechanisms and Functional Implications of Adult Neurogenesis. *Cell* 132: 645–60.
Li Y, Mu Y, Gage FH (2009) Chapter 5. Development of Neural Circuits in the Adult Hippocampus. *Curr Top Dev Biol* 87:149–74.

The identification and characterization of adult NSCs began in the 1990s, when Reynolds and Weiss found that proliferating cells could be isolated from adult mouse brain and expanded in culture when supplemented with epidermal growth factor (EGF). These cells form neurospheres in culture and secondary neurospheres when dissociated. This characteristic fulfilled one criterion for a stem cell: self-renewal. Cells in the neurospheres can differentiate into astrocytes and neurons, as defined by their expression of glial fibrillary acidic protein (GFAP) and neural-specific enolase, respectively. This phenomenon fulfilled the other criterion for a stem cell: multipotency. A collaborative effort between the groups of Weiss and van der Kooy later found that the neurosphere-forming cells reside in the periventricular region of the lateral ventricles.

The periventricular region of the lateral ventricles consists of the ependymal cells lining the ventricles and the SVZ. An observation by Frisen and colleagues suggested that the ependymal cells were the stem cells that gave rise to the proliferating cells in the SVZ. However, independent studies by the groups of Alvarez-Buylla, van der Kooy, and Temple showed that ependymal cells did not form neurospheres in culture and suggested that adult NSCs resided in the SVZ. A lentiviral labeling study by Vescovi, Naldini, and colleagues provided more definitive evidence that cells in the SVZ, but not the ependymal layer, are responsible for the long-term, continuous neurogenesis in the olfactory bulb. Lentiviral vectors can integrate into the chromosomal DNA of the host cells; therefore, the progeny of labeled cells can be traced. Even 3 and 6 months after lentiviral injection into SVZ, labeled cells were found in the RMS. The long-term presence of labeled cells in the RMS was not seen when the virus was injected into the ventricles to label ependymal cells. Therefore, SVZ has been considered to be the site of adult NSCs in the periventricular zone.

Gage and colleagues were the first to characterize NSCs from the hippocampus, the other brain region in which adult neurogenesis occurs. Adult hippocampal progenitor (AHP) is a term that has often been used to describe all proliferating precursor cells in the adult hippocampus. The progenitors/NSCs isolated from hippocampus could be passaged over an extended period of time in monolayer cultures when exposed to mitogens such as basic fibroblast growth factor (bFGF; also known as FGF2). Clonally derived adult hippocampal NSCs in monolayer culture could differentiate into all three neural lineages. In addition, cultured adult rat hippocampal progenitors were capable of neuronal differentiation when grafted into the hippocampus and

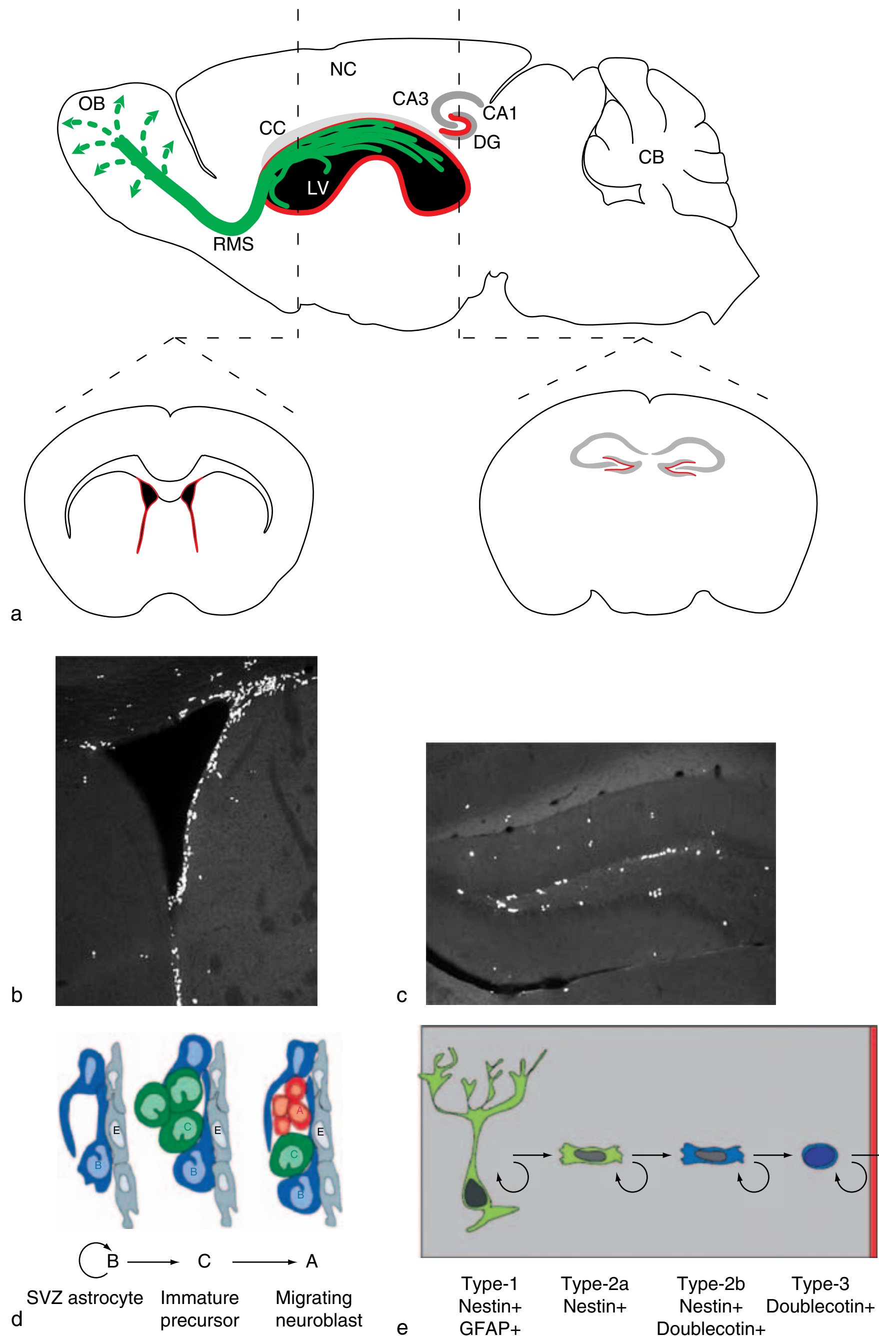

Figure 1 Neurogenesis occurs in the subventricular zone (SVZ) and the subgranular zone (SGZ). (a) Sagittal and coronal views of the mouse brain with the two main germinal zones shown in red. New neurons are generated in the SVZ of the lateral ventricles (LV) and the SGZ of the dentate gyrus (DG) of the hippocampus. Neurons generated in the SVZ migrate through the rostral migratory stream (RMS) and incorporate into the olfactory bulb (OB). Neurons generated in the SGZ migrate a short distance and become granule neurons in the dentate granule cell layer. CB, cerebellum; cc, corpus callosum; NC, neocortex. (b) An example of cell proliferation in the SVZ. Mice were given three shots of BrdU over 24 h and the sample was taken 2 h after the last BrdU administration. Note that more proliferating cells are found on the lateral side of the SVZ. (c) An example of cell proliferation in the SGZ. Images in (b) and (c) were kindly provided by Wei Deng. (d) It has been proposed that three types of cells exist in the SVZ. Type B cells are GFAP-expressing NSCs that give rise to C (immature precursor) cells and B (migrating neuroblast) cells. Reprinted from Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, and Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97: 703–716, Copyright 1999, with permission from Elsevier. (e) Three types of proliferating cells have been proposed to exist in the subgranular layer of the DG. The identification of these cells is mainly based on their specific expression patterns of molecular markers. Adapted from Kempermann G, Jessberger S, Steiner B, and Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. *Trends in Neuroscience* 27: 447–452, Copyright 2004, with permission from Elsevier.

the RMS of the adult rat. Contrary to these findings, van der Kooy and colleagues suggested that the hippocampus does not have multipotential NSCs but only progenitors because they were not able to isolate cells from the hippocampus that form neurospheres; furthermore, they only found neuronal differentiation. However, neurogenesis continues in the dentate gyrus throughout the life of mammals, although at a reduced rate upon aging. Therefore, it is unlikely that these new neurons are derived from cells with limited self-renewal capacity.

Identity of Adult NSCs

Adult NSCs in the SVZ are thought to be slow-dividing cells. Following a high dose of ^{3}H-thymidine to kill proliferating cells in the SVZ, these cells can be repopulated within a few days, suggesting that the proliferating cells are derived from some relatively quiescent cells that are not susceptible to the antimitotic treatment. A similar phenomenon was seen when another antimitotic drug, cytosine arabinoside (Ara-C), was used. The proliferating cells in the SGZ of the dentate gyrus could also recover within 2–3 weeks after the animals were treated with the DNA methylation agent methylazoxymethanol acetate (MAM); however, whether this recovery is caused by the expansion of the remaining proliferating cells or whether slow-dividing NSCs also exist in the SGZ is not known.

Three types of cells have been proposed to exist in the SVZ: type B NSCs, type C transit-amplifying cells, and type A migrating neuroblasts. Type B cells appear to express GFAP and they are the only cells in the SVZ that survived the antimitotic treatment. Type C and A cells recover sequentially soon after cessation of the treatment, leading to a lineage hypothesis from B to C to A cells (**Figure 1(d)**). The identification of B, C, and A types of cells mainly relies on the morphological analyses at the electron microscopic level. In parallel to the SVZ, three types of precursor cells appear to exist in the SGZ: type 1 stem/progenitors and type 2 and type 3 precursor cells. The identification of these different types of cells in the SGZ is mainly determined by the specific expression patterns of molecular markers (**Figure 1(e)**).

Identification of NSCs by Cell Division Markers

One of the features of NSCs is their ability to divide; therefore, the thymidine analogues ^{3}H-thymidine and 5-bromo-2-deoxyuridine (BrdU) are often used to characterize adult NSCs. Both chemicals can be administered systemically, and the labeled cells can be detected through autoradiography and immunohistochemistry. Because NSCs in the SVZ are considered to be slow dividing, and because new neurons generated in the SVZ migrate to the olfactory bulb through the RMS, cells that remain labeled in the SVZ long (1 month) after BrdU or ^{3}H-thymidine labeling are presumably mainly NSCs. In addition to nucleic acid analogues, molecular markers such as the proliferating cell nuclear antigen and Ki67 have been used to recognize mitotic cells by immunohistochemistry. Another way of identifying NSCs is to use retroviruses because retroviral vectors can integrate into the chromosomal DNA and their integration is dependent on mitosis. The ubiquitous distribution of the molecular markers allows detailed morphological analyses of labeled cells. Furthermore, when retrovirus is used to mediate the expression of fluorescent proteins, live cells can be followed in both cell culture and brain slice preparations. All of these methods label both NSCs and their progeny and are biased toward cells with an active cell cycle. Since SVZ NSCs are proposed to be relatively quiescent, the labeling efficiency of NSCs is hypothetically lower than that of rapidly dividing, transit-amplifying cells.

Identification of NSCs by Molecular Markers

Using a transgenic mouse line in which avian leucosis virus-mediated reporter gene transfer only takes place in cells with an active GFAP promoter, Doetsch, Alvarez-Buylla, and colleagues showed that C and A cells in the SVZ were indeed derived from GFAP-expressing B cells. Studies using transgenic mice expressing cre recombinase from the murine GFAP promoter provided consistent results. In addition, in transgenic animals that expressed the herpes simplex virus thymidine kinase (TK) from the murine GFAP promoter, adult neurogenesis in the olfactory bulb and the dentate gyrus was diminished when the animals were given elaidic acid ganciclovir, a drug that can be converted to a toxin by TK. These studies suggest that a subpopulation of GFAP-expressing cells is the precursor of newborn neurons. GFAP-expressing cells have two different morphologies: bipolar or unipolar GFAP cells are considered to be stem cells, and multipolar GFAP cells are astrocytes. Because GFAP-expressing astrocytes are much more abundant than GFAP-expressing NSCs, GFAP has not been considered to be an optimal marker for adult NSCs.

The intermediate filament protein nestin is specifically expressed in the neuroectoderm during development and is highly expressed in the germinal zones in the adult brain. Bartlett and colleagues used fluorescence-activated cell sorting (FACS) to separate cells based on their size ($>12\,\mu m$), low binding to peanut agglutinin, and low level of the heat stable antigen (mCD24a). Approximately 80% of purified cells can form neurospheres, and all of them express nestin.

Sox2 is a member of the Sry-related HMG box transcription factors. It is expressed in totipotent inner cell mass stem cells and in the neuroepithelium of the developing central nervous system. In the adult brain, Sox2 is expressed in both SVZ and SGZ, although its expression is not restricted to these two neurogenic areas. Most Sox2-expressing cells appear to be proliferating neuroblasts. A subpopulation of Sox2 cells also expresses GFAP with one major process spanning the granular cell layer, and these cells have been proposed to be NSCs. Although Sox2 deficiency was shown to cause impaired neurogenesis, no fate mapping studies have been documented using Sox2 as a marker.

A number of cell surface markers have been reported to be able to enrich NSCs through FACS. LeX/ssea-1 (also known as CD15), fucose-containing trisaccharide, was found on the surface of mouse embryonic stem cells and primordial germ cells. Approximately 25% of FACS-sorted LeX+ cells from the adult mouse SVZ generated neurospheres *in vitro* and approximately 18% of LeX+ cells are GFAP+. A monoclonal antibody 473HD that recognizes the chondroitin sulfate epitope was also able to enrich the population of neurosphere-forming cells.

Several other markers have also been reported based on studies in other systems or during early development, including CD133 and Musashi homologue 1. However, these markers have not been extensively characterized in adult NSCs. All of the molecular markers discussed here label heterogeneous populations of cells *in vivo*. Cells that express both GFAP and nestin (or Sox2) have been proposed to be adult NSCs. However, it is not clear whether these cells represent only a subpopulation of adult NSCs.

Identification of NSCs by Genetic Methods

The second intron of the nestin gene contains an enhancer element that is specific for its expression in the central nervous system. This regulatory element has been used either in conjunction with the nestin promoter or with hsp68 minimum promoter to label NSCs. When the nestin promoter and the enhancer from the second intron were used to drive the expression of green fluorescent protein (GFP), GFP expression was more or less restricted to neurogenic regions in the adult brain, including both SVZ and SGZ. GFP was also found in the RMS. FACS-sorted nestin:GFP cells can self-renew and are multipotent. Nestin:GFP cells in the dentate gyrus appear to have two distinct populations, GFAP-positive radial glia-like cells and polysialylated neural cell adhesion molecule (PSA-NCAM)-positive cells. The GFAP+ nestin–GFP cells have been proposed to be stem/early progenitors (type I cells) that give rise to the PSA-NCAM+ nestin–GFP cells (type II cells). Transgenic mice have also been generated using the Sox2 promoter fragment to drive the expression of the reporter β-geo or of GFP.

Regulation of Adult NSCs

Adult NSCs can be regulated at different levels, including the self-renewal and fate determination of NSCs, proliferation and differentiation of their immediate progeny, and cell survival at different stages. All of these forms of regulation may eventually be reflected in a change in adult neurogenesis. Indeed, many conditions have been reported to alter adult neurogenesis. Aging and stress appear to have a negative effect on cell proliferation in both SVZ and SGZ. Hippocampal neurogenesis is enhanced by enriched environment, voluntary exercise, antidepressant treatment, seizure, and many other conditions. It is not clear which component of the process of neurogenesis is affected by these different conditions. Determining the microenvironment in which the NSCs reside (also known as the neurogenic niche) and the intrinsic mechanisms that instruct NSCs will help us better understand the physiological and pathological conditions that are intricately associated with adult neurogenesis.

Extracellular Influences

The behavior of adult NSCs and their progeny is directly influenced by their surroundings – that is, the microenvironment (neurogenic niche) in which they reside. NSCs have been isolated from almost all areas of the adult brain and from the spinal cord. These cells display multipotency in culture; however, neuronal differentiation only occurs in the SVZ and SGZ.

A clear example of the microenvironment playing a critical role in adult neurogenesis came from a transplantation study in which cultured spinal cord NSCs were able to give rise to neurons when grafted into the hippocampus but they showed no neuronal differentiation when grafted back into the spinal cord. Gage and colleagues later found that hippocampus-derived astroglia were able to promote both the neuronal differentiation of AHPs and the maturation of differentiated neurons in culture. Astrocytes from the spinal cord did not have such effects, suggesting that the presence of hippocampal astrocytes might be one of the determinants that permit adult neurogenesis in the dentate gyrus. Wnt-3 might be one of the factors that are responsible for astroglia-induced neurogenesis. Overexpression of Wnt-3 was sufficient to promote neurogenesis, whereas expression of a dominant negative form of Wnt-3 blocked neurogenesis *in vivo*. Neurogenesin-1, a bone morphogenesis protein (BMP)-interacting molecule, has

also been proposed to be a component secreted by hippocampal astrocytes that promote neuronal differentiation.

Dividing cells in the dentate gyrus were found to be closely associated with the vasculature, and possibly coupled with angiogenesis. Endothelial co-culture can significantly increase the number of new neurons in adult SVZ NSC adherent clones. Studies using embryonic cortical NSCs have proposed that factors derived from endothelial cells promote symmetric cell division and, therefore, self-renewal of NSCs. Interestingly, adult mouse NSCs can even transdifferentiate into endothelial lineage when co-cultured with human endothelial cells. Supporting a role for the vasculature niche in adult neurogenesis, the level of vascular endothelial growth factor (VEGF) is closely linked to the level of neurogenesis in the adult hippocampus. VEGF is required for enriched environment- and running-induced neurogenesis in SGZ. VEGFR2 (also known as fetal liver kinase-1 (Flk-1) and kinase insert domain receptor (KDR)) may be expressed by Doublecortin+ cells in the neurogenic zones. Expression of a dominant negative VEGFR2 is able to antagonize VEGF-induced neurogenesis. One study has suggested that, in addition to VEGF, another factor secreted by endothelial and ependymal cells, pigment epithelium-derived factor, can promote self-renewal of adult SVZ NSCs.

Ependymal cells were also reported to express Noggin, an antagonist of BMPs. Noggin might promote SVZ neurogenesis in the adult brain. Ectopic expression of Noggin in striatum was able to promote neuronal differentiation of transplanted NSCs. BMPs promote astrocytic fate in embryonic NSCs. Similarly in the adult, BMP and the leukemia inhibitory factor have been used to induce astrocytic differentiation of cultured AHPs.

The existing neurons may influence adult NSCs through neurotransmitters. Indeed, neurotransmitters such as dopamine, γ-aminobutyric acid (GABA), serotonin, and glutamate have been shown to regulate adult NSCs and/or their progeny. It is controversial whether dopamine promotes or inhibits the proliferation of NSCs in the SVZ. The dopaminergic neurons in the substantia nigra may project to the SVZ and affect SVZ neurogenesis. In the SGZ, GABA could mediate the input to the putative transit-amplifying progenitors and promote the differentiation of these cells and the maturation of the newly generated granule neurons. Antidepressant drugs that act to inhibit serotonin reuptake can consistently upregulate neurogenesis in the SGZ. In addition, glutamate agonist and antagonist could reduce and increase proliferation in the dentate gyrus, respectively. Contrary to these observations, glutamate agonist and antagonist were shown to increase and decrease the proliferation of AHPs *in vitro*.

In addition to the effects of different types of cells within the neurogenic niche, NSCs are subjected to regulation by soluble factors including growth factors, growth hormones, and cytokines. EGF and FGF2 are potent growth factors maintaining SVZ NSCs in culture. In fact, EGF has been shown to convert transit-amplifying cells into multipotent stem cells *in vitro*. EGF also has been claimed to convert adult NSCs to radial glial cells. The morphogen sonic hedgehog (Shh) could induce the proliferation of AHPs in culture. A transgenic study showed that NSCs were responsive to Shh *in vivo*, and that Shh-responsive cells might represent the slow-dividing cells described by van der Kooy and colleagues. Although many factors have been implicated in regulating adult NSCs, it is not clear which cells in the microenvironment secrete these factors.

Intracellular Signaling

It is perhaps not surprising that genes involved in cell cycle regulation, DNA repair, and chromosomal integrity are found to regulate NSCs. SVZ NSCs from p53 knockout mice form more and larger neurospheres compared to wild-type controls. Mice deficient in the cell cycle inhibitor p21 have more NSCs in the SVZ initially, but the number of NSCs decreases after they are 8 months old. It has therefore been proposed that NSCs have a limited number of cell divisions. An intact telomere appears to be important to maintain adult neurogenesis. Reduced proliferation was seen both *in vivo* and *in vitro* in the absence of the telomerase RNA component (Terc) after the mice were bred for four or five generations and the condition worsened when the mice were also deficient in ataxia-telangiectasia mutated (Atm). More interesting, one study suggested that the retrotransposon long interspersed nuclear element-1 (LINE-1 or L1) is actively expressed in adult hippocampal NSCs, and an engineered human L1 was shown to be mobile in the mouse brain.

Adult NSCs are also regulated at the epigenetic level and by transcription factors and their cofactors. Valproic acid, an inhibitor of histone deacetylase, could induce neuronal differentiation in cultured AHPs. Adult NSCs lacking the methyl-CpG binding protein 1 exhibited increased genomic instability and reduced neuronal differentiation. TLX, an orphan nuclear receptor, is required for the maintenance of adult forebrain NSCs. As mentioned previously, sox2 deficiency causes defects in neurogenesis. NeuroD, a neuronal differentiation basic helix–loop–helix transcription factor, is required for the formation of the

dentate gyrus. In addition, the polycomb family transcriptional repressor Bmi-1 was found to be required for the maintenance of both embryonic and adult NSCs. During development, the maintenance and fate specification of embryonic NSCs are defined by a series of bHLH transcription factors. The precise mechanisms of how self-renewal and differentiation are modulated in the adult NSCs still need to be determined.

The Function of Adult NSCs

Adult NSCs have been isolated from many species, including humans. Studies have clearly demonstrated that newly generated neurons in the dentate gyrus and the olfactory bulb are functionally integrated into the preexisting circuitry. By using a retrovirus-mediated labeling approach, Gage and colleagues showed that new neurons born in the adult hippocampus became morphologically indistinguishable from mature granule neurons within 4–8 weeks. More important, the newborn neurons fired action potentials in response to perforant path stimulation. Detailed electrophysiological characterization of new neurons born in the adult SGZ showed that these neurons adopt a developmental process that is similar to that in neurons born during embryonic and postnatal stages. In the olfactory bulb, newly generated periglomerular and granule neurons are also similar to their preexisting counterparts based on their morphological and electrophysiological properties. Interestingly, new neurons in the dentate gyrus have been shown to have a lower threshold for the induction of long-term potentiation at their immature stage, suggesting that the function of adult NSCs might depend on the unique plasticity of newborn neurons before they reach maturation.

Because the hippocampus is required for certain forms of learning and memory, the putative function of adult neurogenesis in the SGZ has been studied extensively in recent years. The level of adult neurogenesis has been well correlated with certain forms of learning and memory. For example, voluntary exercise and enriched environment were both shown to significantly enhance adult neurogenesis, and rodents exposed to these conditions could perform better in a hippocampus-dependent spatial learning task. Similarly, in the olfactory bulb, the survival of new neurons is enhanced by enriched odor exposure.

In addition to the correlative observations, several studies have reported a potential causal relationship between neurogenesis and different forms of learning and memory. Systemic antimitotic treatment with the DNA methylation agent MAM could reduce the level of adult neurogenesis, and the treated animals displayed defects in a trace conditioned eye-blinking task. Low-dose irradiation has also been used to inhibit adult neurogenesis, and treated rats showed impairment in long-term spatial memory and in a nonspatial nonmatching-to-sample task. Furthermore, adult neurogenesis in the SGZ appears to be required for the behavior effects of antidepressant treatment. Localized X-ray irradiation in the hippocampal area significantly reduced cell proliferation in the SGZ, and the irradiated mice failed to respond to the fluoxetine treatment in an anxiety-related, novelty-suppressed feeding test. These studies await corroboration from more spatial- and temporal-specific ablation of adult neurogenesis. In addition, conflicting results have been reported on certain learning paradigms such as the context fear conditioning test. Nevertheless, these are the first results indicating that neurogenesis in the adult brain might be required for hippocampus-dependent learning and memory.

Computational modeling has also been used to predict the function of adult neurogenesis in learning and memory. Among other putative functions in memory formation, an interesting hypothesis is that the continuous neurogenesis in the dentate gyrus might contribute to the formation of time-associated memories.

Conclusion

The discovery of continuous neurogenesis in adult rodent brains was embraced with skepticism for many years. It has now become clear that this phenomenon is conserved in all mammals examined, including humans. The facts that neurons generated in the adult brain functionally integrate into the existing circuitry and that adult neurogenesis is regulated by physiological and pathological conditions suggest that adult neurogenesis has survived evolution to serve some specific functions. More interesting, although NSCs have been purified from both SVZ and SGZ of the human brain, neuronal differentiation has been found in the hippocampus only but not the SVZ in humans. This appears to correlate with the functional differences between the two structures. Compared to rodents, humans rely much less on olfaction for daily activities and probably rely more on the hippocampus for emotions, learning, and memory. With the current advances in the field of adult neurogenesis, it is hoped that in the near future we will understand how new neurons born in the adult brain contribute to the normal brain functions and why the specific functions of adult neurogenesis cannot be fulfilled with the preexisting neuronal population.

The discovery of adult NSCs has triggered new therapeutic studies to treat diseases related to the central nervous system. Such approaches include

grafting NSCs to the site of injury or degeneration and targeting of endogenous stem cells. Indeed, it has been reported that neurogenesis could be induced *in vivo* in some of the nonneurogenic regions. The identification of small molecules that can induce neurogenesis from endogenous NSCs in damaged areas of the adult nervous system will change how we currently view central nervous system therapies.

See also: Neural Stem Cells: Ocular; Neurogenesis in the Intact Adult Brain; Stem Cells and CNS Repair; Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging.

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Neurogenesis in the Intact Adult Brain

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Adult neurogenesis is the development of new neurons from resident neural precursor cells in the adult brain. The term 'adult neurogenesis' encompasses the entire process of neuronal development from the division of the precursor cell to the existence of a functionally integrated new neuron. Adult neurogenesis is rare and locally restricted in mammals but frequent and widespread in lower vertebrates; it seems that over the course of evolution with increasing brain complexity adult neurogenesis became more and more limited. Quantitatively, most adult neurogenesis occurs relatively early in life, but adult neurogenesis persists lifelong on a very low level. There is no evidence that adult neurogenesis would primarily contribute to a neuronal turnover in that it would replace damage or lost neurons. Rather, the new neurons are added to the persisting networks.

In mammals, physiological adult neurogenesis appears to be restricted to two canonical neurogenic regions, the dentate gyrus of the hippocampus and the olfactory bulb (**Figure 1**). Adult hippocampal neurogenesis generates new excitatory granule cells, the principal cells of the dentate gyrus, whose axons form the mossy fiber tract to hippocampal subregion CA3. Adult olfactory neurogenesis produces two types of interneurons in the granule cell layer and the periglomerular regions of the olfactory bulb.

Adult neurogenesis in other brain regions, most notably the neocortex, remains controversial, and if it exists will range on a minute scale. Initial reports about the production of large numbers of cortical neurons were not confirmed by others. In contrast, the question of whether small numbers of cortical interneurons, primarily in the deep cortical layers, might be generated in adulthood is more difficult to prove or disprove. In addition, under particular circumstances (e.g., in the face of locally limited pathology), regenerative or targeted neurogenesis in physiologically nonneurogenic regions seems to be possible. Such exceptions might question a rigid conceptual distinction between neurogenic and nonneurogenic regions. For most practical purposes, the distinction, however, is valid. In contrast to the nonneurogenic areas, the canonical neurogenic regions contain a morphologically distinct neurogenic or stem cell niche that provides a microenvironment permissive for precursor cell function and neuronal development. The niche consists of the precursor cells and their progeny, supporting cells, immune cells, vasculature, and a specialized extracellular matrix, whose exact composition is still unknown. The structure of the precursor cell niches of the adult brain resemble similar niches, for example, in bone marrow, testes, and olfactory epithelium.

Precursor cells can also be found outside the neurogenic regions, albeit at very low density. No structural evidence of a niche is found in these regions and physiologically no (or extremely limited) neurogenesis appears to take place. The function of precursor cells outside the neurogenic regions is unknown. It is also not clear how homogenous the population of neural precursor cells in the adult brain is. Concepts of neuroanatomists like Wilhelm His, Alfred Schaper, and Wilder Penfield, who proposed the existence of a 'spongioblast' in the brain parenchyma, are thereby revived. Currently, the best candidates for the parenchymal precursor cells outside the neurogenic regions are slowly proliferative cells expressing the proteoglycan NG2. The neurogenic precursor cells in the hippocampus and olfactory system, in contrast, are NG2-negative but express markers including nestin, doublecortin, brain lipid binding protein, glial fibriallary acidic protein, Sox2, and others, but neither sensitivity nor specificity are sufficiently clear.

Meaning and relevance of markers that are associated with neurogenesis in the neurogenic regions (nestin, doublecortin, calretinin, etc.) are not known for the nonneurogenic regions. The presence of these markers alone should not be taken as evidence of a neurogenic potential or even ongoing neurogenesis.

Adult neurogenesis also takes place in the olfactory epithelium (as part of the peripheral nervous system), where olfactory receptor neurons are continuously replaced from local precursor cells.

History

Adult hippocampal neurogenesis was first described by Joseph Altman, then at the Massachusetts Institute of Technology, in the early 1960s. Altman also described postnatal neurogenesis in the olfactory bulb in 1969. The response by the scientific community was skeptic, largely for methodological reasons but also because of the fact that no stem cell population was known in the brain and the origin of new neurons thus remained speculative. In the 1980s, Fernando Nottebohm and colleagues described adult neurogenesis in those brain nuclei of canary birds that are responsible for song learning. These studies provided the first link between adult neurogenesis

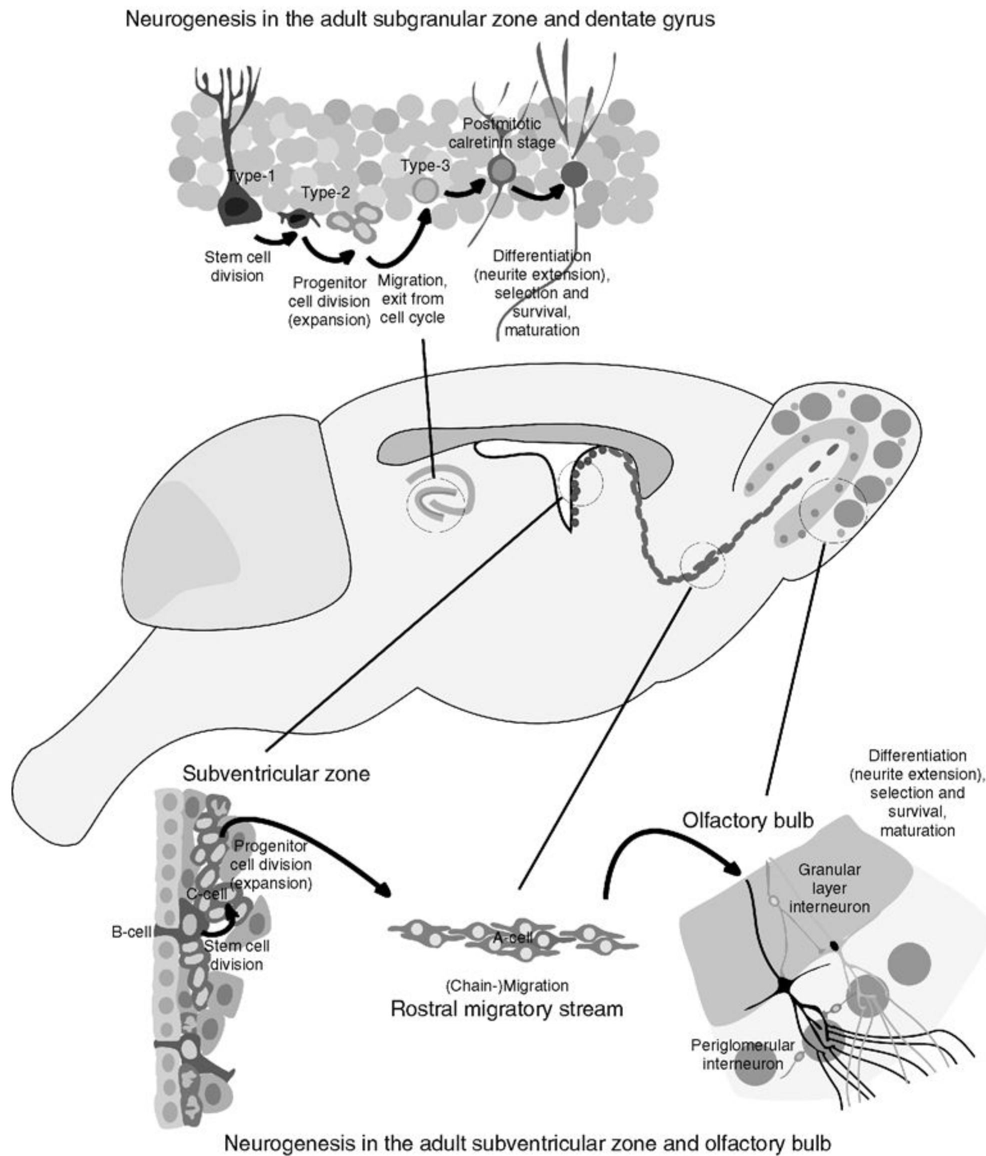

Figure 1 Adult neurogenesis occurs in two canonical neurogenic regions: the olfactory system and the hippocampus.

and behavior. After the discovery of neural stem cells in the adult mammalian brain in 1992, research on adult neurogenesis gained momentum. The isolation of stem and progenitor cells in the olfactory system was first reported by Brent Reynolds and Sam Weiss. Stem cells in the adult hippocampus followed in 1995 with the work of Theo D Palmer, Jasodarah Ray, and Fred H Gage. In the early 1990s, Elizabeth Could and co-workers reported that corticosteroids negatively affect adult neurogenesis. In 1997 and 1998, Fred H Gage's group showed that environmental enrichment and physical exercise positively influence adult hippocampal neurogenesis. The occurrence of adult neurogenesis in the human dentate gyrus was confirmed in 1998 by Eriksson and colleagues.

Adult Hippocampal Neurogenesis

The precursor cells driving adult hippocampal neurogenesis reside in the subgranular zone (SGZ) of the adult dentate gyrus. An astrocyte-like stem cell with radial morphology (type 1 cell) gives rise to highly proliferative intermediate progenitor cells (type 2 cells) and lineage-determined neuroblast-like cells (type 3 cells) that exit from the cell cycle and go through an immature postmitotic stage. During this stage, which coincides with the transient expression of calretinin, the new neurons fully extend their neurites and go through a phase of increased synaptic plasticity. The distinction of the cell types by numbers is preliminary and should one day be exchanged to a functional nomenclature.

Regulation of adult neurogenesis mainly occurs in two stages, an expansion phase on the level of the precursor cells and a phase of selective survival on a postmitotic stage. A transient expression of doublecortin lasts from the type 2 cell level to the calretinin phase. Doublecortin has become a surrogate marker of adult neurogenesis that is even used for quantification. The equalization of doublecortin expression with hippocampal neurogenesis, however, is not without problems because doublecortin expression ends before the cells are fully integrated. The newly generated cells receive first input through GABAergic synapses, which are excitatory. This input is involved in the induction of full maturation. The new cells receive glutamatergic input within days to weeks and go through a transient phase of increased synaptic plasticity (i.e., a lowered threshold for the induction of long-term potentiation). After approximately 7 weeks, the cells have become largely indistinguishable from the older granule cells and remain stably integrated into the network.

Adult Olfactory Neurogenesis

The precursor cells for adult olfactory neurogenesis are found in the subventricular zone (SVZ) of the lateral ventricle. The stem cell of this region has a cilia-bearing process touching the ventricular surface between the ependymal cells. The exact relationship between these stem cells (B cells) and the ependymal cells (E cells) is not clear; in particular, it remains controversial whether under certain conditions ependymal cells can act as stem cells. Undoubtedly, however, the astrocyte-like B cells do serve as stem cells and give rise to highly proliferative progenitor cells (C cells) that generate migratory neuroblasts (A cells). The A cells migrate in a specialized form of migration, homonymic chain migration, through the rostral migratory stream (RMS) to the core of the olfactory bulb, where they change for a radial migratory pattern and reach the granule cell layer and the glomerular and periglomerular regions. Precursor cells with a neurogenic potential can be isolated not only from the SVZ but also from the RMS and the olfactory bulb. All new neurons in the olfactory bulb are inhibitory interneurons and thus GABAergic. A subset of new periglomerular neurons co-expresses dopamine as neurotransmitter. Maturation of the new cells is delayed until they have reached their final position. The new cells in the granular layer express calretinin. The exact lineage relationship between the different types of new olfactory bulb neurons is not clear.

Regulation

Regulation of adult neurogenesis mainly falls into two categories. Numerous relatively nonspecific stimuli can affect the proliferation of precursor cells. Acutely this increase can lead to an increase in net neurogenesis, but this does not necessarily have to be the case. For many factors, the question of whether a particular stimulus has long-term effects on net neurogenesis has not yet been specifically addressed. Many of the nonspecific stimuli are associated with 'activity,' and many examples of pathology (ischemia, trauma, etc.) can upregulate neurogenesis. Other stimuli such as stress and inflammation downregulate precursor cell proliferation and neurogenesis. For many such regulators, complex dose–response relationships might exist so that generalization is difficult. Age can be considered a strong negative regulator early in life but apparently loses this strong impact, because with increasing age adult neurogenesis settles at a very low level.

Stimuli that appear to be of more specific relevance to the hippocampus or the olfactory system act on the selective survival of the new cells. Under physiological conditions, these stimuli tend to be associated with the function of the hippocampus (memory, learning, etc.) or the olfactory system (olfaction). Genetical studies indicate that regulation on the survival and differentiation level has a larger quantitative (and presumably qualitative) impact on the control of adult neurogenesis than the regulation of cell proliferation.

On a mechanistic level, regulation involves several neurotransmitter systems that provide input to the SGZ and SVZ. For the SGZ, the research focus has been on the GABAergic, the serotonergic, and the glutamatergic system. High levels of glucocorticoids acutely suppress neurogenesis. Presumably because the expression of corticosteroid receptor expression changes in the course of neuronal development in the adult, the net effect under prolonged exposure to glucocorticoids is complex. Many situations with elevated levels of glucocorticoids are thus associated with increased levels of neurogenesis. The details of this regulation are unknown. The role of other hormone systems is even more ambiguous.

Growth factors and neurotrophic factors, most notably fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF), strongly affect adult neurogenesis in a complex way. Insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) have been discussed as centrally involved in the activity-dependent regulation of adult neurogenesis. Brain-derived neurotrophic factor (BDNF) is widely proposed to play a major role in controlling adult neurogenesis but experimental evidence supporting this assumption so far remains limited.

Among the short-range-acting signaling molecules with effects on adult neurogenesis contributions of the ephrins, bone morphogenic proteins (BMPs) and

the Wnt system have been described. More are likely to follow. It is assumed that the general and intrinsic developmental principles regulating neuronal development in the adult more or less recapitulate the processes prevalent in the fetus. Extrinsic and activity-dependent regulation, in contrast, are likely to set adult neurogenesis apart from fetal neurogenesis. In addition, differences between the two neurogenic regions might exist with regard to the contribution of individual regulatory factors.

Function

The fact that adult neurogenesis is the exception and not the rule has raised the question of why dentate gyrus and olfactory bulb in contrast to all other brain regions not only accommodate the integration of new neurons but actually call for it in a function-dependent manner. In both systems, adult neurogenesis does not add bulk numbers of new neurons. Rather, the contribution of adult neurogenesis might be qualitative and allow a possibly subtle modification of the existing neuronal network. In the hippocampus, adult neurogenesis allows the activity-dependent optimization of the mossy fiber connection between the dentate gyrus and CA3. Integration is triggered during a phase of increased synaptic plasticity and leads to a cumulative adaptation of the network to a level of complexity and novelty. Alternative hypotheses that mostly suggest a transient functional contribution of the immature neurons in the sense of a direct contribution to memory formation are questioned by the lacking evidence for substantial neuronal turnover and the scarcity of new neurons. Mechanistically, it has been proposed that the new neurons help the hippocampal network to avoid catastrophic interference in the dentate gyrus, that is, the destruction of codings for older information by incoming new information.

Similarly, adult olfactory neurogenesis has been brought into connection with novelty detection, here the coding of novel odors. It seems that new neurons are more sensitive to novel odors and might help to adjust the network to the processing of a new range of olfactory stimuli.

A general principle might be that adult neurogenesis is used in bottleneck situations, where the insertion of new neurons is strategic and allows the incremental optimization of a network that in general tends to remain as lean as possible. Networks in nonneurogenic areas might not exist under this priority of size limitation.

Medical Relevance

Adult neurogenesis might be of medical relevance either because a failure of adult neurogenesis might contribute to brain disease or because a reactive increase in adult neurogenesis might be part of endogenous regenerative attempts. A role for adult neurogenesis in brain pathology, among others, is discussed for temporal lobe epilepsy, major depression, and dementias. In temporal lobe epilepsy, cell proliferation is massively increased, many new neurons are found in ectopic locations, and aberrant projections from the new neurons might support the chronification of the disease. In the context of major depression, a reduction in hippocampal volume has been noted (that is, however, much larger to be explained by a lacking contribution of adult neurogenesis) and all antidepressants have been found to increase adult hippocampal neurogenesis. As the most extreme position in the context, it has been suggested that antidepressants might even require neurogenesis for their action. Dementias might at least partly be explained by a reduction in the contribution that new neurons make to hippocampal function – a concept that is complicated by the fact that the functional relevance of adult neurogenesis itself is not yet fully understood. As yet, no similar concrete links to pathology have been proposed for neurogenesis in the olfactory bulb.

Numerous pathological stimuli, most notably trauma and ischemia, induce the proliferation of precursor cells and in many cases net adult neurogenesis. These effects appear to be indirect because they are found even in the absence of direct damage to the dentate gyrus or SVZ. In addition, after middle cerebral artery occlusion in rats, precursor cells migrated from the SVZ into the striatum and differentiated into local neurons. The massive infusion of growth factors supported the replacement of ischemic CA1 neurons by SVZ precursor cells. Phototoxically induced circumscript cell death in the cortex also led to the replacement of neurons.

Envisioned therapeutic strategies that are based on the transplantation of neurons that have been generated from neural precursor cells benefit from the knowledge accumulating from research on adult neurogenesis, because here nature exemplifies how neuronal development is possible under the conditions of the otherwise nonneurogenic adult brain.

Medical relevance also depends on the occurrence of adult neurogenesis in humans. Adult hippocampal neurogenesis has been demonstrated for humans and nonhuman primates. There is evidence for neurogenesis in the adult olfactory bulb of nonhuman primates but not yet unambiguously of humans. Precursor cells, however, have been isolated from ventricle biopsies and resection specimens from the adult human hippocampus, as well as postmortem brain tissue of infants.

See also: Neural Stem Cells: Adult Neurogenesis; Stem Cells and CNS Repair; Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging.

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Stem Cells and CNS Repair

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Introduction

The myriad neurological pathology that can affect the human brain during prenatal development and throughout life and the brain's limited self-repair capacity call for new therapeutic strategies. Our increasing knowledge about the fundamental biology and therapeutic potential of various stem cell types opened a new chapter in regenerative medicine. The initial work on rodent stem cells during the past two decades is now being successfully continued with stem cells of human origin and from different developmental stages. We have learned about key genes and cellular mechanisms that maintain the status of stem cells or lead to differentiated progeny. We have also learned about the multiple roles of stem cells during development, disease, and aging. It is now well established that stem cells are not only a valuable tool for cell replacement but also equipped with important additional properties that may be harnessed for cell protection, detoxification, and gene therapy ('chaperone effects').

Stem cell biology is being recognized as a continuum of development, and developmental processes are tightly regulated, both temporally and spatially. Better understanding of these developmental events is considered to be a key strategy for the successful use of stem cells (endogenous and grafted) for central nervous system (CNS) repair and functional recovery. For instance, the generation of functional neurons and glial cells during brain development requires a concerted coordination of cell proliferation, migration, cell type specification, and synaptic integration, all of which are also crucial for successful stem cell therapy.

Stem Cell Prototypes

Stem cells give rise to organs and maintain tissue integrity and homeostasis in the adult organism. There are different types of stem cells, including embryonic and somatic (fetal or adult derived), from which new cells can be derived. To fulfill the criteria of a stem cell, as opposed to a 'progenitor' cell, a single clonal cell must have the following functional properties: (1) it should be able to generate the cell types from the organ from which it was derived, and (2) it should be capable of 'self-renewal' (i.e., the ability to produce daughter cells with identical properties). The ability to populate a developing or injured region with appropriate cell types upon transplantation is another important stem cell feature that is well established with hematopoetic stem cells and awaits standardization in other organ systems, including the brain. In the following sections, we introduce two prototypical stem cells, embryonic stem cells (ESCs) and neural stem cells (NSCs), and discuss their potentials for neural repair.

Embryonic Stem Cells

ESCs have been derived from the inner cell mass of blastocysts of different species including human. They are pluripotent and able to yield mature cell types from all different germ layers. Work performed with mouse ESCs has provided proof of principle that pluripotent cell lines can be harnessed for developmental biological studies as well as for new therapeutics. Since significant species differences exist between mouse and human ESCs regarding signaling pathways and molecular regulation of pluripotency, it is pivotal to fully characterize and define the molecular mechanisms in human ESCs. Our understanding of human ESCs is increasing and knowledge is accumulating on improved cell culture conditions, long-term propagation, controlled differentiation, and transplantation into animal models of human disease.

The list of various cell types differentiated from human ESCs (e.g., neurons, cardiomyocytes, and hepatocytes) is continuously increasing. Pluripotent ESCs can be stepwise differentiated in the culture dish by recapitulating aspects of *in vivo* development and the use of relevant epigenetic factors. Importantly, the acquisition of a particular developmental stage of a cell is best characterized by considering morphological, immunophenotypic, and functional criteria. Unlimited access to specific functional human cells is expected to play an important role not only in therapeutic cell replacement but also for disease modeling and drug screening.

Neural Stem Cells

In contrast to pluripotent human ESCs, somatic stem cells are believed to be multipotent and thus capable of generating the major cell types limited to the tissue

of origin. Typically, the NSC is capable of producing neurons, astrocytes, and oligodendrocytes. Somatic/tissue-specific stem cells are the building blocks of organs during development and survive in specialized microenvironments ('stem cell niche') contributing to new cells throughout life. NSCs are multipotent (i.e., they have the ability to yield mature cells in all three fundamental neural lineages throughout the nervous system: neurons, astrocytes, and oligodendrocytes), have the ability to populate a developing region and/or repopulate an ablated or degenerated region of the CNS with appropriate cell types, and undergo self-renewal (i.e., the ability to produce daughter cells with identical properties). NSCs have been identified *in vitro*. No study has demonstrated the existence of multipotent NSCs *in vivo*. NSCs are highly abundant during embryogenesis, with a sharp decline soon after birth. In the adult nervous system, NSCs are confined to the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the lateral ventricles. The newly born neurons in hippocampus have been suggested to improve memory and play a role in mood behavior such as stress and depression. Neuroblasts born in the SVZ migrate along the rostral migratory stream to the olfactory bulbs, where they differentiate into periglomerular and granule neurons. In brain regions such as amygdala, substantia nigra, and cortex, cells with stem cell characteristics *in vitro* have been isolated. Morphologically, NSCs share properties with both astrocytes and radial glia. The main characteristic is a long process that extends radially. Although no definitive marker has been suggested for NSCs, a substantial amount of work shows that they are positive for nestin, an intermediate filament protein, and glial fibrillary acidic protein, traditionally used to identify astrocytes.

NSCs or progenitor cells with a more restricted developmental potential can be generated from human ESCs or directly isolated from the developing CNS as well as from neurogenic regions of the adult brain. Historically, the first established NSC lines exploited knowledge accumulated on tumor viruses and immortalization. These cell lines have been invaluable in expanding our experience on basic stem cell biology and neural repair. Some of these multipotent cell lines, such as the C17.2 NSC line, are still widely used. However, NSCs that have not been genetically modified can also be propagated *in vitro* for extended periods of time using high concentrations of mitogenic factors such as basis fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Neural stem/progenitor cells have been cultured as monolayers on coated substrates or as free-floating spherical aggregated, termed neurospheres.

Stem Cell Repertoire

Chaperone Effects

Initially, stem cells were exclusively considered as tools for cell replacement. However, there is much evidence now for robust additional biological properties (chaperone effects) of stem cells that may be exploited therapeutically. Chaperone effects of stem cells include the natural delivery of neurotrophic, cytoprotective, and anti-inflammatory molecules (e.g., glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor (BDNF), and neurotrophin-3) in order to rescue dysfunctional cells. This concept of stem cell-based chaperone effects was first demonstrated in the brain of aged and Parkinsonian mice and later confirmed and extended to other organ systems and various diseases (e.g., bone marrow-derived mesenchymal stem cells (MSCs) or ESCs for cardiac disease, and umbilical cord cells in stroke).

Environmental Cues

Increasingly, the microenvironments within the CNS are providing insight into the molecular milieu regulating stem cell biology. A specialized microenvironment in the neurogenic regions is responsible for the continued self-renewal and differentiation of the stem cell pool. For example, these regions have a higher density of blood vessels releasing factors, such as vascular endothelial growth factor (VEGF) and BDNF, contributing to increased proliferation and neurogenesis. Different levels of growth factors can have fundamentally different effects on proliferation and differentiation. For example, low levels of insulin-like growth factor-1 (IGF-1) promote neurogenesis, whereas higher levels increase proliferation and oligodendrogenesis. Established protocols for the isolation of NSCs include the growth factors EGF and bFGF that sustain self-renewal of NSCs. Injection of these factors intracranially into mice has been shown to regulate both proliferation and differentiation *in vivo*. Neurotrophins such as BDNF in collaboration with retinoic acid and Wnt signaling regulate neurogenesis, and ciliary neurotrophic factor/leukemia inhibitory factor or bone morphogenic proteins induce gliogenesis. It is also know that the cellular milieu contributes to fate determination and cortical development, specifically through the effects of resident astrocytes in both the SVZ and the hippocampus. Physical exercise and enriched environment have been shown to promote neurogenesis in the SGZ. The effects from physical activity are partly mediated by IGF-1, VEGF, BDNF, and endogenous opioids. The characterization of the stem cell microenvironment will provide the molecular and cellular

scaffold on which stem cell therapy, both endogenous and exogenous, can be built.

Stem Cells as Vectors for Gene Therapy

Brain lesions can be focal and restricted to a certain brain region or widely distributed in the parenchyma. Ideally, both lesion types would be targeted with a specific and efficient delivery of therapeutic molecules and drugs. In fact, efficient delivery is still a major hurdle in gene therapy. The finding that endogenous and grafted NSCs display an extensive migratory potential and tropism toward brain lesions founded the idea that these cells may be used as therapeutic vectors. Proof-of-principle experiments in animal models of lysosomal storage diseases (for a widely distributed brain lesion) and brain tumors (for a focal lesion) have shown that genetically modified NSCs are powerful therapeutics to cross-correct hereditary enzymatic deficiencies or to dramatically reduce a tumor mass. Thus, NSCs hold great promise for both cell and gene therapy.

Stem Cell-Based CNS Repair

It is manifest that stem cells can be used to replace neuronal, astrocytic, and oligodendroglial cells lost due to various brain diseases. However, it is important to note that successful use of stem cell is probably dependent on many factors, including the nature and degree of injury, disease history and age of the patient, primarily affected cell types, the type of stem cell chosen for transplantation, and the site of grafting. Deeper insight into these parameters will be important to tailor patient-specific treatment paradigms in a clinical context.

Stem cells have been explored in a number of rodent and primate models of human neurological diseases. These studies showed successful survival, differentiation, and synaptic integration of grafted stem cells, which led to functional restoration and amelioration of symptoms. In the following sections, we discuss examples of stem cell-based CNS repair and the challenges that remain to be addressed prior to clinical application.

Parkinson's Disease

Parkinson's disease (PD) is characterized by a progressive deterioration and loss of nigrostriatal dopaminergic neurons in the substantia nigra. The consequence of this cell death in the ventral midbrain is a deficient dopamine neurotransmission in the target region, the striatum. Clinically, PD presents with symptoms such as tremor, rigidity, and bradykinesia. Patients transplanted with fetal mesencephalic grafts in the early 1990s have demonstrated that an ectopic transplantation of dopamine-producing cells into the striatum can restore motor function and ameliorate clinical symptoms. Because of the limited availability of fetal tissue, stem cells are expected to provide unlimited numbers of transplantable dopamine neurons.

Several studies using rodent and primate models of PD have demonstrated successful integration and functional improvement after grafting of dopaminergic neurons derived from both ESCs and NSCs. In primate models, monkey ESCs have been transplanted and animals evaluated for behavioral improvements. As with rodent models, functional improvements occur. Furthermore, these behavior assessments can be corroborated with functional neuroimaging (**Figure 1**).

Although considerable progress has been made in stem cell-based treatment of PD inanimals, many challenges remain before clinical translation. Human ESCs differentiate to dopaminergic neurons under various protocols, yet the creation of a purified and homogeneous population of dopaminergic neurons is challenging and needs improvement. Animal models for future investigation should increasingly include primates in order to refine the mechanics and logistics of transplantation. Patients in whom stem cell therapy will be the most effective with the least side effects should be defined. The effects of posttransplantation training and rehabilitation need to be better understood; it appears that these contribute to improved functional outcome in experimental animal models. The clinical experience with fetal grafts suggests that the patient's disease history is an important parameter and that cell therapy will not be the method of choice for every Parkinsonian patient; thus, patient selection will be pivotal for clinical improvements after graft placement. Finally, the adverse side effects such as dyskinesias observed in some patients after transplantation of fetal grafts need careful consideration, and the safety of human ESCs needs to be established prior to clinical transplantation.

Stroke

Arterial occlusion within the brain can lead to ischemia and infarction of brain parenchyma. The current treatment of stroke remains limited and is focused on neuroprotection to limit the expansion of the infarct and to possibly recover the cells within the ischemic penumbra. The use of recombinant tissue plasminogen activator in select clinical situations within a critical time window after the stroke event has led to improved clinical outcome. Unfortunately, the time constraints in which this treatment can be offered limit potential application to a very small group of stroke patients.

Hypoxic/ischemic injury can lead to substantial tissue loss and the formation of infarction cavities, which would be a major therapeutic obstacle for the survival

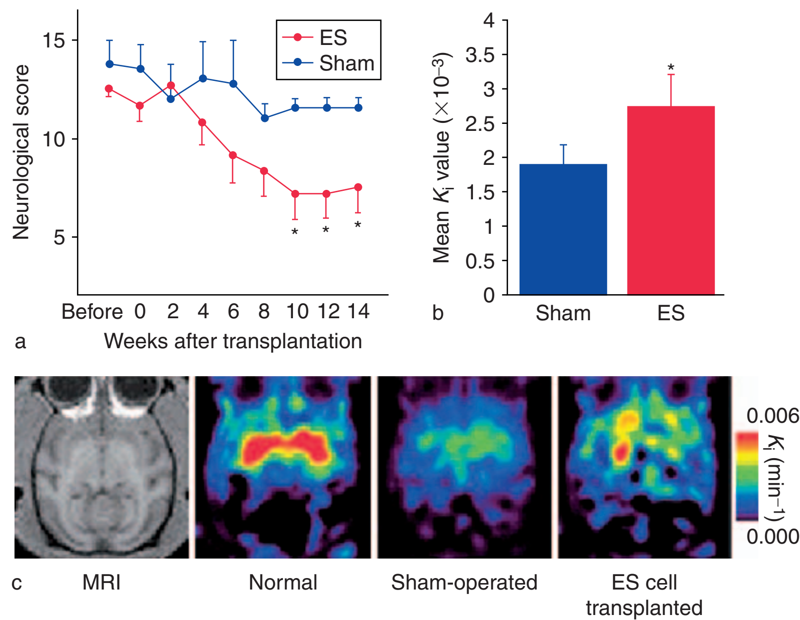

Figure 1 Function of embryonic stem (ES) cell-derived neurospheres in MPTP-treated monkeys. Behavioral scores (a) and PET study (b, c) of ES cell-transplanted ($n=6$) and sham-operated animals ($n=4$). (b) Mean K_i values from the entire putamen. (c) Increased ^{18}F-flourodopa uptake in the putamen of ES cell-transplanted animals. All values are mean ± SD.*$p < 0.05$. Reproduced from Takagi Y, Takahashi J, Saiki H, et al. (2005) Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. *Journal of Clinical Investigation* 115: 102–109.

of newly seeded stem cells. Our group has demonstrated that extensively damaged brain areas can be repaired by the combined use of stem cells and polymer scaffolds that can be placed into the infarction cavity (**Figure 2**). It has been suggested that stem cells may be uniquely suited for stroke therapy given their inherent cytoprotective, anti-inflammatory, and restorative properties. Notably, early studies using other cellular therapies (e.g., human NT-2 teratocarcinoma line) demonstrated some functional improvement in stoke patients. However, the growth of stem cells is better controlled than any other immortalized cell line. Stem cell-derived and implanted neurons were shown to survive for more than 2 years in the human brain. Stem cell use in animal models of cerebral ischemia clearly demonstrates the ability of murine and human NSCs to engraft into the brain and survive, migrate, and differentiate leading to functional improvement. Other studies with murine NSCs have shown the potential of stem cell grafts to promote recovery in ischemic rats and recovery of sensorimotor deficits after transplantation into the striatum and cortex ipsilateral or contralateral to the stroke. In a clinical setting, cell transplants for stroke patients may be feasible even weeks after the ischemic event, allowing the patient to recover from the acute injury. Furthermore, several weeks may be needed to perform detailed neurophysiological and behavioral testing to allow selection of candidate patients. In accordance with a timetable that accounts for the most likely clinical scenario with patients, human somatic NSCs have led to functional recovery from stroke with improvement at both cortical and subcortical levels in various murine models of stroke. ESCs can be differentiated into NSCs following exposure to retinoic acid *in vitro* and have also demonstrated functional recovery in rodents.

Although regenerative cell therapy for stroke appears very promising, the use of stem cells is in its infancy. Clearly, the mechanisms that lead to beneficial effects after stem cell transplantation need to be better understood. For instance, it is important to determine whether the reported improvements are primarily the result of reconstitution of neural circuitry by cell replacement, the enhancement of intrinsic repair mechanisms (including the recruitment of endogenous stem cells), or both. Most likely, grafted and endogenous stem cells are effective through a multitude of mechanisms. It is possible that stem cells may be delivered to the injured brain not only by local intracerebral delivery but also by intravenous or intrathecal routes.

Spinal Cord Injury

Spinal cord injury (SCI) is a devastating ailment with little opportunity for treatment. The injury occurs from mechanical forces in the acute setting and is exacerbated by secondary inflammatory damage, both leading to neuronal death and demyelination. Accordingly, potential therapy would vary depending

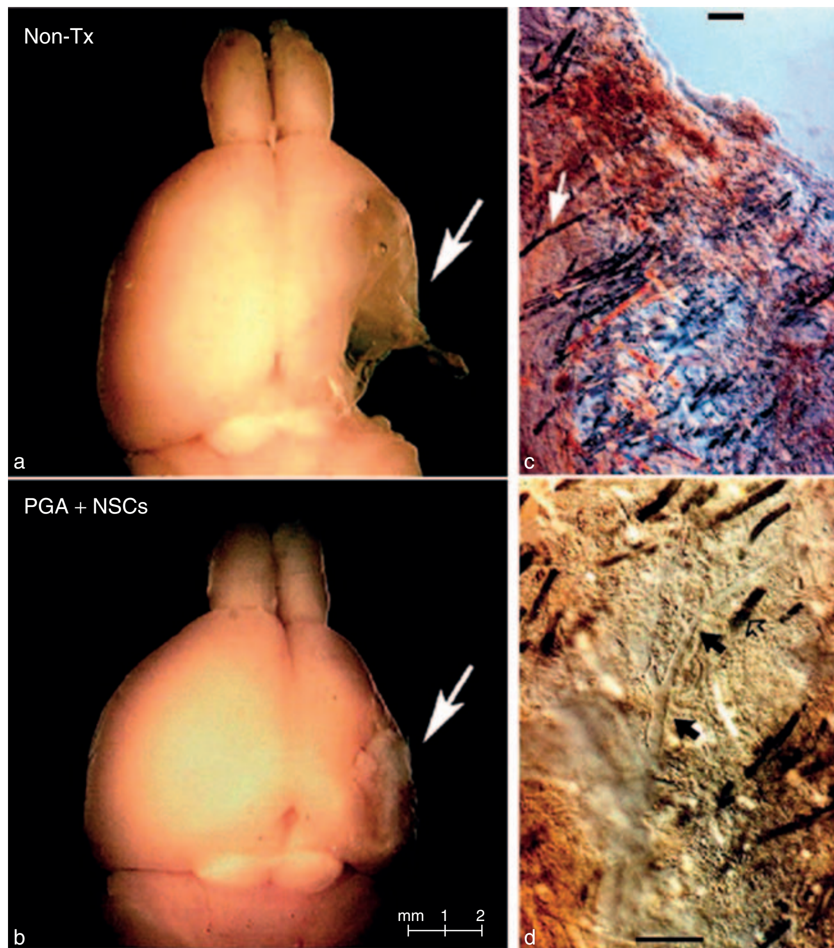

Figure 2 Implantation of neural stem cell–pluripotent embryonic stem cell (NSC–PGA) complexes into a region of cavity formation following extensive hypoxic/ischemic (HI) brain injury and necrosis. (a) Brain of an untransplanted (non-Tx) mouse subjected to right HI injury with extensive infarction and cavitation of the ipsilateral right cortex, striatum, thalamus, and hippocampus (arrow). (b) Contrasting with a, the brain of a similarly injured mouse implanted with an NSC–PGA complex (PGA + NSCs), generated *in vitro* as described in a, into the infarction cavity 7 days after the induction of HI (arrow; $n = 60$). At maturity (age matched to the animal shown in a), the NSC–scaffold complex appears in this whole mount to have filled the cavity (arrow) and become incorporated into the infarcted cerebrum. (c, d) Higher magnification of representative coronal sections through that region, in which parenchyma appears to have filled in spaces between the dissolving black polymer fibers (arrow in c) and even to support neovascularization by host tissues, as seen in d. A blood vessel is indicated by the black arrows in d; the open arrow in d points to degrading black polymer fiber. Scale bar = 100 μm (c, d). Reproduced from Park KI, Teng YD, and Snyder EY (2002) The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. *Nature Biotechnology* 20: 1111–1117.

on the time frame after injury, with minimizing inflammation the primary concern early after injury and regeneration the major goal when injury is in its chronic phase. The pathobiology of SCI is highly dependent on the time course in which the injured spinal cord is examined. This directs the transplantation of NSCs or their derivatives (e.g., oligodendrocytes for myelination) to carefully account for and maximize the timing of transplantation with consideration of cell survival of grafted cells. Studies have addressed some of these issues demonstrating the importance of both timing of transplantation and the role of growth factors in murine models of SCI treated with NSCs. Injured rats received NSC transplants at different time points that would correspond clinically with subacute and chronic SCI, respectively. The administration of growth factors, including EGF, bFGF, and platelet-derived growth factor, resulted in increased numbers of cells grafted into the injured spinal cord by either enhanced survival or increased proliferation. To determine if NSCs together with growth factors can lead to neurological improvement, animals were evaluated using three independent behavioral tasks, all of which showed significant improvements compared to control mice, with even some long-term improvements (**Figure 3**). Human CNS fetal-derived stem cells have been shown to survive, engraft, differentiate, and improve

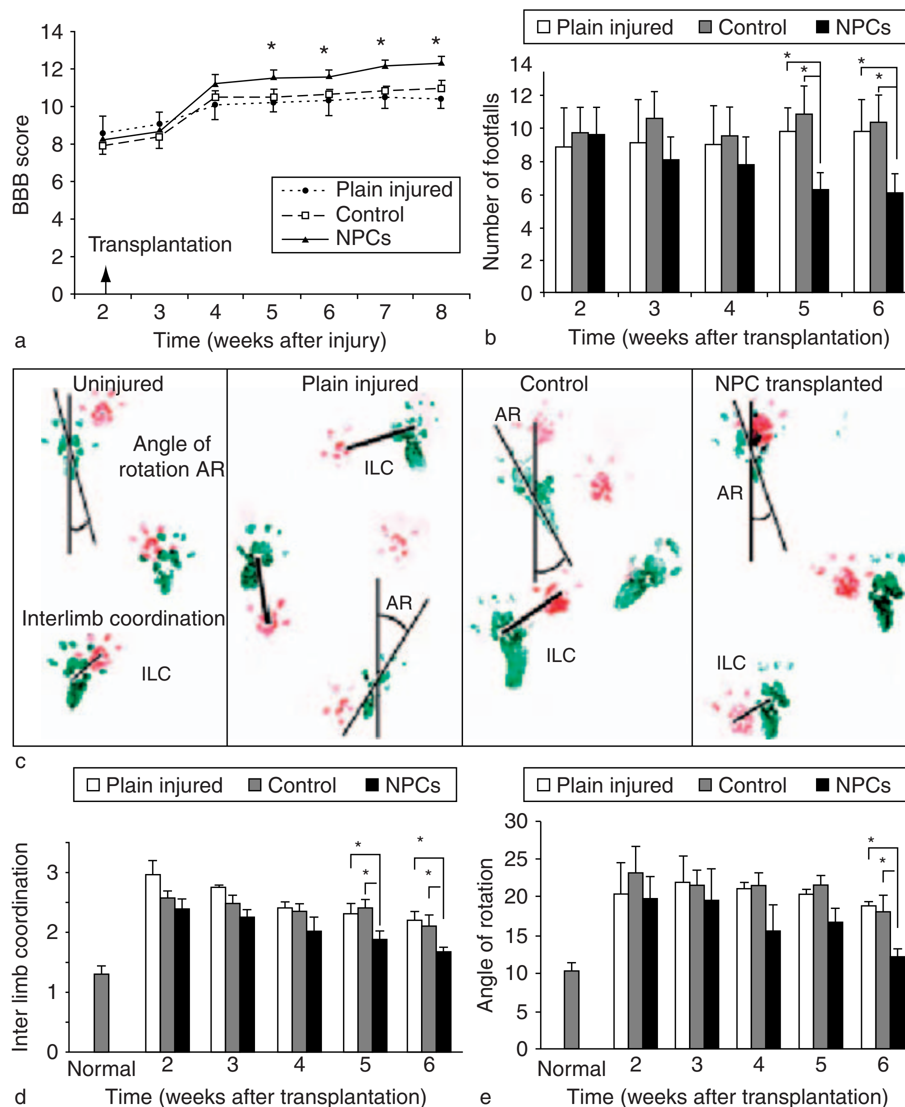

Figure 3 Subacute transplantation of YFP–NPCs resulted in a significant locomotor recovery compared with injured rats in the control group. (a) BBB rating scale showed a significant improvement in the locomotor BBB score in transplanted rats 3 weeks after transplantation compared with plain injured and control groups ($n = 5$ for plain injured group and $n = 8$ for other groups). (b) Using grid-walk analysis, transplanted rats also showed fewer errors in hindlimb placements 5 and 6 weeks after transplantation compared with plain injured and control groups ($n = 5$ for plain injured group and $n = 8$ for other groups). (c) Representative footprints of normal, plain injured, control, and grafted rats ($n = 5$ for plain injured group and $n = 8$ for other groups) show improvement in interlimb coordination as well as angle of rotation in the transplanted group compared with the plain injured and control groups. (d, e) Footprint analysis revealed that transplantation with adult NPCs significantly improved interlimb coordination and reduced the hindlimb angle of rotation 5 and 6 weeks after transplantation. The data show the mean ± SEM. *$p < 0.05$. Reproduced from Karimi-Abdolrezaee S, Eftekharpour E, Wang J, et al. (2006) Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. *Journal of Neuroscience* 26: 3377–3389.

locomotor skills after traumatic SCI in mice. Contusive SCI of the thoracic cord was treated with injection of human NSCs. Functional recovery was assessed and shown to be improved. Also, selective ablation of grafted cells with diphtheria toxin (murine cells are 100 000 times less sensitive to diphtheria toxin than human cells) was used for the targeted killing of human NSCs. This selective ablation led to reversal of symptomatic and behavioral improvement, providing further support that human NSCs can mediate functional recovery in murine SCI models. Despite demonstrating functional recovery in murine models of SCI with both murine and human NSCs, further investigation using primate models is critical prior to any human trials. Indeed, spinal cord anatomy is different in the rodent compared to primates and humans.

Neural regeneration is not without pitfalls; some studies have shown the creation of aberrant axonal

Figure 4 A subset of newborn layer V cortical neurons extends axons to the cervical spinal cord. (a) Both newborn and original layer V corticospinal motor neurons (CSMN) were retrogradely labeled by FG (blue). (b) Field expanded in (c)–(f) showing a BrdUrd$^+$/NeuN$^+$/FG$^+$ triple-labeled adult-born neuron (arrow). (c–e) Individual images show that this BrdUrd$^+$ nucleus (c, red) is located within this neuron, which is retrogradely labeled with FG from the cervical spinal cord (d, blue) and expresses NeuN (e, green). (f) Overlay showing BrdUrd$^+$/FG$^+$/NeuN$^+$ neuron co-localization. (g) Higher magnification overlay of the same neuron from c–f. (h) A separate example of an adult-born neuron with a projection to the spinal cord. Laser-scanning confocal images were combined to produce three-dimensional reconstructions of newborn neurons. Viewing a BrdUrd$^+$/FG$^+$ newborn neuron along its *x* (h′), *y* (h″), and *z* axes (h) unequivocally demonstrates the co-localization of BrdUrd and FG. (i) Quantification of BrdUrd$^+$/FG$^+$ adult-born CSMN extending spinal projections from 12 to 56 weeks after induction of original CSMN apoptosis. Each point indicates the number of adult-born BrdUrd$^+$/FG$^+$neurons/mm^3 in an individual animal; each bar indicates the mean Scale bar = 10 μm (b–e). Reproduced from Chen J, Magavi SS, Macklis JD, et al. (2004) Neurogenesis of corticospinal motor neurons extending spinal projection in adult mice. *Proceedings of the National Academy of Sciences of the United States of America* 46: 16357–16362.

sprouting which led to allodynia hypersensitivity. This may be related to excessive astroglial differentiation, highlighting the need for more controlled differentiation in order to both maximize functional recovery and minimize side effects. Along with increased understanding of cellular and molecular mechanisms, the timing and logistics of transplantation need to be improved, and noninvasive cellular imaging needs to be established.

Endogenous Stem Cell Recruitment for CNS Repair

The alternative or compliment to stem cell transplantation would be the manipulation of endogenous stem cells for therapeutic purposes. The advantages would include using the patient's own cells, not needing an invasive procedure, and obviating concern regarding the immunogenicity of transplanted cells. It appears that adult neurogenesis is restricted to the olfactory bulb and dentate gyrus of the hippocampus, but it is possible that some NSCs exist along the entire adult neuraxis.

Exploiting endogenous NSCs would require successful coordination of cell proliferation, differentiation, migration, and integration, and it does appear that some instructive signals remain in the adult CNS. However, most CNS regions are not permissive for neurogenesis under normal *in vivo* conditions, but some studies suggest that endogenous NSCs are primed to respond to environmental signals that exist primarily during pathological states. Accordingly, one approach to accentuate endogenous stem cell proliferation is the administration of growth factors. Intraventricular infusion of transforming growth factor-α into rodents with lesions of the substantia nigra dopaminergic neurons has led to functional improvements, putatively through recruitment of endogenous stem cells. Other likely bioactive molecules with potential to evoke a proliferative response in regions of the brain with multipotent cells are neurotrophins. Physiologically, neurotrophins are involved in cell cycle regulation, cell survival, and differentiation and are critical during normal development. Growth factor infusion can also promote proliferation of SVZ-derived progenitor cells, which gave rise to hippocampal CA1 pyramidal neurons in rodent stroke models, with

improvements in spatial orientation. Whether the neurogenic response creates neurons with long-term viability remains to be determined. Another candidate with efficacy in stroke models is erythropoietin, which has been shown to induce neurogenesis and functional improvement in rats. Furthermore, endogenous neural precursors can differentiate into new neurons that extend long-distance projections to the spinal cord in the adult rodent. Targeted apoptosis of corticospinal motor neurons was induced, and it was demonstrated that adult-born corticospinal motor neurons were generated extending from the motor cortex to the spinal cord (**Figure 4**).

Regarding neural repair, continued investigation into whether the diseased CNS can be treated with growth and differentiation factors to induce neural repair is required. As more is learned about the molecular signals and environmental cues, endogenous stem cells may prove to be a compliment or even replacement to transplantation of exogenous stem cells.

Other Stem Cells

The use of embryonic or somatic stem cells for brain repair is currently the focus of rigorous scientific investigation. Other stem cells have also been suggested as sources for cell therapy. For instance, some groups have found that mesenchymal stem cells (MSCs) can differentiate into astrocytes and neurons *in vitro* and *in vivo* and may have an advantage over ESCs or NSCs because they are a highly accessible source for the patient's own stem cells. However, there is controversy about the plasticity and developmental potential of MSCs. Some groups suggest that the findings with MSCs may be cell culture artifacts rather than true differentiation into unexpected cell types. Therefore, it is crucial to assay the differentiation of any stem cell into a particular cell type by combining morphological, immunophenotypic, and functional criteria. Currently, MSCs do not appear to be a realistic alternative to the use of ESCs or NSCs for neural repair.

Conclusion

Stem cell biology represents a strong foundation for neural repair. Experimental evidence suggests that this technology may be applicable to treat patients in the future. Since ESCs can be multiplied indefinitely and have the potential to give rise to a variety of functional human cells, it is conceivable that stem cells will play an important role in disease modeling and drug testing. Moreover, since stem cells mimic aspects of normal development, these cells may be used to study early steps of human development which would not be accessible for experimentation otherwise.

We have highlighted current problems in the rapidly progressing stem cell field, which involve safety issues, standardization of the protocols used, development of rigorous assays for characterization, and accumulation of experimental data in primate models of human disease. Realistic candidate diseases and patients who may benefit from stem cell therapy need to be defined before any clinical application. Since clinicians and stem cell biologists share a strong common interest to understand and treat human disease, stem cells have the true potential to transform modern medicine.

See also: Neural Stem Cells: Adult Neurogenesis; Spinal Cord Regeneration and Functional Recovery: Strategies; Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging.

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Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging

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Introduction

Although the postnatal brain had long been thought to lack the capacity to generate new neurons, the past decade's research has radically changed this assumption with ample evidence that new neurons are generated in some regions of the adult brain. This process is referred to as neuronogenesis for the specific production of neurons and more broadly as neurogenesis when all neural cell types may be generated. In many cases the terms neuronogenesis and neurogenesis are used interchangeably, as they will in this article. Neural stem cells and neurogenesis are restricted to specific areas in the adult brain: the subventricular zone/rostral migratory stream/olfactory bulb system and the dentate gyrus of the hippocampus. This spatial restriction means that most of the brain does not support neurogenesis under normal conditions and is thus referred to as nonneurogenic. Understanding the capacity of different regions to support survival and integration of neural stem cells will be of importance for the potential use of stem cells for brain repair, as it is this wider, nonneurogenic portion of the brain that will most frequently need to be the target of repair strategies.

Within the young adult brain, neurogenesis routinely contributes large numbers of new neurons to the olfactory bulb and small numbers of new neurons to the dentate gyrus. Many studies have shown that the rate of neurogenesis in the young adult brain is not fixed, but subject to stimulation by experimental manipulation. Neuropathological studies of the aging central nervous system (CNS) frequently utilize young adult, rather than aged, animals in experimental models or transgenic mutations of senescence-associated diseases, such as Parkinson's, Alzheimer's, or Huntington's diseases. Less is known about the underlying changes leading to the decline in normal (nonpathological) brain aging, yet this characterization will be necessary to interpret the contribution of specific disease pathology to impaired brain function. Here we look at the effects of normal aging on neurogenesis in the adult mammalian brain, the process in which resident neural stem cells give rise to new functional neurons that are incorporated into the circuitry. We discuss how age-induced changes may modulate neural stem cell activity resulting in a significant decline in neurogenesis and the implications of this for the aging brain.

Characterization of Neural Stem Cells

Many postnatal mammalian tissue systems contain cells that possess the features consistent with the definition of a stem cell, that is, the capacity for unlimited self-renewal and the ability of their progeny to differentiate into different cellular types. Stem cells present during embryonic development have considerable flexibility in sculpting tissues; however, this capacity may be restricted in postnatal tissue. In fact, it is a matter of debate as to whether stem cells found in certain adult tissues, such as the CNS, are truly stem cells or would be better termed progenitor cells, reflecting their more restricted proliferate capacity and differentiation potential to the same tissue in which they reside.

At present, there is no single definitive marker by which neural stem cells in tissue can be identified. Identification of stem/progenitor cells in adult tissue is usually accomplished through labeling the proliferating cells and assessing their subsequent lineage outcome. Most *in vivo* studies of neurogenesis have used administration of the thymidine analog bromodeoxyuridine (BrdU) to identify new cells. The duration of BrdU delivery and the interval between administration and examination are both of critical importance in interpreting the cell type under study. For example, sustained delivery of BrdU over several days labels a population of proliferating cells, but obscures the ability to distinguish whether those proliferating cells are slowly dividing or rapidly dividing, or whether the labeled cohort contains a heterogeneous population of both cell types. Therefore, many studies with longer BrdU administration do not provide information discriminating between slowly cycling stem cells and rapidly cycling progenitor cells. The ability to ascertain the frequency of reentry into cell cycle is of importance for understanding the contribution of this parameter to age-related changes in neurogenesis.

Another important parameter to establish when assessing neurogenesis is to extend beyond the quantification of changes in BrdU-positive cell number by also assessing their fate to determine the frequency at which they turn into neurons. As no single definitive marker exists for neural stem cells, identification of progression through lineage commitment is accomplished by combinatorial expression of various

markers. There has been considerable progress in defining the sequential expression of lineage markers in the young adult CNS, but our knowledge of whether the same sequence of markers and duration of their expression are invariably expressed in the aging brain is less than complete. In addition, parameters of neurogenesis differ between mice and rats, suggesting that species differences should be kept in mind when extrapolating results from animal studies.

Neurogenesis Is Decreased with Aging

Neurogenesis does persist in the aged brain, including that of elderly humans. Quantitative studies evaluating age-related changes have consistently reported lower levels of neurogenesis in both the subventricular zone and the dentate gyrus that become statistically significant by mid-age. In aged dentate gyrus of various rat or mouse strains, neurogenesis has been reported to be at the level of 10–20% of young adult animals. Olfactory bulb neurogenesis is significantly reduced to approximately 30% of neuronal production in the young brain and is accompanied by decreased proliferation in both the subventricular zone and the rostral migratory stream to approximately 20–40% of levels in the young brain. The decline of hippocampal neurogenesis is accompanied by a decrease in cell proliferation in addition to a decline in the number of cells expressing doublecortin, a marker associated with early neuronal lineage commitment. However, despite the age-related reduction in neurogenesis, the capacity of newly generated cells in the aged brain to migrate, survive, and differentiate into mature neurons is maintained. Furthermore, early work demonstrated that the age-related decline in dentate neurogenesis could be partially reversed in some conditions, such as environmental enrichment or alteration of glucocorticoid levels. Those reports suggest that neurogenesis in the aged brain is not absolutely diminished, but there remains a capacity to elevate neurogenesis, at least in the hippocampus.

Despite the reduced proliferation of new cells in the aged hippocampus, the proportion of those newly generated cells that differentiate into neurons is comparable to the young hippocampus. One interpretation of these data was that there are fewer neural stem cells in the aged brain generating the basal level of cell production. Studies using BrdU labeling of cell proliferation could not address this possibility, as they fail to discriminate between slowly dividing stem cells and rapidly dividing progenitor cells. However, recent work assessing expression of a stem cell marker, Sox2, in combination with other lineage markers indicates that the subpopulation of Sox2-positive cells that correspond most closely to the likely neural stem cell population is not reduced in the aging brain. These results suggest that the age-related reduction in neurogenesis is not due to there being fewer neural stem cells, but rather that these cells may be more quiescent in the aged CNS. In addition to quiescent stem cells producing fewer new cells in the aged brain, the maturation of newly generated neurons appears delayed and the extent of their dendritic arborization is less in the aged hippocampus.

Modulation of Neurogenesis in the Aged Brain

The observed age-related decrease in neurogenesis may result from a combination of environmental changes and cell-intrinsic limitations with increasing age. The quiescence of neural stem cells in the aged hippocampus may be a cell-autonomous property, but proliferation in aged hippocampus can be stimulated under certain conditions. Thus, there is substantial evidence that environmental factors play an important role in the age-related reduction in neurogenesis.

Overexpression of trophic factors has been shown to enhance neurogenesis in the young brain. Protein levels for some of the key trophic factors (such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF)) promoting neural stem cell proliferation, differentiation, and survival show a decline as early as mid-age, correlating with the observed decrease in the proliferation of stem cells and neurogenesis in the aged brain. These changes may be accounted for by alterations in gene expression and/or posttranslational modification. Enhanced availability of trophic factors has been explored as an attractive target for therapeutic manipulation to promote neurogenesis in the young brain. Increased neurogenesis in the young brain has been reported following delivery of BDNF, FGF-2, IGF-1, VEGF, and epidermal growth factor (EGF). The aged brain, likewise, can be stimulated to increase cell proliferation and possibly neurogenesis following delivery of FGF-2, BDNF, IGF-1, and heparin-binding EGF (HB-EGF). However, in neurogenic regions, any enhancement of neurogenesis appears to be modest and does not restore neurogenesis to the level seen in the young brain. These results suggest that the decline of trophic factor support alone may not be responsible for age-related changes.

Trophic support of cell proliferation and differentiation may be regulated by the availability of both ligand and the receptors to bind to those ligands. Changes in the expression of trophic factor receptors, either on the neural stem cells themselves or on other cells in the neurogenic niche, may contribute to

reduced neurogenesis in the aged brain. Relatively little is known, at present, about possible changes in receptor expression on neural stem cells themselves, but there are indications that alteration of receptor expression occurs in other types of cells residing in the neurogenic niche, suggesting possible secondary effects on neural stem cell proliferation. Age-related changes in receptor expression would support possible cell-autonomous mechanisms for modulating neurogenesis through downstream alteration of the local environment. For example, there is reduced expression of FGFR-2 on astrocytes of the aged neurogenic niche, which may reflect an impairment of these cells to respond to FGF-2 signaling and/or in their autocrine regulation to produce FGF-2. This may, in turn, reduce the paracrine effect of FGF-2 on neural stem cells in the aged neurogenic niche.

Structural plasticity of dendrites and spines in the aging hippocampus can be significantly modified by alteration of hormone levels. Neurogenesis may also be modulated by other factors such as hormones and inflammatory molecules that show age-related changes. Despite the strong evidence for age-related hormone-induced plasticity in the hippocampus, relatively few studies have directly examined modulation of neurogenesis in this context (**Table 1**). Changes in glucocorticoid regulation do appear to influence neurogenesis in the aging brain. Stress has a potent effect on neurogenesis in both the young and aged brain, although the precise linkage with glucocorticoid as the proximal mechanism of that change is a matter of debate. Unfortunately, few studies on the important estrogen-related changes in the aging brain have investigated neurogenesis. There is evidence that neurosteroids, such as allopregnanolone, can promote neurogenesis in an Alzheimer's disease (AD) mouse model, although these mice were still relatively young. The role of hormonal modulation is an active area of investigation with important clinical implications, and assessment of neurogenesis is likely to be incorporated into future reports.

Implications of Decreased Neurogenesis in the Aging Brain

While numerous studies have demonstrated that neurogenesis can be enhanced, the advancement of our understanding about the biology regulating neurogenesis has been hampered by an inability to

Table 1 Reported effects of hormonal alteration on neural stem cell proliferation and neurogenesis in the hippocampal dentate gyrus

Hormone	*Natural age-related change*	*Experimental manipulation of hormonal levels*	*Outcomes*
Corticosterone	Increased	*Rodents*	
		Intact young (exogenous corticosterone)	Decreased neurogenesis
		Intact young (following various stressors)	Decreased cell proliferation and/or neurogenesis
		Intact aged	Decreased neurogenesis
		Intact aged (following psychosocial stress)	Additional decrease in neurogenesis
		Adrenalectomy (decreased corticosterone)	
		Early postnatal; tested young and aged	No effect on neurogenesis at either age
		Young	Increased neurogenesis
		Mid-aged- tested when aged	Increased neurogenesis
		Aged	Increased neurogenesis (reversed by corticosterone)
		Primates	
		Young adult (following psychosocial stress)	Decreased cell proliferation
Gonadal hormones (estrogen)	Decreased in females	*Young female rodents*	
		Ovariectomy (OVX; results in decreased estrogen):	
		OVX	Decreased cell proliferation
		OVX + single estradiol administration	At 1 week, increased cell proliferation At 4 weeks, no effect on cell proliferation
		OVX + single estradiol and progesterone administration	No effect on cell proliferation
		OVX + chronic or cyclic estradiol administration	No effect on cell proliferation
		Aged female rodents	
		OVX ± chronic or cyclic estradiol administration	No effect on neurogenesis reported
		Aged female primates	
		OVX ± chronic or cyclic estradiol administration	No effect on neurogenesis reported

experimentally block neurogenesis. In this regard, studying age-related neurogenesis provides a natural model of reduction that may reveal the key regulatory elements for sustaining the generation of new neurons.

While individual new neurons are generated in the adult brain that assume functional characteristics of mature neurons, the extent to which their integration in a larger neuronal network directly contributes to a functional outcome remains unclear. Numerous studies have sought to establish a link between the generation and addition of new neurons in the olfactory bulb and hippocampus and functional output from those systems. With its natural reduction in neurogenesis, the study of aging animals may provide one approach to addressing the question of function. Unfortunately, studies attempting to correlate the level of impairment in hippocampal-dependent learning tasks and the extent of age-related reduction in neurogenesis have yielded mixed results. Some results have suggested that the aged animals most impaired in behavioral performance had fewer new hippocampal neurons, and other studies have indicated that animals in this group had the highest levels of neurogenesis. However, stimulation of hippocampal neurogenesis in aged animals, such as enriched environment or running, has been reported to correlate with improved performance in hippocampal-dependent tasks. It remains to be demonstrated if this correlation of stimulated neurogenesis and improved performance is causal (i.e., due to increased neurogenesis) or if it is a secondary effect to other biological changes, such as improved blood flow, elevation of circulating cytokines, etc. Although models of hippocampal-dependent learning are relevant to assessing function of hippocampal neurogenesis, there is no evidence to date suggesting that neurogenesis plays a role in age-related mild cognitive impairment in humans.

The incidence of anosmia (decreased or absent sense of smell) is increased in elderly patients with attendant risks for malnutrition and food poisoning. Newly generated olfactory bulb neurons in young animals have been shown to integrate and possibly participate in processing of olfactory input, and it had been postulated that the age-related reduction of olfactory bulb neurogenesis may contribute to the development of anosmia. However, it has been reported recently that humans lack a defined rostral migratory stream, making the extent to which stem cells in the subventricular zone contribute to olfactory bulb neurogenesis in humans unclear. This report also creates uncertainty as to the extent to which age-reduced neurogenesis may contribute to anosmia in elderly humans.

The aging brain is vulnerable to a variety of insults and neurodegenerative disease. With our understanding that neurogenesis persists in the adult and aging brain, there has been enthusiasm for utilizing neural stem cells for structural brain repair, where dysfunctional or dead neurons are replaced by grafted neural stem cells. Much of the work in grafting of neural stem cells has been performed in young animal models. It will be important to assess the physiological changes in the aged brain that may constrain the ability of grafted neural stem cells to survive, differentiate, and integrate into the aged brain. In particular, more information is needed about the ability of nonneurogenic regions of the aged brain to support neural stem cell survival and differentiation, as it is primarily the nonneurogenic regions that will be the target of stem cell therapy. Establishment of these parameters will be critical for discriminating between nonpathological aging and the contribution of pathology in animal models of neurodegenerative disease. Further study of the regulation of neurogenesis in the aged brain should provide useful insights into the development of therapeutic strategies for repairing the aged brain.

See also: Neural Stem Cells: Adult Neurogenesis; Neurogenesis in the Intact Adult Brain; Stem Cells and CNS Repair; Synaptic Plasticity: Neuronal Sprouting.

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Synaptic Plasticity: Neuronal Sprouting

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Introduction

In neuroscience the term 'sprouting' refers to any phenomena invoking neurite growth; sprouting occurs through the life span in the mammalian central nervous system (CNS) and in nonmammalian brains and the peripheral nervous system (PNS). These changes in neurite growth are usually represented by modifications to synapses, evidenced as either alterations in synapse number or junctional area. Such alterations can occur in response to normal and pathological stimuli as part of the overall plasticity of nervous system circuitry, believed to underlie changes in behavioral activity. The structural synaptic changes are referred to as 'synaptic plasticity,' as a reference to the term 'behavioral plasticity.' The latter term was first defined by William James in 1890 as any meaningful change in behavior of an organism.

Synaptic plasticity was first recognized during observations of reactive synaptogenesis and sprouting that occurred in response to extension lesions. It was demonstrated that in response to neuritic loss the nervous system was able to form new synapses, and undamaged neurons were able to form new branches (sprouts) with the capability of making new synaptic connections. This led directly to the examination of synaptic plasticity in neurodegenerative diseases and injury. Although the field started with the examination of plastic responses to neuronal injury, it is clear that plasticity occurs throughout life as a normal response and it is recognized that synaptic plasticity is more pervasive than just a compensatory mechanism. It is a subtle, ongoing process in the normal organism, responding to the environment and endogenous rhythms.

Normal Adult Plasticity

The nervous system is very dynamic, even down to the level of its synaptic connections. Proper mental function is based upon a dynamic organization of synaptic structure. The lability of these neuronal networks is highest in the phylogenetically young brain structures involved in realization of perception and self-awareness, which rely upon continual readjustment and refinement. This lifelong self-optimization process underlying neuronal complexity is remodeled to meet environmental demands. Experience-dependent learning combines both additive and subtractive processes. In response to an organism's attempt to control aspects of a new, complex environment there is the creation of new synaptic connections. Then, through the process of pruning, the brain selects the strongest neural circuits, fine-tuning the connections in a subtractive process. Thus experience-dependent learning couples sprouting with selective pruning, with a net gain of synapses. This constant, dynamic synaptic turnover makes it possible to adapt to complex, changing environments. Thus these synaptic connections are in a constant state of flux, responding to hormonal and environmental alterations with adaptive changes in neural circuitry and resulting behavioral alterations. However, the selective dynamic stabilization and destabilization of synaptic connections are accompanied by increasing inherent potential of failure and neuronal vulnerability during the aging process.

Neuronal plasticity mediated by sprouting can involve alteration in dendritic spine number and/or size or alterations in neuritic complexity (length and branching). Dendritic spines are micrometer-sized protrusions of the dendritic membrane that serve as the postsynaptic component for the vast majority of central nervous system excitatory synapses. Spine synapses, in contrast to shaft synapses, act to create spatially isolated compartments, confining biochemical signals and membrane trafficking to localized regions, allowing for fine-tuning of individual synaptic responses. Spine turnover and morphological changes in existing spines are important for the modulation of neuronal circuits during development and plasticity. Dendritic spines are highly dynamic in size, shape, and number, with highest density occurring during late development and decreasing to a relatively stable level throughout adulthood. Changes in spine number and shape are observed in response to high-frequency synaptic activity, behavioral stimuli, or endogenous hormonal cycles.

Further refinement of the neuronal circuitry can be imposed by the function of neighboring glial cells. Soluble glia-derived factors are also important in synaptogenesis, synapse maturation, and plasticity. Neuronal sprouting is dependent upon astrocyte-derived apolipoprotein E (apoE) and cholesterol, which complex into apoE-containing lipoproteins involved in membrane remodeling, repair, and lipid redistribution. Further, neuritic sprouting is impaired by activated glial cells and can be enhanced by reduction in the expression of glial fibrillary acidic protein (GFAP). Increased astrocyte contact with neurons and coverage

of neuronal surface area can modify synaptic activity by controlling local neurotransmitter concentrations and by spatially restricting neuron–neuron contact.

The structural plasticity described by synaptic plasticity is beginning to be linked to functional plasticity. Such functional plasticity can be seen as alterations of synaptic transmission that are strengthened or weakened in response to previous activity. These alterations in synaptic transmission can be of short duration, as in paired-pulse depression and posttetanic potentiation, or long-lasting, as in long-term depression and long-term potentiation (LTP). LTP, generated in the hippocampus, is thought to be a synaptic component of learning and memory, one form of behavioral plasticity. LTP is expressed as an increase in synaptic strength in response to a stimulus train, with increased amplitude being observed after 10–20 s and for many hours, or even up to days or weeks. Accompanying the change in synaptic transmission there is an expansion of the area of the synaptic junction. Although the number of synapses and the shape of dendritic spines do not change after the induction of LTP, stimuli that alter spine number or shape have been shown to also regulate LTP generation. Thus, the functional plasticity represented by LTP is tightly linked to structural changes associated with synaptic plasticity.

Synapse turnover is a constant process of loss and replacement of synapses, with total synapse number determined by the relative rates of synaptogenesis and synaptic regression. One set of stimuli may result in a net increase in synapse number by increasing synaptogenesis. Likewise, competing stimuli may induce synaptic regression, resulting in a net decline in synapse number. The subtle balance between stimuli may represent a mechanism underlying the synaptic plasticity observed as part of the normal life cycle, dependent upon developmental stage, experience, and age.

Neurons have the capacity to respond to perturbations to maintain their function within normal physiological range, such that the neuron can regulate the function of each impinging synaptic terminal, decreasing or increasing the strength of each synapse, resulting in a normal sum total excitation despite inappropriate innervation. Such homeostatic regulation of neuronal activity would maintain the robust function of the nervous system during the restructuring and refinement of neural circuits during the postembryonic developmental period. Such homeostatic regulation would establish limits beyond which activity-dependant changes in synaptic number would not reasonably alter cellular activity. This allows for maintenance of cellular physiological responses during the tumultuous periods of synaptic pruning required for establishment of synaptic structure and function. As the dendrites of the central nervous system, preexisting synaptic connections grow physically and chemically more removed from the cell soma, and more surface area is exposed for the inclusion of new synaptic contacts, leading to new functionality imposed by the changing response properties of the neuron.

The continual turnover of synapses, coupling sprouting with selective pruning underlying experience-dependent learning, allows for efficient memory storage in the brain, balancing the energy and metabolic demands of synapse maintenance with the extra energy cost of synaptic overgrowth required to obtain selective removal. Such a scheme allows for the judicious refinement of neural circuits required for proper memory storage within a highly adaptable context that is modifiable by endogenous and extrinsic environmental cues. However, the reliance of this system on exquisite balance between continual synaptogenesis and synaptic regression is vulnerable to maladaptive alterations in either side of the equation, leading to declines in cognitive function. The importance of correct pruning of the neural circuitry is observed in seizure patients. In the dentate gyrus, aberrant development of recurrent collaterals branching from granule cell axons (the mossy fibers) may contribute the scrambling of neural circuitry and to the epileptogenic activity in patients with temporal lobe epilepsy.

Puberty, Reproduction, and Sex Steroids in Synaptic Sprouting

Synaptic density peaks in the early postnatal period; this is followed by an extended time period of continuous synaptic turnover balanced toward synaptic removal or pruning, leading to a gradual decline to adult levels of synapses by puberty. Puberty is the point of transition, marking the metamorphosis of the child into the adult, with accompanying changes in reproductive maturity and alterations in body growth and composition, as well as profound changes in brain function. Not only does puberty mark the end of the extensive period of synaptic pruning that occurs during development, but it is also represented by the initiation of adult patterns of synaptic plasticity and sprouting. Neuronal connectivity is not fixed once development is complete but instead continues to change throughout life. However, there is a reduction in synaptic elimination, shifting the balance of the continuous synaptic turnover from a state of pruning to a state of maintenance.

Most dramatic are the alterations in synaptic connectivity in the hypothalamic centers governing sexual physiology and behavior. Here, steroid hormones both remodel and activate neuronal circuits during

adolescent brain development, establishing recurrent interactions between steroid hormones and the central nervous system. The release of gonadotropin-releasing hormone (GnRH) from GnRH neurons, a process that couples brain activity and gonadal function, is accomplished by these recurrent interactions, establishing a controlled cyclic gametogenesis and gonadal hormone production. In females, the midovulatory gonadotropin surge that stimulates ovulation is preceded by an estrogen-inducible retraction of axosomatic synapses in the arcuate nucleus. Following ovulation there is a recovery to baseline levels of synaptic connections, representing defined cyclical synaptic plasticity under control of sex hormones governing reproductive physiology. Due to the lack of alterations in spine or neurite morphology, these events are not sprouting *per se*; however, they remain reminiscent of the sex hormone-induced sprouting involved in the development of these same neural circuits.

Although the ovarian cycle-related synaptic changes in the hypothalamus do not involve neuritic outgrowth, in the hippocampus and prefrontal cortex there is an accompanying alteration in dendritic spine density. Specifically, there is significantly lower spine density during estrus than during proestrus. In addition, removal of circulating gonadal steroids via ovariectomy results in a significant decrease in spine density of the hippocampus in adult female rats, and spine density increases upon replacement of estrogen. This effect is reminiscent of the estrogen-induced sprouting during development of hypothalamic circuits underlying reproductive physiology and behavior.

Further supporting a role of sex hormones as environmental regulators of synaptic sprouting in the adult animal, gonadal steroids have been shown to be involved in regulating lesion-induced axonal sprouting. Although lesion-induced sprouting is more akin to pathological alterations in neuronal function, it is a useful paradigm to study the interaction of aging and environmental factors on normal aging-related synaptic plasticity and sprouting. These effects are often too subtle to isolate the mechanisms underlying normal synaptic turnover that are revealed by magnifying the effect via reactive regenerative processes. Sprouting is increased in the presence of sex hormones and in response to environmental enrichment or exercise *in vivo* following perforant path lesions of the hippocampus, and *in vitro* in the wounding-in-a dish model. The role of glial cells in the sprouting response is supported by the inverse relationship between astrocytic glial fibrillary acidic protein expression and neuritic sprouting. However, the influence of sex steroids on synaptic sprouting invites another point of vulnerability to the proper maintenance of the synaptic circuitry with the decline in hormonal control in aged animals, emphasized by the loss of sex hormone production in reproductive senescence, as discussed later.

In addition to the intrinsic regulation of synaptic density by gonadal hormones, extrinsic environmental cues can support sprouting as well. Environmental enrichment and exercise have been well established as one means of promoting neuritic sprouting and the incorporation of newly generated neurons into existing neuronal circuits. Exposing rodents to an enriched environment, consisting of large cages with toys ladders, mazes, and social interactions, results in increased performance on spatial learning tasks, compared with rodents housed in impoverished conditions. Similar results are obtained with increased physical exercise. These behavioral modifications are complemented by physical brain changes consisting of increased synaptic density and increased complexity of dendritic branching. While these adaptive responses are most pronounced in the young brain, environmental stimulation produces effects in the aging brain as well.

Neuronal Sprouting and Aging

Aging is associated with a decline in cognitive function that can be explained in part by alterations in cellular response directly affecting plasticity, in which the ability to learn new tasks decreases with age. On the cellular level, synaptic contacts, synaptic strength, and plasticity are reduced with age. These changes are much more subtle than are the dramatic alterations in neuronal morphology and survival that occur in age-associated neural disorders such as Alzheimer's disease and Parkinson's disease. In contrast to these pathological states, neuron loss does not appear to be an important contribution to age-related functional decline. Rather, subtle shifts in dendritic branching and spine density occur in region-specific patterns.

Although there is little evidence of significant neuronal loss during normal aging, alterations in dendritic extension of the neuronal soma occur in a large number of cells during aging. Many neurons show progressive restriction and atrophy of their more peripheral dendrite branches and, especially in cortical pyramidal cells, among the basilar shafts. The remaining dendritic branches often demonstrate beaded swellings, in correlation with the irregular dendritic spine loss. However, during these aging-related losses to dendritic systems, the potential for neuronal growth is not lost. Possibly as a compensatory response to increase available synaptic area, other neurons grow further dendritic extensions.

Even though there are these dendritic alterations in many neurons, they do not represent a gross regression of dendrites and remain region specific. There is

a reduction in dendritic branching with age in the prefrontal cortex in humans and rodents. In contrast, the hippocampus of humans and rodents does not experience significant changes in dendritic length with age, with net stabilization of dendritic extent in CA1, CA2, CA3, and the subiculum. In fact, in many brain regions, including the parahippocampal gyrus and the dentate gyrus, the aging brain is associated with increased dendritic branching, until very old age, when there is a consistent regression of the dendritic tree back to that of mature young adults. The region specificity of the dendritic losses indicates that the declines in neuronal plasticity are not merely consequences of wear and tear or global aging. This variability in aging that occurs within subpopulations of neurons reflects the different demands throughout the life span.

The observed region-specific increases in dendritic plasticity may be a compensatory response to partial cell loss that occurs during normal aging. New connections would be formed by healthy neurons within the population, assuming parallel functions. Such an extension of existing inputs would maintain circuit function, but would limit the amount of redundancy in the system, making it more vulnerable to further alterations in synaptic connectivity. Alternatively, the new connections could be formed by fibers from converging pathways that can act to boost weakened signals and maintain functional stability. This response may be a recapitulation of the developmental environment in which many neurons are programmed to form a certain number of terminals or to synapse, and if they cannot do so in one region, they will tend to increase terminal growth elsewhere. The increase in available synaptic area in response to the progressive regression of neighboring dendritic systems works to maintain the total postsynaptic surface per neuron. This leads to a concept of two neuronal responses to aging, one involving dendritic retraction and one involving reactive dendritic expansion. This two-stage response of dendritic spines (loss of total number but enhanced sprouting of remaining spines) represents a neuronal response to the decreased allostatic load, defined as a decrease in the capacity of neurons to oppose the damaging effects of strong, excessive stressors, compatible with full function at normal levels of load. The decreased allostatic load, or homeostatic reserve, is a characteristic of normal aging, resulting from continual or repeated stress.

Even though the structural and behavioral plasticities associated with sprouting are present in the normally aged brain, functional decline and its associated synapse loss are still observed. The aged rodent brain has a remarkable capacity for sprouting and synaptogenesis. However, aged synapses respond to plasticity-inducing stimuli differently than do young synapses, and the degree of sprouting is often blunted with respect to young animals. Further, even though in many brain regions the absolute number of spines is often not altered, there are significant changes in the shape and distribution of synaptic contacts. With increasing age there is a shift from L-type (lollipop shape) to N-type (nubby-like shape) synapses. The spine head becoming smaller, the spine shaft thickening, and a decrease in spine length characterize this shift. Cognitive-sparing diet alterations have been demonstrated to prevent the loss in L-type synapses. The aging-related shift in spine makeup and response is demonstrated by the interaction between vulnerability to aging and sex steroids in female rats. The estrogen-induced spine increase seen in CA1 of young rats does not occur in response to estrogen in aged rats. However, in the aged rat, estrogen increases the number of *N*-methyl-D-aspartate (NMDA) receptor per synapse, restoring the more youthful receptor profile that is lost in normal aging. Thus estrogen may help preserve hippocampal function in the context of a decreased synaptic density. This is another case in which the aged response to environmental stimuli maintains the circuit function, but with a decrease in the system's redundancy. This alteration in estrogen responsiveness is species specific. In contrast to the rodent, the CA1 region of the aged monkey hippocampus is still as responsive to estrogen-induced spine density and synapse number increases as is the young monkey.

Many of the other age-related changes in neuronal plasticity are generally subtle and may only be observationally manifested under conditions of perturbation. This was first observed in 1978 by Scheff and colleagues, who demonstrated that there is a clear reduction in collateral sprouting and regeneration in the aged nervous system. This limited response of reactive sprouting in aged animals has been well characterized by many groups since. Following entorhinal lesion, both young and aged rats can replace synapses lost in the dentate molecular layer of the hippocampus. However, this reinnervation, which begins very rapidly in young animals, is delayed in aged animals, with impairment in the initial phases of synaptic sprouting. Thus the rate of reactive sprouting is decreased as a function of age.

The age-related alterations in neuronal plasticity are emphasized again with the interplay between sex steroids and reactive sprouting. As discussed earlier, estrogen greatly enhances neurite outgrowth in various models of sprouting. However, in the aged female rat the induction of neuronal sprouting is impaired and, unlike in young adults, the sprouting is not sensitive to estradiol. This effect may be due to the increased glial activation associated with aging,

as evidenced by an increase in the activation marker GFAP. GFAP expression is usually regarded as secondary to neurodegenerative processes. However, glia become activated early in aging without concurrent clinical manifestations of pathology. Since neuritic sprouting is inversely related to GFAP expression, normal aging-related glial activation may have a profound impact on synaptic functions. Increased glial activation and GFAP expression may shift the balance of sprouting and retraction unfavorably during the ongoing synaptic turnover, leading to age-related declines in cognitive function.

Age-related alterations in synaptic plasticity may be due to changes in the properties of individual neurons or due to changes in the nervous system itself, as the individual neurons exist within a highly organized system. The decrements in neuronal plasticity could be due to inherent changes in the neuron's ability to extend neurites or form new spines. Alternatively, the decrement could be due to alterations in the complex system of extrinsic signals that trigger the requisite outgrowth. However, there has not been a consistent observation of age-related changes in neurotrophic factors to support the latter scenario. Another alternative to intrinsic changes to the aged neuron diminishing its capacity for growth involves alterations in the target neural circuitry in a way that lessens the accommodation or stimulation of sprouting. This is a model supported by the fact that the aged hippocampus supports less neuron ingrowth than does young hippocampus, whereas aged transplants show robust innervation in young host hippocampal tissue. This is an effect that does not appear to be due to reductions in baseline substrate properties that promote neurite outgrowth. Further support is provided by the observation that sprouting in the wound-in-a-dish model is diminished when neurons are co-cultured with astrocytes from aged animals, as compared to astrocytes from young animals. Thus, in the target region preventing efficient sprouting, there are likely changes that are marked by increased glial activation. This increased glial activation could result from the increase in allostatic load imposed by the results of cumulative reactive oxygen species (ROS) production or excessive stress. Both increased oxidative load and stress (or its hormonal effector, glucocorticoid) have been shown to reduce neuronal plasticity, LTP, and behavioral learning. Further implicating oxidative load in aging-associated cognitive decline, spine losses can be attenuated by superoxide dismutase overexpression (antioxidant defense).

In addition to reduced oxidative load and hormonal modification, synaptic sprouting can be altered favorably by other means. Calorically restricted diets can retard synapse loss, mostly through maintenance of L-type synapses, rather than preventing a decrease in total spine number. The processes of synaptic plasticity appear to be strengthened by increased neuronal activity, driven by enriched environment or behaviors such as exercise. Such age-associated dendritic morphologies are also reflected in the region-specific alterations in spine density. This continued structural plasticity of the aged brain is reflected behaviorally, in that age-related loss in speed of mental processing can be offset by continual environmental enrichment and challenge so as to promote synaptic plasticity. The fact that aging-vulnerable circuits are still responsive to environmental stimuli indicates that the aged synapse remains plastic. This is in contrast to cognitive deficits due to neuronal loss, in which case little can be done to restore circuit function. If the cognitive deficit of normal aging is due to the frailty of the synapse, the mechanisms of synaptic plasticity might be harnessed to for therapeutic intervention.

See also: Synaptic Plasticity: Neuronogenesis and Stem Cells in Normal Brain Aging.

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Axonal Regeneration: Role of Growth and Guidance Cues

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Introduction

Compared to other organ systems, the central nervous system (CNS) in the adult human has one of the lowest regenerative capacities following pathological insults, and within the phylogenetic tree the mammalian nervous system is the least capable to renew itself. Besides traumatic nerve injury, a great number of neurodegenerative diseases exhibit marked axonal pathologies, which often precede the histologically more impressive neuron cell death and possibly represent an early stage in disease progression. A functional restoration in a structure as complex as the nervous system therefore requires not only the preservation of cell bodies, but also the maintenance and/or reconstruction of axonal connections to their physiological target regions.

Increased life expectancy has resulted in a continuous rise in the incidence and prevalence of neurodegenerative diseases. The understanding of basic cellular and molecular mechanisms underlying the lack of CNS regeneration is thus not only of interest for the life science community but also of major socioeconomic relevance. Clearly, a deeper insight into mechanisms of axonal de- and regeneration is indispensable for the identification of future treatment strategies in the field of clinical neurosciences.

In this article, developmental mechanisms of axon guidance which regulate axonal pathfinding during embryogenesis are discussed. Interestingly, numerous molecules with developmental roles persist throughout adulthood and may take new functions in the lesioned nervous system, inhibiting or fostering axonal regeneration.

Developmental Guidance Cues: Orchestrating a Symphony of a Thousand

During development, a multitude of signals are required in order to shape naive cells to form adult tissue. Molecular gradients of the so-called morphogens have been identified to regulate cell fate by the induction of transcriptional changes, which, for example, results in cell-type specification according to a cell's position in the gradient. Cellular processes, such as axons, similarly react to molecular gradients of guidance molecules, which act to direct motile structures toward a target area. Four major groups of largely conserved developmental guidance molecules are known to date: ephrins, semaphorins, netrins, and slits. Recent evidence, however, suggests that morphogens are able to route growth cones in a similar way, in addition to their classical function in the commitment of cell fate. The versatile calcium ion influences growth cone development in a precise spatiotemporal manner, including the mediation of pathway cross-talk.

Classical Morphogens with Novel Old Functions

Morphogens are signaling molecules that during development have a locally confined expression in a specific source region from where a concentration gradient is established. The exact mechanism of gradient formation is still a matter of debate, and evidence supports diffusive mechanisms as well as repeated cycles of endo- and exocytosis. Cells positioned in morphogen gradients react to concentration differences by modification of their differentiation program inducing cell fate specification. In addition to this classical morphogen role, evidence is growing for morphogens as axon guidance molecules (**Figure 1**).

Sonic hedgehog (Shh) is a member of the hedgehog protein family of which two other members are known in mammals. It is produced in the notochord and in the ventral midline of the CNS – the floor plate cells, where it is involved in shaping the dorsoventral axis of the neural tube. Signaling through its receptor patched (Ptc), Shh induces the release of the G-protein-associated mediator smoothened (Smo), which eventually activates zinc finger transcription factors of the Ci/GLI family.

As a midline-derived molecule, Shh has been shown to function as a chemoattractant for commissural neurons, guiding their axons toward the midline. This could be inhibited by the inactivation of the Shh signaling mediator Smo, suggesting a direct action of Shh on the axons rather than the induction of secondary guidance cues. In contrast to commissural axons of the spinal cord, retinal ganglion cells (RGCs) are prevented from crossing the midline by continuous expression of Shh. Here, Shh signaling has been shown to induce an inhibition of cyclic AMP (cAMP) production in the cell, which is known to result in growth inhibition. The opposing effects of the same guidance cue on axonal growth of different cell types remain puzzling. It is likely to assume different signaling pathways resulting in diametrical effects or an interplay with other guidance cues present at the same time. A context-specific effect of Shh, for example, dependent on intracellular cAMP levels, is also proposed.

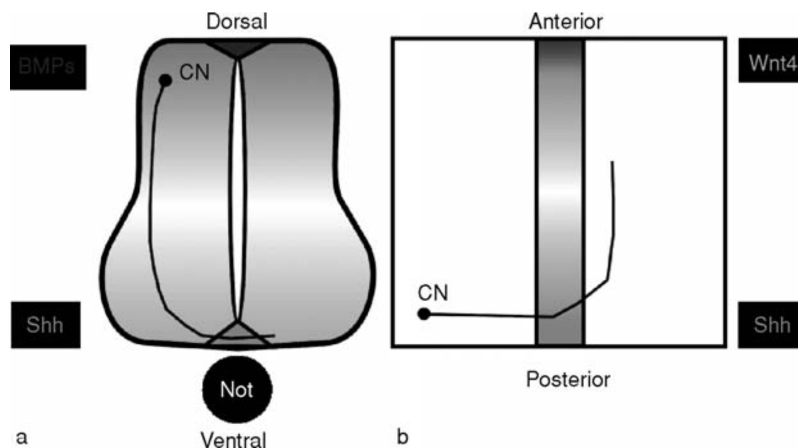

Figure 1 Morphogens involved in commissural axon guidance during embryonic development. Gradients of bone morphogenetic proteins (BMPs) and sonic hedgehog (Shh) shape the dorsoventral axis, while gradients of Shh and Wnt4 regulate the posteroanterior guidance. Cross-section of developing spinal cord (a) and 'open-book' conformation (b). CN, commissural neuron.

Similar to the regulation of the fate of ventral neurons by Shh, members of the bone morphogenetic protein (BMP) family regulate the fate of dorsal interneurons and commissural neurons. BMPs as well as the related growth and differentiation factors (GDFs) belong to the large superfamily of transforming growth factors-β (TGF-β) and signal through type I and type II TGF-β receptors. Through receptor-regulated SMADs and co-SMADs, the signal is transduced to the nucleus, where transcription is activated. BMPs have been shown to equally act as guidance cues for commissural axons after their initial role as morphogens. Heterodimers of GDF7 and BMP7 guide axon growth of commissural neurons, which could be shown by defective outgrowth of commissural axons in knockout mice lacking GDF7, BMP7, or both. A role for BMPs in the development of RGC axons has been suggested by studies in which a deletion of the BMP receptor Ib resulted in misrouting of ventral RGC axons, enabling them to enter the optic nerve head.

The Wingless/Wnt family of morphogens is expressed by roof-plate cells and participates in the developmental specification of dorsal interneurons. Binding to the Frizzled (Fz) receptors is a common denominator for members of the Wnt family, but signaling pathways that are employed downstream of Fz can be diverse: Wnt binding to the Fz receptor and its coreceptor LRP5/6 eventually results in the inhibition of glycogen synthase kinase 3β (GSK3β). In the absence of Wnt signaling, GSK3β phosphorylates β-catenin, which is then ubiquitinated and targeted for degradation by the proteasome. In this so-called canonical pathway, Wnt signaling thus induces stabilization of β-catenin. Stabilized β-catenin induces transcriptional activation of Wnt target genes via association with the lymphoid enhancer factor (Lef/Tcf). Additionally, GSK3β phosphorylates microtubule-associated proteins (MAPs), and Wnt signaling therefore may directly alter the cytoskeletal structure. Other Wnt signaling pathways involve c-Jun-N-terminal kinase (JNK) activation (the so-called planar cell polarity pathway), directing cytoskeletal organization and coordinated cell polarization as well as the release of intracellular calcium and consequent calcineurin activation (Wnt/Ca^{2+} pathway).

Similar to other morphogens, Wnt family members can act as guidance cues for developing neurons. For example, Wnt5 has been shown to repel anterior commissural axons in *Drosophila*. In mouse embryos, Wnt4 acts as a chemoattractant for postcrossing commissural axons. Again, molecules from the same family show opposing effects on axon outgrowth, which may be explained by utilization of different signaling pathways or, as in the case of Wnt5 signaling in *Drosophila*, even the employment of the unconventional receptor Derailed (Drl).

Neurotrophins

As early as in 1939 limb bud transplantation experiments performed by V Hamburger in chick embryos suggested the existence of a target-derived soluble factor that guides the developing axons of sensory neurons. The protein, now known as nerve growth factor (NGF), was later identified by S. Cohen and R. Levi-Montalcini and turned out to serve as growth and survival factor for sympathetic neurons. Besides NGF, the family of neurotrophins – named after their survival and growth-promoting effects on neurons – today comprises three other structurally related proteins in mammals: brain-derived neurotrophic factor (BDNF), neurotrophin 3, and neurotrophin 4/5. All neurotrophins share a common signaling mechanism involving binding to receptor tyrosine

kinases (trk) and the so-called low-affinity neurotrophin receptor p75NTR.

Upon neurotrophin binding, the appropriate trk receptor dimerizes and induces the activation of multiple signaling pathways, of which protein kinase A (PKA), phospholipase C-γ (PLC-γ), and phosphatidylinositol-3-kinase (PI3K) are the most important ones. Neurite elongation and axon guidance mediated by neurotrophins are likely to be transduced via the actin depolymerizing factor (ADF)/cofilin pathway and consequent f-actin rearrangement (**Figure 2**).

NGF is able to precisely control neurite outgrowth via local action on distinct cellular segments. Targets of sympathetic and sensory neurons secrete NGF and thus a chemoattractant function of the molecule was suggested. Later, NGF was shown to foster sensory axon elongation and arborization. In culture, NGF induces outgrowth of dorsal root ganglion (DRG) neurons.

Very similar to NGF, other members of the neurotrophin family have effects on neuronal growth, survival, and axon guidance. BDNF, for example, is able to promote the elongation of RGC axons *in vivo*. Motor neurons show an expression of all neurotrophin receptors during development, and this coincides with the expression of all neurotrophins in their muscular target tissue, promoting the hypothesis of neurotrophins as target-derived guidance cues for developing motor neurons. Interestingly, neurotrophins may act in a chameleon-like manner, changing their chemoattractant properties into a repulsive character, depending on the

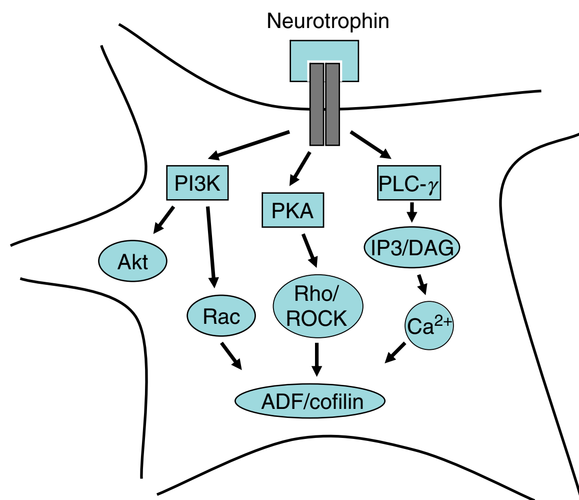

Figure 2 Signaling pathways of neurotrophins involved in axon guidance. Neurotrophin binding to the trk receptor results in activation of the phosphatidylinositol-3-kinase (PI3K), protein kinase A (PKA) or phospholipase C (PLC). Signaling through intermediate molecules results in decreased phosphorylation and thus activation of actin depolymerizing factor (ADF)/cofilin inducing neurite growth.

preconditioning of the neuron: NT-3 acted as a chemoattractant for DRG neurons, except when the culture was pretreated with NT-4/5. Preconditioning of DRG cultures with NT-3 rendered them unsusceptible toward NGF-mediated attraction. Furthermore, growth cones of DRG neurons pretreated with NGF were collapsed by local application of BDNF. The activation and cross-talk of different intracellular signaling pathways as well as a regulation of receptor expression in preconditioned cultures may be discussed as a reason for the differential effects mentioned.

Besides the neurotrophin family, many other growth factors may act as guidance cues, of which only two examples are given: glial cell line-derived neurotrophic factor (GDNF), one of the most potent survival factors for dopaminergic neurons, has chemoattractive functions on peripheral neurons via activation of cyclin-dependent kinase 5 (Cdk5). Studies in rat explant cultures identified hepatocyte growth factor/scatter factor (HGF/SF) as a mesenchyme-derived chemoattractant for developing motor neurons. In addition to its function as survival factor, HGF/SF thus was able to promote axon growth into the limb bud after binding to the c-Met trk.

Ephrins

A model system for the study of axonal guidance cues in the developmental period is the retino-tectal projection. Axons of RGCs follow a precise topographical projection pattern innervating their target areas in the tectum: RGCs in the dorso-ventral axis terminate along the medio-lateral axis of the tectum, while the temporo-nasal distribution in the retina finds its tectal representation in the antero-posterior axis. The involvement of a chemical gradient derived from the target tissue which regulates the targeted outgrowth of RGC axons was suggested by stripe explant experiments. RGC axons originating from the temporal half of the retina would preferentially grow into stripes derived from the rostral part of the tectum, while stripes derived from the caudal colliculus induced growth cone collapse. A similar preference was observed for RGC axons from the dorsal retinal part, which preferred the lateral tectum stripes and vice versa. When the stripes were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) this effect was no longer observed, suggesting that the guidance molecule is membrane bound via a GPI anchor. The guidance molecules responsible for this part of retinal pathfinding are now known as ephrins: two of them, ephrinA5 and ephrinA2, play a pivotal role in the establishment of the topographical map in the retinotectal projection. Studies with knockout mice for either ephrinA5 or ephrinA2 showed a

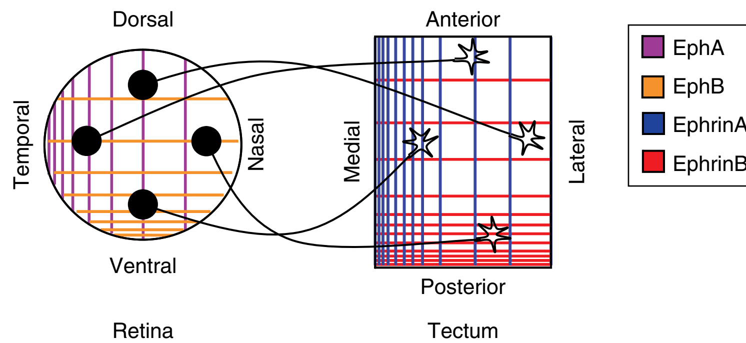

Figure 3 Establishment of retinotectal projections by gradients of Eph receptors and ephrins. Temporonasal gradients of EphA and dorsoventral gradients of EphB define RGCs projecting to the tectum. Anteroposterior gradients of ephrinB and mediolateral gradients of ephrinA result in a retinotopical termination of RGC axons in the tectum.

disorganization of the retinotectal projection, while the double knockout shows an even more prounounced disturbance of axonal wiring. Two families of ephrins are known to date: the GPI-anchored ephrinAs and the transmembrane domain-linked ephrinBs. Trks, termed EphA and EphB, respectively, are responsible for the mediation of the Ephrin signal. It is now confirmed that ephrinA/EphA gradients establish the antero-posterior tectal architecture, while ephrinB/EphB gradients determine the mediolateral projections (**Figure 3**).

Semaphorins

The large family of semaphorins comprises proteins that are either secreted or membrane-bound and that were initially identified for their ability to collapse growth cones of cultured neurons. Up to now, more than 20 semaphorins have been identified and classified into eight subclasses. In invertebrates, subclasses 1 and 2 are expressed, while vertebrates express subclasses 3–7. Nonneurotropic DNA viruses encode for V class semaphorins. All semaphorins are characterized by the so-called sema domain, which is located in the N-terminal region. Semaphorins of classes 3 and 4 homodimerize, which appears to be required for their function.

Neuropilins were the first receptors identified to bind class 3 semaphorins, and they establish target selectivity by formation of multimeric complexes. As is now known, neuropilins bind only to the secreted class 3 semaphorins. However, because of the shortness of their cytoplasmic domain, neuropilins alone are not capable of transducing the signal and therefore form complexes with another type of semaphorin receptors – the plexins. Four subfamilies of plexins have been identified in mammals, termed plexin-A through plexin-D. In contrast to neuropilins, plexins do not need to heteromerize for signal transduction. In their monomeric form plexins represent the receptors for membrane-bound semaphorins of classes 1, 4, 5, 6, and 7.

Additionally, the transmembrane cell adhesion molecule L1 has also been shown to form a complex with neuropilin-1 and act as a semaphorin receptor, modulating the effects of Sema3A: in its soluble form, L1 is able to block the Sema3A-induced growth cone collapse, while L1-deficient mice do not respond to Sema3A-mediated repulsion (**Figure 4**).

Netrins

In the search for factors responsible for the pathfinding of commissural axons in the spinal cord, the biochemical purification revealed two largely homologous proteins – Netrin-1 and -2. To date, Netrins 1–4, which are secreted, and Netrin-G, which is GPI-anchored, are known to exist in vertebrates. Mammalian netrins show homologies to the *Caenorhabditis elegans* UNC-6 protein, which in turn is related to laminin. Two protein families, DCC (deleted in colon cancer) and UNC-5, give rise to netrin receptors.

Netrins seem to have specialized in modeling bilateral symmetry via either chemoattraction (via DCC) or repulsion (via UNC-5). Netrin-1 stimulates outgrowth of commissural axons at different levels in the CNS, and *netrin-1*-deficient mice show severe defects in the establishment of the forebrain commissures. In addition, Netrin-1 is required for developmental pathfinding of RGC axons as they enter the optic nerve, and genetic ablation of Netrin-1 leads to optic nerve hypoplasia. Thalamocortical and corticospinal as well as hippocampal and cerebellar projections equally depend on Netrin-1 signaling for their proper development. One of the intriguing netrin features is its ability to change from attractant to repellent depending on developmental state and intracellular cAMP levels.

Slits

Similar to the midline-forming activity of netrins, the secreted and chemorepellent slits are involved in the

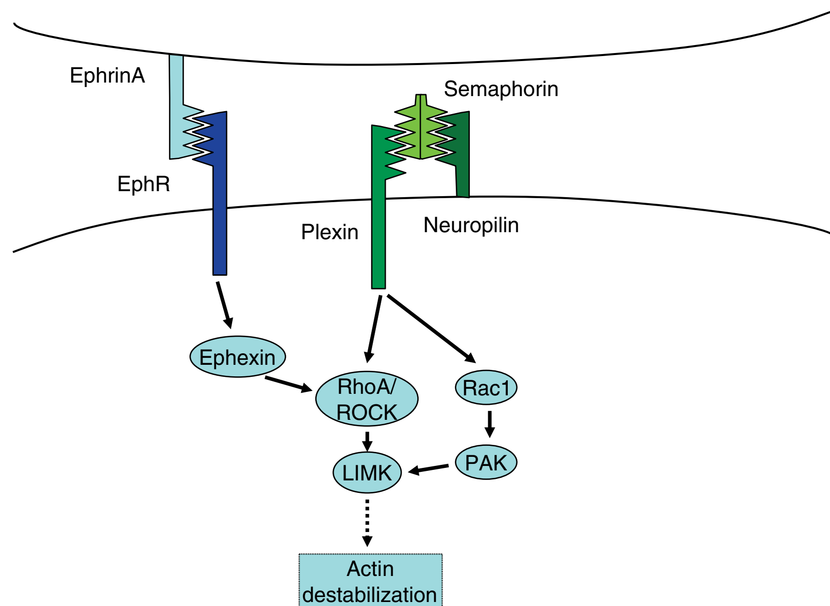

Figure 4 Guidance cue signaling activating growth inhibitory pathways. Membrane-bound ephrins bind to Eph receptors while soluble semaphorins bind to neuropilin/plexin receptors. Both receptor types can elicit growth inhibitory signaling via activation of the RhoA/ROCK cascade, finally inducing actin destabilization and growth inhibition. PAK, p21-activated kinase.

formation of symmetrical structures, such as the optic chiasm. Once bound to axonal Roundabout (Robo) family receptors, slits drive axons away from the midline. Commissural axons initially express low levels of Robo but upregulate the receptor after they successfully crossed to the contralateral side, which prevents them from recrossing the midline. Slits 1–3 have been identified in vertebrates so far, which are able to bind to all three known receptors: Robo 1, Robo 2, and Rig-1.

Left to say, that most of the molecules described above have functions outside the nervous system. Vasculogenesis represents one of the other major processes, where semaphorins, ephrins, netrins, and slits exert their function as guidance cue in development and pathology.

Calcium

The divalent calcium ion is a multifunctional player with roles as a second messenger in the transduction of intracellular signals and as mediator of the action potential. Intracellular cytoplasmic calcium levels ($[Ca^{2+}]_i$) seem to regulate growth cone motility. It has been shown that increased $[Ca^{2+}]_i$ decreases growth cone motility and a reduction of $[Ca^{2+}]_i$ promotes it. However, continuous elevation of $[Ca^{2+}]_i$ can result in adaptation of growth cone behavior, suggesting that targets of Ca^{2+} signaling are downregulated or adjust their sensitivity to the stimulus.

Growth cone motility is not only regulated by baseline cytoplasmic calcium levels, but largely responds to local fluctuations of $[Ca^{2+}]_i$, the so-called calcium transients. Such temporally and locally confined $[Ca^{2+}]_i$ shifts can be generated by calcium flux through ion channels and/or calcium release from intracellular stores. Numerous neurotransmitters are known to regulate calcium channels and thus calcium influx, which in turn can lead to repulsion or attraction of growth cones. For example, the inhibitory neurotransmitter γ-aminobutyric acid (GABA) can act as an attracting stimulus for growth cones, when binding to $GABA_A$ receptors, while exerting repulsive properties, when acting through the $GABA_B$ receptor. The precise spatial and temporal regulation of calcium levels within the growth cone appears to be crucial for the regulation of filopodial dynamics. A proposed binary response to calcium transients, however, greatly oversimplifies the complexity of the growth cone responses. For example, filopodial protrusion can be stimulated by short local Ca^{2+} elevations, but the repeated induction of calcium transients results in the opposite effect.

Downstream targets of calcium include proteins regulating actin dynamics, such as myosin family members, fodrin, α-actinin, gelsolin, and ADF/cofilin. Intracellular calcium sensor proteins, such as calmodulin (CaM), bind calcium and regulate diverse signaling cascades via Ca^{2+}/CaM-dependent phosphatases and kinases. One of the most prominent family members is the Ca^{2+}/CaM-dependent protein kinase II (CaMKII), which shows isoform-specific calcium sensitivity and controls developmental neurite extension.

Guidance Cues in the Adult and Lesioned Nervous System – Play It Again, Sema!

For a long time, the function of axonal guidance cues seemed limited to the developmental period, where neuron–target connection have yet to be established. After successful target innervation, the role of guidance cues seemed obsolete. There is, however, a growing body of evidence that molecules acting as guidance cues and that initially have been attributed classical developmental roles persist throughout adulthood. Even more importantly, experimental data suggest their involvement in the pathophysiology of the lesioned CNS. Next to commonly known inhibitory molecules derived from CNS myelin, the persistence or reexpression of repulsive guidance cues seems to be at least partially responsible for the insufficient regenerative response of the CNS. On the other hand, molecules with neurotrophic properties during embryogenesis may help to overcome regenerative inhibition and support the survival and outgrowth of lesioned neurons.

Myelin-Based Inhibitors of Axon Growth

In contrast to developing axons, neuronal processes in the adult CNS are ensheathed by oligodendrocytes allowing for fast, saltatory conduction of the action potential. Oligodendrocyte-derived myelin has been established as one of the most important inhibitors of axonal growth, while Schwann cells derived from peripheral nerves showed to be permissive toward regenerating axons.

Myelin-based inhibitors of axon growth, that is, Nogo, oligodendrocyte-myelin glycoprotein (OMgp), and myelin-associated glycoprotein (MAG), were initially proposed to account for most of the myelin-derived inhibitory activity. If this were the case, knockouts for one or several of the known myelin-based inhibitors should have markedly improved regenerative capacities in CNS lesion models. However, mutant mice lacking one or all three Nogo isoforms (Nogo-A, B, and C) showed only moderate to no increase in regeneration after spinal cord injury. A similar failure to increase regeneration was observed in MAG knockout mice.

The Nogo receptor (NgR1) transduces the Nogo signal by binding of the 66-amino-acid extracellular domain of Nogo. For signaling, NgR trimerizes with the p75NTR, a versatile receptor initially identified as the low-affinity neurotrophin receptor, and the recently identified LINGO-1, a leucine-rich repeat transmembrane protein. Besides Nogo, MAG and OMgp signal through the trimeric NgR1/p75NTR/LINGO-1 receptor complex. Similar to Nogo knockouts, NgR-deficient mice show a persistent axonal growth inhibition by myelin *in vitro*, while axons from p75NTR-deficient mice were less inhibited, suggesting a more influential role for p75NTR in the regenerative response. Nevertheless, regeneration of corticospinal tract axons after spinal hemisection was not improved either in NgR- or p75NTR-deficient mice, questioning the major role of Nogo/NgR in the mediation of regenerative inhibition. Several hypotheses have been brought forward to explain the persistent lack of regeneration in mice deficient for MAG, Nogo, p75NTR, or NgR. Myelin-based inhibition may employ pathways different from NgR/p75NTR-receptor signaling to induce inhibition. For example, the tumor necrosis factor receptor family member TROY, which is highly expressed in the adult cortex, is able to form a trimeric complex with NgR and LINGO-1 and thus substitute for p75NTR (**Figure 5**).

Next to the 'classical' myelin-based inhibitors, a number of other outgrowth-inhibiting molecules are known to be expressed in the adult CNS. Chondroitin sulfate proteoglycans (CSPGs), such as versican and brevican, are present on differentiated oligodendrocytes and participate in the inhibition of the regenerative response.

Neurocan and phosphacan are two nervous system-specific CSPGs that show high-affinity binding to the extracellular matrix protein Tenascin-C and other cell adhesion molecules, such as Ng-CAM/L1, N-CAM, and TAG-1/axonin-1. Tenascin-R is yet another extracellular matrix protein, which is expressed on oligodendrocytes of adult mice and is upregulated after lesion injury *in vivo*, strongly suggesting an inhibitory role in axonal regeneration.

Developmental Guidance Cues in the Adult

In spite of the overwhelming presence of myelin-derived regeneration inhibitors in the adult, it was equally tempting to suggest that molecules known for their repulsive function as guidance cues during developmental axonal pathfinding additionally act as inhibitory substrates in adulthood.

As outlined previously, ephrins are involved in developmental patterning and axonal guidance and exert their action after binding to transmembrane trks, the Eph receptors. *In situ* hybridization studies in mice revealed that ephrins and Eph receptors are also widely expressed in the adult CNS, although the expression is attenuated compared to the embryonic tissue. Especially, the EphA4 receptor shows a high expression pattern throughout the CNS, while ephrinBs and EphB receptors are highly expressed in regions of known plasticity, such as the olfactory bulb, the hippocampus, and the cerebellum.

A graded expression pattern of ephrinAs in the superior colliculus similar to that during development,

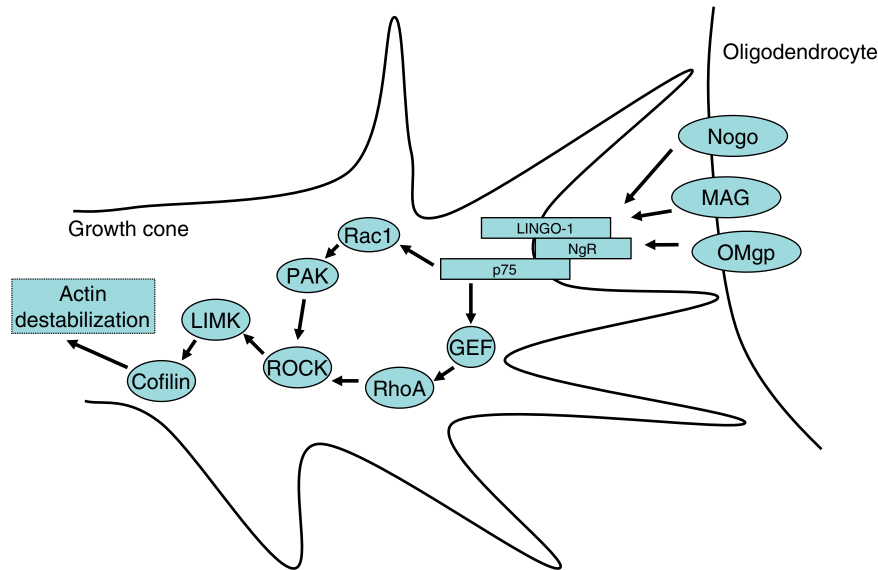

Figure 5 Inhibitory pathways triggered by myelin-based growth inhibitors. Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) signal through the trimeric NgR/LINGO-1/p75 receptor. RhoA activation via guanine nucleotide exchange factors (GEFs) induces activation of Rho-associated protein kinase (ROCK) and LIM kinase (LIMK). LIMK phosphorylates cofilin, inducing actin destabilization and growth inhibition.

was observed in the adult mouse before and after differentiation by axotomy. This suggests a requirement for topographic guidance information for functional regeneration of the retinocollicular projection.

Spinal cord hemisection induced an upregulation of EphA4 in astrocytes at the lesion site of wild-type mice. Knockout mice lacking EphA4 were shown to better regenerate corticospinal and rubrospinal axons after spinal cord hemisection than their wild-type littermates. In this paradigm, the regeneration response was improved even though the 'classical' inhibitory signaling cascade via NgR1/p75 was not affected. Myelinating oligodendrocytes in the adult spinal cord further show an expression of ephrinB3 and postnatal cortical neurons express the EphA4 receptor showing sensitivity toward ephrinB3. These data strongly suggest a persistent inhibitory function for developmental repellent molecules.

In analogy to ephrins, a permanent role for semaphorins in the adult CNS has been recently described. Oligodendocytes and astrocytes of postnatal rat optic nerves show expression of multiple semaphorin family members, and RGCs express the neuropilin-1 semaphorin receptor. Of all semaphorins tested, Sema5A had the most pronounced inhibitory effect on growth cone collapse of RGCs. When RGCs were seeded on P8 optic nerve explants, outgrowth was significantly increased after treatment with a Sema5A neutralizing antibody, suggesting an inhibitory role for Sema5A in the regeneration of adult RGC axons. Sema4D/CD100 is expressed on oligodendrocytes of adult mice and acts as a strong inhibitor for postnatal sensory and cerebellar granule cell axons. After spinal cord lesion, Sema4D expression was transiently highly upregulated in oligodendrocytes at the periphery of the lesion. Class 3 semaphorins show an upregulation in the scar tissue of spinal cord-lesioned rats, and receptors for semaphorins are expressed on lesioned cortico- and rubrospinal tract axons.

The involvement of netrins and slits in regenerative paradigms of the adult CNS is less clear. Recently, however, netrin-1 has been shown to promote outgrowth of dopaminergic neuron axons via its DCC receptor, while slit-2 repelled dopaminergic axon growth via its Robo receptor *in vitro*. Whether axonal regeneration of dopaminergic neurons *in vivo* is equally regulated by netrins and slits remains to be determined.

One common denominator of inhibitory molecules seems to be the signaling via the Rho/ROCK pathway. Similar to NgR1/p75/LINGO-1, EphA4 activates the small GTPase RhoA, which in turn leads to an activation of the Rho kinase (ROCK). Rho kinase activates LIM kinase-1 (LIMK1), which phosphorylates cofilin at its serine 3 residue and thus inactivates cofilin. As a potent regulator of actin filament dynamics, the inactivation of cofilin results in actin polymerization and reduced axonal growth.

The role of classical morphogens for axonal regeneration in the lesioned system has not been studied to a great extent yet. However, there is experimental evidence to suggest neuroprotective effects. Supranigral administration of Shh provided an increased survival of dopaminergic neurons after lesion with the selective dopaminergic neurotoxin

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in common marmosets and improved their locomotor activity. Adenovirally administered Shh has also been shown to protect motor neurons after axotomy of the facial nerve in adult rats.

Neurotrophic Factors Promoting Axonal Regeneration in the Adult

In contrast to the presence of inhibitory molecules, trophic support derived from neurotrophic factors seems rather limited after CNS lesions. In the mechanically lesioned spinal cord of adult rats, for example, only a marginal upregulation of BDNF and GDNF and its appropriate receptors was observed. Schwann cells of corresponding nerve roots, however, showed a much more pronounced increase in NGF and GDNF mRNA levels, indicating the better regenerative capacity of the peripheral nervous system.

The administration of neurotrophic factors in a therapeutical approach thus seems promising. In the visual system, regeneration of RGC axons can be studied in a crush model of the optic nerve, where the integrity of the nerve is conserved and the transected axons face a nonpermissive environment for regeneration. Several growth factors have been shown to promote not only the survival of RGCs after crush, but equally improve the regrowth of RGC axons into the optic nerve.

Ciliary neurotrophic factor (CNTF) has been shown to promote regeneration of RGC into peripheral nerve grafts, and axotomized RGCs express the appropriate receptors CNTF receptor α and leukemia inhibitory factor receptor (LIFR). The application of CNTF and a Nogo-neutralizing antibody was even synergistic toward promotion of the regenerative response. GDNF, a member of the TGF-β superfamily and potent survival factor for dopaminergic neurons, has been tested for proregenerative properties in spinal cord injury. Mini-guidance channels filled with GDNF and placed into a hemisection gap lesion promoted the regrowth of spinal cord axons into the implant and increased the number of propriospinal neurons.

The combination of growth factors has been the subject of several studies to promote axonal regeneration. For example, fibroblast growth factor-2 (FGF-2), Neurotrophin-3 (NT-3), and BDNF had a synergistic effect on regeneration of RGC axons following crush axotomy, being more effective than treatment with either growth factor alone. BDNF and aFGF promote regeneration of spiral ganglion cell axons into the lesioned organ of Corti. BDNF and NT-3 enhanced propriospinal axonal regeneration in the adult rat spinal cord. The effects of NT-3 and BDNF on regeneration have been shown to be at least partly mediated by enhanced polymerization of f-actin in growth cones.

Intrinsic Regenerative Capacity

The proregenerative effect of neurotrophic factors decreases with the age of neurons. Several factors have been brought forward to be responsible for age-dependent susceptibility to neurotrophic factors and decreased intrinsic regenerative capacity. Differential downregulation of neurotrophin receptors on adult neurons and the increased signaling of inhibitory pathways are likely to contribute to this. An age-dependent reaction of the nervous system toward lesion and a better restoration ability of the peripheral nervous system has been well known from clinical experience. The marked difference between regeneration responses of central and peripheral neurons in the adult CNS may also be explained by differential regulation of transcriptional programs upon lesioning. Pseudo-unipolar DRG neurons, for instance, show a pronounced regenerative response in their peripheral branch, while the ascending central branch is unable to regenerate after injury. Expression of the transcription factor c-Jun, in DRG neurons, is markedly upregulated after lesion of the peripheral branch, while central lesions induce only weak c-Jun upregulation. This correlates with the restricted regeneration potential of the central branch. However, placing a dorsal root into the hemisection site, promoting regeneration into the transplant, again induced marked c-Jun expression in DRG neurons. A very similar effect was shown on the upregulation of growth-associated protein 43 (GAP-43) expression, which is increased equally after lesion of the peripheral branch and almost nonexistent after central lesion. The upregulation of c-Jun and GAP-43 in paradigms of regeneration suggested that these proteins are involved in shaping the cell-intrinsic answer to successful regeneration. Therefore, an overexpression, for example, by viral vectors, has been proposed. Several studies, however, showed that simple overexpression of GAP-43 may not be sufficient to induce regeneration. In the current picture, GAP-43 and c-Jun are standing at the end of a transcriptional program involving the expression of a whole plethora of other so-called regeneration-associated genes (RAGs). However, c-Jun may also act as a two-sided sword in regeneration and death signaling. It has been shown that c-Jun is upregulated after optic nerve axotomy. Moreover, proximal axotomy resulted in stronger c-Jun upregulation and in fewer surviving RGCs than distal optic nerve transection. The arguments for c-Jun as an important apoptosis mediator and potential therapeutical target are supported by the fact that siRNA-mediated downregulation of c-Jun may indeed protect RGCs from axotomy-induced cell death. Thus, the reactivation of growth-associated, developmentally

regulated programs is a necessary prerequisite for axonal regeneration. However, it also renders the adult neuron more susceptible to apoptotic cell death.

In striking contrast to the mammalian nervous system, the CNS of amphibians and fish shows a vigorous regeneration reaction following traumatic lesion. Goldfish, for example, readily regrow their axons after optic nerve transection to functionally reinnervate the tectum and even restore vision. Fish RGC do not degenerate after axotomy, but show a pronounced hypertrophy with dense nucleoli, suggesting an activation of protein and RNA synthesis. Similar to mammals, trophic factors, like NGF, stimulate the outgrowth of explanted fish RGC *in vitro*. Proteins secreted by the sheathing cells of the optic nerve (axogenesis factor-1 and -2) have also been found to promote regeneration. Myelin-based inhibitors of axon growth are also present in the fish optic nerve. However, their composition seems to be different from mammalian myelin: RGC explants from fish regenerate readily on fish myelin, but fail to regenerate on rat myelin. Finally, lesion-induced intrinsic transcription programs in fish and amphibians may lead to expression of different target genes than in mammals. For example, the cell adhesion molecule Contactin 1 (Cntn1) is duplicated in fish and activated after optic nerve lesion, suggesting a regeneration-promoting role similar to L1, N-CAM, or TAG-1. The retinol-binding protein purpurin was shown to be upregulated following axotomy and promoted neurite outgrowth of retinal explants. In summary, differences in the intrinsic regenerative capacity by activation of specific transcriptional programs in fish and amphibians may be at least as important for successful regeneration as a more permissive environment.

Preconditioning lesions to the peripheral branch of DRG axons induced an increased regenerative response in the central branch of the DRG in the dorsal column. This was accompanied by an elevation of intracellular cAMP and inhibition of PKA. Increased regeneration in response to application of neurotrophic factors has also been shown to be mediated by elevated levels of cAMP. As could be demonstrated in cerebellar neurons, priming with BDNF or GDNF incurred resistance against MAG-induced outgrowth inhibition via cAMP elevation and activation of PKA. This involves inhibition of phosphodiesterase by activation of the extracellular-signal regulated kinase (ERK). Direct injection of a cell-permeable cAMP analog (dibutyryl-cAMP; db-cAMP) into the dorsal column of a lesioned spinal cord fostered regeneration. Elevated cAMP levels may modulate the response to lesion via activation of the cAMP response element binding protein (CREB) transcription factor. However, CREB activation alone may be insufficient, as was implicated in a study combining neurotrophin treatment and cAMP in a model of spinal cord injury (**Figure 6**).

Taken together, axonal regeneration in the adult mammalian CNS is restrained by a multitude of intrinsic and extrinsic factors: an inhibitory environment consisting of repulsive guidance cues and myelin-derived growth inhibitors paired with lack of

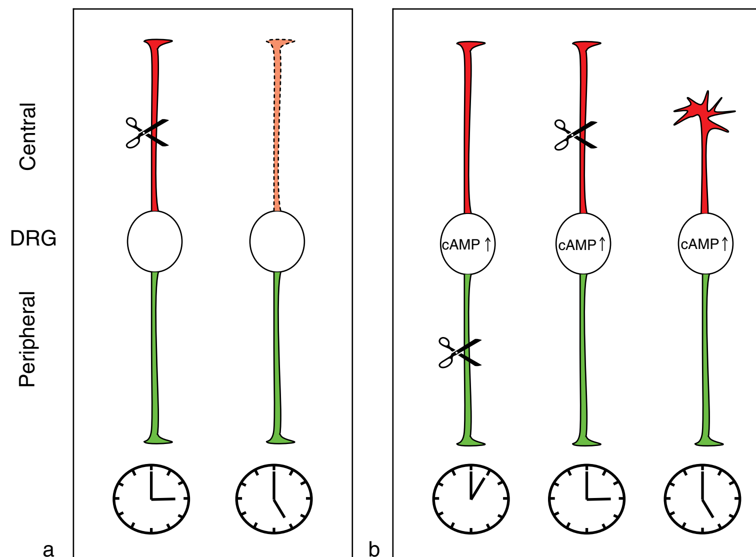

Figure 6 Preconditioning may change intrinsic growth capacity via induction of growth-associated genes. Traumatic lesion to the central branch of the dorsal root ganglion (DRG) results in its degeneration. Preconditioning lesions to the peripheral branch of the DRG and subsequent lesion of the central branch induces a regenerative response in the central branch. This is accompanied by an elevation of intracellular cyclic AMP (cAMP) levels.

trophic support and pro-regenerative transcriptional programs result in insufficient functional renewal capacity. Strategies to overcome regenerative failure will therefore most likely be based on a combination of approaches, targeting several pathways simultaneously.

See also: Axon Guidance: Building Pathways with Molecular Cues in Vertebrate Sensory Systems; Peripheral Nerve Regeneration: An Overview.

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Spinal Cord Regeneration and Functional Recovery: Strategies

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Overall Strategies for Spinal Cord Repair

Spinal cord injury (SCI) disconnects the cord distal to the injury from descending motor inputs, and prevents ascending sensory information from reaching the brain. Lesions above the thorax also block sympathetic outflow, and the sacral parasympathetic output is affected in most patients. In addition, the local damage at the region of injury affects motor neurons and other spinal circuits at that level. Most spinal injuries are incomplete, so in the majority of patients some function in some of these modalities remains. The aim of spinal cord repair is to replace the functions that have been lost, and by doing so to allow the patient to regain as much useful function as possible. The patients' own priorities for return of function depend on their level of the injury. For patients with high cervical injuries affecting the diaphragm, the priority is to regain sufficient motor ability to get off the ventilator, while for patients with slightly lower C4–C5 injuries the priority is to regain some voluntary arm movement. For patients with thoracic injuries, who have normal arm and hand control, the priorities are very different, with sexual function and bowel and bladder control heading the list. There is no treatment at present that comes close to promoting complete repair of the damaged spinal cord. However, there are various interventions currently under development that will probably bring back some useful function to patients. The rationale for these interventions is described in this section, then more detail is provided later.

The earliest stage of intervention after SCI is to attempt to protect the cord from further damage. Considerable additional damage can be caused by unskilled handling of patients by emergency teams, but the standard of training and equipment is now excellent in most countries. Following injury, there is an extended period of lesion extension, with death of neurons and glia caused by excitotoxic, inflammatory, and other mechanisms. There has been a long search for effective neuroprotective treatments, but none is yet proven to be effective in human patients. The National Acute Spinal Cord Injury Studies (NASCIS) trial suggested that high-dose methyl prednisolone, if given within 8 h of injury, had some protective action. However, recent studies suggest that the benefits and adverse effects of this treatment are evenly balanced, and it is not used in many countries.

Axon regeneration is a major target for spinal cord repair. The potential benefits of promoting axon regeneration through and beyond spinal cord injuries are obvious. In current animal experiments axon regeneration has been seen for a maximum of about 4 cm. This could produce a useful motor improvement over three or four spinal segments below the injury. However, there is also a possibility that motor axons regenerating through an injury might connect to intraspinal circuits, leading to a relay of their influence further down the cord. The extent to which these intraspinal relay circuits are functional in the human cord is at present uncertain. For sensory axon regeneration, it is unlikely using present technology that axons can be made to regenerate as far as the sensory nuclei of the medulla. However, as with motor circuits, many sensory axons do not connect directly to the medulla, but make their first synapse close to the level at which they enter the cord, making it possible that information from regenerating sensory axons could be relayed to the brain.

Plasticity is an important target for spinal cord repair. Most spinal injuries are incomplete, leaving some axons passing through the lesion. Promotion of plasticity may therefore allow the remaining connections to have greater effects. Some indication of the potential of this form of treatment can be inferred by studying spontaneous recovery after spinal cord injury in young children. The spinal cord, as with most parts of the nervous system, has a period of great plasticity after birth, which becomes much reduced with maturation. Young children show much greater recovery after equivalent SCI than adults do, demonstrating the potential effects of greater plasticity on spinal cord injury.

In addition to biological treatments to enhance regeneration and plasticity, there will be a considerable part to play for prosthetic devices. Biological treatments are likely to have their greatest effect on the spinal levels just below the injury, so enhancing functions further below these levels may require prosthetic devices.

Protecting the Cord from Further Damage

The mechanisms that increase the size of spinal injuries over time are probably similar to those that expand the size of strokes and other injuries. Excitotoxicity, inflammation, mitochondrial damage, and

other mechanisms are involved. In parallel with attempts to develop neuroprotective strategies for stroke, treatments have been applied to spinal injury models. Following encouraging animal data, high-dose methylprednisolone entered trials with the NASCIS consortium. Some benefit was shown in those patients that received the treatment 3–8 h after injury. In some countries this became the standard of care, but in recent years adverse effects from the treatment have emerged, and in most countries the treatment is now not routinely given. Good preclinical data promoted a clinical trial of the *N*-methyl-D-aspartate (NMDA) receptor blocker gacyclidine, but this did not show clinical benefit. Potential anti-inflammatory and other treatments are undergoing preclinical development, but currently there is no treatment that is generally accepted to be useful for neuroprotection after SCI.

Axon Regeneration Strategies

After lesions in the spinal cord or elsewhere in the central nervous system (CNS) there is no long-distance axon regeneration, unlike in the peripheral nervous system (PNS), where axons can regenerate over long distances. At the site of injury a glial scar forms, and the abortive axon growth terminates in this structure.

Why Does Axon Regeneration Fail in the Damaged Spinal Cord?

Myelin inhibitory molecules Where the spinal cord is damaged, myelin is disrupted, and degenerating axons beyond the lesion also generate myelin debris. This debris is cleared very slowly compared with myelin clearance in the damaged PNS, and remains for months after the injury. This and other differences between PNS Schwann cells (which promote axon regeneration) and oligodendrocytes led to studies examining the effects of CNS myelin on axon regeneration. Myelin from several mammalian species is very inhibitory to axon growth, and removal of oligodendrocytes from the region around a spinal cord injury promotes some axon regeneration. Four types of inhibitory molecules have now been identified. Nogo-A is a member of the reticulon family and is found in oligodendrocytes and some axons. It acts on the Nogo-66 receptor and also on a currently unidentified second receptor. Myelin-associated glycoprotein (MAG) is one of the most plentiful myelin proteins and also has inhibitory effects via the Nogo-66 receptor and via gangliosides. Oligodendrocyte myelin glycoprotein (OMgp), a third myelin molecule, also produces inhibitory effects via the Nogo-66 receptor. The Nogo receptor associates in *cis* with p75, LINGO-1, and TROY to produce inhibitory signaling, much of it via the small guanine triphosphate hydrolase (GTPase) Rho. In addition ephrin-B3 is expressed on oligodendrocytes, inhibiting growth through interactions with Eph receptors on axons. There is therefore a plethora of inhibitory molecules on oligodendrocytes. Many experiments have modified inhibition from just one of these molecules and shown some axon regeneration, but the effects of multiple treatments are not yet established.

The glial scar Wherever the CNS is damaged a glial scar forms, first evident within hours of injury and developing over a period of weeks. Initially microglia, oligodendrocyte precursors, and astrocytes hypertrophy and multiply. Later meningeal cells from the surface or from major blood vessels enter to form a fibroblastic lesion core, which also contains vascular endothelial cells and inflammatory cells. Within this structure there are several inhibitory influences. In the glial tissue surrounding the lesion core several inhibitory chondroitin sulfate proteoglycans (CSPGs) are upregulated, produced by astrocytes and oligodendrocyte precursors. This, together with upregulation of tenascin, leads to a highly inhibitory extracellular matrix through which axons would have to regenerate. Astrocytes also upregulate inhibitory ephrin-B2 and EphA4. In the lesion core the meningeal cells express high levels of members of the semaphorin 3 family, and also slit proteins.

Axon growth ability Axon growth cone activity is influenced by the integration of many signaling pathways. Many of the inhibitory molecules act via the small GTPase Rho, which in turn has effects on the axonal cytoskeleton through the control of actin polymerization and depolymerization. However, there are also positive effects on axon growth which can come from neurotrophins, integrins, adhesion molecules, and other sources. To some extent inhibitory influences from the previously discussed molecules can be counteracted by growth-promoting effects of adhesion molecules and tropic molecules and vice versa. In the damaged CNS the environment surrounding axons is overwhelmingly inhibitory, with the various inhibitory influences overwhelming any growth-promoting effects. In addition, axons vary greatly in their ability to regenerate, with adult CNS axons exhibiting lower growth ability than embryonic axons. Also, some CNS axons regenerate better than others. For instance, in the spinal cord the central branches of sensory axons generally regenerate more vigorously than do other damaged axons.

Treatments to Promote Spinal Cord Axon Regeneration

Neutralizing myelin inhibitory molecules Historically, Nogo-A was the first inhibitory molecule to be discovered in the CNS, and treatments based on its neutralization are therefore well advanced. Many experiments have shown that administration of antibodies able to block the inhibitory effects of Nogo-A can promote the regeneration of axons in the damaged spinal cord, and can promote functional recovery. Only a small proportion of the cut axons have been seen to regenerate, and only for distances of between 1 and 2 cm, but this has been associated with substantial functional recovery. The most recent experiments have used two monoclonal antibodies that bind to the middle section of the molecule, which does not interact with the Nogo-66 receptor. These have been applied to macaque partial cervical injuries and have produced considerable functional recovery. Other Nogo-related treatments have focused on the Nogo-66 receptor and the part of the Nogo-A molecule that interacts with it. Thus a soluble form of the receptor and a peptide that competes with Nogo-A for binding to it both promote axon regeneration after spinal cord injuries. Signaling from the Nogo-66 receptor involves p75, TROY, and LINGO-1. Therapies based on these molecules have not yet been tested in spinal cord injuries.

Overcoming inhibition in the glial scar Much of the inhibition of axon regeneration in the glial scar comes from the various CSPG molecules that are upregulated after injury. The inhibitory properties of these molecules depend largely on the sulfated sugar glycosaminoglycan chains attached to the core proteins. Bacterial enzymes called chondroitinases digest these glycosaminoglycans into disaccharides, and this removes much of the inhibitory activity from the CSPGs. This strategy has been applied to rodent spinal cord injuries, resulting in a degree of axon regeneration comparable to that seen after anti-Nogo-A antibody treatments, with good return of function. Mice lacking expression of EphA4 exhibit enhanced axon regeneration after injury, but there are at present no reagents that can be applied to the injured spinal cord to neutralize Eph/ephrin mechanisms or semaphorin 3 inhibition.

Treatments affecting the regenerative ability of axons Many of the inhibitory mechanisms discussed in the preceding sections converge on a small number of signaling pathways, which in turn affect the activity of axon growth cones via controls on cytoskeletal motility mechanisms. There have therefore been attempts to affect axon regeneration by manipulating these signaling processes. Many of the inhibitory molecules signal through Rho, and blocking Rho from its effector kinase can therefore block inhibitory influences on axon growth. Rho kinase inhibitors and the bacterial Rho ribosylation enzyme C3 have been used for this purpose. The C3 enzyme has been attached to a cell-permeant peptide, allowing it to enter cells, and this compound, named Cethrin, has recently started clinical trials. During development the regenerative vigor of axons declines, and this is associated with a decrease in cAMP levels. Treatments that increase neuronal cAMP have enhanced axon regeneration in tissue culture models and in the damaged rodent spinal cord. Other signaling pathways in which intervention has led to axon regeneration in animal spinal injury models are conventional forms of protein kinase C and the epidermal growth factor (EGF) receptor, and the target of inosine, serine–threonine kinase (N-kinase). In addition to treatments affecting signaling pathways, axon regeneration has been promoted by the application of neurotrophins, which stimulate axon regeneration. Neurotrophins applied at the injury site or beyond it have increased the number of regenerating axons and have acted as chemotropic attractants, bringing axons through the injury site. Neurotrophins have effects both on axons and at the level of the cell body, where they induce expression of regeneration-promoting genes. Brain-derived neurotrophic factor (BDNF) applied to the cell bodies of descending spinal cord tracts has prevented neuronal atrophy and promoted axon regeneration.

Transplantation of permissive cells Axons regenerate over long distances after peripheral nerve damage in association with Schwann cells. This observation led to experiments in which peripheral nerve tissue and Schwann cells were implanted into the CNS, with the result that many CNS axons are stimulated to regenerate in this Schwann cell environment over long distances. Regeneration into these Schwann cell grafts has been considerably enhanced by increasing cyclic adenosine monophosphate (cAMP) levels. However, a problem is that these axons are unwilling to cross the sharp boundary that forms between the implanted Schwann cells and the CNS astrocytes, and to leave the Schwann cell environment and reenter the CNS. To some extent this problem has been solved by using olfactory ensheathing cells, which are similar to Schwann cells and promote CNS axon growth. These cells have some ability to mix with astrocytes and migrate from the transplant site into the host CNS. Regenerating axons are able to cross these transplants and continue on into the host CNS, in some cases with good return of neurological functions. A third strategy has been to transplant embryonic CNS tissue

into spinal cord lesions, on the rationale that axons grow in this environment during development. These transplants have promoted regeneration of various classes of CNS axon, again with return of function. This form of transplant has an added dimension, which is that the neurons in the transplant can receive inputs from regenerating host axons and grow their own axons into the host tissue, thereby acting as relays.

Bridging the gap Human spinal cord injuries can be large, with damaged tissue extending over more than one spinal level. Even the most common injuries due to fracture dislocation produce damage over 1 cm or so. In most human injuries the cord lesion is incomplete, with some remaining spinal cord tissue continuing across the lesion. Experience from animal studies shows that regenerating axons generally grow through this remaining tissue rather than through the fibroblastic lesion core. However, for all lesions, and necessarily for complete lesions, it may be necessary to provide a bridge of permissive material to carry axons across the lesion area. Schwann cells or olfactory ensheathing cells are the obvious candidates for this permissive bridging material. However, given the size and disorganization of the lesion it will probably be necessary to implant them in some form of matrix. Discussion of potential designs is beyond the scope of this article, but progress has been made with oriented fibronectin fibers and with self-aggregating peptide fibrils.

Promoting CNS Plasticity

Most spinal cord injuries are incomplete, leaving some axons passing through the lesion site. In over half of patients these carry some useful function. A potential form of treatment is to enhance the natural plasticity of the spinal cord so that these remaining connections increase their efficacy. A possible problem with this concept is that remaining axons induced to sprout might make inappropriate connections and produce maladaptive changes. As described in the following sections, the general finding is that this has not been a problem and that the spinal cord is able to make good use of the new connections, and function is improved. However, sprouting of small sensory axons induced by nerve growth factor (NGF) has been shown to produce enhanced pain sensitivity.

Decline in Plasticity with Age

A general feature of the CNS is that there is a period of great plasticity immediately after birth, known as the critical period. These critical periods terminate in rats at around 35 days after birth, and in humans around 5 years, depending on the brain area. During these critical periods the compensation for injury and functional recovery from various forms of CNS damage, including SCI, is considerably greater than in later life. It is therefore a reasonable treatment objective to attempt to return to the adult CNS the degree of plasticity that was present in the young. The factors that terminate critical periods are not well understood, and much of what is known comes from investigations into ocular dominance plasticity in the visual cortex. It is generally accepted that increased inhibitory influences from GABAergic inhibitory interneurons plays a part, but removing this inhibition results in epileptic attacks and so is not a practicable treatment option. Recently two other mechanisms have been discovered. At the time that plasticity terminates many neurons become surrounded by condensed extracellular matrix coats known as perineuronal nets (PNNs), which contain several inhibitory CSPGs together with tenascin and hyaluronan. Removal of these structures by digestion with chondroitinase or prevention of their formation results in enhanced plasticity. There is also evidence for an involvement of Nogo-A in plasticity, because anti-Nogo antibodies have been shown to promote spinal cord plasticity, and animals lacking the Nogo-66 receptor display prolonged ocular dominance plasticity.

Treatments That Enhance Plasticity

The adult CNS displays a degree of plasticity which allows some functional recovery after damage. Recently three interventions have been developed that enhance this process: chondroitinase, anti-Nogo-A antibodies, and inosine. Chondroitinase digests the glycosaminoglycan chains on CSPGs and also has activity against hyaluronan. Following chondroitinase treatment of the brain or spinal cord, many of the PNN molecules are removed. This treatment has been shown to return plasticity both to the visual cortex and the spinal cord. Following spinal cord injury, chondroitinase treatment led to a rapid improvement in sensory–motor performance, at least some of which must have been due to promotion of plasticity, and the treatment has promoted recovery in a model in which plasticity must have been the mechanism after peripheral nerve repair with inaccurate connection of the motor and sensory axons. Chondroitinase also promotes sprouting of sensory axons, but without producing hyperalgesia. Anti-Nogo-A was applied to animals with lesions of one corticospinal tract, leading to sprouting of the remaining tract across the midline. The antibody

also produced rearrangement of topography in the motor cortex and recovery of function in stroke models, presumably by promoting plasticity. Manipulation of the Nogo-66 receptor has also promoted sprouting, and prolonged visual cortex plasticity is seen in mice lacking the Nogo-66 receptor. Inosine, whose action is thought to be via N-kinase, was given to rats with partial spinal injuries and promoted both axon regeneration and sprouting, and recovery of function in a stroke model.

Rehabilitation Treatments and Plasticity

As with other conditions in which there is physical damage to the CNS, rehabilitation therapy can improve the abilities of patients. After spinal cord injury, patients usually receive intensive physiotherapy to maximize useful functions. There is also extensive experience of the beneficial effects of physical therapy in other CNS disorders such as stroke. These treatments probably work in part by stimulating and guiding plasticity in the CNS, allowing intact parts of the CNS to take over lost functions. Constraint therapy, in which strapping of the unaffected limb is used to improve function in a disabled arm after stroke, has this aim. It is probable that the effectiveness and speed of action of physical therapy could be improved by treatments, as described previously, that promote plasticity. There is direct evidence for this from the use of cortical stimulation to promote plasticity in stroke models coupled with rehabilitative therapy.

Prostheses and Other Devices

Regeneration and plasticity therapies of the type described in the preceding sections are starting to enter clinical trials, and will eventually become an accepted part of the treatment for spinal injury. However, it seems probable that they will have their maximum effect in the spinal levels immediately below the lesion. Moreover, there is currently no treatment under development that promises to induce a complete repair of a spinal cord injury. Patients will therefore continue to suffer from disabilities for which they require treatment. Alongside advances in regeneration and plasticity treatments have been advances in prosthetic devices designed to aid bladder, bowel, arm, and hand function and devices to aid with standing and walking. Discussion of these approaches is

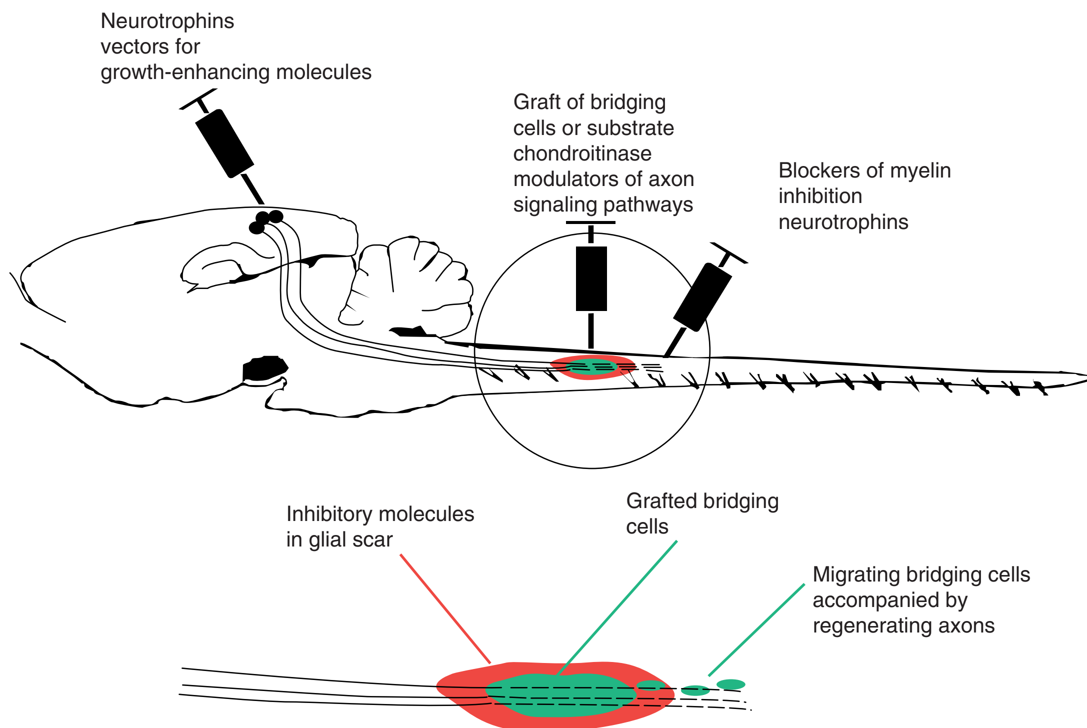

Figure 1 Summary of the classes of treatment that have been developed to promote axon regeneration in the damaged spinal cord. Some treatments, such as bridge grafts and treatments affecting the glial scar, must be applied to the site of injury. Myelin inhibitors are found throughout the central nervous system as well as at the injury site, and need to be applied both to the injury and more distally. Neurotrophins exert a major part of their effect through changes in gene expression in the neuronal cell body, and have been most effective applied here as well as at the injury site.

beyond the scope of this article. However, advances in the field are being made, as are improved methods for interfacing microelectronics with the nervous system. It is probable that in the foreseeable future spinal injury treatment will involve both regenerative treatments and prosthetic devices.

Clinical Trials

Of the treatments that have been successful at promoting recovery of function after spinal cord injury in animal models, two have now begun clinical trials, the Rho inhibitor Cephrin and a humanized anti-Nogo-A monoclonal antibody. Other treatments are in late preclinical development. The development of new regeneration and plasticity-inducing treatments, which have been effective in animal models of spinal cord injury, has necessitated the urgent development of methods for conducting clinical trials. Because of the unpredictability of outcome soon after injury, the limited number of patients available, and the difficulties in assessment, the development of trial protocols is challenging. This area is beyond the scope of this article, and readers are referred to the report by Steeves, Fawcett, and Tuszynski of the first international meeting on clinical trial design in SCI that was held in 2004 in Vancouver, Canada.

Future Prospects

For many years there have been progressive advances in the basic science of axon regeneration and plasticity. These have now led to several treatments that have promoted functional recovery in animal models of SCI, and the first of these treatments have now entered phase I clinical trials. Over the coming years more treatments will reach this point, and information on efficacy will come from phase II and phase III trials, the methodology for which is currently under development. It is probable that some of these treatments will show efficacy and will become part of the standard treatment for the condition. A condition of the complexity of SCI will require more than one intervention for optimum treatment, and it will be necessary to find out how best to combine therapies. These efforts will be preceded by animal experiments on combination treatments. Alongside this will go advances in physical therapy, probably aided by treatments to promote plasticity, and the development of new prostheses.

See also: Axonal Regeneration: Role of Growth and Guidance Cues.

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Peripheral Nerve Regeneration: An Overview

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Spontaneous Recovery of Function in the Central Nervous System

Following axonal injury in the adult mammalian nervous system, some neurological function is usually regained spontaneously over a period of months, despite the absence of functional axonal regeneration. The importance of alternative pathways in such recovery of neurological function can be demonstrated by a second lesion, after initial recovery of function. For example, after lesion of the deep cerebellar nuclei, monkeys initially develop tremor and lose coordination, and then slowly recover. This recovered function is then irretrievably lost if somatosensory regions of the cerebellar cortex are ablated at a second intervention. The efficacy of compensation by spared neural elements varies among neurological pathways and among functions mediated by given pathways. For example, after division of the corticospinal tract in rats, locomotion is transiently impaired whereas fine reaching movements of the forelimb never recover.

The formation of new synapses in the adult mammalian brain was convincingly proved for the first time in an electron microscopic study in 1969. New synapses from spared afferents are formed on deafferentated postsynaptic sites in the septal nucleus, hippocampus, red nucleus, spinal cord, and many other neuronal nuclei in the central nervous system (CNS) and are electrophysiologically functional. A second pattern of sprouting is seen proximal to the site of axonal transection, analogous to growth after horticultural pruning. In the spinal cord, new connectivity can provide a polysynaptic pathway to replace a monosynaptic pathway. The capacity to form new connections through extensive axonal elongation, over micrometers rather than millimeters, accounts for the recovery of function that is anticipated after partial spinal cord injury but which is exceptional after clinically complete loss of function.

Other forms of synaptic plasticity are demonstrable. With contemporary imaging techniques, is now possible to visualize in real time retraction and extension of dendritic spines in hippocampal slice preparations. Synaptic plasticity may be molecular rather than morphological, a classic example being long-term potentiation in the hippocampus, a brain-derived neurotrophic factor-dependent phenomenon whereby postsynaptic responses are enhanced by previous electrical activity. This type of synaptic plasticity underlies learning, and presumably relearning, after neural injury. Disinhibition of synapses within minutes of injury can unmask previously silent connections. For example, after unilateral cervical spinal cord injury, activity in the ipsilateral phrenic nerve ceases only to reappear immediately after a second injury to the contralateral phrenic nerve. In the 'crossed phrenic reflex,' preexisting connections between contralateral descending fibers and phrenic motorneurons are believed to be activated by a hypoxic stimulus.

Another potential mechanism of spontaneous recovery of function is remyelination of axons demyelinated by inflammation or trauma. Demyelinated axons not only fail to conduct impulses but also are vulnerable to Wallerian degeneration because of loss of trophic support. Endogenous oligodendrocytes are capable of remyelination in toxic, inflammatory, and traumatic animal models of demyelination.

Morphological and molecular synaptic plasticity and, to a lesser extent, remyelination are major mechanisms of recovery of function after neural injury. The potential to enhance such spontaneous recovery by electrical activity is demonstrated by observations that locomotion in cats with spinal cord lesions can be improved by early assisted training on a treadmill.

Peripheral Nerve Regeneration

Cellular events underlying Wallerian degeneration and axonal regeneration have been well characterized for many years. Distal to the site of injury, axons degenerate and myelin disintegrates over several days and the debris is phagocytosed by macrophages within 2 weeks (in the rat). The environment created by Schwann cells, macrophages, fibroblasts, and other endoneurial cells supports axonal regeneration at a rate of several millimeters per day.

During Wallerian degeneration, Schwann cells increase in number and become aligned in bands of Büngner. They stop synthesizing mRNAs for myelin protein, ciliary neurotrophic factor, and other proteins and start to synthesize another group of mRNAs, such as those for p75 nerve growth factor receptor and monocyte chemoattractant protein-1. The Schwann cell phenotype during Wallerian degeneration, distinct from that of immature Schwann cells or myelinating or unmyelinating Schwann cells in an intact nerve, is determined by inflammatory cytokines and the loss of axonal signals to Schwann cells. If Schwann cells are deprived of axons for months,

they eventually atrophy and die and the endoneurium ceases to support axonal regeneration well. The prototypic, but not unique, axon-to-Schwann cell signaling pathway, comprising neuregulin and its erbB2–erbB3 receptor dimer, stimulates Schwann cell survival, mitosis, and myelin formation. Two classes of molecules in the endoneurium promote axonal regeneration. Components of the extracellular matrix, in particular laminin, guide and stimulate axons through interaction with their integrins. Diffusible neurotrophic molecules such as nerve growth factor, brain-derived neurotrophic factor, and leukemia inhibitory factor have local actions on the growth cone and retrogradely influence the nerve cell body: their synthesis is strongly increased during Wallerian degeneration. The ability of Schwann cells to redifferentiate in response to axonal degeneration is one critical component of the capacity for regeneration in the peripheral nervous system.

Macrophages in the endoneurium of injured nerves increase in number through recruitment of macrophages and proliferation of resident cells. Macrophages also accumulate around axotomized neurons in dorsal root ganglia. Leukocyte infiltration into the peripheral nervous system after injury is selective for monocytes/macrophages, with minimal involvement of lymphocytes and polymorphonuclear neutrophils: it is regulated by chemokines and cell adhesion molecules that mediate interactions between leukocytes and the vascular endothelium, in particular, monocyte chemoattractant protein-1. Macrophages phagocytose axonal and myelin debris and contribute to the induction of neurotrophic factors, in particular, nerve growth factor. If macrophage invasion into the distal nerve segment is prevented, then axonal regeneration is impaired.

Another series of morphological changes is observed in the cell bodies of axotomized neurons in dorsal root ganglia or in the spinal cord or brain stem. Neurons may die after division of their major peripheral axons, the likelihood being greater if the injury is close to the cell body or if the animal is young. 'Chromatolysis' of neurons refers to the dispersion of ribosomes and peripheral displacement of the nucleus. That inducible events in the nerve cell bodies contribute to axonal regeneration is demonstrated by a variety of 'conditioning' phenomena whereby axonal regeneration is accelerated by a previous or additional axonal injury. Regeneration of the peripheral axons of primary sensory neurons is accelerated by a more distal nerve injury 1 week before the crush injury and regeneration of the central axons of these bipolar neurons is strongly enhanced by a concomitant injury to the corresponding peripheral axons. The propensity for regeneration induced in neurons by axonal injury persists for months, but not indefinitely. Changes in gene expression in neurons following nerve injury include the appearance of messenger ribonucleic acids (mRNAs) for regeneration-associated genes and alteration in either direction of neuropeptide mRNAs. These changes are triggered by changes in retrograde axonal transport. For example, the retrograde influence of nerve growth factor is decreased at least until axons encounter a compensatory source in the distal nerve segment, and this deprivation accounts for a decrease in neurofilament mRNA and many of the fluctuations of neuropeptide mRNAs. The retrograde axonal signals that induce regeneration-associated genes are less well defined. The products of regeneration-associated genes in axotomized sensory neurons include transcription factors (c-Jun), growth factors (brain-derived neurotrophic factor and interleukin-6), growth cone proteins (growth-associated protein-43), and proteins of less well-known function, such as small proline-rich repeat 1A, and their induction is believed to underlie the propensity for regeneration in these neurons. The ability of neurons to redifferentiate is a second factor in the capacity for regeneration in the peripheral nervous system (PNS).

A major limitation to the results of nerve repair stems from the lack of guidance cues to lead axons to their former destinations. Functional recovery is much better after crush injury with preservation of endoneurial architecture than after repair by nerve suture or nerve graft when this physical guidance is lost. Although some cues specific to motor or sensory axons appear to exist, their ability to restore appropriate connections with the periphery is limited. The most important determinant of recovery following nerve repair in human beings is the nature of the injured nerve. For example, the radial nerve with relatively few sensory fibers and an uncomplicated pattern of muscle innervation recovers much better than does the ulnar nerve, a mixed nerve innervating diverse muscles.

Peripheral nerve injury evokes changes in connectivity in the spinal cord, thalamus, and cerebral cortex. Particularly after partial nerve injury, synaptic rearrangement in the dorsal horn of the spinal cord may lead to painful phenomena, including allodynia and hyperesthesia. After a neurotization procedure providing novel peripheral connections (for example, between the intercostal nerve and the biceps), plasticity in the cerebral cortex may improve functional recovery.

Neural Repair in the CNS

Cellular and molecular strategies to repair the brain or spinal cord have been devised. Cellular therapies aim to replace either neurons or glial cells, and

molecular therapies aim to stimulate the nerve cell body or counteract inhibitory influences in CNS glia.

Replacement of neurons, appropriate to the treatment of neurodegenerative disorders, was pioneered by Bjorklund and Stenevi. Following extensive animal studies, fetal mesencephalic tissue was transplanted into the striatum of patients with Parkinson's disease. The cells survived and yielded modest, long-lasting clinical improvement: possible mechanisms of action include integration of grafted neurons into host circuits, release of dopamine by the grafted cells, and beneficial humoral actions of fetal neural tissue. More limited success has been obtained through the grafting of fetal neural tissue into the brains of patients with Huntington's disease. Because of the considerable practical difficulties in obtaining human fetal nervous tissue for transplantation, attempts have been made to generate neuronal cell lines from embryonic stem cells. Also, efforts are under way to enhance the proliferation and differentiation of stem cells indigenous to the adult mammalian brain. Although proof of the principle has been obtained that neuronal replacement can improve or arrest decline of function in neurodegenerative disease, neuroprotective measures such as local administration of neurotrophic factors perhaps hold more hope for future success.

Glial cell transplantation to promote axonal growth in the CNS is based on the premise that such growth could be supported better by replacing astrocytes and oligodendrocytes with more permissive cell types. Reports a century ago that CNS axons could regenerate into a peripheral nerve graft were confirmed many years later by new axonal tracing techniques. It is now apparent that axons from many but not all classes of CNS neurons can grow into the environment provided by Schwann cells and other endoneurial cells. However, two factors have severely limited the success of this strategy in promoting functional recovery. First, the percentage of axons from any given CNS fiber tract that grow into a peripheral nerve graft is small, particularly if the injury is distant from the nerve cell body. Second, axons that enter such grafts have limited ability to exit from them and form new synapses. Olfactory ensheathing cells hold promise to overcome the second obstacle. They are a mixed population of Schwann cells and other cells which support olfactory system axons with the unique ability to grow from the PNS into the CNS in adult mammals. Whereas axons regenerating in dorsal spinal nerve roots fail to penetrate the PNS–CNS interface at the root entry zone, axons regenerating in the olfactory nerve successfully enter and form synapses in the olfactory bulb. Although the cellular and molecular differences between cells in the endoneurium of peripheral nerves and olfactory ensheathing cells have not been fully defined, the unique ability of the latter cells to shepherd axonal growth into the CNS provides a rationale for using them in repair of the CNS. Indeed, transplantation of olfactory ensheathing cells after partial spinal cord injury in rats has led to remarkable recovery of motor function. The rapidity of behavioral improvement, within 10 days, suggests that the recovery is likely due to short-distance sprouting rather than to long-distance regeneration. Other peripheral ensheathing cells – for example, from immature animals – may also support axonal growth across CNS injury sites better than do adult Schwann cells. A less daunting challenge for glial cell transplantation is to restore conductivity to nerve fibers demyelinated by inflammation or trauma. Schwann cells, olfactory ensheathing cells, and oligodendrocyte precursor cells have all shown some promise in this regard.

Two major classes of molecules that inhibit axonal growth in adult CNS glial tissue are myelin-associated proteins and chondroitin sulfate proteoglycans. Three proteins associated with CNS myelin, myelin-associated glycoprotein, Nogo, and oligodendrocyte myelin protein, each strongly inhibits neurite growth *in vitro*. All three act through a receptor complex comprising the Nogo receptor and p75 neurotrophin receptor, which activates the small GTPase Rho and its downstream target Rho-associated kinase, which, in turn, inhibits growth cone extension. Local administration of antibodies to the Nogo receptor and of Rho-associated kinase inhibitors have been found to promote regeneration and recovery of function after spinal cord injury in rodents. Curiously, mice with null deletion of the Nogo receptor do not show enhancement of regeneration of spinal axons. Likewise, delivery of chondroitinase to the injured spinal cord improves both functional recovery and axonal regeneration. For both types of treatment, the improvement of functional recovery precedes and exceeds the enhancement of axonal regeneration and may be mediated through increased synaptic plasticity.

A second impediment to axonal regeneration in the CNS is the difficulty in raising a strong regenerative response in axotomized neurons. Some success has been achieved in enhancing regenerative responses in CNS neurons through neurotrophic factors and small molecules. For example, infusion of brain-derived neurotrophic factor in the red nucleus induces regeneration-associated genes in the neurons and increases the regeneration of rubrospinal axons into a peripheral nerve graft, and injection into the globe of a viral vector delivering ciliary neurotrophic factor increases axonal regeneration from retinal ganglion cells. In general, neurotrophic factors must be

delivered locally in the vicinity of nerve cell bodies, as their side effects upon systemic administration are prohibitive. Cyclic adenosine monophosphate (cAMP) also increases the propensity of neurons to regenerate *in vitro* and *in vivo*, and the need for local delivery can be obviated by systemic administration of a phosphodiesterase inhibitor. Research in the past generation has revealed unexpected plasticity and capacity for recovery in the adult mammalian CNS and has characterized some of the underlying molecular, cellular, and physiological mechanisms.

See also: Axonal Regeneration: Role of Growth and Guidance Cues; Neurogenesis in the Intact Adult Brain.

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Subject Index

Notes

Cross-reference terms in italics are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space). Readers are also advised to refer to the end of each article for additional cross-references – not all of these cross-references have been included in the index cross-references.

The index is arranged in set-out style with a maximum of three levels of heading. Major discussion of a subject is indicated by bold page numbers. Page numbers suffixed by T and F refer to Tables and Figures respectively. vs. indicates a comparison.

This index is in letter-by-letter order, whereby hyphens and spaces within index headings are ignored in the alphabetization. Prefixes and terms in parentheses are excluded from the initial alphabetization.

A

A2B receptor, netrin binding function 477–478
α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors *see* AMPA receptor(s)
Aβ peptide *see* Amyloid beta (Aβ)
Accessory oculomotor nucleus *see* Edinger-Westphal nucleus
Accessory olfactory bulb (AOB)
  VNO input 226
Acetylcholine receptors (AChRs)
  fetal *vs.* adult forms 530, 534–535
  neuromuscular junction *see* Neuromuscular junction, acetylcholine receptors
  self-aggregation 531–532
Acetylcholinesterase (AChE)
  neuromuscular junction
    basal lamina component 541
    collagen-tailed (ColQ) 541
    expression changes during development 532
    gene expression changes during development 532
    redistribution during maturation 531*f*, 533
    see also Neuromuscular transmission
achaete-scute gene/protein
  cell cycle regulation 596–597
  complex enhancers 135–136
Acrania *see* Cephalochordates
Actin/actin filaments
  axonal growth and guidance
    axonal branching 382
    growth cones 381–382
      filopodia 422
  integrin-mediated signaling 542
  models 383
  motor proteins *see* Myosin(s)
  polymerization 480
  postsynaptic development 492
    dendritic spines
      dynamic interaction 492–493
Actin-associated proteins
  apical constriction and neural plate bending 9
Actin-binding proteins
  motor proteins *see* Myosin(s)
  PSD 499
α-Actinin
  postsynaptic density 499
'Actin treadmill,' axonal growth cones 382
Action potential(s)
  muscle(s)
    early developmental 545–546
    see also Neuromuscular transmission
Activator protein-2 (AP-2)
  AP-2α protein, Schwann cell differentiation 307
  Hox gene expression 66–67
Activator protein 2α (APα2), Schwann cells 282–283
Active zone (AZ) 512
  components 512, 517
    cytoskeleton/cytomatrix *see* Cytoskeleton of the active zone (CAZ)
    proteins
      scaffolding 512
    regulatory functions 517
    structural 517
  development 502
    see also Presynaptic development
  synaptic vesicle release 512, 517
    see also Neurotransmitter release; Synaptic vesicle(s)
Activity-dependent ECS volume changes *see* Extracellular space (ECS)
ADAM cleavage 146
  Notch activation 144, 145
Adenomatous polyposis coli protein (APC)
  Wnt signaling 40–41
Adenosine
  neuromuscular transmission
    astrocytes and 570
Adenosine triphosphate, transmitter role
  gliotransmission
    astrocytic release 570
  neuromuscular transmission
    AChR induction at vertebrate NMJ 547
    astrocytes and 570
Adhesion complexes, growth cones 480–481, 481*f*
Adhesion molecules *see* Cell adhesion molecules (CAMs)
Adolescence
  behavioral changes
    gonadal hormones and 350
  brain development *see* Brain development in adolescence
  reproductive system and 350
Adrenal gland(s)
  medulla
    development 338–339
β-Adrenergic receptors
  β2 receptors
    deficiency, visual pathway development 442
β-adrenoceptors *see* β-Adrenergic receptors
Adrenomedullary hormonal system *see* Adrenal gland(s)
Adult hippocampal progenitor (AHP) 641–643
Adult neurogenesis *see* Neurogenesis, adult
Afferent neurons *see* Sensory afferent(s)
African clawed frog *(Xenopus laevis) see Xenopus laevis*
Age/aging
  adult stem cell effects 644
  of brain *see* Brain aging
  enteric nervous system
    neurotrophic factors 632
  glial cell growth factors 639
Aggrecans, optic tectum retinotopy development 92–93
Agrin
  NMJ and *see* Agrin, neuromuscular junction role
  receptor *see* Muscle-specific kinase (MuSK)
  splice variants 548, 549*f*
Agrin, neuromuscular junction role 541, 548, 549*f*
  binding partners 541
  functional importance 541
  interactions 541
  NMJ development and AChR aggregation 509, 531, 535–536, 541, 544, 548
    aggregation induction 546
    changing views on role 544–545
    convergent signaling pathways 550
    ectopic application studies 549–550, 549*f*
    gain-of-function mutants 549

Agrin, neuromuscular junction role (*continued*)
knockout animals 546, *546f*, 548–549
neurocentric model and 551
original identification of role 546
signaling pathways 546, 550
in vivo genetic studies 548
Albinism
optic pathway development 431
tyrosine-negative 431
Allen Brain Atlas, molecular anatomy of mammalian brain 202
ALS *see* Amyotrophic lateral sclerosis (ALS)
Alteplase *see* Tissue plasminogen activator (TPA)
Alternative splicing
agrin 548, *549f*
Trk receptors 622
Altman, Joseph, adult neurogenesis 648–649
Alzheimer's disease, animal models
autophagy and 619–620
Alzheimer's disease, pathology/pathogenesis
amyloid β deposition *see* Amyloid beta (Aβ)
glia role
growth factors 639
endothelin-1 639
hepatocyte growth factor 639
TGF-β1 639
neurodegeneration and
autophagy and 619–620
see also Amyloid beta (Aβ)
Alzheimer's disease, therapy 625–626
neurotrophins 625–626
nerve growth factor 625–626
Amacrine cells
generation 259, *261f*
Math3 262
NeuroD 262
Pax6 262
Six3 262
location 259
retinal development 257, 262
Ammon's horn *see* Hippocampus
AMPA receptor(s)
endocytosis and *see* AMPA receptor trafficking
expression/distribution
synaptic localization 491
PSD complexes 498
adaptor/scaffolding proteins 497
expression and 'desilencing' silent synapses 517–518
synaptic plasticity
activity-dependent maturation 456
long-term depression *see* Long-term depression, glutamate role
long-term potentiation *see* Long-term potentiation, glutamate role
trafficking *see* AMPA receptor trafficking
AMPA receptor trafficking 456–457
Amphibian(s)
brain structure
regeneration, retinal 268
neural regeneration
CNS axonal regeneration capability 679
retinal 268
ocular stem cells
ciliary marginal zone 267–268
regenerative capacity 268
retinal pigment epithelium 268
Amphiphysin
presynaptic development and 506
Amplification
axonal growth gradient guidance models 384
Amygdala
anatomy/organization
sexual dimorphism 350, 356
sexual dimorphism 350, 356
Amyloid beta (Aβ)
Alzheimer's disease 619–620
FGF-2 induction 639
interleukin-1β induction 639
Amyotrophic lateral sclerosis (ALS)
animal models
SOD1 knockout mice 605
synapse elimination and 556, 559
etiology/pathogenesis
cell death mechanisms
programmed cell death 605
genetic factors
SOD1 mutations 558–559
ubiquitin-proteasome system 558
see also Familial amyotrophic lateral sclerosis (ALS)
familial *see* Familial amyotrophic lateral sclerosis (ALS)
management/therapeutic strategies
neurotrophin therapy 626
Anacusis *see* Hearing loss
Anaphase-promoting complex (APC)
cell fate/determination role 595
cyclin proteolysis and cell cycle control 595
Androgen(s)
masculinization and 348, 352
see also Sexual differentiation
receptors *see* Androgen receptors
Androgen receptors
sexual differentiation 359, *360f*
complete androgen insensitivity syndrome 352
telencephalic nuclei 356
Animal cap assays 10
neural induction studies 10
problems/disadvantages 10
Anosmia
aging brain and decreased neurogenesis 665
Antennapedia (Antp) gene, mutation 61, *62f*
Anterior commissure
sexual dimorphism 352
Anterior neural border (ANB) 100
specification/patterning
anterior ventral endoderm role in 107, 109
induction 109
local organizers 109
midline patterning/holoprosencephaly role 109
posteriorization signals 80
Shh-FGF signaling 109
telencephalic specification role 109
Wnt antagonists 102, 109
Anterior neural ridge (ANR) 100
posteriorization signals 80
Anterior–posterior mapping, topographic map development 368
gradient-determined repulsion 368
opposing gradients 368, *369f*
Anterior visceral endoderm (AVE), as non-neural organizer of neural plate
ANB specification 106, 109
AP patterning of neural plate 106–107
BMP antagonists 106
forebrain specification 106, *107f*
neural induction 12–13, 106
Anterolateral quadrant (ALQ) of spinal cord *see* Spinal cord
Anteroposterior (AP) patterning 95
Hox genes *see* Hox genes/proteins
key features 100, *101f*
midbrain-forebrain boundary *see* Midbrain-forebrain boundary
midbrain-hindbrain boundary *see* Midbrain-hindbrain boundary (MHB)
neocortical development 122
organizers (signaling centers) *see* Developmental organizers
posteriorization *see* 'Caudalization' (posteriorization)
retina development *see* Retinal development
signals
extrinsic 73–74
see also Morphogen(s)
intrinsic 74
see also Transcription factors (TFs)
somites
segmentation and *see* Segmentation, AP somite patterning
spinal cord *see* Spinal cord development
Anteroventral periventricular nucleus (AVPV)
inputs 356, 357–359, *358f*
sexual dimorphism 349, 355
adolescents 350
arcuate nucleus innervation 359
olfaction role 356
sex steroid effects on axon growth 357–359, *358f*
testosterone-induced cell death 349, 357
Antiapoptotic genes
testosterone effects 357
Anti-disialosyl antibody, perisynaptic Schwann cell death 564
Anti-Müllerian hormone, sexual differentiation and 348
Anti-Nogo-A antibodies, spinal cord injuries 684–685
AP5 studies, visual pathway development 440–441, *442f*
APC protein *see* Adenomatous polyposis coli protein (APC)
Aphakia mutant mice, DA neuron development studies 223

Apical–basal axis
  retinal development 255
Apo-E–cholesterol, glial-mediated synaptogenesis 514, 568
Apolipoprotein E (ApoE)
  ApoE4 *see* Apolipoprotein E4 (ApoE4)
  functional roles
    synaptic plasticity 666–667
Apolipoprotein E receptor 2 (ApoER2)
  cortical lamination and 194
Apoptosis 603–605, 607
  apoptosis-necrosis dichotomy 615–616
  autonomic neurons *see* Apoptosis, autonomic nervous system
  autophagy and 617
  developmental
    neurotrophin effects on 336–337
    sexual differentiation 351, 357
  molecular mechanisms 607
  neurotrophin effects 607, 624
    mutant mouse studies 608
  p75NT receptor 622–624
Apoptosis, autonomic nervous system **607–614**
  enteric nervous system **607–614**
    neuron number determination 610*t*, 612
    receptor expression 612–613
      mutant mice 612–613
  parasympathetic nervous system 611
    ciliary neurotrophic factor 611–612
  sympathetic nervous system
    Ret signaling 611
    superior cervical ganglion 607
    Trk ligands 609
    Trk receptors 609
Apoptosis, nervous system injury
  enteric nervous system 613
Apterous (Ap) neurons 213
  axon pathfinding 213–214
  cluster generation 214, 214*f*
  genetic specification mechanisms 213
    cascades 213–214
    transcription factors associated 213
  neurotransmitter expression 213
  patterning events 214–215
  remodeling 214*f*, 215
Apterous, as LIM transcription factor 188
Arborization nuclei, neuromuscular junctions 561
Archicortex 116
Arcuate nucleus (ARC)
  development *see* Hypothalamic development
  sex steroids and sexual differentiation 359
Arfgef2, cortical neuronal migration and 194
Arrestin(s)
  β-arrestins
    signal transduction
      Notch signaling 146
Arrow gene/protein, Wnt signaling 39–40
Artemin (ARTN)
  ENS development role 630
  expression 611
    SCG defects 611
  receptor (GFRα3) 630
  sympathetic neurons
    axonal outgrowth 336
Ascl1 *see* Mash1 transcription factor
Association neurons *see* Interneuron(s)
Astrocyte(s)
  axon guidance and *see* Axonal guidance, glial cells and
  classes 218
  communication *see* Astrocytes, communication and signaling
  development 218
    differentiation
      CNTF 636
      FGF-1 636
      FGF-2 636
      glial fibrillary acidic protein (GFAP) 636
      growth factors 636
      leukemia inhibitory factor receptor 636
      PDGF 636
      region-specific 636
      see also Gliogenesis
    macroglial lineage and 218
    precursor expression markers 219
    stages 219
  functional roles 635
  GFAP *see* Glial fibrillary acidic protein (GFAP)
  growth factors 635, 639*t*
    axonal regeneration 640
    CNTF 635
    fibroblast growth factor-2 635
    insulin-like growth factor 1 635
    leukemia inhibitory factor 635
    NGF 635
    NT-3 635
    NT-4 635
    TGF-α 635
    TGF-b 635
  receptor expression *see* Astrocytes, communication and signaling
  signaling *see* Astrocytes, communication and signaling
  synaptic functions of *see* Glial cells, synaptic functions
  transmitter release *see* Astrocytes, communication and signaling
Astrocyte-derived basal laminae, axon guidance role 393
Astrocytes, communication and signaling
  gliotransmitters/neuromodulators
    ATP release 570
    D-serine release 570
    glutamate 570
  see also Glial cells, neurotransmission modulation
Astrocytoma(s) 638
  growth factor receptors 638
Astroglia *see* Astrocyte(s)
Asymmetrical divisions
  retinal development 263, 264*f*
Atg gene family, autophagic cell death 617
Atm gene/protein, adult NSC regulation 645
Atoh7 *see* Math5 (Atoh7) gene/protein
Atoh1 gene/protein, hair cell differentiation 247
Auditory system(s)
  development
    dendritic arbor formation 458
Auerbach's plexus *see* Myenteric plexus
Aurora A, cell fate determination 593
Autapses 514–515
Autonomic cell culture
  enteric neurons
    neurotrophic factors and 631
Autonomic nervous system, development **321–332**
  enteric nervous system *see* Enteric nervous system, development
  gangliogenesis 333
  molecular signals 333–335, 334*f*, 335*t*
  neural crest 322*f*, 333, 338
    chick-quail orthotopic transplantation 321
    growth factors 321
    homeobox gene expression 321
      clusters 321
  in vitro/*in vivo* analysis 328*f*
    migratory morphogenesis 322
      enteric ganglia 330
      gangliogenesis 323
      microenvironment influences 325*f*
      molecular control 322
      parasympathetic ganglia 328
      sympathetic ganglia 324
    origin role 321
    sympathetic ganglia
      choline transporter expression 326–327
      HAND2 deletion 326
      Phox2b deletion 326
      vesicular acetylcholine transporter 326–327
  origin 321
  plasticity *see* Autonomic neuroplasticity, developmental
Autonomic nervous system, neurochemistry
  acetylcholine *see* Cholinergic neurons/systems
  developmental plasticity 338
  multiple transmitters/neuromodulators 338
    see also Enteric circuits, neural coding
  see also Autonomic neuroeffector junction
Autonomic neuroeffector junction
  plasticity and *see* Autonomic neuroplasticity
  structure
    variation between organs 337–338
Autonomic neuroplasticity
  developmental *see* Autonomic neuroplasticity, developmental
  NGF and 333, 336–337, 338
  utility of study 333
Autonomic neuroplasticity, developmental **333–340**

Autonomic neuroplasticity, developmental (*continued*)
- definition 333
- gangliogenesis 333
  - migration 335
  - molecular signals 333–335, 334*f*, 335*t*, 338–339
  - neural crest cells 333, 335
- neurochemical plasticity 338
  - acetylcholine synthesis machinery 339
  - molecular signals 338–339
  - transmitter 'phenotype switching' 339
- neuroeffector pathways 336
  - axon outgrowth 336
    - injury-induced plasticity 336, 337*f*
    - neurotrophic factors 336
    - postganglionic axons 336
    - preganglionic axons 336
    - somatic mesoderm 336
  - neurotrophic hypothesis 336
    - NGF and 336–337
  - target innervation regulation 337
    - BDNF role 338
    - propulsive *vs.* repulsive 338
    - transmitter phenotype and 339
    - TrkA *vs.* p75 balance 338

Autonomous specification, programmed cell death 600

Autophagic cell death **615–621**
- apoptosis and 617
- definition 615
- historical aspects and conceptual origin 615
  - early beliefs 615
  - 'suicide bag' hypothesis 615–616
  - supporting evidence 616
- macroautophagy and 615, 616*f*
- neuronal death and degeneration 615, 617
  - 3-MA studies 618*t*
  - developmental 618, 619*f*
  - lysosomal storage disease 620
  - neurodegenerative disease 619
  - neurological disease 618, 620*f*
- prevention strategies
  - genetic 617
  - pharmacological 616, 618*t*

Autophagic vacuole 615

Autophagosomes 615

Autophagy 603–605, 615
- cell death and *see* Autophagic cell death
- chaperone-mediated 615
- genetic prevention 617
- macroautophagy 615, 616*f*
- microautophagy 615
- pexophagy 615
- pharmacological prevention 616
- types 615

Avian ciliary ganglion 329–330
- developmental influences 331*t*

Axin (scaffold protein) 40–41

Axogenesis factor-1 679

Axogenesis factor-2 679

Axon(s)
- action potential propagation *see* Action potential(s)
- development
  - neurogenesis *see* Neurogenesis
  - NMDA receptors 445–446
- dorsal root ganglia (DRG) *see* Dorsal root ganglia (DRG)
- elimination/pruning
  - CST development 403, 405–406
- growth *see* Axonal growth/guidance
- microtubule organization 480
- morphology/structure
  - remodeling, Wnt proteins 42, 42*f*
- myelination control 242
- Schwann cell relationship **293–297**
- transport along *see* Axonal transport

Axonal arbor(s)
- definition 456
- retinotopic map refinement 370

Axonal growth, extracellular matrix role 399
- effects 399
- growth cone migration 422, 423*f*, **480–486**
  - adhesive interactions 480, 481*f*, 483
    - cadherins 482, 485
    - fibronectin 484
    - IgCAMs 482, 485
    - integrin adhesion receptors 482
    - integrins 422, 484
    - L1 485
    - laminins 483
    - modulation 481*f*, 482–483
    - NCAMs 485
  - axonal regeneration 485
  - cytoskeletal dynamics 480
- phosphacan 401
- proteoglycans 400
- receptor tyrosine phosphatases 401
- tenascin gene family 399
- *see also* Axonal guidance cues

Axonal growth cones 381, **422–426**
- adhesion complexes 480–481, 481*f*
- collapse 395, 396*t*
- cytoskeletal reorganization 423–424, 480
  - actin filaments 381–382, 423–424
    - 'actin treadmill' 382
    - polymerization and steering 424
  - depolymerizing drug effects 423–424
  - filopodia 422
  - microtubules 382, 423–424, 424*f*
  - myosin role 480
  - *see also* Cytoskeleton
- ECM guidance *see* Axonal growth, extracellular matrix role
- extracellular guidance 472
- functional roles 422
- guidance cues *see* Axonal guidance cues
- guidepost cells *see* Guidepost cells
- historical aspects 426
- migration/motility 422, 460, 472
  - axon extension and branching models 382–383, 422, 423*f*
  - cytoskeletal reorganization *see above*
  - differential tensile forces 422–423
  - ECM role *see* Axonal growth, extracellular matrix role
  - guidance molecules *see* Axonal guidance cues
  - netrin-1 and response regulation 476
  - polarized turning effects 470
  - surface topology effects 422–423
- morphology/structure 381–382, 382*f*, 422, 423*f*
  - filopodia *see* Filopodia
  - lamellipodia 422
  - polarized nature of 422–423
  - sonic hedgehog effects 470
- motor neurons, NMJ development 502
  - ACh release 529
- plasma membranes 480–481
- presynaptic development role 502–503
- signaling cascades 480–481
- steering 422
  - *see also* Axonal growth/guidance

Axonal growth/guidance 365, **381–386**, 422, 473*f*
- autonomic neuroeffector junction development 336
- blueprint hypothesis 393
- branching *see* Axon branching
- cAMP role 476–477, 478
- collateral branching and
  - *see also* Axon branching
- CST neurons 404
  - midline crossing 404, 405*f*, 406*f*
- cytoskeleton role *see* Axonal growth cones
- definition 381
- experimental data 381
- extracellular matrix and *see* Axonal growth, extracellular matrix role
- future work 386
- GDNF/GFRa1
  - Ret signaling 611
- glial cell role in guidance *see* Axonal guidance, glial cells and
- growth cones *see* Axonal growth cones
- guidance molecules *see* Axonal guidance cues
- historical research 472
- invertebrates
  - *Drosophila* development
    - neuromuscular junction 522
- models 381, 382
  - axon–axon interactions 385
    - descriptive models 385
    - guidance cue secretion 385
  - axon extension and branching 382
    - actin polymerization 382

growth cone behavior 382–383
microtubule transport 382
axon turning and guidance 383
contact-mediated guidance 383, 384
filopodia 384–385
gradient guidance 383
amplification 384
filopodia role 384
Rho-GTPase signaling network 384
spatial-sensing strategy 383–384
growth cone morphology 383
actin dynamics 383
filopodia modeling 383
guidance cue patterning 385
detection problems 385
diffusion 385
scaling problems 385
topographic map formation 385
retina/optic tectum/superior colliculus map 385
molecules (guidance cues) *see* Axonal guidance cues
morphogens **460–464**
motor neurons
NMJ development 502, 529
pathfinding 190
optic pathway development 429
perisynaptic Schwann cells 566
presynaptic development and 502–503, 506
protein kinase C role 476–477
sex steroids and sexual differentiation 357, 358*f*
sonic hedgehog role *see* Sonic hedgehog (Shh)
target recognition 365
thalamocortical afferents 119
UNC-6 472
Axonal guidance, glial cells and **393–402**
astrocytes 393
basal laminae 393
corpus callosum development 393
ECM effects 399
evidence 393
monolayers 393
three dimensions 393
cell adhesion molecules 393, 394*t*
cadherins 394
calcium-dependent adhesion 394
immunoglobulin superfamily 394
integrins 395
CNS developmental compartments 396
hindbrain rhombomeres 396
prosencephalon prosomeric model 397
specialized cells 397
evidence 395
growth inhibition 395
Eph kinases 395
ephrins 395
growth cone collapse 395, 396*t*
myelin-based inhibitors 396, 397*t*
neuropilins 395
Nogo inhibitors 396, 397*t*
semaphorins 395
midline glial cells 394*t*, 397
genetic control 397
neural tube floor plate 398
optic nerve chiasm 398
neuron-glial interactions 393, 394*t*
Axonal guidance cues 381, 382, **387–392**, 424, 480
attractive (chemoattractant) 425–426, 425*f*, **465–471**
hedgehog family 465
netrin-1 470, 474
Wnt proteins 464
axonal regeneration *see* Axonal regeneration
axonal trajectories 387–388
axon–axon interactions 385
calcium 382
cAMP 382
cGMP 382
contact-mediated 388
developmental expression 671
adults 676
diffusible 388
distance acting over 426
Drosophila NMJ development 523
functional roles 422
glial-derived *see* Axonal guidance, glial cells and
hedgehog family
sonic hedgehog *see* Sonic hedgehog
inhibitory (repulsive) 366, 425–426, 689
BMP7 462
myelin-based 676, 677*f*, 689
netrin-1 476
sonic hedgehog effects *see* Sonic hedgehog (Shh) signaling
Wnt proteins 462
midline crossing 404
EphA4/Ephrin B3 405, 406*f*
L1-CAM 404–405, 405*f*
Robo/Slit 405
molecular families 425
morphogens and signaling molecules 425
netrin-1 476, 638
chemoattractive actions 425–426, 474
'deleted in colorectal cancer' 474
inhibitory (repulsive) actions 476
role 474
neural network establishment 387–388
optic tectum retinotopy and 93
permissive cues 424–425
physical features 424–425
receptor signaling 425, 426
Rho signaling 426
sensory systems **415–421**
see also Axonal growth, extracellular matrix role; Chemoaffinity hypothesis; Guidepost cells
Axonal injury, neuronal response
early reaction
retrograde transport blockade 688
Axonal regeneration 485–486
central *vs.* peripheral neurons 678–679
endoneurium molecules in 687–688
glial cell-derived growth factors 639–640
astrocytic 640
growth cone migration 485
L1 486
growth/guidance cues **671–680**, 688
adults 676
lesioned nervous system 676
netrin-1 478
see also Axonal growth/guidance; Axonal guidance cues
intrinsic regenerative capacity 678
preconditioning lesions 679, 679*f*
macrophages in 688
neurotrophic factors 676
Nogo-A in *see* Nogo-A
previous injury and 688
spinal cord injuries *see* Spinal cord regeneration/repair
see also Axonal injury, neuronal response; Axon branching
Axonal transport
endosomes *see* Endosome(s)
presynaptic development and 507
Axon branching
corticospinal development 406–407, 407*f*, 408*f*
evoked responses and 406–407, 409*f*
motor neurons 529, 553
see also Axonal growth cones
Axon guidance/pathfinding *see* Axonal growth/guidance
Axosome shedding 569–570
Axosomes, NMJ synapse elimination 556

B

BACE1 *see* Beta-site APP cleaving enzyme 1 (BACE1)
Bacterial artificial chromosomes (BACs)
molecular anatomy of mammalian brain 199
β-amyloid *see* Amyloid beta (Aβ)
Barhl1, hair cell differentiation 249
Basal lamina
components 538
growth cone migration 483–484
NMJ postsynaptic basal lamina *see* Neuromuscular junction, basal lamina
Basement membrane
retinal development 255
Basic helix-loop-helix (bHLH) transcription factors **150–155**
class I factors 150
E2a 150
HEB 150

Basic helix-loop-helix (bHLH) transcription factors (*continued*)
  class II factors 150, 151*t*
    Mash1 (Ascl1) 151*t*
      oligodendrocyte implications 152–153
    Math1 (Atoh1) 151*t*
      ectopic expression effects 153
    Math3 (Neurod4) 151*t*
    Math5 (Atoh7) 151*t*, 154
    Ngn1 (Neurog1) 151–152, 151*t*
    Ngn2 (Neurog2) 151*t*, 152
    Ngn3 (Neurog3) 151*t*
    Olig1 151*t*
    Olig2 151*t*
    Olig3 151*t*
    Ptf1a 151*t*
      GABAergic neurons 154–155
  class VI factors 150
    Hes *see Hes* genes/proteins
  Drosophila 150
    achaete–scute genes 150
  E-box binding 150, 156
  Hes family *see Hes* genes/proteins
  motor neuron specification
    LIM factor interactions 189
    progenitor expression 185
  neural stem cell maintenance 160
  neuronal differentiation 150
    Notch signaling 152*f*
  neuronal subtype specification 153, 153*f*
    neurotransmitter phenotype 153–154
  neurons *vs.* glia 152
  Notch signaling 150, 152*f*
  protein expression control 154
    BMPs 154
  protein family 150
  retinal cell specification 261
    activators 262
    bipolar cell generation 262
    repressors 262
  Schwann cell development 310
  spinal cord development 175
    cross-repressive interactions 173–174
    function control 175
    see also Motor neuron specification (vertebrate)
  structure 150
  Xenopus 151–152
Basket cells
  plasticity 437
Bassoon 506–507, 517
  see also Piccolo
Bate, Michael, guidepost cells 387
Bauplan 95
Bax protein
  programmed cell death association 602
Bcl-2 family proteins
  cell death associations 603*t*
  programmed cell death association 600–601, 602
  testosterone effects during sexual differentiation 351, 357
BDNF *see* Brain-derived neurotrophic factor (BDNF)
Bearded (Brd) gene family, E(spl) complex, role 135
Beaten path (Beat1c), *Drosophila* motor neuron development
  cell identity and 522
  defasciculation and 522–523
Bed nucleus of stria terminalis (BNST)
  sexual dimorphism 352, 356
    AVPV projections 357–359, 358*f*
    testosterone and cell death regulation 357
    ventromedial hypothalamus innervation 359
Behavioral plasticity 666
Bergmann glia 218
  expression 219
  glial-synapse relationship 571
  growth factors 636, 639*t*
    IGF-1 636
Beta-amyloid *see* Amyloid beta (Aβ)
Beta-site APP cleaving enzyme 1 (BACE1)
  myelination role 301–302
bHLH *see* Basic helix-loop-helix (bHLH) transcription factors
Bicortical (partial) lissencephaly *see* Subcortical band
    heterotopias (SBHs)
Bidirectional modification, NMDA receptors in development 447
Bidirectional movements, retinal development 255
Bienenstock–Cooper–Munro (BCM) plasticity model 437
Bim, programmed cell death 600–601
Binocularity studies, plasticity 436
Binocular vision *see* Stereoscopic vision
Bipolar cells (local circuits) *see* Interneuron(s)
Bipolar cells (retina)
  generation 259, 261*f*
    basic helix-loop-helix (bHLH) transcription factors 262
    Chx10 262
    Mash1 262
    Math3 262
  location 259
  in retina development 262
  retinal ganglion cell connections *see* Retinal ganglion cells (RGCs)
Bird(s)
  vision/visual system
    ocular stem cells
      ciliary epithelium 268–269
      CMZ 268
      Müller glia 269
      regenerative capacity 268
Biregional Cdon binding protein (Boc)
  expression 467–468
  hedgehog signaling 471
  mutational effects 467–468
  sonic hedgehog role 467
    binding 467–468
  structure 467
Blueprint hypothesis, axon guidance 393
BMP2 gene/protein
  olfactory neurogenesis 234
  retina dorsoventral axis development 128
BMP4 gene/protein 45–46
  autoactivation 45–46
  DV organization 51–52
  hair cell differentiation 246
  neural crest development 271–272
  olfactory neurogenesis 234
  retina dorsoventral axis development 127–128
BMP7 gene/protein
  chemorepellant effects 462
    GDF7 heterodimers 462
  olfactory neurogenesis 234
BMPs *see* Bone morphogenetic proteins (BMPs)
Boc
  Hedgehog signaling regulation 34–36, 35*f*
  RNA interference experiments 467–468
Bone morphogenetic proteins (BMPs)
  adult neurogenesis and 650–651
    adult NSC regulation 644–645
  antagonists 11–12
    Chordin 106
      holoprosencephaly and 111
      timing of expression and evidence against default model 11–12
    Follistatin, timing of expression and evidence against
        default model 11–12
    neural induction 106, 107
    Noggin 106
      holoprosencephaly and 111
      timing of expression and evidence against default model 11–12
    timing of expression 11–12
  as axon guidance cue 672
  developmental role *see* Bone morphogenetic proteins
      and development
  proneural basic helix-loop-helix proteins 154
Bone morphogenetic proteins and development
  antagonism in neural induction 10, 11*f*, 106, 107
    see also Neural induction
  AP patterning 80
  autonomic nervous system development 338–339
    enteric nervous system 628–629
    gangliogenesis 333–335
  DV patterning 10, 33–34, 109
    retina development 127–128
  forebrain specification 100
  mesoderm specification 10
  midline specification 111
    antagonism in neural induction 107
    epidermal ectoderm and 107
    eye specification 103
    HPE associations 111
    HPE phenotypes 112

roof plate expression and dorsal patterning 109
roof plate induction and 107
Shh-FGF8 signaling regulation 113
sites and functions 109*f*, 111–112
neocortical arealization 121
neural crest induction 312
neuroectoderm signaling **45–54**, 46*f*
antagonists 45, 47–48
Drosophila, graded gene repression 48
evolutionary conservation 45
historical aspects 45–46
phylogenetic comparisons 50, 51*f*
expression gradients 46–47, 48–49
extracellular components 46, 47*f*
homologous genes, roles 45
vertebrates
dorsoventral patterning 45, 57, 172–173
dorsoventral polarity 52
opposing graded BMP/Hh signals 46
olfactory neurogenesis 234
oligodendrocyte precursor generation control 238–239
retinal ganglion cell development 672
roof plate
expression and dorsal patterning 109
forebrain induction 107
midbrain induction 89
sonic hedgehog signaling and neural patterning 27
BoNTs *see* Botulinum toxin(s)
'Border genes'
function 272–273
neural crest 272
Botox *see* Botulinum toxin(s)
Botulinum toxin(s)
regeneration and
sprouting 565
Boundary formation (development) *see* Segmentation (developmental)
Boutons *see* Synaptic bouton(s)
Bowel(s) *see* Intestine(s)
Brachyury, neural induction in chicks and cell fate specification 14
Brain(s)
aging of *see* Brain aging
connectivity
see also Neural connectivity
development *see* Brain development
disorders
transcriptional silencing issues 180–181
see also Neurological diseases/disorders
evolution *see* Brain evolution
gonadal steroids and *see* Gonadal hormone(s)
human *see* Human brain
mammalian *see* Mammalian brain
neurons 457–458
see also Neuron(s)
receptors *see* Receptor(s)
size *see* Brain volume/size
trauma *see* Traumatic brain injury (TBI)
volume *see* Brain volume/size
Brain aging
ApoE and *see* Apolipoprotein E (ApoE)
biochemical/metabolic changes
extracellular space *see* Extracellular space (ECS)
dendritic branching 668–669
neuroplasticity and
sprouting 668
Brain-derived nerve growth factor *see* Brain-derived neurotrophic factor (BDNF)
receptor binding
p75 receptor(s) *see* p75 receptor(s)
Brain-derived neurotrophic factor (BDNF)
adult neurogenesis and 650
autonomic nervous system
enteric nervous system 628
development role 630
gastric motility and 632
postnatal development role 631
target innervation role 338
axonal regeneration 689–690
developmental role
cell differentiation/morphology 625
DA neuron development 223
postnatal ENS development 631
synapse elimination at NMJ 557–558
synapse formation role 514
disease/dysfunction and 639
glial cell production 639*t*
Müller glial cells 636
oligodendrocytes 635
historical aspects 622
neural regeneration and
peripheral nerve regeneration 640
sexual differentiation and 351
synaptic function 625
synaptic plasticity role 437
therapeutic use 626*t*
Brain development
adolescence *see* Brain development in adolescence
adulthood to middle-age
sexual differentiation and 350
cerebral cortex *see* Cortical development
definitions 95
forebrain *see* Forebrain
hindbrain *see* Hindbrain
midbrain *see* Midbrain
midline eye patterning 27
models 95
closed neural tube and 95
see also Neural tube
columnar 96
chemiarchitectonic correlation 98
diencephalic divisions and 97
gene expression studies and 98
limitation (straight brain axis) 97
sensory afferents and 96–97
gene expression studies and 98
neuromeric **95–99**, 97*f*, 103
definitions 95
gene expression studies and 98
His's contribution to 96, 98*f*
historical aspects 96
Orr's contribution to 96, 97*f*
re-emergence of 98
utility 95
myelination and *see* Myelination
NMDA receptors *see* NMDA receptors, developmental role
positioning of primordia 80
posteriorization 80
rostral neural tube origin 3
see also Neurulation
sexual differentiation *see* Sexual differentiation
sonic hedgehog and patterning 27
see also Sonic hedgehog (Shh) signaling
see also Neurodevelopment
Brain development in adolescence
sex differences and sexual dimorphism 350
Brain evolution
brain size
expansion *see* Brain evolution, expansion/volume increases
cortical
neocortex and 116
see also Cerebral cortex
expansion and *see* Brain evolution, expansion/volume increases
Brain evolution, expansion/volume increases 116
Brain Gene Expression Database 202
Brain Gene Expression Map 202
Brain size *see* Brain volume/size
Brain volume/size
evolutionary expansion *see* Brain evolution, expansion/volume increases
extracellular space and *see* Extracellular space (ECS)
human
sexual dimorphism 352
Branchiostoma, midbrain patterning 93
BrdU (5-bromo-2-deoxyuridine)
adult NSC identification 643
neural stem cell labeling 662
Brinker (Brk) (transcriptional repressor) 49
Brn1/Brn2, cortical lamination and 194
Brn2 protein, Schwann cell development 308
Brn3b transcription factor
ganglion cell differentiation 262
retinal development 257, 257*f*
Bromodeoxyuridine (BrdU) *see* BrdU (5-bromo-2-deoxyuridine)
Btg1 (transcription factor), *Hox* gene expression 68
α-Bungarotoxin
sprouting effects 565

C

Cable properties/cable theory
  cortical neuronal migration and 195, 195*f*
M-Cadherin(s), NMJ basal lamina 542
N-Cadherin(s)
  axon growth/guidance
    domains 394
    growth cone migration
      cytoplasmic tails 482, 484*f*
      expression 485
  neural crest cell migration 273–274
NMJ basal lamina 542
synaptic stabilization 517
Cadherin(s)
  axon growth/guidance 394
    domains 394
    growth cone migration 482, 485
      cytoplasmic tails 482, 484*f*
      receptor regulation 482–483
      signaling pathway activation 482
  calcium binding 485
  cell migration
    melanoblast migration and 318
    neural crest cell migration 273–274
  distribution 485
  expression 482, 485
  NMJ basal lamina 542
    catenin interactions 542
  optic tectum retinotopy development 92–93
  postsynaptic density and 498
  presynaptic development 509, 517
  see also Protocadherin(s)
Cadherin 19, Schwann cells 282–283
Caenorhabditis elegans
  development
    axon growth/guidance
      egg-laying behavior 391
      UNC-6 472
    LIM homeodomain transcription factors 188
    presynaptic development 509–510
  RPM-1
    see also PHR (Pam/Highwire/RPM-1)
  Wnt signaling 463
Ca^{2+} ions *see* Calcium ions (Ca^{2+})/calcium signaling
Cajal *see* Ramón y Cajal, Santiago
Cajal–Retzius cells
  synapse formation function 390
    cell lesion experiments 390
Calcineurin
  plasticity role 436
Calcitonin gene-related peptide (CGRP)
  AChR induction at vertebrate NMJ 547
Calcium (Ca^{2+}) *see* Calcium ions (Ca^{2+})/calcium signaling
Calcium/calmodulin-dependent kinase(s) (CaMKs)
  transcriptional silencing 180–181
Calcium/calmodulin-dependent kinase II (CaMKII)
  activation 43
  functional roles
    PSD protein 499
    retrograde signaling at invertebrate NMJ 527
  synaptic plasticity/learning and memory 436
Calcium/calmodulin-dependent serine protein kinase (CASK)
  neurexin binding 515
  presynaptic development role 515–516
    synaptic vesicle tethering and 516
  RIM1 interactions 517
Calcium ions (Ca^{2+})/calcium signaling
  axonal growth/guidance 382, 426, 675
    cell adhesion 394–395
    downstream targets 675
    growth cone motility regulation 675
  CaMKs and *see* Calcium/calmodulin-dependent kinase(s) (CaMKs)
  intracellular
    neuronal death and *see* Neurodegeneration/neurodegenerative disease
  neural patterning and, Wnt signaling 43, 43*f*
Calcium signaling *see* Calcium ions (Ca^{2+})/calcium signaling
CaMKs *see* Calcium/calmodulin-dependent kinase(s) (CaMKs)
cAMP response-element-binding protein *see* CREB (cyclic AMP response
    element-binding protein)
cAMP signaling *see* Cyclic AMP
Cancer
  autophagic cell death and 617
  ErbB receptor function 303–304
  RE1-silencing transcription factor dysregulation 209
capricious gene/protein
  Drosophila motor neuron target selection 524
CA1 region *see* Hippocampus
CA2 region *see* Hippocampus
CA3 region *see* Hippocampus
Cash1 transcription factor, ANS gangliogenesis 333–335, 334*f*, 335*t*
CASK *see* Calcium/calmodulin-dependent serine
    protein kinase (CASK)
Caspase(s) 603*t*, 607
  activation
    extrinsic pathway 607
    intrinsic pathway 607
Caspase recruitment (CARD) domain 602
CAT 301, embryonic development 400–401
β-Catenin
  neural patterning
    phosphorylation 41
    Wnt signaling 40
  nuclear import
    chromatin remodeling 41
    proposed processes 41
    transcription factor interactions 41
REST regulation 208–209
Catenin(s)
  αN-catenin, postsynaptic density 499
  NMJ 542
'Caudalization' (posteriorization) 80
  anterior visceral endoderm role 106–107
  boundaries and lineage restriction 83
  competence
    predetermination of cellular fate 84, 84*f*
    signal integration 83
  forebrain specification 100
  neural plate *see* Neural plate
  organizers 81*f*, 82
    function 82, 84*f*
    functional range 85, 85*f*
    lineage restriction 83
    see also Developmental organizers
  prevention mechanisms 100
  signals/pathways 80, 184
    BMP signaling 80
    FGF signaling 80, 82
    hedgehog signaling 81–82, 84
    Irx signaling 80–81
    retinoic acid, *Fgf8* expression 58
    retinoic acid role 55
    Wnt signaling 80–81, 82
  spinal cord formation 3
  see also Anteroposterior (AP) patterning
CaVs *see* Voltage-gated calcium channels (VGCCs)
CAZ *see* Cytoskeleton of the active zone (CAZ)
Cdc2, cell fate determination 593, 594–595
Cdc42
  activation 476
  agrin signaling 550, 551*f*
Cdk2
  cell-fate determination 594–595
  MITF transcription factor interactions 317–318
Cdk5
  cell-fate determination and 595
  neuronal motility/migration
    cortical lamination and 194
      pathway 195, 195*f*
Cdo, Hedgehog signaling regulation 34–36, 35*f*
Cdon proteins *see* Cell adhesion molecule-related/downregulated by
    oncogenes (Cdon) proteins
Cdx homeobox(es) 64–65
Ceh10 transcription factor, retinal development 256
C. elegans see Caenorhabditis elegans
Cell(s)
  polarity
    retinal development 255
  specification *see* Cell-type specification
Cell adhesion
  molecules involved *see* Cell adhesion molecules (CAMs)
  retinal development 257

Cell adhesion molecule-related/downregulated by oncogenes (Cdon) proteins
biregional (BoC) *see* Biregional Cdon binding protein (Boc)
mutations 467
sonic hedgehog binding 467–468
structure 467
Cell adhesion molecules (CAMs)
axonal targeting signals 393, 394*t*
cadherins 394
calcium-dependent adhesion 394
immunoglobulin superfamily 394
midline crossing 404–405
cardiac neural crest 277
melanoblast specification and 315*t*
synapses 512, 513*f*
synaptogenesis 514, 516*f*
presynaptic development 507, 509
Cell body *see* Soma
Cell–cell junctions
nonsynaptic 512
synapses *see* Synapse(s)
Cell contact, glial-mediated synaptogenesis 568
Cell culture
autonomic nervous system *see* Autonomic cell culture
synapse formation in 514–515
Cell cycle 594, 594*f*
cell-fate/differentiation and *see* Cell cycle, terminal differentiation role
genes
cell fate and 593
regulation 594, 594*f*
APC and cyclin degradation 595
cdk inhibitors *see* Cyclin-dependent kinase inhibitors (cdkis)
cdks *see* Cyclin-dependent kinase(s) (cdks)
cell differentiation/patterning factors and 596
cyclins *see* Cyclin(s)
geminin 596
p53 family and 596
Rb phosphorylation and 594, 595
restriction point 594, 594*f*
terminal differentiation and *see* Cell cycle, terminal differentiation role
Cell cycle, terminal differentiation role **592–598**, 593*f*
cell cycle genes and 593
APC 595
cell cycle progression 594
cyclin-dependent kinase inhibitors 594
cyclin-dependent kinases 595
cyclins 595
geminin 596
p53 family 596
Rb protein 595
'cell cycle length hypothesis' 592
cell cycle stage and 593
asymmetrical division of determining factors 593
chromatin structure role 593
transcription factor expression 593
cortical specification 593
neural progenitors, interkinetic nuclear migration 585
S phase and cell-fate determination 587
differentiation/patterning factor effects on cell cycle 596
cofactor requirement 597
hedgehog signaling 597
Notch signaling 597
tissue-specific transcription factors 596–597
'transcription factor clock' 597
Wnt signaling 597
Drosophila nervous system 592
retinal specification 253–254, 255–256, 259–260, 594
Cdkis and 595
cyclins and 595
prox1 and 593
Cell death
apoptosis-necrosis dichotomy 615–616
autophagy *see* Autophagic cell death
necrotic *see* Necrosis
programmed
apoptosis (type 1) *see* Apoptosis
autophagy (type 2) *see* Autophagic cell death
sex steroids and sexual differentiation 357
Cell differentiation
corticospinal development 403
melanoblasts 318
neocortex *see* Neocortical arealization (area differentiation)
NMDA receptors 439
retina *see* Retinal development
see also Cell-type specification
Cell division
asymmetrical divisions, neural stem/progenitor cells **585–591**, 586*f*
markers, adult NSC identification 643, 650
nervous system development 592
Cell fate decisions
retinal development 253–254
see also Cell-type specification
Cell migration
melanoblasts *see* Melanoblast(s)
neurons/neural precursors *see* Neuronal migration
retinal development 254–255, 255*f*
Cell replacement therapy 646–647
CNS repair **653–661**
endogenous stem cell recruitment 659
Huntington's disease *see* Huntington's disease (HD), therapy
Parkinson's disease *see* Parkinson's disease, therapy
Cell surface markers, adult NSC identification 644
Cell-type specification
cortical *see* Cortical cell-type specification
evolutionary conservation 590
mechanisms
bone morphogenic proteins 45, 49*f*, 50*f*
cell cycle role *see* Cell cycle, terminal differentiation role
extrinsic determinants 587, 592
embryonic cell position and 184
motor neuron progenitors 184
see also Signaling pathways
hedgehog family proteins 465
intrinsic determinants 587, 592
Drosophila 590
motor neuron progenitors 184
see also Transcription factors
neuronal **213–217**
combinatorial coding *vs.* master regulators 216
feed-forward loops 216
remodeling 216
target-derived instructive signals 216
melanocytes **312–320**
see also Melanoblast(s)
motor neurons in vertebrates *see* Motor neuron specification (vertebrate)
positional information and 73
anterior-posterior (rostrocaudal) *see* Anteroposterior (AP) patterning
dorsal-ventral *see* Dorsoventral (DV) patterning
progressive restriction of progenitors 586, 590, 592
retinal *see* Retinal development
spinal cord transcriptional networks 174
Central nervous system (CNS)
aging *see* Brain aging
axon growth/guidance
developmental compartments 396
hindbrain rhombomeres 396
prosencephalon prosomeric model 397
phosphacan 401
receptor tyrosine phosphatases 401
see also Axonal growth/guidance
BAC library *see* Bacterial artificial chromosomes (BACs)
brain *see* Brain(s)
enteric nervous system interaction
development 341
hormone effects
sexual dimorphism and *see* Sexual dimorphism
injury
spinal cord *see* Spinal cord injury (SCI)
neurotrophins and 625
see also Neurotrophin(s)
plasticity
aging effects 684
constraint therapy 685
enhancement treatments 684
rehabilitation treatments 685
spinal cord injuries 684
see also Neuroplasticity
regeneration/repair 688
axonal regeneration 485–486
cellular therapies 688–689
glial cell growth factors 639
molecular therapies 688–689
neuron replacement therapies 689
regeneration response strength 689–690

Central nervous system (CNS) (*continued*)
stem cells **653–661**
see also Neural stem/progenitor cells (NSPCs)
see also Neural regeneration/repair
rostral, transcription factors associated 397
spinal cord *see* Spinal cord
spontaneous functional recovery 687
alternative pathways 687
axon remyelination 687
crossed phrenic reflex 687
new synapse formation 687
synapses *see* Synapse(s)
Cephalochordates
nervous system
midbrain patterning 93
Cerebellar radial fibers *see* Bergmann glia
Cerebellum
anatomy/physiology
afferent systems
Bergmann glia and 571
climbing fibers 571
astrocyte-synapse relationship and 571
development
glial precursors 219
Irx2 transcription factor (iroquois) and 84–85
Cerebral cortex 585
adult neurogenesis 648
see also Neurogenesis, adult
anatomy *see* Cerebral cortex, anatomy/organization
development *see* Cortical development
evolution 116
localization of function 192
malformation *see* Malformations of the cerebral cortex (MCCs)
neocortex *see* Neocortex
organization *see* Cerebral cortex, anatomy/organization
Cerebral cortex, anatomy/organization 192, 585
cell types
neurons *see* Cortical neuron(s)
circuits/connectivity
thalamocortical 116–117
columnar *see* Cortical columns
folding
loss of *see* Lissencephaly
interneurons 585
laminae 585
layer-specific morphology 585
neuronal density 585
neurons *see* Cortical neuron(s)
pyramidal cells (projection neurons) *see* Pyramidal neuron(s)
regions/classification
archicortex 116
neocortex *see* Neocortex
paleocortex 116
species-specific 192
Cerebral palsy
activity-dependent CST refinement and 409–410, 411*f*
rehabilitation and 413–414
Cerebrohepatorenal syndrome *see* Zellweger syndrome
Cerebrovascular accident (CVA) *see* Stroke
cGMP *see* Cyclic GMP (cGMP)
CGRP *see* Calcitonin gene-related peptide (CGRP)
Chaperone-mediated autophagy 615
Charcot–Marie–Tooth disease
myelination function 303–304
Chemical synapse(s) 512
see also Neurotransmitter(s)
Chemoaffinity hypothesis **365–371**
graded molecular guidance cues 366
topographic map development 366
anterior–posterior mapping 368
dorsal–ventral mapping 368
models 365, 366, 367*f*
phases 366
retinotopic map refinement 370
termination zones 366–368, 369
Chemoattractants (axon pathfinding) *see* Axonal guidance cues
Chemorepellant signaling (axon pathfinding) *see* Axonal guidance cues
Chicken(s)
development
motor neuron development studies 72
neural induction *see* Neural induction
neurulation *see* Neurulation
Chloride ions (Cl^-)
GABAA receptor depolarization 573
intracellular concentration 573, 574*f*, 580–581
neural development and 573, 574*f*
Chloride transporters
neural circuit development 574, 574*f*
Cholesterol
esterification, defective *see* Niemann-Pick disease
glial-mediated synaptogenesis 514, 568
synaptic plasticity 666–667
transport proteins *see* Apolipoprotein E4 (ApoE4); *Apolipoprotein E (ApoE)*
Cholinergic neurons/systems
at motor nerve terminals *see* Neuromuscular transmission
optic tectum 453
Cholinergic receptors *see* Acetylcholine receptors (AChRs)
Chondroitinase
spinal cord injuries 684–685
Chondroitin sulfate proteoglycans (CSPGs)
axonal growth/guidance 398
extracellular matrix components 400
growth inhibition 676
outgrowth inhibition 401
phosphacan/DSD-1-PG 401
optic tectum retinotopy development 92–93
structural organization 400–401
Chordin (Chd)
holoprosencephaly and 111
neural induction 45–46
neural induction and 106
signaling conservation 46
timing of expression and evidence against default model 11–12
Choroid plexus
ciliary epithelium relationship 268–269
melanocytes 312
Chromatin 178
euchromatin (active form) 178, 179*f*
heterochromatin (repressed form) 178, 179*f*
remodeling/modification *see* Chromatin remodeling
transcriptional silencing *see* Transcriptional silencing
Chromatin remodeling
cell cycle regulation of cell fate 593
developmental complexes and lineage-specific expression
retinal development 256
spinal cord development 174
transcriptional silencing 178, 179–180
see also Histone(s)
Chromatolysis
axonal injury 688
Chromatolytic reaction *see* Chromatolysis
Chronic idiopathic intestinal pseudo-obstruction (CIIP) 346
Chronotopic fiber reordering, optic pathway development 429*f*, 430
Chronotropic maps, optic pathway development 430
Churchill
induction by FGF8 during chick neural induction 13–14
neural induction in chicks and cell fate specification 14
Chx10, bipolar cell generation 262
Ci/Gli transcription factors, chemoattractants 465
Ciliary body
anatomy 267*f*, 268–269
ocular neural stem cells 268
Ciliary epithelium
choroid plexus relationship 268–269
ocular neural stem cells 267*f*, 268
Ciliary ganglion neurons
innervation targets 329*f*
survival 611–612
Ciliary marginal zone (CMZ), ocular neural stem cells 267, 267*f*
amphibia 267–268
birds 268
mammals 268
regenerative capacity 268
teleost fish 267–268
Ciliary neurotrophic factor (CNTF)
ENS development role 630
glial cells 639*t*
astrocytes 635
differentiation 636
neurodegenerative disease and 639
neuronal regeneration 678
peripheral nerve 640
parasympathetic nervous system apoptosis 611–612
Schwann cells 634

Cip1 (p21), cell-fate determination 594
Clathrin-mediated endocytosis (CME)
  Notch signal transduction 142–144
'Clock and wavefront' model, somitogenesis 58–59
'Clock-like' mechanisms, retinal development 254
Clonal analysis
  cortical cell-type specification 587
CNS *see* Central nervous system (CNS)
Cocaine
  histone modification 181
Cochlea
  anatomy 245
  otoacoustic emissions *see* Otoacoustic emissions (OAEs)
Cognition
  sex differences 348
Colinearity, Hox genes/proteins 75
Colitis, neurotrophin effects 632–633
Collagen(s)
  type IV
    binding partners 539
    α chains 538–539
      NC1 domain 538–539
      trimer formation 539
    genes 538–539
    meshwork formation 539, 539*f*
    MMP cleavage 539, 543
    NMJ basal lamina 538
  type XVIII, NMJ basal lamina 541
Collagen-tailed acetylcholinesterase (ColQ-AChE) 541
  neuromuscular junction 541
Collateral branching *see* Axon branching
ColQ acetylcholinesterase *see* Collagen-tailed acetylcholinesterase (ColQ-AChE)
Columnar brain development models 96
  failure/limitations 97
Column of Terni (TC), motor column specification 74–75
Combinatorial (cross-fiber) coding
  neuronal cell type specification mechanisms, master regulators *vs.* 216
Commissural axons/fibers
  BMP7 effects 462
  guidepost cells 388
  netrin-1 effects 460
  projection 460–461, 461*f*
  sonic hedgehog
    Boc receptor function 467
    role 465, 468
      chemorepulsion 461
    turning effects 461
Commissural plate (CoP), FGF8 expression 121
  Sp8 effects 120–121
Comm proteins, axon guidance 389
Competence changes, retinal progenitor cell multipotency 259
Competency model, retinal development *see* Retinal development
Competitive arbor rearrangement, visual pathway development 441–442
Complete androgen insensitivity syndrome (CAIS), sexual behavior/sexual dimorphism and 352
Conditioned specification, programmed cell death 600
Cone photoreceptor(s)
  generation 259, 261*f*
  location 259
Cone-rod dystrophy-2 263
Congenital adrenal hyperplasia (CAH)
  sexual behavior/sexual dimorphism and 352
Congenital fibrosis of the extraocular muscle 2 (CFEOM2) 91
Congenital megacolon *see* Hirschsprung disease
Connectin, *Drosophila* motor neuron target selection 524
Convergent extension
  neural plate shaping 3
    cellular mechanism 7
    molecular mechanism 8
  planar cell polarity pathway and 8
  Wnt signaling and 8–9
Cooperative near-neighbor input activity, visual pathway development 442
Corpus callosum
  anatomy 393
  glia and
    astrocyte-dependent cortical connections 393
Cortical cell-type specification 586
  historical aspects 585
  inside-out pattern 193, 586, 593
  intrinsic factors 589
  cell cycle role 587, 593
    chromatin structure and 593
  cleavage plane and 587–588
  Drosophila model 587, 590, 593
  extrinsic factors *vs.* 587
  identification of 587
  Notch signaling 587, 588
  Numb and homologs 587, 588
  temporal determinants
    Fezl 589–590
    Foxg1 589
  progenitors
    glial generation 588–589
    'interkinetic nuclear migration' 585
    interneuron generation 117
    progressive restriction 586, 590
    projection neuron generation 117, 588
    radial glia as 193, 585–586
      see also Radial glial cells
    restricted population 586
    SVZ and VZ segregation and 588
    symmetric *vs.* asymmetric cell division 586*f*, 587
      cleavage plane and 587–588
      early *vs.* late 587
      molecular determinants 587, 593
  spatial determinants (SVZ *vs.* VZ) 588
  temporal determinants (birthdate and laminar fate) 586
    ^{3}H thymidine birthdating studies 586, 589
    clonal analysis 587
    intrinsic determinants 589
    transplantation experiments 587, 593
Cortical columns 585
  brain development models 96
Cortical development 116, **585–591**
  arealization, neocortex *see* Neocortical arealization (area differentiation)
  autophagic cell death 618
  cell-type specification *see* Cortical cell-type specification
  corticogenesis, neocortex 117, 118*f*
  disorders 192
    epilepsy and 192–193
    holoprosencephaly *see* Holoprosencephaly (HPE)
    neuronal migration disorders 192–193, 195–196
      lissencephaly *see* Lissencephaly
    proliferation and patterning disorders 192–193
    see also Malformations of the cerebral cortex (MCCs)
  evolutionary perspective, SVZ emergence 588–589
  historical aspects 585
  lamination (layer formation) 192–193
    inside-out generation 193, 586, 593
    molecular mechanisms 194
    timing 193
  neocortex *see* Neocortical development
  neural plate differentiation 116
  neural tube vesicle formation 116
  neuronal migration 192–193, 585–586
    disorders 192–193
      lissencephaly *see* Lissencephaly
      radial migration phase and 194
    molecular pathways 194, 195*f*
    neocortical 117, 193
    radial 193
      phases 193–194
    radial migration
      doublecortin (DCX) role 194
      filamin A role 194
      LIS1 role 194
    tangential 193
  proliferation and differentiation 192–193
  regionalization
    boundary formation *see* Segmentation (developmental)
    neocortical areas and 122
  steps 192–193
Cortical neuron(s)
  cholinergic neurons *see* Cholinergic neurons/systems
  postsynaptic differentiation, receptor transport 488–489
  pyramidal cells (projection neurons) *see* Pyramidal neuron(s)
Cortical plate (CP) 117
  cytoarchitecture 118
  projection neuron generation 117
    selective axon elimination 118–119
Corticogenesis, neocortical 117, 118*f*
Corticospinal development **403–414**

Corticospinal development (*continued*)
brain stem/spinal cord projections (CST formation) 403, 404
collateral branching 405
decussation and midline crossing 404
EphA4/Ephrin B3 405, 406*f*
L1-CAM 404–405, 405*f*
Robo/Slit 405
pathfinding 404
connectional specificity within spinal cord 403, 405
activity-dependent refinement 408, 413
deviations/vulnerability and 413
hemiplegia and 409–410, 411*f*
ipsilateral terminations and 408–409, 411*f*
level and pattern of 408, 410*f*
rehabilitation role 413–414
unilateral augmentation effects 409, 411*f*
topographic refinement 405–406
axonal pruning 403, 405–406
bilateral terminations 408, 409*f*
branch growth/bouton formation 406, 407*f*, 408*f*
evoked responses and 406, 407*f*, 409*f*
postnatal 406–407
threshold reduction 408, 409*f*
timing 405–406
transient terminations 405–406, 407*f*
use-dependent development of CST 410, 412*f*
cortical motor map development 403, 408*f*, 409*f*, 412, 413*f*
factors affecting 403
neuronal differentiation 403
Fezl role 403, 404*f*
Otx1 role 403
phases/time course 403
postnatal development 403
topographic refinement and 406–407
study methods 403
rehabilitation implications 413
skilled motor behavior development 410
CS refinement prevention and 410*f*, 411, 412*f*
difficult of establishing link 410–411
independent finger movements 410–411
postnatal motor cortex blockade and 411
Corticospinal system (CS)
cortical origins 403
motor maps 412
see also Somatosensory cortex
definition 403
development *see* Corticospinal development
Ryk expression 463
spinal cord projections *see* Corticospinal tract (CST)
see also Skilled movements
Corticospinal tract (CST) 403
development *see* Corticospinal development
origins 403
COUP-TFI transcription factor 120
neocortical arealization 120
AP gradation 122
expression patterns 120
FGF8 and 121
regulatory gene interactions 121
CpG islands, transcriptional silencing 179
Cranial nerve(s)
development
columnar models 96–97
disorders 91
oculomotor patterning and 91
Craniofacial dysmorphisms
cranial neural crest defects 274–275
Craniofacial motor neurons
specification
anteroposterior (AP) signals 184
oculomotor patterning and 91
Crb, retinal development 255
CREB (cyclic AMP response element-binding protein)
development role
activity-dependent development of *Drosophila* NMJ 525–526
melanoblast specification 314
olfactory system axon guidance 418–419, 419*f*
Schwann cell proliferation 307
sexual differentiation 359–360
synaptic plasticity role 436–437
transcriptional silencing 180–181
Cre/Lox recombination system
oligodendrocyte precursor fate mapping 243
Cre recombinase
commissural axon guidance 466–467
Critical periods
sexual differentiation 348, 354–355
Crohn's disease
neurotrophin effects 633
Crossed phrenic reflex 687
Cross-repression, spinal cord transcriptional networks 173
Crx transcription factor
photoreceptor cell specification 263
retinal development 266
CSL genes/proteins, Notch signaling 141*f*, 147
transcriptional repression 147
Cux2, cortical neuronal cell fate 589–590
CVax gene/protein, retina dorsoventral axis development 127–128
CWN-1 (Wnt signaling molecule), expression 463
Cyclic adenosine monophosphate (cAMP) *see* Cyclic AMP (cAMP)
Cyclic AMP (cAMP)
axonal regeneration 679, 679*f*, 689–690
axon growth/guidance role 425*f*, 426, 476–477, 478
growth cones 382
development role
activity-dependent development of *Drosophila* NMJ 525
inhibition
Sonic hedgehog 671
Cyclic AMP-dependent protein kinase A *see* Protein kinase A (PKA)
Cyclic AMP response element-binding protein *see* CREB (cyclic AMP response element-binding protein)
Cyclic GMP (cGMP)
axonal growth cones 382
Cyclin(s)
APC-mediated proteolysis 595
cell cycle progression role 594, 594*f*
cell fate/differentiation and 595
retinal progenitor cell regulation 252–253
Cyclin D1
cell-fate determination and 595
Cyclin D3
cell-fate determination and 595
Cyclin D2, cell-fate determination and 595
Cyclin-dependent kinase(s) (Cdks)
Cdk5 *see* Cdk5
cell cycle progression role 594, 594*f*
cell fate/differentiation and 595
retinal progenitor cell regulation 252–253
Cyclin-dependent kinase inhibitors (cdkis)
cell-fate role 594
retinal ganglion cells 259–260
Cyclopamine
axon guidance role 469
Smo inhibition 27–28
Cyclopia 27
holoprosencephaly and 105
sonic hedgehog signaling and 27
Cyp26c1 gene, retinoic acid-mediated expression 56–57
Cyp26 family, retina dorsoventral axis development 128
Cytochrome *c*
apoptosis role 607
Cytokine(s)
glial cells
neurotransmission 570
Schwann cells 634, 687–688
neuropoietic, ENS development role 629*t*, 630
Wallerian degeneration 687–688
Cytomatrix assembled at the active zone (CAZ) *see* Cytoskeleton of the active zone (CAZ)
Cytoskeleton
actin *see* Actin/actin filaments
axonal growth cones
dynamics and migration 480
see also Axonal growth cones
microtubule-associated proteins *see* Microtubule-associated protein(s) (MAPs)
microtubules *see* Microtubule(s)
MITF modulation of 318
presynaptic development 510
see also Cytoskeleton of the active zone (CAZ)
PSD and 499
remodeling
developmental, NMDA receptors and 445–446
Cytoskeleton of the active zone (CAZ) 502

D

Dab1 signaling
  cortical lamination and neuronal migration 194, 195, 195*f*
Dachsous molecule, positional information 19
DAN protein family, neural induction function 45–46
Dark-reared animals
  neuroplasticity 433
DAT *see* Dopamine transporter (DAT)
Dbx1
  BMP signaling 46–47
  DV neural tube expression 32*f*
    cross-repression 33
  tectal-tegmental boundary 92
Dbx2
  BMP signaling 46–47
  DV neural tube expression 32*f*
    cross-repression 33
DCC (deleted in colorectal cancer)
  axonal growth and guidance 381, 415, 460
    chemorepulsion 470
    expression 476, 478
    extracellular domain 474–475
    intracellular domain 474–475
    loss of function 474
    netrin-1 474, 478–479
    netrin receptor function 474
    P2 domain 474–475
    P3 domain 476
    Rho GTPase activation 476
  enteric nervous system development 346–347
DCT, adult melanocyte stem cell regulation 319
DCX *see* Doublecortin (DCX)
Deafness *see* Hearing loss
Decapentaplegic (Dpp) gene/protein
  autoactivation 45–46
  cell fate specification 48–49
  dosage-dependent gene expression 49
  positional information 18–19
  vnd gene expression repression 50
Decussating pathways
  midline crossing by the CST 404, 405*f*
  optic pathway development 429
'Default model' of neural induction 10
  evidence against 10–11, 12*f*
  evidence for 10, 11*f*
Degenerative diseases *see* Neurodegeneration/neurodegenerative disease
Delamination, neural crest 273–274
'Deleted in colorectal cancer' *see* DCC (deleted in colorectal cancer)
Delta ligand *see* DSL (Delta, Serrate, Lag-2) ligand family
Delta signaling *see* Notch signaling pathway
Deltex, Notch signaling 146
Demyelination
  leprosy 304
  MS *see* Multiple sclerosis (MS)
  multiple sclerosis *see* Multiple sclerosis (MS)
Dendrites/dendritic arbor
  age-related changes 668–669
  definition 456
  development **456–459**
    branching capacity 457–458, 458*f*
    lifespan 457, 457*f*
    synaptic input function 458, 459*f*
    see also Dendritic spines; Synaptogenesis
  filopodia *see* Filopodia
  ion channels
    glutamate receptor blockade effects 458
  morphology/structure
    motor neurons 190
      development 190
    see also Dendritic spines
  motor neurons 190
  sex steroids and sexual differentiation 359
  spines *see* Dendritic spines
Dendritic spines
  endocytosis *see* Dendritic spines, endocytic traffic
  filopodia *see* Filopodia
  morphology/structure
    changes 666
      age-related 669
    filopodia *see* Filopodia
  neural circuit development, GABAergic primate neurons 578
  remodeling/plasticity
    synaptic plasticity *see below*
    turnover 666
  synapses
    formation
      filopodia 512–513
  synaptic plasticity and
    aging 669
    estrogen effects 668, 669
Dendritic spines, endocytic traffic
  postsynaptic glutamate receptors and plasticity
    NMDA receptors
      developmental 444
Denervation
  neuromuscular junction and
    partial denervation
      at birth and synapse elimination 554
Dentate gyrus (DG)
  adult neurogenesis *see* Neurogenesis, adult
  synaptic plasticity
    neural circuitry scrambling 667
    see also Hippocampal long-term potentiation (LTP)
  see also Hippocampus
Depolarizing bipolar cells (DBCs) *see* Bipolar cells (retina)
Derailed receptor(s) (drl), Wnt5 signaling 462–463
  expression 463
  mutants 462–463
  repulsive activity 463
Derepression, spinal cord transcriptional networks 173
Descriptive models, axon–axon interactions 385
Deuterostomes
  BMP phylogeny 51, 51*f*
  midbrain patterning 93–94
Development
  autonomic nervous system plasticity *see* Autonomic neuroplasticity
  autophagic cell death and 618, 619*f*
  critical periods *see* Critical periods
  disorders of *see* Neurodevelopmental disorders
  enteric nervous system (ENS) *see* Enteric nervous system, development
  hypothalamus *see* Hypothalamic development
  neurological *see* Neurodevelopment
  neuronal homeostasis *see* Neuronal homeostasis
  postnatal *see* Postnatal development
  progressive cell fate restriction 10
  signaling pathways 10
  vasoactive intestinal polypeptide (VIP) *see* Vasoactive intestinal peptide (VIP)
Developmental neuroplasticity 333
  autonomic *see* Autonomic neuroplasticity, developmental
  visual systems
    neural activity in *see* Visual development, neural activity role
Developmental organizers
  forebrain midline *see* Forebrain midline development
  local (neural/secondary)
    defining characteristics 82
      ectopic cell fate generation 83
    forebrain specification/patterning 100
      AP patterning 100
      DV patterning 100–101
      midline development 108
    functional range and receiving fields 85, 85*f*
      planar transcytosis 85–86
      restrictive clearance 85
    functions 82, 84*f*
    positions 81*f*
      neural plate constrictions and 83
    signals 80, 81*f*
      FGFs 80
      hedgehog signaling 81–82
      response to (competence) 83–84
      Wnts 80–81
    tissue competence and
      definition 83–84
      predetermination of cellular fate 84
      signal integration 83
  non-neural neural plate organizers
    ANB specification 107
    anterior visceral endoderm 106
    epidermal ectoderm 107
    forebrain midline development 105
    forebrain specification 106, 107*f*

Developmental organizers (*continued*)
Hensen's node 106
hypothalamus morphogenesis 108
neural induction 106, 107
prechordal plate 108
roof plate induction 107
Diabetic neuropathy
autonomic
enteric 632
Diazepam
neuroplasticity studies 437
Dicer
deletion in DA neurons 225–225
Dickkopf family proteins, Wnt signaling 40
Diencephalic neuroepithelium, optic pathway development 430
Diencephalon
development 116
columnar models and 97
local organizer *see* Zona limitans intrathalamica (ZLI)
specification and early development 103
hypothalamus *see* Hypothalamus
optic pathway 427
structural division 96–97
columnar models and 97
thalamus *see* Thalamus
Diencephalon–midbrain boundary (DMB) *see* Midbrain–forebrain boundary
Differential splicing *see* Alternative splicing
Dll1 recycling defects, Notch signaling 144
DIMM gene/protein
neuronal cell type specification 216
Diphtheria toxin, oligodendrocyte precursor ablation 239–240
Diptera (flies)
Drosophila melanogaster see Drosophila melanogaster
notum cell division 136
dishevelled (Dsh) gene/protein
axis specification 41
phosphorylation, signaling effects 40
Disparity, stereoscopic depth *see* Stereoscopic depth
Dispatched (Disp) protein, sonic hedgehog signaling 22–23
Dlx transcription factors
neural crest development 272
DMBX1 transcription factor, invertebrate midbrain patterning 93
DNA binding-dependent mechanisms, retina anteroposterior axis development 126
DNA binding-independent mechanisms, retina anteroposterior axis development 126
DNA methylation
de novo 181–182
transcriptional repression 179
Dok-7, MuSK regulation and NMJ maturation 532–533
Dominance concept, morphogen gradients and polarity 16–17
Dopamine (DA)
adult NSC regulation 645
anatomy *see* Dopaminergic neurons/systems
neurotransmission *see* Dopaminergic neurons/systems
reuptake/transport
transporter proteins *see* Dopamine transporter (DAT)
see also Dopaminergic neurons/systems
Dopaminergic neurons/systems
afferent connectivity
midbrain
see also Midbrain dopaminergic neurons
cell replacement therapy *see* Cell replacement therapy
development
adolescence and
see also Puberty
axonal development control 223
mesencephalic *see* Midbrain dopaminergic neurons
differentiation **221–225**
medical importance 221
recent advances 225–225
tissue oxygen pressure 224
in vitro 224
midbrain tracts *see* Midbrain dopaminergic neurons
pathways
midbrain *see* Midbrain dopaminergic neurons
populations 221
puberty and *see* Puberty
Dopamine transporter (DAT)
enteric nervous system development 343
Dorsal (Dl) morphogen, DV polarity 48–49, 49*f*, 50
Dorsal epidermal mass, neuroectoderm formation 46–47, 48*f*
Dorsal horn *see* Spinal cord, dorsal horn
Dorsal root ganglia (DRG)
neurons
axon guidance, CSPG-mediated inhibition 401
sodium channels *see* Voltage-gated sodium channels (VGSCs)
Dorsal sensory classes of interneurons (dI), precursors 172–173
Dorsolateral hinge points (DLHPs), neurulation 3–5, 5*f*
Dorsoventral (DV) patterning **29–38**
BMPs and 10, 33–34, 45, 109
axis inversion 48*f*, 49, 52–53, 53*f*
dorsal sensory classes of interneurons 172–173
forebrain specification/patterning 100–101
midline formation
dorsal 109
ventral 108
mesoderm-secreted signals 29
additional signals 31–33
sonic hedgehog *see* Sonic hedgehog (Shh) signaling
midbrain *see* Midbrain patterning
perspective 38
retina *see* Retinal development
spinal cord
retinoic acid 57
transcriptional networks 172, 173*f*
sonic hedgehog role 172
see also Spinal cord development
Xenopus 41
Wnt signaling 41
Dorsoventral mapping, topographic map development 368
Eph B 369
ephrin B1 expression 369
Wnt signaling 369
Double cortex *see* Subcortical band heterotopias (SBHs)
Doublecortin (DCX)
adult hippocampal neurogenesis 650
gene mutations
initiation role 194
lissencephaly and 194, 196, 197
knockout mice 197
neuronal migration 194
protein functions 197
DP5, programmed cell death association 600–601
Drebrin
postsynaptic density 499
DRG *see* Dorsal root ganglia (DRG)
Driesch, Hans 16
Drosophila development
apterous neurons
metamorphosis 214*f*, 215
ventral nerve cord 213, 214–215
axon growth/guidance, midline glial cells 397
BMPs and neuroectodermal signaling
DV axis inversion 48*f*, 49, 52–53, 53*f*
graded neural gene repression 48
Sog expression 45–46
cell-type specification 132–133
cell cycle role 592
intrinsic determinants 590
Notch signaling and 587
Numb and Prospero 587, 593
LIM factors 188
NB7-1 lineage 590
transcription factor expression 592
as internal 'clock' 597
see also Cell cycle, terminal differentiation role
cortical, border formation 122–123
ganglion mother cell 592
daughter cell fates 593
Hox genes and
Antennapedia gene mutation 61, 62*f*
clusters 68–69
macrochaete development 135
morphogens, positional information 18, 19
motor system
embryonic *vs.* pupa 520
neuromuscular junction *see Drosophila* neuromuscular junction, larval
neuroblasts (multipotent precursors) 592
asymmetric division (mother cell production) 592
cell cycle effects 592
sequential TF expression 597

neurogenesis 131
cell fates 132–133
organizer signaling, functional range 85–86
planar cell polarity (PCP) pathway 8
functions 8
Wnt signaling 8–9
synapse elimination, glial cells and 569
synaptogenesis
presynaptic development 509–510
Drosophila melanogaster
basic helix-loop-helix proteins 150
commissural neurons, projection 461*f*, 462
development *see Drosophila* development
gene expression
developmental *see Drosophila* development
Highwire *see* PHR (Pam/Highwire/RPM-1)
netrin secretion 462
neuromuscular junction *see Drosophila* neuromuscular junction
signaling pathways
Notch signal transduction 144–145
receptor 140
Wnt signaling
neuromuscular junctions 42–43
porcupine gene, palmitoylation and 39
Wnt5 proteins 462
see also Drosophila development
synaptic transmission
neuromuscular *see Drosophila* neuromuscular junction
transcriptional silencing, heterochromatin effects 181
unc5 expression 476
Drosophila neuromuscular junction 520
development *see Drosophila* neuromuscular junction, larval
larval *see Drosophila* neuromuscular junction, larval
muscle innervation
larval abdominal muscles *see Drosophila* neuromuscular junction, larval
neuromuscular receptors
metabotropic glutamate 520
postsynaptic ionotropic glutamate 520
subunit expression 520
retrograde signaling
larval NMJ development and 526, 526*f*
synapse development 526, 526*f*
structure-function relationship 525
utility as model 520
Drosophila neuromuscular junction, larval **520–528**
activity-dependent development 524
early functions 524
receptor localization 524
synaptic refinement 524
NMJ growth and 525
FasII role 525
GluR induction and 525
neurotransmission effects 525
pre- and postsynaptic specialization 525
peristaltic contractions and 524
size-activity relationship 525
cAMP and 525
CREB and 525–526
dunce mutants 525
FasII and 525
functional plasticity and 526
GluR expression/localization 526
hyperactive mutants 525
tetraspanins and 524
cellular determination and axon guidance 522
defasciculation 522
Beaten path 522–523
MN axon CAMs 522
muscle-derived cues 523
receptor tyrosine phosphatases 523
motor neuron identity 522
dorsal factors 522
downstream effectors 522
ventral factors 522
see also Axonal growth/guidance
general features 520, 521*f*
glutamate receptors 520
induction 525
localization 526
subunit expression 520
glutamatergic innervation 520
peripheral nerve branches 520
presynaptic *vs.* postsynaptic 520, 522*f*
muscle innervation pattern 520, 522*f*
fidelity 521–522
ganglionic peripheral nerve 520
type 1b (big) innervation 520–521, 521–522
type 1s (small) innervation 520–521
type 2 innervation 521
type 3 innervation 521
target selection 523
molecular recognition of synaptic targets 523, 523*f*
combinatorial model 524
myopodia 523
transsynaptic signaling 525
anterograde (Wnt signaling) 527
retrograde 526, 526*f*
CaMKII and 527
canonical pathway 526–527
GluRII mutation and 526
Highwire and 527
noncanonical pathway 527
TGFβ/BMP signaling 526–527, 526*f*
DSL (Delta, Serrate, Lag-2) ligand family 140, 142*f*
endocytosis 142
epsin-dependent 145
function 140–141
hair cell differentiation, prosensory specification 245
ligand recycling 144
Notch activation 131–132, 133*f*
mechanotransduction 145
proteolysis 141*f*, 144, 146
Notch-Delta signaling 131
internalization 131
Notch dissociation 144–145
proteolytic processing 140
regulation 141
solubility 140, 145
structure 140
ubiquitination 142–144, 146
zebrafish homologs 138
'Duail' embryos 275–276, 276*f*
Duchenne muscular dystrophy (DMD)
dystroglycan complex 541–542
dunce gene/protein
activity-dependent development of *Drosophila* NMJ 525
Dye(s)
optic nerve development 429
Dynamin(s)
Notch signaling 141–142, 145–146
presynaptic development and 506
Dynein(s)
regulation
LIS1 and 196–197
α-Dystrobrevin
dystroglycan complex components 541
α-Dystroglycan 541
β-Dystroglycan 541
Dystroglycan complex
components 541
Duchenne muscular dystrophy and 541–542
dystrophin/utrophin binding 541–542
NMJ basal lamina 541
Dystrophin
dystroglycan complex binding 541–542

E

E3 enzymes *see* Ubiquitin ligases (E3)
E-box 318
Ecdysozoa, BMP phylogeny 51, 51*f*
Ectoderm, transcription factor expression 56
Edinger–Westphal nucleus 87
Efferent neurons *see* Motor neuron(s) (MNs)
EGF *see* Epidermal growth factor (EGF)
EGFR *see* Epidermal growth factor receptor (EGFR)
Egg-laying behavior
guidepost cells 391
hermaphroditic specific neurons 391
synaptic guidepost protein-1 391
synaptic guidepost protein-2 391
EGL-20 (Wnt signaling molecule), expression 463

Elderly people *see* Age/aging
Electron microscopy (EM)
  optic nerve development 429
Embryonic (E) days, neural crest 321
Embryonic field concept 17
Embryonic stem cells (ESCs)
  CNS repair 653, 656*f*
    see also Cell replacement therapy
  DA neuron development studies 224
Emx2 gene/protein 120
  neocortical arealization 120
    AP gradation 122
    area identity and 123*f*
    expression patterns 120
    FGF8 and 121
    regulatory gene interactions 121
  telencephalon specification/development 102
Emx homeobox genes, retina dorsoventral axis development 127
En-1 transcription factor
  DA neuron development 222
spinal cord development 174–175
En-2 transcription factor
DA neuron development 222
Endocytosis
  AMPA receptors *see* AMPA receptor trafficking
  clathrin-mediated *see* Clathrin-mediated endocytosis (CME)
  dendritic spines *see* Dendritic spines, endocytic traffic
  dynamins *see* Dynamin(s)
  FGF8 signaling and AP patterning 85, 85*f*
  Notch signaling 142, 143*t*
Endoneurial fibroblasts, Schwann cell precursors 289
Endosomal–lysosomal system
  neurodegenerative diseases/disorders 619
  see also Endosome(s)
Endosome(s)
neurological disease and 619
Endothelin-1
  Alzheimer's disease 639
Endothelin-3
  melanocyte development 280
Endothelin receptor-B (EDNRB), melanoblast specification role 314
Endothelin receptors
  melanoblast specification role 314
Enhanced green fluorescent protein (EGFP)
  GENSAT project 201–202
Enhancer of Split (E(spl))
  enhancer elements associated 133–135, 133*f*
  Notch signaling 133, 147
  proneural expression 131–132
Entactins (nidogens), basal lamina 540
Entelechy concept, history 16
Enteric circuits, neural coding
  circuits
    complexity 628
Enteric ganglionitis (plexitis)
  neurotrophins and 632
Enteric inflammatory neuropathy *see* Enteric ganglionitis (plexitis)
Enteric nerves *see* Enteric nervous system
Enteric nervous system (ENS)
  anatomy/physiology *see* Enteric nervous system, anatomy/physiology
  definition 612
  development *see* Enteric nervous system, development
  disorders *see* Enteric nervous system disorders
  functional role 612
  neural coding *see* Enteric circuits, neural coding
  neurotrophic factors and 629*t*, 631
    developmental effects *see* Enteric nervous system, development
    disease role 630, 632
    expression patterns in adult gut 631
      neurotrophins 631–632
      receptors 632
    growth factors as 629
    neuropeptides as 629
    role in adult and aging gut 632
      gastrointestinal motility effects 632
      protective effects 632
  physiology *see* Enteric nervous system, anatomy/physiology
Enteric nervous system, anatomy/physiology
  complexity 628
  ganglia features 323–324
  neural circuits *see* Enteric circuits, neural coding
  neurochemistry
    neuropeptides *see* Enteric nervous system, neuropeptides
    serotonin role 629
  neurotrophin receptor expression 628
Enteric nervous system, development **341–347**
  autologous events 345
  BMP signaling 628–629
  differentiation 331
  disorders/defects 346, 630
    chronic idiopathic intestinal pseudo-obstruction 346
    dysplasia, genes implicated 325*t*
    Hirschsprung disease *see* Hirschsprung disease (HSCR)
    intestinal aganglionosis, mutations associated 612–613
    intestinal neuronal dysplasia 346
    nectrin effects 346–347
    NT3 346
  Hand2 345
  Hedgehog signaling 628–629
  motility control 341
  neural crest precursors 322
    glial cell line-derived neurotrophic factor 322–323
  neural crest regions 344, 344*f*
    chick embryo quail cells 344, 344*f*
  neurotrophic factors **628–633**
    adult ENA and 631
    glial-derived neurotrophic factor 345
    growth factors as 629
    neuropeptides as 629
    postnatal development role 630
      actions on enteric neurons and glia 631
      expression patterns in gut 631
      neural crest-derived stem cells 630–631
      neuropeptide expression and 630–631
    prenatal development role 629
      artemin 630
      BDNF 630
      developmental disorders 630
      GDNF 629
      knockout animals 629–630
      neuropoietic cytokines 630
      neurturin 629
      NGF 630
      NT-3 630
    receptor expression 628
    types found 629*t*
  nonautologous events 345
  organization 341, 342*f*
    central nervous system 341
    enterochromaffin cells 342, 342*f*
    interstitial cells of Cajal 341
    intrinsic primary afferent neurons 342
    vagal sensory fibers 341
  peristaltic reflex 341
  Ret 345
  secretory behavior control 341
  serotonin 342
  vagal neural crest 277, 278*f*
Enteric nervous system, neuropeptides
  biological actions
    enteric circuits *see* Enteric circuits, neural coding
    as neurotrophins 629
  gene expression changes
    developmental 630–631
  neural coding *see* Enteric circuits, neural coding
  types found
    PCAP
      developmental changes 630–631
    VIP
      developmental changes 630–631
Enteric nervous system disorders
  developmental disorders *see under* Enteric nervous
    system, development
  IBD *see* Inflammatory bowel disease (IBD)
  inflammatory neuropathies *see* Enteric ganglionitis (plexitis)
neurotrophic factors and 630, 632
Enteric neurons
  apoptosis *see* Apoptosis, autonomic nervous system
  cell number determination 610*t*
  myenteric 612, 613
  nervous system injury 613
Enteric neuropathy, inflammatory *see* Enteric ganglionitis (plexitis)
Enterochromaffin cells (ECs)
  ENS development 342, 342*f*

Entorhinal cortex (EC)
  anatomy/organization
    afferents
      axon guidance 390
Environmental factors
  retinal development 260
Ependymal cells
  growth factors 635
Ependymal glia *see* Ependymal cells
Ependymoglia *see* Ependymal cells
Ependymoglial cells *see* Ependymal cells
EphA4 gene/protein
  CST crossing at the midline 405, 406*f*
  retina anteroposterior axis development 126
EphA5 gene/protein
  retina anteroposterior axis development 126
EphA6 gene/protein, retina anteroposterior axis development 126
EphA7 gene/protein
  retina anteroposterior axis development 126
  retinal axon repulsion 368
EphA receptors 673–674
  optic tectum retinotopy development 93, 416–417, 417*f*
  retinal development 125
EphB1 gene/protein, ventrotemporal (VT) retina development 126
EphB2 gene/protein
  retina dorsoventral axis development 127–128
EphB3 gene/protein, retina dorsoventral
    axis development 127–128
EphB receptors 673–674
  optic tectum retinotopy development 93, 416–417
  PSD and 498
  retinal development 125
  trunk neural crest cells 279
Eph kinases, axon guidance, growth inhibition 395
  Sperry hypothesis 395
ephrinA2 gene/protein 366
  optic tectum 453
  retina dorsoventral axis development 128
  retinotectal projection 673–674
ephrin A5 gene/protein 366
  optic tectum 453
  retina anteroposterior axis development 126
  retinotectal projection 673–674
EphrinA ligands
  adult CNS 676–677
  optic tectum retinotopy development 93, 416–417, 417*f*
  retinotectal projection 673–674
ephrinB1 gene/protein
  retina dorsoventral axis development 127–128
  reverse signaling 367*f*, 369
  somite expression 279
ephrinB2 gene/protein
  DA neuron development 224
  retina dorsoventral axis development 127–128
ephrinB3 gene/protein
  axon guidance 398–399
  CST crossing at the midline 405, 406*f*
EphrinB ligands
  optic tectum retinotopy development 93, 416–417
  PSD and 498
Ephrins *see* Ephrins/Eph receptors
Ephrins/Eph receptors 673
  cell responses
    inhibitory (repulsive) guidance cues 381, 395, 673, 674*f*, 675*f*
      adult CNS 676
      model systems 673–674
      Sperry hypothesis 395
  classes 373
  Eph receptors 366, 381, 425, 673–674, 674*f*
    adult CNS 676
    DA neuron development, topographic mapping 224
    signaling 366
  glial-mediated synaptic stability and 570–571
  postsynaptic development role 490
  signaling 366
  trunk neural crest cells 279
  visual system development
    LGN gradients 417
    optic chiasm decussation decisions 429–430
    optic tectum retinotopy development 93, 416–417, 417*f*
    optic tract development 430–431
    retina development 125
Eph tyrosine kinases 381
Epiblast, differentiation 56
Epidermal ectoderm, as non-neural organizer of
    neural plate 107
  BMP antagonism and neural induction 107
  roof plate formation 107
Epidermal growth factor (EGF)
  adult neurogenesis and 650
  retinal development 266
  Schwann cells 634
Epidermal growth factor receptor (EGFR)
  signaling 135–136
Epidermis formation, BMP role 45–46
Epigenetic regulation
  adult NSC regulation 645–646
  chromatin modification *see* Chromatin remodeling
  DNA methylation *see* DNA methylation
Epilepsy
  etiologies
    cortical malformations 192–193
Epithalamus (habenula), development 116
Epithelial apposition, neural fold 5–6, 6*f*
Epithelial delamination, neural fold 5–6, 6*f*
Epithelial kinking, neural fold 5–6, 6*f*
Epithelial–mesenchymal transition (EMT), neural crest 271
  mediation 273–274
  timing, species differences 274
Epithelial ridging, neural fold 5–6, 6*f*
Epsin(s)
  DSL endocytosis 145
  interactions associated 145
'Equipotentiality'
  morphogen gradients and polarity 16
Erb4 (Her 4) receptors, thalamocortical axons 417
ErbB1 receptors *see* Epidermal growth factor receptor (EGFR)
ErbB3 (Her 3) receptors, Schwann cell development 307
ErbB receptor(s)
  AChR induction at vertebrate NMJ 547, 548*f*
  cancer 303–304
  conditional inactivation 303
  dimerization 298–299
  neural crest role *see* Neural crest
  neuregulin ligands 547
  NMJ-specific expression 550–551
  pathological conditions 303
  peripheral nervous system 303
  Schwann cells
    perisynaptic 563, 564
    precursors 299–300
      loss 300
      transgenic mouse studies 300, 301
  structure 298–299, 299*f*
ERK signaling *see* Extracellular signal-related kinase
    (ERK) signaling
ERNI (early response to neural induction) 11
  induction by FGF8 13–14
  as preneural marker 12–13
ESCRT proteins, Notch signaling 146
Estrogen(s)
  axon guidance and 357–359
  gene expression regulation 354–355
  neurogenesis and 357
  receptors *see* Estrogen receptor(s) (ERs)
  sexual differentiation *see* Sexual differentiation
  synaptic plasticity 668, 669
Estrogen receptor(s) (ERs)
  sexual differentiation and 359, 360*f*
  signaling pathways 359–360
Ets transcription factors, motor neuron specification
  dendrite patterning 190
  motor pool specification 187
  sensory neuronal inputs and 190
  soma migration and 189–190
Euchromatin 178, 179*f*
Eumelanin 312
Euscelis, morphogen gradients and polarity 17
even-skipped gene/protein
  cortical border formation 122–123
  Drosophila motor neuron identity and 522
  unc5 expression and 476
Evolutionary conservation
  cell-type specification mechanisms 590

Evx1 mutant mice, spinal cord development 174–175
Excitatory synapses 487, 496*f*
  glutamatergic *see* Glutamate/glutamatergic transmission
  postsynaptic membrane 495
    postsynaptic density *see* Postsynaptic density (PSD)
Excitatory-to-inhibitory shifts, GABA circuit development
  actions 573
  chloride transporters 574
  developmental stages associated 575
  giant depolarizing potentials 577
  hippocampus 575
  insult pathogenesis 579
  ontogenesis 579
  potassium/chloride cotransporter 2 574–575, 574*f*
Excitotoxicity
  molecular mechanism
    cell death pathways
      apoptosis *see* Apoptosis, nervous system injury
      autophagic neuronal death 618–619
  see also Excitotoxicity
Expectation maximization (EM) studies 524
Experimental colitis, neurotrophin effects 632–633
Extracellular matrix (ECM) **480–486**
  axonal growth/guidance *see* Axonal growth, extracellular matrix role
  developmental organizer signals and 85
  functional roles 538
  glycoproteins 399
  melanocyte development
    melanoblast migration and 318
    melanoblast specification and 315*t*
  neural crest cell migration 273–274
  presynaptic development and 502–503
  synapses 538
    CAMs and *see* Cell adhesion molecules (CAMs), synapses
    NMJ *see* Neuromuscular junction, basal lamina
Extracellular signaling, adult NSC regulation 644
Extracellular signal-related kinase (ERK) signaling
  synaptic plasticity 436–437
  see also MAP kinase signaling pathway
Extracellular space (ECS)
  sonic hedgehog signaling 23, 26*f*
  see also Extracellular matrix (ECM)
Extrinsic apoptotic pathway *see* Apoptosis
Eye(s)
  development *see* Eye development
  melanocytes 312
  as photoreceptors *see* Photoreceptor(s)
Eyeblink patterns, somatic marker of prenatal androgen exposure 352
Eye development
  DV axis inversion model 52–53
  eye fields 102
    gastrulation 102–103
    retinal development 266
    Shh-FGF8 feedback loop and 108
  gene expression
    Hes5 gene 162
    'master control genes' 102–103
  optic vesicles 102, 103
    retinal development 266
  retina *see* Retinal development
  signaling pathways
    BMP signalling 103
    canonical Wnt pathway 102–103
    hedgehog signalling 103
    nodal signalling 103
  specification and early development 101*f*, 102
Eye fields **125–130**
  see also Retinal development
Eye field transcription factors (EFTFs)
  retinal development 266
  Shh-FGF8 feedback loop and 108
Eye-specific segregation, NMDA receptors 440–441, 441*f*
Ezrin-moesin-radixin (ERM) proteins, growth cone migration 482

F

Familial amyotrophic lateral sclerosis (ALS)
  gene mutations
    SOD1 558–559
Fascia dentata, GABAergic synapses 575–576
Fasciclin II (FasII)
  axon guidance role 390
  Drosophila motor neuron target selection 523–524
Fasciclin III (FasIII), *Drosophila* NMJ development
  motor neuron identity and 522
  motor neuron target selection 524
  NMJ growth role 525
  size-activity relationship and 525
Fas/Fas ligand
  programmed cell death association 601
Fast axonal transport *see* Axonal transport
Fate mapping, retina anteroposterior axis development 125
Fat molecule, positional information 19
Female(s)
  sex-specific cognitive abilities 348
  sexual differentiation *see* Sexual differentiation
Fezl gene/protein
  corticospinal development 403, 404*f*
  temporal determinant of cortical cell-type specification 589–590
Fibroblast growth factor(s) (FGFs)
  developmental role
    AP patterning 80, 82–83
      anterior neural border induction 109
      Hox gene expression and motor neuron specification 75, 76
    DV patterning 33–34
    forebrain specification 100
      FGF8 role *see* Fibroblast growth factor 8 (FGF8)
      telencephalic 102
    mesoderm induction role 10
    motor neuron specification and 75, 76, 185
    neural crest induction 271–272, 312
    olfactory neurogenesis 232
    retinal development 253, 266
    synapse formation/maturation 514
  Hox gene regulation 64, 65
    signaling gradients 65
    tyrosine kinase receptors 64–65
  negative feedback regulation 83–84, 113
  neural injury and repair
    retinal regeneration 268
      progenitor regulation 252–253
  oligodendrocyte precursor generation 239
  receptors *see* Fibroblast growth factor receptors (FGFRs)
  signal transduction 83–84
    ECM coreceptors 85
    see also Fibroblast growth factor receptors (FGFRs)
Fibroblast growth factor 1 (FGF1)
  astrocyte differentiation 636
  radial glia differentiation 638
Fibroblast growth factor 2 (FGF2)
  adult neurogenesis and 650
  β-amyloid induction 639
  ENS role 629
  glial cells 639*t*
    astrocytes 635
      differentiation 636
    gliomas 638
    Müller glial cells 636
    radial glia differentiation 638
    Schwann cell differentiation 637
  neurodegenerative diseases 639
  neuronal survival 638
  olfactory neurogenesis 234
  oligodendrocyte precursor proliferation 241–242
Fibroblast growth factor 8 (FGF8)
  AP patterning and posteriorization 80, 82
    functional range
      endocytosis role 85, 85*f*
      HSPGs and 85
    retinal development 125
    rostral (posterior) midline *see midline specification/patterning (below)*
    signal transduction 83–84
  Hox gene expression regulation 64–65
  mesencephalic dopaminergic development 221, 224
  midbrain patterning 88, 89*f*
    genetic studies 88
    isthmic organizer and 82, 88, 88*f*
    midbrain-forebrain boundary 82
    midbrain-hindbrain boundary 80, 82, 85
    tissue competence and 83–84
  midline specification/patterning

ANB and rostral (posterior) midline 102, 109, 111, 112*f*
feedback regulation of Shh signaling 112, 112*f*
HPE associations 111
HPE phenotypes 111
sites and functions 111
dorsal midline defects and 113
as dorsal-ventral intermediary 114
FGF8-roof plate pathway 112, 112*f*
Shh-FGF8 feedback loop in 112, 112*f*
evidence 112
eye field signaling 113
negative regulation by BMPs 113
positive regulation by Six3 111, 113
neocortical arealization 121
commissural plate expression 120–121
transcription factor interactions 121
Sp8 120–121
neural induction in chicks 12–13
Churchill induction and 13–14
ERNI induction and 13–14
Sox3 induction and 13–14
olfactory neurogenesis 228, 232–234, 233*f*
Sox2 coexpression 230–231, 232–234
optic tectum 453
retinoic acid antagonism 58
signal transduction 83–84
ECM coreceptors and 85
Fibroblast growth factor 15 (FGF15), tectal-tegmental boundary formation 92
Fibroblast growth factor 18 (FGF18), olfactory neurogenesis 232–234
Fibroblast growth factor receptors (FGFRs)
Hox gene regulation 64–65
signal transduction 83–84
Fibronectin
binding partners 540
expression 484
growth cone migration 484
NMJ basal lamina 540
AChR aggregation and 540
receptors *see* Integrins
structure 484
Field concept, morphogen gradients and polarity 17
Filamin A (FLNA) gene/protein
cortical neuronal migration and 194
initiation role 194
Filopodia
axonal
growth cones 383, 384–385, 422
actin cytoskeleton 422
migration 480–481
dendritic spines
extension 512–513
extension
dendritic spines 512–513
'Fine-tuning'
visual development 433
Finger(s)
length, prenatal androgen exposure 352
movement control *see* Finger movements, control
Finger movements, control
corticospinal refinement and 410–411
see also Grasp/grasping
Fish(es)
axonal regeneration capability 679
myelin-based inhibitors 679
optic tectum, behavioral correlates 454
stratum marginal elements 449
teleost *see* Teleost fish
vision/visual system
retinotopic maps 434
Floor plate 29
DV patterning of neural tube 29, 74
forebrain development 100–101
midbrain patterning *vs.* 89
formation requirements 29
induction 21–22
midbrain (rFP)
induction 89, 89*f*
midbrain dopaminergic neuron formation and 91
midline glial cells, axon guidance 398
netrin-1 460
sonic hedgehog expression 21–22, 24*f*
Fluorescence-activated cell sorting (FACS)
adult NSC identification 643
FMRFamide gene expression 213, 215*f*
Focal adhesion kinase (FAK)
growth cone migration 482
Folding, neural plate *see* Neural plate
Follistatin, timing of expression and evidence against default model 11–12
Forebrain
development *see* Forebrain development
taste role
limbic forebrain 239
telencephalon *see* Telencephalon
Forebrain development 3, 27, 116, 239
compartmentalization models (lineage restriction) 83
'default model' of 106–107
diencephalon *see* Diencephalon
disorders, holoprosencephaly *see* Holoprosencephaly (HPE)
early **100–104**
AP patterning
'caudalization' 100
'organizers' and 100
'Wnt signalling' 100, 101–102
diencephalon 103, 116
DV patterning 100–101
eye 102
gastrulation 100
hypothalamus 101
key features 100, 101*f*
local signaling centers (organizers) 100
AP patterning and 100
DV patterning and 100–101
midline formation *see* Forebrain midline development
see also Developmental organizers
neural induction/caudalization 100
neural plate regionalization 100
telencephalon 102, 116
glial precursors 219
oligodendrocytes 239
hypothalamus *see* Hypothalamic development
midbrain boundary formation *see* Midbrain-forebrain boundary, neural patterning
midline formation *see* Forebrain midline development
non-neural *vs.* local (neural) organizers 105–106
prosomere (neuromere) model **95–99**, 103, 397
competing columnar models 96
definitions 95
historical aspects 96
re-emergence of 98
see also Brain development, models
telencephalon *see* Telencephalon
Forebrain-midbrain junction, development 88
Forebrain midline development **105–115**
disorders of *see* Holoprosencephaly (HPE)
local (neural) organizers at the midline 108, 108*f*, 109*f*
anterior neural border patterning 109, 109*f*
ANB induction 109
holoprosencephaly links 109
telencephalic specification/patterning and 109
Wnt antagonists/FGF signaling 102, 109
dorsal patterning 109
BMPs and Wnt signaling 109
dorsal midline induction 110
MIH holoprosencephaly and 110
roof plate induction 109, 113
dorsal-ventral patterning link (FGF8) 114
ventral patterning 108
holoprosencephaly pathogenesis 109
hypothalamic morphogenesis/induction 101, 101*f*, 108
Nodal/Shh 101, 109, 110, 112
molecular mechanisms/signaling pathways 110, 110*t*
HPE-related genes and identification 110
interactions (genetic networks) 112, 112*f*
FGF8 as intermediary between dorsal and ventral patterning 114
FGF8-roof plate pathway regulation by FGF8 113
holoprosencephaly phenotypes and 114
Nodal-Shh pathway in ventral midline 112
Shh-FGF8 pathway in ventral/rostral midline 112
Shh-FGF8 pathway regulation by BMPs 113
Shh-FGF8 pathway regulation by Six3 111, 113
Zic2-roof plate pathway in dorsal midline 113
pathways involved 110

Forebrain midline development (*continued*)
BMPs 107, 109, 111
FGF8 102, 109, 111
Nodal 101, 108, 109, 110
Shh 101, 108, 109, 110
Wnt signaling 101–102, 107, 109, 114
regulation via mRNA level 114
non-neural organizers of the neural plate 105, 107*f*
epidermal ectoderm 107
BMP antagonism in neural induction 107
roof plate induction 107
Hensen's node/AVE 106
anterior neural border specification 107
forebrain specification 106
neural induction 106
morphogen antagonists and 106
prechordal plate 108
classical holoprosencephaly link 108
formation 108
hypothalamic morphogenesis and 108
organizers
functional roles 105
neural *see above*
non-neural *see above*
timing 105
unanswered questions/future directions 114
Four-jointed molecule, positional information 19
FoxD1 gene/protein
retina AP axis development 125–126
ventrotemporal (VT) retina development 126
FoxD3 gene/protein
neural crest expression 272–273
Schwann cell development 307
FoxG1 gene/protein
retina anteroposterior axis development 125, 126
retina dorsoventral axis development 128
temporal determinant of cortical cell-type specification 589–590
Frizzled (Fz) proteins 39–40, 463
positional information 19–19
receptor complexes 39–40, 40–41
agonists 40
antagonists 40
Frizzled nuclear import (FNI) pathway 42–43, 43*f*
Frog(s)
ephrin B reverse signaling 369
Frizzled (Fz) proteins, optic chiasm decussation decisions 430
neuromuscular system
neuromuscular junctions 561, 562*f*
PSC ablation 563
PSC sprouting 565
topographic map development
ephrin B reverse signaling 367*f*
retinotopic map refinement 370
vision/visual system
optic tectum 454
retinotopic maps 434
Fruit fly *see Drosophila melanogaster*
F-spondin
trunk neural crest 279
Functional plasticity 667
Furrowing, neural plate 7
Fyn tyrosine kinase
postsynaptic development pathways 492

G

GABA (γ-aminobutyric acid) *see* GABA/GABAergic transmission
GABA, development role **573–581**
cortical networks, activity establishment 580
excitatory-to-inhibitory shift
actions 573
chloride transporters 574
developmental stages associated 575
giant depolarizing potentials 577
insult pathogenesis 579
growth cone attractant 675
NMDA receptor coactivation 573
primate neurons, developmental curve 578
proneural basic helix-loop-helix proteins 154–155
receptor establishment 579
synapse development
GABAergic *vs.* glutamatergic 575
paracrinic action 579
transmitter release 579
transport 579
trophic actions 579
$GABA_A$ receptor(s)
activation 573
synaptic functions
postsynaptic membrane 499–500
$GABA_C$ receptor(s)
postsynaptic membrane 499–500
GABA/GABAergic transmission 495
adult stem cell regulation 645
development role *see* GABA, development role
fast transmission 499–500
interneurons 573, 576
synapse formation function 390
maturation 573
nonprincipal cells *see* Interneuron(s)
receptors *see* GABA receptor(s)
slow transmission 499–500
synapses/synaptic functions
fascia dentata 575–576
postsynaptic currents (PSCs) 575–576, 580–581
pyramidal neurons 576
see also Inhibitory synapses
GABA receptor(s)
antagonists 573
seizure induction 575–576
$GABA_A$ *see* $GABA_A$ receptor(s)
$GABA_C$ *see* $GABA_C$ receptor(s)
ionotropic and fast transmission 499–500
metabotropic and slow transmission 499–500
Galleria, morphogen gradients and polarity 17
Gallus domesticus see Chicken(s)
γ-aminobutyric acid *see* GABA/GABAergic transmission
Gangliogenesis 333, 334*f*, 335*t*
Ganglion cell layer (GCL) 259
see also Retinal ganglion cells (RGCs)
Ganglion mother cell
cell-fate of daughter cells 593
production from asymmetrical neuroblast division 592
GAP-43 *see* Growth-associated protein-43 (GAP-43)
Gap junction(s)
neuromuscular junction *see* Neuromuscular junction (NMJ)
NMDA receptors 439–440
Gastrointestinal motility
neurotrophin effects 632
Gastrulation 55, 56
developmental organizers 81*f*
see also Developmental organizers
forebrain specification 100
eye fields 102–103
hypothalamus 101
initiating signals 14
neural induction and 12–13, 14
neurulation *see* Neurulation
Gata2 transcription factor
autonomic nervous system development 338–339
gangliogenesis 333–335
Gata-3, hair cell differentiation 246
Gbx1 gene/protein, AP patterning and 80
Gbx2 gene/protein
optic tectum 453
retina anteroposterior axis development 125
gdf11 gene/protein
Hox gene expression and 65
knockout animals 254
retinal development 254
GDNF *see* Glial cell line-derived neurotrophic factor (GDNF)
GDNF family ligands (GFLs)
GFRα coreceptors *see* GFRα
RET *see RET* gene/protein
Ret activation 608–609
Geminin
cell cycle regulation 596
cell fate/differentiation role 596
Gene Expression Nervous System Atlas (GENSAT)
molecular anatomy of mammalian brain 201, 201*f*
Gene-specific regression 178
Genetic networks, forebrain midline development 112, 112*f*

Genetic studies
  adult NSC identification 644
Genomics Institute of the Novartis Research Foundation (GNF) 202–203
GENSAT (Gene Expression Nervous System Atlas) *see* Gene Expression Nervous System Atlas (GENSAT)
Gfi1, hair cell differentiation 249
GFRα
  autonomic gangliogenesis 335
  c-ret complex and GDNF binding 608–609, 608*f*, 611, 628
  enteric nervous system 628
    adult expression 632
    postnatal expression 631
    prenatal expression 629
  parasympathetic axon outgrowth 336
GFRα2
  enteric expression
    adult gut 632
    postnatal 631
    prenatal 629–630
  neurturin (NTN) receptor 629–630
GFRα3, artemin receptor 630
GGF-1, Schwann cell differentiation 637
Gb6 gene/protein, retina anteroposterior axis development 126
Giant depolarizing potentials (GDPs)
  characteristics 577
  kinetics 577–578
  neural circuit development
    excitatory-to-inhibitory shifts 577, 578–579
    GABA 577, 577*f*
    propagation, hippocampal preparations 577
GKAP/SAPAP protein family
  postsynaptic density 497
Glass bottom boat (Gbb), retrograde signaling in *Drosophila* larval NMJ 526–527, 526*f*
Glia *see* Glial cells (glia)
Glial cell line-derived neurotrophic factor (GDNF)
  autonomic nervous system
    enteric nervous system *see* Glial cell line-derived neurotrophic factor, ENS role
    gangliogenesis 335
    parasympathetic 611–612
      axon outgrowth 336
      neuronal survival and 336–337
  developmental role
    axonal growth/guidance
      parasympathetic axon outgrowth 336
      Ret signaling 611
    DA neuron development 222, 225–225
    as guidance cue 673
    neuronal cell type specification 216
  glial cell production 639*t*
    gliomas 638
    Schwann cells 634–635
  neurodegenerative disease and 639
  peripheral nerve regeneration 640
  receptor complex 628, 629
    see also GFRα; *RET* gene/protein
  as survival/neuroprotective factor
    ENS neuropathy prevention 632
    ROS-induced apoptosis and 632
  synapse elimination at the NMJ 557–558
  therapeutic use/gene therapy
  see also GDNF family ligands (GFLs)
Glial cell line-derived neurotrophic factor, ENS role 612–613
  adult expression 631–632
  development and 322–323, 345, 629, 629*t*
    Hirschsprung disease and 628
    postnatal expression 631
  neuroprotection
    neuropathy prevention 632
    ROS-induced apoptosis and 632
Glial cell line-derived neurotrophic factor-related ligands (GFLs) *see* GDNF family ligands (GFLs)
Glial cells (glia)
  astrocytes *see* Astrocyte(s)
  axon guidance *see* Axonal guidance, glial cells and
  development
    differentiation 238
    enteric nervous system 344
    gliogenesis *see* Gliogenesis
    precursors
      macroglial lineages 219
      protein expression 219–220
  growth factors *see* Glial growth factors (GGFs)
  microglia *see* Microglia
  neuromodulatory functions *see* Glial cells, neurotransmission modulation
  olfactory system 226
  oligodendrocytes *see* Oligodendrocyte(s)
  phagocytosis, synapse elimination and 569
  production *see* Gliogenesis
  synaptic functions *see* Glial cells, synaptic functions
  transplantation/replacement 689
  tumors
    growth factors and 638
Glial cells, neurotransmission modulation 570
  cytokines and 570
  heterosynaptic depression and 570
  transmitter/neuromodulator release 570
    ATP/adenosine release 570
    D-serine release
      NMDAR-mediated LTP 570
    glutamate release 570
Glial cells, synaptic functions **567–572**
  astrocytes 567, 568*f*
    ablation effects 571
    synapse formation/maturation 514, 569*f*, 571
    synaptic stability and 570, 571
    synaptic transmission and *see* Glial cells, neurotransmission modulation
  cytokines and
    microglial 570
  microglia
    cytokines and plasticity 570
  neurotransmission modulation *see* Glial cells, neurotransmission modulation
  at the NMJ *see* Perisynaptic Schwann cells (PSCs)
  Schwann cells *see* Perisynaptic Schwann cells (PSCs)
  synapse formation/maturation 514, 567
    contact role 568
    GABAergic hippocampal neurons 568
    PSCs and NMJ formation 567–568
    RGC synaptogenesis 514, 567, 569*f*
    secreted factors 568
      Apo-E-cholesterol 514, 568
      thrombospondins 514, 568
    synapse induction in multiple neuronal classes 567
  synapse location 570
  synapse pruning/elimination 569
    Drosophila development 569
    PSCs and NMJ development 558, 569–570
  synapse structure/stability 570
    Eph receptors/ephrins and 570–571
    glial ablation effects 571
    hormonal responses and 571
  synaptic sprouting 668, 670
  synaptic strength/plasticity *see* Glial cells, neurotransmission modulation
  'tripartite synapse' 567, 568*f*
Glial cell transplantation 689
Glial fibrillary acidic protein (GFAP)
  adult NSC identification 643
  aging and
    synaptic plasticity 669–670
  astrocyte differentiation 636
  synaptic plasticity 666–667
Glial growth factors (GGFs) **634–640**
  aging 639
  Alzheimer's disease 639
  axonal regeneration 639–640
  biological roles 636, 637*f*
  brain tumors and 638
  CNS neural regeneration 639
  definition 634
  in development 638
  myelination control 242
  neurodegenerative disease 639
  peripheral nerve regeneration 640
  puberty 639
  radial glia differentiation 638
Glial replacement therapy 689
Glial sling 393
Glioblastoma multiforme (GBM) 638
  PDGF 638
Glioblastomas *see* Glioblastoma multiforme (GBM)

Gliogenesis
  adult CNS, macroglial lineages 219
Glioma(s) 638
  FGF-2 638
  GDNF 638
Gli3 protein
  DV patterning of the neural tube 36–37
  sonic hedgehog repressor activity 23*f*, 25
Gli proteins
  AP patterning of the neural tube 84
  DV patterning of the neural tube 36, 37*f*
    Gli1 36
    Gli2 36
    Gli3 36–37
  mesencephalic dopaminergic development 221
  sonic hedgehog transduction 23*f*, 24–25, 26*f*, 36, 84, 221
Global ischemia *see* Ischemic stroke (cerebral ischemia)
Glucocorticoid(s)
  glial cells and
    Schwann cell proliferation 307
Glutamate *see* Glutamate/glutamatergic transmission
Glutamate excitotoxicity *see* Excitotoxicity
Glutamate/glutamatergic transmission
  adult NSC regulation 645
  dysregulation/dysfunction 454
    excitotoxicity *see* Excitotoxicity
  excitotoxicity *see* Excitotoxicity
  glial cells
    astrocytes
      release and neuromodulation 570
  neurotoxicity *see* Excitotoxicity
  neurotransmitter role
    peripheral transmitter evidence 454
  optic tectum 453
  receptors *see* Glutamate receptor(s)
  synapses
    formation 513–514
    organization 496*f*
      PSD *see* Postsynaptic density (PSD)
Glutamate neurotoxicity *see* Excitotoxicity
Glutamate receptor(s)
  activity-dependent maturation
    'silent' synapses 456–457, 457*f*, 491
  clustering at the PSD 495
    AMPA receptors 495
    NMDA receptors 495
    scaffolding/adaptor proteins
      AMPA receptor-linked 497
      NMDA receptor-linked 496
      Shanks 497
Glutamate receptor-interacting protein (GRIP)
  AMPA receptors and
    scaffold protein 497
Glutamatergic neurons *see* Glutamate/glutamatergic
    transmission
Glutamatergic synapses *see* Glutamate/glutamatergic transmission
Glutamatergic transmission *see* Glutamate/glutamatergic transmission
Glycoprotein(s)
  extracellular matrix 399
Glycosylphosphatidylinositol (GPI) anchor, immunoglobulin superfamily
    and axon guidance 394–395
Glycosylphosphatidylinositol-linked cell surface receptor α(GFRα)
  see GFRα
Goldfish
  CNS regeneration capability 679
Golgi apparatus
  synaptic vesicle production 507
Golgi epithelial cells *see* Bergmann glia
Gonadal hormone(s) 348
  sexual differentiation *see* Sexual differentiation
  synaptic plasticity and sprouting 667
Gonadotropin-releasing hormone (GnRH)
  synaptic plasticity and 667–668
G-protein(s)
  G_{olf} (olfactory specific) 418–419
    mutations 418–419
  receptor-linked *see* G-protein-coupled receptor(s) (GPCRs)
G-protein-coupled receptor(s) (GPCRs)
  olfactory (odorant) receptors 227
Gradient-determined repulsion, AP mapping 368
Gradient guidance *see* Axonal guidance cues
Grasp/grasping
  corticospinal refinement and 411, 412*f*
  see also Reaching
Grasshoppers
  guidepost cells 387
Green fluorescent protein (GFP)
  adult NSC identification 644
  retinal ganglion cell generation 260
Grg-4, optic tectum 452*f*
GRIP *see* Glutamate receptor-interacting protein (GRIP)
GRO-alpha, oligodendrocyte precursor proliferation 241
Groucho/TLE cofactor, spinal cord development, cross-repression 174
Growth and differentiation factor 7 (GDF7)
  BMP7 heterodimers 462
  knockout mutants 462
Growth and differentiation factor 11 (GDF11)
  olfactory neurogenesis 234, 235*f*
  RGC generation 260–261
Growth and differentiation factors (GDFs)
  as axon guidance cue 672
  signaling 672
Growth-associated protein-43 (GAP-43)
  axonal regeneration 678–679
  overexpression 678–679
Growth cones *see* Axonal growth cones
Growth cone turning assay 461
Growth factor(s)
  adult neurogenesis and 650
  development role
    cardiac neural crest 277
    melanoblast specification and 315*t*
    retinal development 266
  ENS actions 629
  glial *see* Glial growth factors (GGFs)
  receptors
    melanoblast specification and 315*t*
  see also Neurotrophin(s)
Gsh gene expression, BMP signaling 46–47
Guanine nucleotide exchange factors (GEFs)
  NMDA receptors 448
Guanylate kinase-associated proteins (GKAPs)
  PSD-95 interactions 490
Guidance cues (axonal growth) *see* Axonal guidance cues
Guidepost cells **387–392**
  features 387
  historical studies 387
    cell ablation 387
  midline 388
    adhesion 389
    attraction 388
    commissural axons 388
    netrins 388–389
    repulsion 389
  synapse formation function 390
    Caja-Retzius cells 390
      cell lesion experiments 390
    GABAergic interneurons 390
    visual cortical circuit maturation, subplate neurons 390
    vulval epithelial cells 391
Gustatory nuclei *see* Gustatory system(s)
Gustatory system(s)
  central pathways (vertebrate)
    forebrain contribution
      limbic forebrain 239
Gut *see* Gastrointestinal tract

H

Hair cell differentiation **245–250**
  Barhl1 249
  cell cycle exit 247
    cyclin-independent kinase inhibitor role 247
      $p19^{ink4A}$ 247
      $p27^{kip1}$ 247
  cell survival 248
    myosin-VI 248–249
    myosin-VIIa 248–249
  commitment of hair cells 247
    Atoh1 role 247
      HES1 overexpression effects 247–248
    transdifferentiation 248
  Gfi1 249

inhibitors of differentiation 248
Pou4f3 249
prosensory patches 245
prosensory specification 245
BMP4 246
Gata-3 246
Lfng 246
notch signaling role 245
jagged1 245
Notch1 245
serrate expression 245
Pax2 246
signaling molecules 245, 246*f*
SOX2 246
retinoic acid signaling 249
thyroid hormone signaling role 249
prestin effects in hypothyroid animals 250
Hairy/enhancer of split genes *see Hes* genes/proteins
Hand2, ANS development role 338–339
deletion effects 326
enteric neurons 345
gangliogenesis 333–335
Harmonious equipotential system, morphogen gradients and polarity 16
HB9 (Hb9) transcription factor
Drosophila motor neuron identity and 522
spinal cord development 174–175
Hearing loss
melanocyte depletion and 312
Hedgehog (Hh) signaling pathway 29
cell cycle interactions 597
cell fate specification 465
chemoattractants 465
enteric nervous system development 628–629
eye development 103
morphogen gradients and positional information 18–19
posteriorization and 81–82
hypothalamic development and 101, 108
ZLI and 81–82, 82–83, 84
posttranslational modification 34
retinal progenitor cell regulation 252–253
tissue patterning 465
BMP/Hh signaling 46
Hedgehog-interacting protein (Hip1) 34
neural patterning role 24
Shh signaling and DV patterning 34, 35*f*
Helix-loop-helix proteins, basic *see* Basic helix-loop-helix (bHLH) transcription factors
Hemichordates
BMP phylogeny 51–52
nervous system
organization 51–52
Hemiplegia
activity-dependent refinement of the CST 409–410, 411*f*
rehabilitation and 413–414
Hensen's node 11
'caudalizing signals' 184
as non-neural organizer of neural plate
ANB specification 107
forebrain specification 106
neural induction 106
neural plate induction 3
Heparan sulfate proteoglycans (HSPGs)
axon guidance 400
FGF coreceptors 85
NMJ basal lamina 540
agrin 541
collagen XVIII 541
perlecan 541
unique features 540–541
Wnt protein transport 39
Hepatocyte growth factor/scatter factor (HGF/SF)
Alzheimer's disease 639
as guidance cue 673
Herceptin 304, 304*f*
Hermansky-Pudlak syndrome (HPS), optic pathway development 431–432
Hermaphroditic specific neurons 391
Herrick, CJ
columnar model of brain development 96–97
Hes1 gene/protein
boundary formation 162
expression 156–157, 158*f*, 159–160, 159*f*, 163*f*
proneural bHLH implications 162–163
eye formation 162
retinal specification 262
Müller glial cells 262
hair cell differentiation 247–248
Mash1 gene 156
mechanisms 157*f*
neural stem cell maintenance 160
neuroepithelium development 160–161, 162*f*
structural defects 161*f*
Notch signaling 147–148
spinal cord development 175
Hes3 gene/protein 156
expression 159–160
Hes3a 156
Hes3b 156
neural stem cell maintenance 160
Hes5 gene/protein
boundary formation 162
expression 156–157, 159–160, 159*f*
eye formation 162
Müller glial cells 262
neural stem cell maintenance 160, 161–162
Hes6 gene/protein 156
Hes7 gene/protein 158
Hes genes/proteins **156–164**
boundary formation regulation 162
CNS development 163*f*
factors 156
features 157*f*
gene expression regulation 156
Deltalike (*Gll*) 159–160
Notch1 159–160
notch signaling 156–157, 157*f*
nervous system development 158
expression patterns 159*f*
radial glial cells 158–159
neural stem cell maintenance 160
bHLH genes 160
radial glial cells 160
retinal development 266–267
structure 156
E-box binding 156
transcriptional activity 156
Heterochromatin 178, 180*f*
Drosophila melanogaster 181
Heterosynaptic long-term depression (LTD)
astrocytes and 570
Higher vocal center (HVC)
sex steroid effects on axonal growth/guidance 357–359
High-voltage activated (HVA) calcium channels *see* Voltage-gated calcium channels (VGCCs)
Highwire *see* PHR (Pam/Highwire/RPM-1)
Hindbrain
development 3, 116
AP patterning, retinoic acid and 56
rhombomeres 396
Hip1 gene/protein
expression 461, 469
sonic hedgehog binding 469
Hippocampal damage, stress-related
acute stress
neurogenesis and 648–649
Hippocampal formation *see* Hippocampus
Hippocampal long-term potentiation (LTP)
mechanisms
glial cells and 570
NMDA receptors and
D-serine and 570
Hippocampus
adult stem cells/neurogenesis *see* Neurogenesis, adult
age-related changes
neurogenesis 663
anatomy/physiology
neurons
postsynaptic differentiation 488
postsynaptic differentiation, proteins 488–489
stress-induced changes *see* Hippocampal damage, stress-related
pyramidal cells *see* Pyramidal neuron(s)
dentate *see* Dentate gyrus
development
guidepost cells 390
disease/dysfunction

Hippocampus (*continued*)
glucocorticoid-induced *see* Hippocampal damage, stress-related
ischemia/infarction
adult neurogenesis and 651
see also Stroke
seizures 579
stress-related *see* Hippocampal damage, stress-related
excitatory-to-inhibitory shift 575
learning role *see* Hippocampus, learning and memory role
memory role *see* Hippocampus, learning and memory role
neurogenesis (adult) *see* Neurogenesis, adult
Hippocampus, learning and memory role
adult neurogenesis and 646
Hirschsprung disease (HSCR) 322, 346, 347
GDNF/ret signaling role 628, 630
gene defects 278
c-ret mutation 628
His, Wilhelm, neural model 96, 98*f*
Histone(s) 178
H1 178
H2A 179*f*
H2B 179*f*
H3 179*f*
cocaine effects 181
H4 179*f*
cocaine effects 181
modification
neuroactive drug effects 181
transcriptional silencing 178
replacement, transcriptional silencing 179–180
Histone acetyltransferase(s) (HATs)
transcriptional silencing 178–179
Histone deacetylase(s) (HDACs)
transcriptional silencing 178–179
HNF3β (transcription factor) 47
Holoprosencephaly (HPE) 27, **105–115**, 467
ANB patterning and 109
classical 105
ANB role 109
Noggin/Chordin mutants 112
prechordal plate role 108
local organizers *vs.* 109
subtypes 105
definitions/classification 105
genetics
Noggin/Chordin mutants 112
incidence 105
middle interhemispheric (MIH) 105, 106*f*
dorsal patterning and 110, 114
Noggin/Chordin mutants 112
molecular mechanisms 110, 110*t*
HPE genes 110
signaling pathways 110
BMPs 111
FGF8 111
Nodal 110
Shh 110
Tgif role 110, 114–115
Wnts and 114
neuropathology 105, 106*f*
phenotypes 105, 106*f*
prechordal plate role 108
ventral patterning and 109
scientific fascination with 105
unanswered questions/future directions 114
Homeobox (homeoitic) genes *see* Hox genes/proteins
Homeodomain-encoding genes/proteins *see* Hox genes/proteins
Homeoitic (homeobox) genes *see* Hox genes/proteins
Homer proteins 497
coiled-coil (CC-Homer) 497
PSD-95 interactions 490
Homo sapiens see Human(s)
Homosexuality, prenatal androgen exposure and homosexuality in women 352
Horizontal cells (retina)
bipolar cell connections *see* Bipolar cells (retina)
development/specification
Math3 263
Pax6 263
Prox1 263
Six3 263
distribution 259
generation 259, 261*f*
in retina development 263
Hormone(s)
astrocyte-synapse relationship and 571
Horner's syndrome
injury-induced developmental plasticity and 336, 337*f*
House mice *(Mus musculus) see* Mice
Hoxa1 gene, function 61–62
Hoxb1 gene
function 61–62
induction 56–57
RARE associations 64, 65*f*, 66*f*
regulation 56–57, 67
Hoxb3 gene, activation 64
Hoxb4 gene
activation 64
competition 68–69
expression 65
posttranscriptional regulation 69
Hoxb8 gene, expression 66–67
Hox genes and motor neuron specification 61–62
anteroposterior (AP) patterning 74, 77, 185
mechanisms 77
motor columns 74–75
motor pools 74–75, 77
sequential activation by morphogens 75
cross-inhibitory interactions
motor column specification 76
motor pool identity 78, 185
downstream pathways 78
LIM proteins *see* LIM homeodomain transcription factors
motor columns 75, 75*f*
AP axis 74–75
cross-inhibitory actions 76
FGF signaling 76
molecular markers 75–76
motor pool identity 76, 187
AP specification and 77
cross-inhibitory interactions 78, 185
early expression patterns 76
intrasegmental diversity and 77
lineage analysis 77–78
mechanisms 77, 78
motor axon connectivity and 77
organizational features and 76, 77*f*
positional boundaries and 77
spinal circuitry and 78–79
unanswered questions 78
Hox genes/proteins
Antennapedia (Antp) gene mutation 61, 62*f*
anteroposterior (AP) patterning 61, 64, 70
spinal cord transcriptional networks 176
cell fate determination
LIM proteins *see* LIM homeodomain transcription factors
motor neuron specification *see* Hox genes and motor neuron specification
Shh signaling in neural tube 31
chromosomal arrangement and spatial colinearity 61, 69, 74*f*, 75
gene clusters 61, 62*f*, 68–69
definition 61
expression 61, 62*f*, 63*f*, 177
initiation 70
function 61
pharmacological studies 61–62
segmental identity 61–62
hindbrain neural patterning 56–57
interneuron specification 78–79
midbrain-hindbrain boundary formation 84
neural tube development 21
paralogs, cofactor binding 67–68
regulation **61–71**
competition 68
global regulatory elements 69
experiments 69
posttranscriptional 69
miRNAs 69
sharing 68
signaling pathways associated 64, 65
fibroblast growth factor *see* Fibroblast growth factor(s) (FGFs)
retinoic acid *see* Retinoic acid (RA)

    spinal cord transcriptional networks 176
    transcription factors 65
      autoregulation 67
      cofactors 67
      crossregulation 67
      initiation *vs.* maintenance 68
  retinal development 261, 266
  sensory neuron specification 78–79
  spinal cord development 174
    dorsoventral patterning 57
    MN specification *see* Hox genes and motor neuron specification
    transcriptional networks 176
Hox responsive elements (HREs) 67, 68
Hrs proteins, Notch signaling 146
5-HT *see* Serotonin (5-HT)/serotonergic neurons
5-HT receptors *see* Serotonin (5-HT) receptor(s)
Human(s)
  brain *see* Human brain
  ocular stem cells, ciliary epithelium stem cells 269
Human brain
  adult hippocampal neurogenesis 651
    see also Neurogenesis, adult
  volume/size *see* Brain volume/size
Hunchback, cell-type specification 590
  cell cycle and 597
Huntingtin gene/protein
  RE1-silencing transcription factor (REST) and 209
Huntington's disease (HD)
  huntingtin *see* Huntingtin gene/protein
  pathology/pathogenesis
    autophagy and 619
    see also Huntingtin gene/protein
  therapy
    cell therapy
      fetal neural tissue grafting 689
    neurotrophins 626
HVC *see* Higher vocal center (HVC)
Hydra, morphogens and positional information 17–18
5-Hydroxytryptamine (5-HT) *see* Serotonin (5-HT)/serotonergic neurons
Hyperglycemia
  enteric neuropathy and 632
Hypoblast, neural induction role 12–13
Hypomyelinating neuropathies, congenital 309
Hypopigmentation
  optic chiasm decussation decisions 430
Hypothalamic development
  specification and early development 101
    BMP signaling and 101–102
    gastrulation 101
    local organizers 100–101, 101*f*, 108, 110
    nodal/Shh signaling 108
    nodal/Shh signalling 101
    prechordal plate role 108
      classical holoprosencephaly and 108
    transcription factor expression 102
    Wnt antagonism 101–102
Hypothalamus
  development *see* Hypothalamic development
  sex differences
    sexually dimorphic nuclei 352, 355, 357–359, 358*f*
      dendritic morphology 359
    see also Sexual differentiation
  synaptic plasticity, sex hormone effects 667
Hypotheses
  paradigms *vs.* 95–96
Hypoxia-ischemia (HI)
  autophagic neuronal death 618–619

I

Id-family proteins, spinal cord development 175
Imaging studies *see* Neuroimaging
Immunoglobulin cell adhesion molecules (IgCAMs)
  expression 482
  growth cone migration 482, 485
    ERM proteins 482
    L1 485
    receptor regulation 482–483
Immunoglobulin superfamily
  axon guidance 394
    domains 394–395
    glycosylphosphatidylinositol anchor 394–395
    neurite outgrowth 395
    signal transduction 395
ind gene/protein, neuroectoderm signaling 49
  associated gene repression 49–50, 49*f*
Infantile pyloric stenosis
  neurotrophins and 630
Inflammatory bowel disease (IBD)
  neurotrophic factors and 632–633
  see also Crohn's disease; Ulcerative colitis
Inhibitors of differentiation (Ids) 248
Inhibitory synapses 487
  GABAergic transmission *see* GABA/GABAergic transmission
  postsynaptic membrane 495, 499
  see also GABA/GABAergic transmission
Injury
  brain trauma *see* Traumatic brain injury (TBI)
  nervous system *see* Nervous system injury
Inner ear 245
  auditory components *see* Cochlea
  melanocytes 312
Inner nuclear layer (INL) 259
Inner plexiform layer (IPL) 259
Inosine, central nervous system plasticity 684–685
in situ hybridization (ISH)
  molecular anatomy of mammalian brain 200
Insulin-like growth factor-1 (IGF-1)
  adult neurogenesis and 650
  CNS neuron regeneration 639
  glial cells 639*t*
    astrocytes 635
    Bergmann glial cells 636
    oligodendrocytes
      differentiation 637
$\alpha 3\beta 1$ Integrin, netrin binding function 477–478
$\alpha 6\beta 4$ Integrin, netrin binding function 477–478
Integrin(s)
  actin cytoskeleton and 542
  axon growth/guidance 395
    growth cone migration 422, 484
  cytoplasmic domains 482
  expression 482
  kinase regulation by 542
  melanoblast migration and 318
  netrin binding 477–478
  subunits/heterodimer formation 542
  synapses *see* Integrins, synaptic functions
Integrin adhesion receptor(s), growth cone migration 482
  binding specificity 482
β_1 Integrins
melanoblast migration and 318
Integrins, synaptic functions
  glial-mediated synaptogenesis 568
  neuromuscular junction
    postsynaptic basal lamina 542
      α7 subunit and 542
      intracellular signaling 542
    presynaptic role 542
  postsynaptic density and 498
Interkinetic nuclear migration
  cortical cell-type specification 585
  neural plate shaping 7
  neural progenitors 585
  retinal progenitor cells (RPCs) *see* Retinal progenitor cells (RPCs)
Interleukin-1β (IL-1β)
  β-amyloid induction 639
Internal capsule (IC)
  thalamocortical projections 417
Interneuron(s)
  cortical 585
    development 117
  development
    cortical 117
    Hox gene specification 78–79
    spinal cord
      DV patterning 29, 30*f*
Interphase DNA, transcriptional silencing 180
Interstitial cells of Cajal (ICCs)
  enteric nervous system development 341
Interstitial collateral axon branching *see* Axon branching

Intestinal aganglionosis, mutations associated 612–613
Intestine(s)
  neuronal dysplasia 346
Intracellular calcium *see* Calcium ions (Ca^{2+})/calcium signaling
Intracellular signaling
  adult NSC regulation 645
  see also Signaling pathways
Intracellular sorting
  neurotrophins 622
Intraflagellar transport (IFT), retinal development 256
Intraretinal output, intrinsically photosensitive RGCs *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
  retinal patterning 129–130
Intrinsic excitability
  neuronal homeostasis *see* Neuronal homeostasis
Intrinsic primary afferent neurons (IPANS), enteric nervous system development 342
Invertebrate(s)
  development
    midbrain patterning 93
  GPCRs *see* G-protein-coupled receptor(s) (GPCRs)
  neuromuscular junctions *see* Neuromuscular junction, invertebrate
ipRGCs *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
Iris
  melanocytes 312
Iroquois (Irx) transcription factor(s)
  DV neural tube expression 32*f*
    cross repression 33
  midbrain patterning 88
    midbrain-forebrain boundary, ZLI and 80–81
    midbrain-hindbrain boundary 84–85
Irritable bowel syndrome (IBS)
  5-HT_3 receptor
    antagonist effects 343
  5-HT_4 receptor
    agonist effects 343–344
IRSp53, postsynaptic density 499
Irx2 transcription factor (iroquois), midbrain-hindbrain patterning 84–85
Irx3 transcription factor (iroquois)
  DV neural tube expression 32*f*
    cross repression 33
  hypothalamic specification/development 102
  midbrain patterning 88
    midbrain-hindbrain boundary 84–85
  retina anteroposterior axis development 125
Ischemic stroke (cerebral ischemia)
  autophagic neuronal death 618–619
  global
  RE1-silencing transcription factor 208
ISL1 transcription factor
  oculomotor complex patterning 91
  spinal cord development 174–175
Islet transcription factor, *Drosophila* motor neuron identity and 522
Isolated lissencephaly sequence (ILS) 196
Isthmic organizer 87
  establishment 87, 88*f*
    OTX2 expression and 87–88
  interaction with other midbrain organizers 89
  optic tectum retinotopy and 93
  signaling
    FGF8 and 82, 85, 88, 88*f*
    tissue competence and signal integration 83–84
    transcription factor expression 88, 88*f*
Isthmo-optic nucleus, autophagic cell death 618
Isthmus, Fgf8 expression 162

J

Jacobson's organ *see* Vomeronasal organ (VNO)
Jagged (Notch ligand)
  hair cell differentiation 245
  oligodendrocyte precursor differentiation 242
Jaw muscles
  proprioception, development 92
Johnston, J B
  columnar model of brain development 96–97
c-Jun gene/protein
  axonal injury/regeneration and 678–679
  Schwann cell development 307, 309
c-Jun N-terminal kinase (JNK) signaling pathway
  programmed cell death association 600–601

K

Kainate
  seizure induction 579
Kemp echoes *see* Otoacoustic emissions (OAEs)
Keratan sulfate proteoglycans (KSPGs)
  axon guidance 400
  structural organization 400–401
Kinase(s) *see* Protein kinase(s)
Kinesin(s)
  axonal transport 493
  postsynaptic development, KIFs 493
Kip1 (p27), cell-fate determination 594
Kip2 (p57), cell-fate determination 594
c-KIT
MAP kinase signaling 314
KIT ligand *see* Stem cell factor (SCF)
Knockout animal(s)
  atoh7 gene/protein 254
  developmental studies
    neocortical arealization 119, 120
    NMJ
      agrin/MuSK 542, 546, 546*f*, 548–549
      ERBB receptors 547, 548*f*
      NRG1 547
    Rb knockouts and neural development 596
    retina development
      BMP4 gene/protein 127
      FoxD1 gene/protein 126
      Shh and RGC generation 260–261
  enteric nervous system
    neurturin knockouts and development 629–630
  gdf11 gene/protein 254
  growth and differentiation factor 7 (GDF7) 462
  laminin β_2 knockout mice 540
  Math5 (Atoh7) gene/protein 254
  neurodegenerative disease
    myelination disorders, *Olig1* knockouts 240
  neurotransmission
    Munc-13 517
  nicotinic acetylcholine receptors, retinotopic maps 434
  serotonin transporter *see* Serotonin transporter (5-HTT/SERT)
  synaptogenesis
    TSP and glial-mediated 568
Kreisler (transcription factor), *Hox* gene expression 66, 68
Krox 20 (transcription factor)
  Hox gene expression 66
  Schwann cell development 307, 308–309
    myelination regulation 309
Kruppel gene/protein, cell-type specification 590
  cell cycle and 597

L

L1-family cell adhesion molecules (L-CAMs)
  corticospinal axon midline crossing 404–405, 405*f*
  growth cone migration 485
    axonal regeneration 486
  RE1 activator role 211
Lag-2 *see* DSL (Delta, Serrate, Lag-2) ligand family
Lamellipodia
  growth cone migration 422, 480–481
Laminin(s)
  distribution/expression pattern 483–484
  growth cone migration 483
  NMJ 539
    extrajunctional *vs.* synaptic cleft 540
    retrograde signaling in formation 509
  receptors *see* Integrins
  structure 472, 473*f*, 483–484
  subunit structure 539–540
Laminin-1, axonal growth/guidance 399
  growth cone migration 482, 484
Laminin α_1, retinal development 255
Laminin β_2
  knockout mice 540
  postsynaptic NMJ basal lamina 540
  presynaptic development 509
Landmark cells *see* Guidepost cells
Large GTPases *see* Dynamin(s)
Laser capture microdissection (LCM)
  molecular anatomy of mammalian brain 200

Lateral ganglionic eminence (LGE)
oligodendrocyte precursors 239
Lateral geniculate nucleus (LGN)
development 430–431
axon guidance, molecular cues 417
thalamocortical projections 417
ephrin gradients 417
pigment-related gene mutations 431
topographic mapping 417
eye-specific maps 434
pharmacological manipulation 435
retinal activity 435
tetrodotoxin studies 434–435
transgenic animals 435
visual pathway development 441
Lateral inhibition
Notch signaling **131–139**
Notch signaling
Bearded (Brd) gene family and 135
binary cell fate bias 136
cell progeny fates 135
Enhancer of Split (E(spl)) and 133
failure 131–132
outcome bias 134*f*, 135
proneural clusters, cell selection 131, 135
Lateral motor columns (LMCs) *see under* Motor columns
Lateral olfactory tract (LOT)
cell projections 418, 419–420
chemorepulsion 420
α-Latrotoxin 489
L-cones *see* Cone photoreceptor(s)
Leber congenital amaurosis 263
Lectican(s)
trunk neural crest 279
LEF1/TCF
adult melanocyte stem cell regulation 319
melanoblast specification role 314
Leprosy
demyelination 304
Leukemia inhibitory factor (LIF)
ENS development role 631
postnatal 631
glial cell production 639*t*
astrocytes 635
Schwann cells 634
peripheral nerve regeneration 640
receptor, astrocyte differentiation 636
Lewy bodies
Parkinson's disease/parkinsonism 619
Lfng
hair cell differentiation 246
LGN *see* Lateral geniculate nucleus (LGN)
Lhx3 gene/protein
neuronal development 176
LIF *see* Leukemia inhibitory factor (LIF)
Lim3 transcription factor
Drosophila motor neuron identity and 522
Limb(s)
motor neuron specification, anteroposterior (AP) signals 184
somatic marker of prenatal androgen exposure 352
Limbic forebrain, taste role 239
LIM homeodomain transcription factors
C. elegans 188
D. melanogaster 188
evolution 188
isthmic organizer induction 88
LIM motif 188, 188*f*
mechanism of action 188, 188*f*
bHLH transcription factor interactions 189
neuron-specific protein-protein interactions 188–189
nuclear LIM interactor 188, 188*f*
spinal cord transcriptional networks (post-mitotic) 175
expression 176
LIM domain functions 175
nuclear LIM interactor 175
vertebrate development 188
combinatorial actions 188–189
motor neuron specification *see* Motor neuron specification (vertebrate)
oculomotor complex patterning 91
LIM motif 188, 188*f*
Lin11 gene/protein 188
Lineage/birth date relation, retinal development 253, 253*f*, 254*f*
Lipid(s)
posttranslational modification, hedgehog proteins 34
Lipoprotein receptor-related proteins (LRPs), Wnt signaling role 39–40
LIS1 (PAFAH1B1) gene/protein
cortical neuronal migration and 194, 195*f*
initiation role 194
lissencephaly and 194, 196–197
mouse knockouts 196
developmental expression patterns 196–197
protein functions 195, 196–197
Lis1$^{+/-}$ mice, nuclear migration studies 196
Lissencephaly (LIS) **192–198**, 193*f*
bicortical (partial) *see* Subcortical band heterotopias (SBHs)
classical (type I) 192–193, 195–196
gene associations 196
clinical aspects 195
cobblestone (type II) 195–196
incidence 195–196
inheritance 195–196
microlissencephaly 192–193
molecular mechanisms 194
genes/proteins 195, 196
cdk5 194
classical LIS and 196
DCX *see* Doublecortin (DCX)
filamin A 194
LIS1 *see LIS1 (PAFAH1B1)* gene/protein
MAPs 194
reelins/reelin receptors 194
pathway 195, 195*f*
stage-specific 194
neuronal migration defect 192
partial (bicortical) *see* Subcortical band heterotopias (SBHs)
phenotypes
cobblestone variant 195–196
compound 192–193, 195–196
MDS *vs*. ISL 196
related syndromes 192–193, 195–196
utility of study 197
Lmx-1a (transcription factor), DA neuron development 222, 224
Lmx-1b (transcription factor)
DA neuron development 222
isthmic organizer induction 88
LMX transcription factors
midbrain patterning
isthmic organizer induction 88
roof plate induction 89
Local organizers (developmental) *see* Developmental organizers
Locus coeruleus (LC)
anatomy/physiology
adolescence and sexual dimorphism 350
Long-term depression (LTD)
AMPA receptors and *see* Long-term depression, glutamate role
glutamate receptors *see* Long-term depression, glutamate role
heterosynaptic
astrocytes and 570
mGluRs and *see* Long-term depression, glutamate role
NMDA receptor-dependent *see* Long-term depression, glutamate role
postsynaptic receptors, knockout mice 491–492
Long-term depression, glutamate role
NMDA receptors and
developmental role 447
NR2A complexes 447
Long-term potentiation (LTP)
AMPA receptors and *see* Long-term potentiation, glutamate role
dendritic spine structural plasticity 667
glutamate receptors and *see* Long-term potentiation, glutamate role
hippocampal *see* Hippocampal long-term potentiation (LTP)
neurotrophins 625
NMDA receptor-dependent *see* Long-term potentiation, glutamate role
Long-term potentiation, glutamate role
hippocampal *see* Hippocampal long-term potentiation (LTP)
NMDA receptors and
developmental role 447
NR2B complexes 447
glial release of D-serine and 570
see also NMDA receptor(s)
Lophotrochozoa, BMP phylogeny 51–52
Lou Gehrig's disease *see* Amyotrophic lateral sclerosis (ALS)
Low-voltage activated (LVA) calcium channels *see* Voltage-gated calcium channels (VGCCs)
LTD *see* Long-term depression (LTD)

LTP *see* Long-term potentiation (LTP)
LY294002, prevention of autophagic cell death 616
Lysosomal storage diseases (LSDs)
autophagy role 620

M

Macroautophagy 615, 616*f*
Macrochaetes, development 135
Macroglial cells
lineages **218–220**
glial restricted precursors 219
gliogenesis, adult CNS 219
neuroepithelium 218
radial glia 218
regional variations 219
subtypes 218
Macrophage(s)
axonal regeneration 688
see also Microglia
Maf protein family (transcription factors), *Hox* gene expression 68
Magnocellular red nucleus (RNm) *see* Red nucleus
MAGUKs (membrane-associated guanylate kinases) 496
NMDA receptors 447, 448
PSD-95 *see* PSD-95
Male(s)
sex-specific cognitive abilities 348
sexual differentiation *see* Sexual differentiation
Malformations of the cerebral cortex (MCCs) 192
compound phenotypes 192–193
epilepsy association 192–193
holoprosencephaly *see* Holoprosencephaly (HPE)
migration disorders 192–193, 195–196
lissencephaly *see* Lissencephaly
radial migration phase and 194
proliferation and patterning disorders 192–193
Mammal(s)
brains *see* Mammalian brain
netrin expression 472
vision/visual systems
ocular stem cells
ciliary epithelium 269
CMZ 268
retinotopic maps *see* Retinotopic maps
Wnt signaling 463
Mammalian brain
cerebellum *see* Cerebellum
molecular anatomy **199–205**
current large-scale projects 201, 203*t*
Allen Brain Atlas 202
Brain Gene Expression Database 202
Brain Gene Expression Map 202
Gene Expression Nervous System Atlas 201
microarray-based brain gene expression databases 202
SymAtlas 202
TeraGenomics 202
genomic technology integration 199
in situ hybridization 200
microarrays 199
molecular relationships 203
developing brain studies 203–204
human studies 204–205
mouse studies 204
transcription factor role 203
tissue collection methods 200
dissection 200
voxelation 200
MAP kinase signaling pathway
components
FGF receptor signaling, posteriorization and 83–84
KIT/KIT ligand and 314
sexual differentiation 359–360
see also Extracellular signal-related kinase (ERK) signaling
MAPK signaling *see* MAP kinase signaling pathway
MAPs *see* Microtubule-associated protein(s) (MAPs)
Martinotti interneurons 585
Mash1 transcription factor 151*t*
autonomic nervous system development 338–339
gangliogenesis 333–335, 334*f*, 335*t*
Hes1 and 156
olfactory progenitor cells 226–227
established neurogenesis and 230*f*
primary neurogenesis and 229*f*, 231
oligodendrocyte implications 152–153
retinal cell specification 262
bipolar cell generation 262
Mastermind (MAM) proteins, Notch signaling 147, 148*f*
'Master neuronal regulator hypothesis'
combinatorial coding *vs.* 216
RE1-silencing transcription factor 207–208
Maternal bonding, oxytocin *see* Oxytocin
Math1 (Atoh1) 151*t*
ectopic expression effects 153
Math3 transcription factor 151*t*
amacrine cell generation 262
bipolar cell generation 262
horizontal cell specification 263
Math5 (Atoh7) gene/protein 151*t*, 154
ectopic expression 254
ganglion cell differentiation 262
knockout mice 254
retinal development 254, 266
Matrix metalloproteinase(s) (MMPs)
neural crest cell migration 273–274
NMJ basal lamina 543
collagen IV cleavage 539
M-box, MITF transcription factor binding 318
MCCs *see* Malformations of the cerebral cortex (MCCs)
M-cones *see* Cone photoreceptor(s)
MDGA1 gene/protein, neocortical expression patterns 123–124
Mec-3 188
MeCP2 211
Medial forebrain bundle (MFB)
DA axonal development 223
Medial ganglionic eminence (MGE)
oligodendrocyte precursors 239
Medial longitudinal fasciculus (MFL)
development 90
Medial preoptic area (MPOA)
inputs 356
sexual dimorphism 355
olfaction and 356
testosterone and cell death regulation 357
Median hinge point (MHP), neurulation 3–5, 5*f*
Medullary cord, secondary neurulation 3
Medulloblastoma
histopathology
molecular genetics
REST dysregulation 209
Meinox/TALE proteins, *Hox* gene expression 67–68
Melanin 312
optic tract development and 431
synthesis 312
types 312
Melanoblast(s) 312–313
adult melanocyte stem cell similarity 318–319
differentiation 318
dendritic phenotype acquisition 318
melanization and 312–313, 318
MITF and 318
self-renewal *vs.* 319
generation in neural crest 312–313, 313*f*
migration 313*f*, 317
β_1 integrin role 318
cadherin expression and 318
pathways/targets 312–313
proliferation during 312–313, 318
protein expression during 312–313
timing 312–313
proliferation 317
during migration 312–313, 318
MITF role 317–318
specification
cell number and location 316
pre-*vs.* postmigratory commitment 317
small numbers, evidence 316–317
uneven head-tail distribution 316–317
molecular mechanisms 313, 315*t*
CREB 314
endothelin receptor-B 314
interacting mechanisms 314, 316*f*
KIT role 314
LEF1/TCF 314

MITF role 314
PAX3 314
SOX10 314
Wnt signaling 314–316
Melanocyte(s)
adult stem cells 318
niches 318–319
regulation 319
development 280, **312–320**
general principles 312, 313*f*
melanoblasts *see* Melanoblast(s)
questions raised by 313
signaling pathways 280
functional roles 312
locations 312
melanosomes 312
pigment *see* Melanin
Melanoma
MITF as 'lineage addiction oncogen' 317–318
Melanopsin-expressing RGCs *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
Melanosomes 312
transfer of 312
Memapsin 2 *see* Beta-site APP cleaving enzyme 1 (BACE1)
Membrane-associated guanylate kinases *see* MAGUKs (membrane-associated guanylate kinases)
Membrane stripe assay 366
Mental conditions *see* Psychiatric disorders
Mental disorders *see* Psychiatric disorders
Mental illness *see* Psychiatric disorders
Mesencephalic dopamine neurons *see* Midbrain dopaminergic neurons
Mesencephalic trigeminal nucleus, midbrain patterning 92
Mesencephalon *see* Midbrain
Mesencephalus lateralis dorsalis nucleus 87
Mesenchymal stem cells (MSCs), CNS repair 660
Mesoderm
induction
signaling pathways
fibroblast growth factors 10
Nodal 10
see also Neural induction
spinal cord patterning 74, 74*f*
AP (caudalizing signals) 184
DV (ventralizing signals) 29, 30*f*
alternative signals 33
sonic hedgehog *see* Sonic hedgehog (Shh) signaling
Metamorphosis
apterous neurons 214*f*, 215
Metazoans
BMP phylogeny 51, 51*f*
3-Methyladenine (3-MA), prevention of autophagic cell death 616, 618*t*
Methylation, DNA *see* DNA methylation
Methyl-CpG-binding protein 2 *see* MeCP2
Methyl-CpG-binding proteins (MBDs)
transcriptional silencing 179
Mice
development
retinotopic map refinement 370
Microarray analysis
molecular anatomy of mammalian brain 199
Microautophagy 615
Microcephaly 192–193
Microenvironment, adult NSC regulation 644
Microfibril-associated glycoprotein (MAGP), DSL-induced Notch signaling 145
Microglia 218
cytokine release
synaptic modulation and 570
Microlissencephaly 192–193
MicroRNA *see* miRNA (microRNA)
Microtubule(s)
associated proteins *see* Microtubule-associated protein(s) (MAPs)
axonal development
axonal branching
models 382
axonal growth cones 382
dynamics
polymerization 480, 481*f*
functional roles
axonal development *see axonal development (above)*
neuronal migration and, LIS1 regulation 196–197
see also Axonal transport
transport 493
Microtubule-associated protein(s) (MAPs)
mutations and disease
lissencephaly 194
neuronal migration role 194
Microtubule-associated protein 1B (MAP1B)
neuronal migration role 194
phoshorylation 42
Midbrain 87
anatomy/organization
arcs 90, 90*f*
cerebral peduncle 87
dopaminergic neurons *see* Midbrain dopaminergic neurons
location 87
posterior 87
mesencephalicus lateralis dorsalis nucleus 87
torus semicircularis 87
substantia nigra *see* Substantia nigra (SN)
tectum (dorsal midbrain) 87
optic tectum (nonmammalian animals) 87
retinal input and retinotopy 87
superior colliculus (mammals) *see* Superior colliculus (SC)
tegmentum (ventral midbrain) 87
nuclei 87
development 3, 87, 116
forebrain junction 87
hindbrain junction 87
see also Midbrain-hindbrain boundary, neural patterning
patterning *see* Midbrain patterning
invertebrate homologs 93
non-mammalian vertebrates *see* Optic tectum
oculomotor control
superior colliculus *see* Superior colliculus (SC)
optic pathway 427
somatosensory processing 87
Midbrain arcs 90, 92*f*
Midbrain dopaminergic neurons
development
extrinsic factors associated 221
intrinsic factors associated 222
midbrain patterning 91
subtypes 223
Midbrain–forebrain boundary
neural patterning 87, 88, 90, 104
FGF8 role 82
lineage restriction 83
Pax6 repression and 82
organizers 103–104
Fez/Fezl signaling 80–81
hedgehog signaling 81–82
Irx signaling 80–81
Wnt signaling 80–81
see also Zona limitans intrathalamica (ZLI)
transcription factor boundaries 104
Midbrain–hindbrain boundary (MHB)
DA neuron development
co-culture experiments 223
FGF-8 expression 221–222
neural patterning **80–86**, 87
lineage restriction 83
midbrain nuclei of non-midbrain origin 91
neural induction and posteriorization 80
specific markers 80, 81*f*
primordia positioning and 80
tissue competence
predetermination of cellular fate 84, 84*f*
signal integration 83
transcription factor expression 84, 84*f*
organizers 87, 88*f*, 100
functional role 82
receiving field and ECM role 85
see also Isthmic organizer
Midbrain patterning **87–94**
adult structure and 87
congenital disorders, oculomotor complex 91
dorsal patterning 92
mesencephalic trigeminal nucleus 92
optic tectum 92, 92*f*
tectal-tegmental boundary 92
early patterning, boundaries and signals 87
BMP-mediated patterning 89
FGF8-mediated patterning 80, 88, 89*f*

Midbrain patterning (*continued*)
floor plate induction 89, 89*f*, 91
forebrain-midbrain junction 87, 88
gene expression studies 88*f*
interactions between organizers 89
isthmic organizer *see* Isthmic organizer
midbrain-hindbrain junction *see* Midbrain-hindbrain boundary, neural patterning
roof plate induction 89
Shh-mediated patterning 89, 89*f*, 90–91, 92
ZLI and 81–82
Wnt-mediated patterning 80, 82, 88, 89, 90
ZLI and 80–81
invertebrates 93
Shh signaling 92
ventral patterning 90
dopaminergic neurons 91
medial longitudinal fasciculus 90
midbrain arcs/interarcs 90, 90*f*
identification/classification 90
Shh signaling 90–91
ventricular zone 90
Wnt signaling 90
non-midbrain-derived nuclei and 91
oculomotor complex 91
red nucleus 91
sonic hedgehog and 89, 89*f*
Middle interhemispheric (MIH) holoprosencephaly 105, 106*f*
dorsal patterning and 110
Midline
crossing
corticospinal development 404, 405*f*, 406*f*
glial cells and axon guidance 394*t*, 397
neural tube floor plate 398
optic nerve chiasm 398
molecular mechanisms 404
EphA4/Ephrin B3 405, 406*f*
L1-CAM 404–405, 405*f*
Robo/Slit 405
formation, forebrain *see* Forebrain midline development
Miller-Dieker syndrome (MDS) 195–196
17p13.3 deletions 196
phenotype 196
Miniature endplate potentials (mEPPs) 565
Minimal Information About a Microarray Experiment (MIAME) 202
Mir133b (microRNA) 225–225
miRNA (microRNA)
Hox gene regulation 69
REST expression 207
MITF transcription factor
adult melanocyte stem cell regulation 319
CDK2 interactions 317–318
cell shape and 318
as 'lineage addiction oncogen' 317–318
M-box binding 318
melanocyte development role 280
melanoblast differentiation 318
melanoblast proliferation 317–318
melanoblast specification 314
KIT-MITF interactions 314
transcription factors involved in expression 314, 318
Wnt signaling and 314–316
posttranslational modification 318
Mitochondria
cellular functions
apoptosis role 603*t*
induction 607
see also Apoptosis
Mitogen-activated protein kinase (MAPK) signaling *see* MAP kinase signaling pathway
Mitogens
adult neurogenesis and 641–643, 650
polypeptides, oligodendrocyte precursor development 241
see also Growth factor(s)
Mitral cells
axon guidance 418, 419–420
MKP3 repressor, FGF negative feedback regulation 83–84
MMPs *see* Matrix metalloproteinase(s) (MMPs)
m-Numb, retinal cell specification 263
Models
belief in 96
brain development *see* Brain development, models
coexistence 95–96
discarding obsolete models 95–96
paradigms *vs.* hypotheses 95–96
parsimony and 95
utility and why we use models 95
Modulatory signaling receptors, NMDA receptors *see* NMDA receptor(s)
Molecular guidance cues
axonal guidance and *see* Axonal guidance cues
chemoaffinity hypothesis 366
Monocular deprivation *see* Visual deprivation
Monosynaptic stretch reflex *see* Stretch reflex
Morphogen(s) 73–74, 671
antagonists and, forebrain specification 106
as axon guidance molecules **460–464**, 671, 672*f*
see also Axonal guidance cues
cell responses 465
definition 16, 465
functional role 74
gradients 16
hedgehog *see* Hedgehog (Hh) signaling pathway
history **16–20**
gradients and polarity 16
harmonious equipotential system 16
positional information 17
neocortical development *see* Neocortical arealization (area differentiation)
signaling centers *see* Developmental organizers
Morphogenesis *see* Ontogenesis (morphogenesis)
Morphogenetic signaling centers *see* Developmental organizers
Motor columns
column of Terni (TC) 74–75
molecular markers 75–76, 75*f*
lateral (LMC) 72, 176*f*, 187
brachial level 72
gene expression pattern 77
intrasegmental MN diversity 78
generation/specification 74–75, 75*f*
lateral MNs (LMCl) 72
lumbar level 72
medial MNs (LMCm) 72
molecular markers 75–76, 75*f*
median (MMC) 187
organization 72, 73*f*
specification 72, 176–177, 187, 188–189, 190
axon pathfinding 190
Hox expression and 75, 75*f*
AP patterning and 74–75
cross-repression and 76
FGF signaling role 76
see also Hox genes/proteins
LIM-HD factors and 74–75, 187–188
motor pools and 187
see also Motor pools
Motor control
corticospinal development and 410
see also Corticospinal development
fine movements
corticospinal development and 410–411
finger movements *see* Finger movements, control
grasping *see* Grasp/grasping
reaching *see* Reaching
Motor cortex
developmental blockade, corticospinal development and 410*f*, 411
finger movements and *see* Finger movements, control
motor maps *see* Motor maps
Motor endplate *see* Neuromuscular junction (NMJ)
Motor experience
components 410
corticospinal development and 410, 412*f*
Motor ganglia, neurodevelopment 333
Motor maps 403
development 403, 408*f*, 409*f*, 412, 413*f*
importance for skilled movements 412
Motor neuron(s) (MNs) 553
anatomical organization 72, 73*f*
columns *see* Motor columns
pools *see* Motor pools
diversity 72
endplate *see* Neuromuscular junction (NMJ)
midbrain 87
morphology

axon branching 529, *553*
dendritic arbors 190
motor unit component *536*, *553*
activity-dependent modulation *see* Motor unit, activity-dependent modulation
see also Motor unit(s); Neuromuscular junction (NMJ)
properties/characteristics 72, *536*
proprioceptive feedback 190
Schwann cells and 532
Motor neuronal firing patterns 536
see also Motor unit, activity-dependent modulation
Motor neuron development 72, 176, 176*f*, 184
autophagic cell death 618
axon branching 529, *553*
birth 529
cell-type specification
Drosophila larvae 522
classic chick studies 72
growth cones and axonal pathfinding 190, 502, 529
Hox gene expression and *see* Hox genes and motor neuron specification
motor columns 176–177
myelination 532
NMJ formation and target innervation 502, 529
ACh release from growth cones 529
agrin release 531
axon outgrowth and identity specification 529
Drosophila larval NMJ 522
myelination and 532
precision 72, *538*
Schwann cells and 532
see also Neuromuscular junction development, mammalian
rhombomeres 396–397
ventral neural tube origin 184
Motor neuron disease (MND) *see* Amyotrophic lateral sclerosis (ALS)
Motor neuron specification (vertebrate) **184–191**
anteroposterior (AP) patterning 74*f*, 184
classic chick experiments 72
extrinsic signals (morphogen gradients) 74, 184
intrinsic signals (transcription factors) 74
Hox gene expression and *see* Hox genes and motor neuron specification
retinoic acid role 57
axon pathfinding 190
dendrite patterning and synaptogenesis 190
motor pool morphology 190
proprioceptive feedback and 190
dorsal projecting motor neurons (d-MNs)
LIM-HD factor expression 185–187, 186*f*, 188
progenitor transcription factor expression 185
Shh signaling and 184–185
soma migration 189*f*, 190
dorsoventral (DV) patterning 74*f*
extrinsic signals (morphogen gradients) 74, 184–185
intrinsic signals (transcription factors) 74, 184–185
extrinsic signals 73–74, 74*f*, 184
anteroposterior (AP) signals 184, 185
dorsoventral (DV) signals 31, 184–185
FGFs 75, 76, 185
functions 74
retinoic acid 74
sonic hedgehog 74, 184–185
see also Morphogen(s)
Hox expression and *see* Hox genes and motor neuron specification
intrinsic signals 73, 74, 74*f*, 185
bHLH factors 185
homeodomain factors 74, 185
LIM proteins *see below*
Hox proteins 185
reciprocal inhibition 185
see also Transcription factors (TFs)
LIM factors 186*f*
axon growth/guidance and 190
bHLH factor interactions 189
combinatorial actions 188–189
dMN *vs.* vMN specification 185–187
evolution 187
expression patterns 188
LIM code and MN diversification 186*f*, 187, 190
molecular mechanisms 188, 188*f*
motor column specification 74–75, 187–188
neuronal migration and 189–190
motor columns *see* Motor columns
motor pools *see* Motor pools
positional information and 73
progenitors 29
pMN region of ventricular zone 185
soma migration 189, 189*f*
stepwise progression 185, 186*f*
ventral-exiting motor neurons (v-MNs)
axon growth/guidance 190
LIM-HD factor expression 185–187, 186*f*, 188–189
motor columns 187, 188–189, 190
motor pools 187
progenitor transcription factor expression 185
Shh signaling and 184–185
soma migration 189–190, 189*f*
somatic motor neurons 187, 189–190
visceral motor neurons 187, 189–190
Motor pools
clustering 72
definition 72
classical 76
transcription factor patterns 76
organization 72, 73*f*, 76, 77*f*
specification 72, 73*f*, 184, 185, 187
dendritic morphology 190
transcription factor expression 187
Ets genes/proteins 187
Hox genes/proteins *see* Hox genes/proteins
spinal cord transcriptional networks 176
Motor unit(s)
adaptation/plasticity *see* Motor unit, activity-dependent modulation
definition *553*
force production
factors regulating *553*
maturation (mammalian) *536*
firing patterns *536*
muscle fiber homogeneity *536*
precision *538*
transmission efficacy *536*
modulation *see* Motor unit, activity-dependent modulation
size *553*
reduction by synapse elimination *553*
evidence *554*
see also Motor neuron(s) (MNs); Muscle(s)/muscle fiber(s); Neuromuscular junction (NMJ)
Motor unit, activity-dependent modulation
MN firing patterns and 536
maturation/development role 536
Mouse *see* Mice
Mpp5, retinal development 255
msh gene/protein, neuroectoderm signaling 49
associated gene repression 49–50, 49*f*
Msx genes/proteins
BMP signaling 46–47
DA neuron development 222
neural crest development 272
Müller cells (glia) 218
expression 219
generation 259, 261*f*
Hes1 262
Hes5 262
growth factors 636, 639*t*
BDNF 636
FGF-2 636
NGF 636
NT-3 636
TNF-α 636
VEGF 636
intrinsic stem cells 269
location 259
retinal development 262
Multiple sclerosis (MS)
Schwann cells 634
Multipotency, stem cells 641
Munc13 genes/proteins
knockout mice 517
see also other UNC proteins
Munc18 gene/protein 517
Muscle(s)/muscle fiber(s)

Muscle(s)/muscle fiber(s) (*continued*)
action potential generation
early development 545–546
see also Neuromuscular transmission
classification/types
development 530
development
activity-dependent 536–537
differences between muscle types 553
Hox genes and innervation 177
see also Hox genes and motor neuron specification
myoblast specification 536–537
myotubes *see* Myotube formation
NMJs (mammalian) *see* Neuromuscular junction development, mammalian
receptor expression 530, 545
skeletal muscles 529
synaptic nuclei clustering 550
force/effort
factors regulating 553
see also Motor neuron(s) (MNs)
motor units *see* Motor unit(s)
neural control (innervation)
Hox genes and 177
see also Hox genes and motor neuron specification
see also Neuromuscular junction (NMJ)
neuromuscular junction *see* Neuromuscular junction (NMJ)
polyaxonal innervation *see* Polyaxonal (polyneuronal) muscle innervation
stretch
reflex *see* Stretch reflex
Muscle-specific kinase (MuSK) 42–43
neuromuscular junction development 531, 535–536, 542
knockout mice and 542, 546, 546*f*, 548–549
neurocentric model and 551
signaling pathways 546, 550
convergent pathways 550
positive feedback loop 550
synaptic nuclei and 550
unified model and 552
transcription regulation (N-box) 550
see also Agrin
MuSK *see* Muscle-specific kinase (MuSK)
Mus musculus see Mice
Mute synapses *see* Silent synapses
Myelin-associated glycoprotein (MAG)
axonal growth/guidance 396
axon growth inhibition 676, 689
Myelinating glial markers 561
Myelination 242–243
disorders
degenerative *see* Myelinopathies
Olig1 knockout mice 240
glial markers 561
motor neurons axons and NMJ maturation 532
optimal myelin thickness 301
regulation/control
BACE1 role 301–302
glial growth factor 242
hypermyelination 301, 302*f*
hypomyelination 301–302
neuregulins 637
neuregulin-1 242
NFκB role 308
peripheral nervous system 300
phosphatidylinositol 3-kinase 301
Schwann cells 290, 308
axon relationships 294, 295*f*
onset 290
myelin-related transcription factors 290
negative regulation 291
β-neuregulin role 290
related transcription factors 290
see also Schwann cell(s)
see also Oligodendrocyte(s); Schwann cell(s)
Myelin-based inhibitors, axon growth 396, 397*t*, 676, 677*f*
Myelinopathies
MS *see* Multiple sclerosis (MS)
peripheral 310
see also Demyelination
Myelin protein zero (MPZ), Schwann cell differentiation 306, 309
Myenteric plexus
apoptosis 612, 613
inflammation *see* Enteric ganglionitis (plexitis)
Myoblast(s)
differentiation, RE1-silencing transcription factor role 208
fusion (myotube formation) 529
specification 536–537
MyoD, cell cycle effects 596–597
Myonuclei clustering 535–536
Myosin(s)
myosin II
actin filament flow 480
myosin VI
hair cell differentiation 248–249
myosin VIIa
hair cell differentiation 248–249
Myotube formation 529, 545
AChR expression 545
contraction and AP generation 545–546
kinetics 529
primary *vs.* secondary 529

N

Nab1 protein, myelination regulation 309
Nab2 protein, myelination regulation 309
Narp
postsynaptic development role 490
Nasal septum, development 228–229
Nasal turbinates, development 228–229
Na_v *see* Voltage-gated sodium channels (VGSCs)
N-box 550
NC1 domain, type IV collagen a chains 538–539
NCAMs *see* Neural cell adhesion molecules (NCAMs)
NDE1, neuronal migration 195
Ndel1 gene/protein
neuronal migration 195, 195*f*
Necrosis 603–605
apoptosis-necrosis dichotomy 615–616
Nedd E3 ubiquitin ligase(s), Notch signaling 146
Negative feedback regulation
developmental role
fibroblast growth factors and 83–84, 113
sonic hedgehog and DV patterning 34, 35*f*
retinal ganglion cell generation 260
Negative gene regulation *see* Transcriptional repressors/repression
Nematostella vectensis, netrin expression 473*f*, 474, 475*f*
Neocortex 116
adult neurogenesis 648
anatomy/organization
cortical areas (fields) *see* Neocortical areas (fields)
laminar patterning (layers) 116, 193
development *see* Neocortical development
evolution/size increases *see* Neocortex evolution
Neocortex evolution
neocortical areas (fields) conservation 116
size increases 116
see also Brain evolution
Neocortical arealization (area differentiation) 117, 118*f*
area-specific TCA input and 119
functional specialization and 119
mitogenic activity 119
TCA pathfinding 119
CP architecture and projection neuron distribution 118
selective axon elimination 118–119
extrinsic mechanisms 117–118
genetic regulation 117–118, 122*f*
'area identity' and 123, 123*f*
differential expression criteria 120
genetic manipulation experiments 119, 120
indirect evidence 119
morphogens/signaling molecules 121
BMPs 121
FGF8 120–121
signaling/patterning centers 119–120, 121, 122*f*
Wnts 121
spinal cord development *vs.* 123
transcription factors 120
anteroposterior gradation 122
border formation and 122

COUP-TFI 120, 121, 122
Emx2 120, 121, 122
expression patterns 120, 122*f*
interactions between 121
Pax6 120
Sp8 120
intrinsic mechanisms 117–118
regionalization relationship 122
Neocortical areas (fields) 116
borders 116
development *see* Neocortical arealization (area differentiation)
evolutionary conservation 116
genes expressed 123–124
primary motor area (M1) 116
primary sensory areas 116
visual *see* Primary visual cortex (V1: striate cortex)
spatial relationship 116
thalamocortical afferents (TCAs and) 116–117, 119
Neocortical development **116–124**, 118*f*
AP patterning 122
arealization *see* Neocortical arealization (area differentiation)
cortical plate (CP)
corticogenesis 117
cytoarchitecture 118
selective axon elimination 118–119
corticogenesis (histogenesis) 117, 118*f*
cell-type specification 586
interneuron generation 117
projection neuron generation 117
see also Cortical cell-type specification
cortical plate (CP) 117
disorders 192
neuronal migration 117, 193
preplate (PP) 117
regionalization
arealization relationship 122
boundary formation *see* Segmentation (developmental)
Neogenin receptors, optic tectum retinotopy development 93
Nerve growth factor (NGF)
apoptotic role 607
cell differentiation/morphology 625
clinical applications 625–626, 626*t*
Alzheimer's disease 625–626
peripheral neuropathy 626–627
developmental role
enteric nervous system 630
discovery 628
enteric nervous system 628
development role 630
IBD role 633
glial cells 639*t*
astrocytes 635
Müller glial cells 636
oligodendrocytes 635
Schwann cells 634
historical aspects 622
identification 672–673
neural injury and regeneration
peripheral nerves 640
see also see also clinical applications (above)
satellite cells 634
sympathetic neurons
development 336–337
plasticity and 333
targeted deletion 624
Nerve injury *see* Nervous system injury
Nerve terminals
motor nerves *see* Neuromuscular junction (NMJ)
retraction 564
Nervous system(s)
alternative splicing *see* Alternative splicing
central *see* Central nervous system (CNS)
injury *see* Nervous system injury
peripheral *see* Peripheral nervous system (PNS)
programmed cell death functions 599, 600*t*
Nervous system injury
adult neurogenesis
nonneurogenic regions 648
see also Neurogenesis, adult
apoptosis and *see* Apoptosis, nervous system injury
excitotoxicity *see* Excitotoxicity
glial responses
perisynaptic Schwann cells 565
Schwann cells *see* Schwann cell(s)
neuromuscular transmission and
perisynaptic Schwann cells 565
see also Neuromuscular transmission
peripheral *see* Peripheral nerve injury
see also Neural regeneration/repair; Neurodegeneration/neurodegenerative disease
N-ethylmaleimide-sensitive fusion proteins *see* NSFs (*N*-ethylmaleimide-sensitive fusion proteins)
Netrin(s) **472–479**, 674
adult CNS 677
axonal growth/guidance 381, 388–389
chemoattractant signaling 425–426, 474
chemorepellent signaling 476
growth cone response regulation 476
bilateral symmetry modeling 674
in development 474, 674
enteric nervous system development 346–347
expression 462, 474, 478
Nematostella vectensis 473*f*, 474, 475*f*
tissues associated 478
family members 472, 473*f*
historical research 472
receptors associated 477
signal transduction 474
structure 472, 473*f*
C-terminal C domain 473–474
domain V 472–473
domain VI 472–473
Netrin-1
axonal growth/guidance 398–399, 415, 474
axonal pathfinding 638
chemoattractant signaling 474
chemorepellent signaling 470, 476
growth cone response regulation 476
expression 478
tissues associated 478
floor plates 460
knockout mice 460, 465–466
loss of function 474
orthologs 474, 475*f*
receptors 381
Netrin-3 474
Netrin-4 474
tissues associated 478
Netrin-G(s) 474
Neu-Laxova syndrome 192–193
Neurabin, postsynaptic development 492–493
Neurabin II (spinophilin)
postsynaptic density 499
postsynaptic development 492–493
Neural cell adhesion molecules (NCAMs) 485
expression 485
growth cone migration 485
olfactory sensory neurons 226–227, 229*f*, 230*f*
presynaptic development 509–510
Neural cells
glial cells *see* Glial cells (glia)
neurons *see* Neuron(s)
Neural connectivity
sexual differentiation and 357
Neural crest **271–281**
autonomic nervous system development 322*f*
cell types derived from 333, 338
enteric ganglia 330
enteric nervous system development 344, 344*f*
stem cells 630–631
gangliogenesis 323, 333, 335
parasympathetic ganglia 328
sympathetic ganglia 324
cardiac 277, 277*f*
gene expression 277
signaling pathways 277
cell migration
melanoblasts 312–313, 317
migration staging area 312–313
cranial 274
cell potentiality 273
craniofacial development 275–276, 276*f*
function 274–275
migration 275, 275*f*

Neural crest (*continued*)
prespecification 276–277
transplantation studies 275–276, 276*f*
definition 312
delamination 273–274
embryonic (E) days 321
epithelial-mesenchymal transition *see* Epithelial-mesenchymal transition (EMT), neural crest
gene expression 272, 274*f*
hindbrain 275, 275*f*
historical aspects 271
induction/inducing signals 271, 312
BMPs 312
FGFs 312
Wnts 312, 314–316
melanocyte development **312–320**
see also Melanoblast(s)
microenvironment influences 325*f*
neuregulin-1-ErbB pathway and PNS specification **298–305**
components 298
as master myelination regulator 300
research directions 304
Schwann cell lineage regulation 299, 306
potentiality 273
premigratory phase cells 273
sacral 272*f*, 280, 281*f*
Schwann cell development 282, 283*f*, **306–311**
AP2α 307
maintenance of immature state 307
myelination initiation 308
neuregulin-1-ErbB pathway 299, 306
terminal differentiation 307
structures derived from 335
subdivisions 271, 273*f*
trunk 278
guidance cues 279
migratory pathways 278–279, 279*f*
dorsolateral 280
ventral 279, 279*f*
plasticity 280
undifferentiated stem cells 335–336
vagal 273*f*, 277
Neural degeneration *see* Neurodegeneration/neurodegenerative disease
Neural epithelium, regionalization *see* Segmentation (developmental)
Neural folds 271
Neural-glial switch 240–241
Neural groove 3
formation (neural plate bending/folding) *see* Neural plate
Neural induction
animal cap assays 10
BMP inhibition and 'default model' 10, 106, 107
evidence against 10–11, 12*f*
chick embryos 11–12
evidence for 10, 11*f*
cell fate and decision making 14
chick as model system **10–15**
advantages 11
BMP inhibition alone as insufficient for induction 11–12, 14
FGF8 as hypoblast inducing signal 12–13
Hensen's node 11
preneural markers preceding gastrulation 12–13, 14–15
time course of events 11, 12–13, 12*f*, 13*f*
as complex process 10
forebrain specification 100
neurulation *see* Neurulation
organizers *see* Developmental organizers
positioning of brain primordia 80
posteriorization 80
proteins associated 45–46, 46*f*, 56
timing 12
gastrulation and 12–13
Spemann and Mangold experiments 12–13, 14
as unsolved problem 14
Xenopus 45–46
misexpression studies 10
Neural injury *see* Nervous system injury
Neural patterning
anteroposterior *see* Anteroposterior (AP) patterning
dorsoventral *see* Dorsoventral (DV) patterning
midbrain–forebrain boundary *see* Midbrain–forebrain boundary
midbrain–hindbrain boundary *see* Midbrain–hindbrain boundary (MHB)
retinoic acid *see* Retinoic acid (RA)
rostrocaudal *see* Anteroposterior (AP) patterning
sonic hedgehog signaling *see* Sonic hedgehog (Shh) signaling
Wnt pathway *see* Wnt signaling
Neural plasticity *see* Neuroplasticity
Neural plate 80, 218
bending (neural groove/folds) 3–5
actin-binding proteins and apical constriction 9
apposition 3–5, 5*f*
cellular mechanisms
folding 7, 8
furrowing 7, 8
cranial *vs*. spinal levels 3–5
extrinsic factors 8
hinge points 3–5, 5*f*, 7
intrinsic factors 8
molecular mechanisms 8
neural fold morphogenesis 5–6, 6*f*
NTDs and 8, 9
epidermal ectoderm and 107
formation/induction 3, 46–47, 48*f*, 100
cellular mechanism (palisading) 7
as default state 8
fusion (neural tube formation) 3, 6–7
extrinsic factors 8
gene regulatory and signaling interactions 271–272, 274*f*
neural tube defects and 8, 9
organizers *see* Developmental organizers
posteriorization (caudalization) 80, 100
anterior borders 80
anterior visceral endoderm and 106–107
signaling molecules 80
see also Anteroposterior (AP) patterning; 'Caudalization' (posteriorization)
regionalization
cortical development 116
forebrain development 100, 101*f*
shaping 3, 4*f*, 8
convergent-extension movements 3
cellular mechanism 7
molecular mechanism 8
intrinsic factors 8
NTDs and 8
planar-cell polarity pathway and 8
Wnt signaling pathway 8
Neural progenitors *see* Neural stem/progenitor cells (NSPCs)
Neural regeneration/repair
adult neurogenesis in nonneurogenic regions 648
axonal *see* Axonal regeneration
ischemic stroke *see* Ischemic stroke (cerebral ischemia)
peripheral nervous system *see* Peripheral nerve regeneration/repair
perisynaptic Schwann cells 565
neuregulin-1 565
similarity to developmental NMJ synapse elimination 555
spinal cord *see* Spinal cord regeneration/repair
see also Neural stem/progenitor cells (NSPCs)
Neural retina leucine zipper (Nrl), photoreceptor cell specification 263
Neural stem/progenitor cells (NSPCs) 662
adult *see* Neural stem/progenitor cells (NSPCs), adult
cell division
asymmetrical divisions 592
cortical progenitors **585–591**
symmetrical divisions 592
characteristics 662
CNS repair and 653
see also Cell replacement therapy
cortical *see* Cortical cell-type specification
criteria 641
definitions 641
glial cells
macroglial lineages 219
oligodendrocytes *see* Oligodendrocyte progenitor cells (OPCs)
protein expression 219–220
identification in adult tissue 662
multipotency 641
neural crest and 335–336
neuroepithelial cells 592
neuronogenesis **662–665**
ocular *see* Ocular neural stem cells
olfactory system 226, 227*f*

postnatal gut 630–631
spinal cord development 172
delineation 173–174, 173*f*
temporal change to characteristics 162*f*
therapeutic use *see* Cell replacement therapy
Neural stem/progenitor cells (NSPCs), adult **641–647**, 648, 649*f*
definition 641
discovery 648–649
functions 646
computational modeling 646
learning and memory 646
new neuron integration into existing circuits 646
historical aspects 641
identification 662
identification/characterization 643, 648
cell division markers 643, 650
culture methods 641
genetic methods 644
molecular markers 643
inhibition of neurogenesis 646
locations 641, 642*f*, 649, 650
ocular *see* Ocular neural stem cells
SGZ *see* Subgranular zone (SGZ)
SVZ *see* Subventricular zone (SVZ)
mammalian *vs.* nonmammalian vertebrates 266
mitogen effects 641–643, 650
niche environment 648
in nonneurogenic regions 648
regulation 644, 650
epigenetic 645–646
extracellular signaling 644
intracellular signaling 645
retinal *see* Ocular neural stem cells
rodents *vs.* humans 646
SVZ *vs.* SGZ cells 646
Neural tube 21, 22*f*
AP patterning **80–86**
classic chick experiments 72
posteriorization 80
signals 80, 81*f*
caudal
forebrain *see* Prosomeres
spinal cord formation 3
cortical development 116
defects *see* Neural tube defects (NTDs)
DV patterning **29–38**
mesoderm-secreted signals 29
additional signals 31–33
sonic hedgehog *see* Sonic hedgehog (Shh) signaling
midbrain *see* Midbrain patterning
spinal cord *see* Spinal cord development
ventral organization 29, 30*f*
floor plate cells 29, 30*f*
midline glial cells and axon guidance 398
see also Spinal cord, dorsoventral (DV) patterning
formation *see* Neurulation
homeodomain proteins 21
see also Hox genes/proteins
midbrain patterning *see* Midbrain patterning
motor neuron development 184
see also Motor neuron specification (vertebrate)
neuromeres 3
brain development model (neuromere model) 96
see also Brain development, models
caudal (forebrain) *see* Prosomeres
hindbrain (rhombomeres) 95
midbrain (mesomeres) 95
see also Midbrain, development
spinal (myelomeres) 95
oligodendrocyte precursor cell specification 238
organization 29
organizers *see* Developmental organizers
progenitor cells 21, 23*f*, 172
progenitor domains 21
rostral 218
brain formation 3
segmentation 96
sonic hedgehog signaling 25
subventricular zone 218
ventricular zone 218, 238
Neural tube defects (NTDs)
gene defects 8, 9
neurulation defects 8, 9
prevention 9
Neuregulin(s) (NRGs) 298
AChR induction at the vertebrate NMJ 547
changing views on role 544–545
convergent signaling pathways 550
neurocentric model and 551
NRG1 and
ErbB receptors 547
neuromuscular development role 547
in vitro studies 547
in vivo genetic studies 547, 548*f*
ErbB signaling 306
AChR induction at the vertebrate NMJ 547
components 298
γ-secretase-dependent proteolysis 298–299
Schwann cells 300, 306
lineage regulation 299
myelination 298, 300
see also ErbB receptor(s)
glial cells 639*t*
oligodendrocyte differentiation 637
Schwann cells
ErbB and *see above*
survival and 532
transcriptional control of development 306
myelination 637
trunk neural crest migration 279
Neuregulin-1 (NRG-1)
AChR induction at the vertebrate NMJ 547
ErbB receptors 547
neuromuscular development role 547
EGF-like domain 298–299
isoforms 298, 299*f*
type III 298, 300, 301, 302*f*, 303*f*
myelination regulation 242
posttranscriptional modification 301–302
NRG-1α
oligodendrocyte differentiation 636
Schwann cell differentiation 637–638
NRG-1β
oligodendrocyte differentiation 636
Schwann cell differentiation 637–638
proteolytic cleavage 298
Schwann cells
differentiation 307
β-neuregulin 1
neural crest 284
precursors 286, 287*f*
perisynaptic
neuroregeneration 565
synaptogenesis 563
Neurexin(s) 489
CASK interactions 515–516
neuroligin recognition *see* Neurexin–neuroligin complex
presynaptic development 509, 515
synapse stabilization 516–517
Neurexin–neuroligin complex 515
PSD 498
Neurite(s)
outgrowth 399–400
immunoglobulin superfamily 395
see also Axon branching; Dendrites/dendritic arbor
Neuritis *see* Neuropathy
Neuroactive steroids *see* Neurosteroids
Neuroblast 5-6T, apterous neuron clusters 214
Neuroblast division, Notch-Delta signaling 135
Neurocan
axon growth inhibition 676
NeuroD
amacrine cell generation 262
motor neuron specification, LIM factor interaction 189
olfactory neurogenesis 231–232
Neurod4 *see* Math3 transcription factor
Neurodegeneration/neurodegenerative disease
autophagy role 619
endosomal-lysosomal system involvement 619
in epilepsy *see* Epilepsy
excitotoxic *see* Excitotoxicity
growth factors/neurotrophic factors

Neurodegeneration/neurodegenerative disease (*continued*)
BDNF 639
CNTF 639
fibroblast growth factor-2 (FGF-2) 639
GDNF 639
glial cell growth factors 639
see also Growth factor(s); Neurotrophin(s)
NMDA receptor stimulation *see* Excitotoxicity
perisynaptic Schwann cells 563
prevention *see* Neuroprotection/neuroprotective agents
synapse elimination *vs.* 555
Wallerian *see* Wallerian degeneration
Neurodegenerative disease *see* Neurodegeneration/neurodegenerative disease
Neurodevelopment
anteroposterior patterning *see* Anteroposterior (AP) patterning
cell death role 599–600, 601*f*
autophagic cell death and 618, 619*f*
major causes/mechanisms 618
see also Developmental neuroplasticity
differentiation *see* Neuronal differentiation
disorders *see* Neurodevelopmental disorders
dorsoventral patterning *see* Dorsoventral (DV) patterning
glial cells *see* Gliogenesis
midbrain patterning *see* Midbrain patterning
neural crest *see* Neural crest
neural migration *see* Neuronal migration
neural plate *see* Neural plate
neural stem cells *see* Neural stem/progenitor cells (NSPCs)
neural tube *see* Neural tube
neurogenesis *see* Neurogenesis
neurotransmitter ‘phenotype switching’
autonomic development 339
injury effects 339
neurulation *see* Neurulation
plasticity *see* Developmental neuroplasticity
segmentation *see* Segmentation (developmental)
see also Brain development; Spinal cord development
Neurodevelopmental disorders
cortical development 192
holoprosencephaly *see* Holoprosencephaly (HPE)
lissencephaly *see* Lissencephaly (LIS)
Neuroectoderm
bone morphogenic signaling *see* Bone morphogenetic proteins (BMPs)
neural crest *see* Neural crest
neural patterning 55
neural tube *see* Neural tube
Neuroepithelium/neuroepithelial cells (NE) 218
apical cell junctions, retinal development 255
asymmetric *vs.* symmetric division 592
glial restricted precursors 219
macroglial lineages 218
Hes genes and 160–161, 162*f*
multipotency 218, 592
cell cycle and 592
progenitor domains 238, 239*f*
sonic hedgehog (Shh) gradient sensing 184–185
Neurofibrillary tangles (NFTs)
Alzheimer’s disease 619–620
Neurog1 *see ngn1* gene/protein
Neurog2 *see ngn2* gene/protein
Neurog3 151*t*
Neurogenesis
adult brain *see* Neurogenesis, adult
autonomic gangliogenesis 333–335
definition 662
Drosophila 131, 132–133
estrogen and 357
olfactory system *see* Olfactory system, neurogenesis
radial glial cells 238
spinal cord transcriptional networks 175
neuronal differentiation 175
precursor cells 175
see also Neural stem/progenitor cells (NSPCs)
Neurogenesis, adult 641, 642*f*, **648–652**, 649*f*
aging effects 644, 663
hippocampus 648–649, 663
implications 664
anosmia 665
neural stem cells, grafting of 665
studies 665
modulation and 663
and stress 664
trophic factors, overexpression of 663
negative effects of corticosteroids 648–649
olfactory bulb 663
quantitative studies 663
Sox 2 663
see also Hippocampal damage, stress-related; Stress, neuroplasticity and
definition 648
dysfunction 651
functional roles 648, 651
qualitative 651
see also Neuroplasticity
hippocampal (dentate gyrus) 641, 642*f*, 648, 649, 649*f*
adult hippocampal progenitor (AHP) 641–643
functions 646
types 649
IGF-1 and 650
integration 650
location *see* Subgranular zone (SGZ)
mitogen effects 641–643
regulation 650
stress effects 648–649
aging and 648–649
see also Hippocampal damage, stress-related; Stress, neuroplasticity and
stress effects
aging and 644
historical aspects 648
inhibition effects 646
markers 648
medical relevance 651
neocortical, controversy 648
in nonneurogenic regions 648
ocular **266–270**
see also Ocular neural stem cells
olfactory system *see* Olfactory system, neurogenesis
regulation 644, 650
epigenetic 645–646
expansion stage 650
extracellular signaling 650
extracellular signaling 644
intracellular signaling 645
neurotransmitter systems 650
nonspecific *vs.* specific stimuli 650
postmitotic survival 650
stress effects 644
hippocampal (dentate gyrus) 648–649
aging and 644, 648–649
hippocampus 648–649
negative effects of corticosteroids 648–649
see also Hippocampal damage, stress-related
see also Neural stem/progenitor cells (NSPCs), adult
Neurogenic regions 266
ocular *see* Ocular neural stem cells
SGZ *see* Subgranular zone (SGZ)
SVZ *see* Subventricular zone (SVZ)
Neurogenin
cell-fate determination 593
neuogenin1, olfactory neuronal precursors 226–227, 231
established neurogenesis 230*f*
primary neurogenesis 229*f*
Neurogenin-2, DA neuron development 222
Neuroglioform interneurons 585
Neuroimaging
dopamine *see* Dopaminergic neurons/systems
visual pathway development 441–442, 444*f*
Neuroligin(s)
neurexin recognition 498, 515
postsynaptic density and 498
differentiation 489
aggregation 489
clustering effects 490
isoforms 489
knockout mice 489
NMDA receptor recruitment 491
presynaptic development 509, 515
synapse stabilization 516–517
Neuroligin-1, postsynaptic differentiation 488
scaffold role 490
Neuroligin-2
postsynaptic differentiation 489, 490

Neurological diseases/disorders
autophagy role 618
degenerative *see* Neurodegeneration/neurodegenerative disease
NeuroM, motor neuron specification 189
Neuromere(s) 3
brain development model (neuromere model)
definition 95
doubts over and columnar model 96–97
His's model and 96, 98*f*
historical aspects 96
Orr's model and 96, 97*f*
see also Brain development, models
caudal forebrain *see* Prosomeres
definition 95, 96
Neuromuscular junction (NMJ) 529, 539*f*, 553
acetylcholine receptors *see* Neuromuscular junction, acetylcholine receptors
acetylcholine release *see* Neuromuscular transmission
acetylcholinesterase role *see* Acetylcholinesterase (AChE)
action potential thresholds 536
agrin *see* Agrin, neuromuscular junction role
arborization nuclei 561
basal lamina *see* Neuromuscular junction, basal lamina
botulinum toxin and *see* Botulinum toxin(s)
cell types 538, 558
motor neurons *see* Motor neuron(s) (MNs)
muscle fibers *see* Muscle(s)/muscle fiber(s)
Schwann cells *see* Perisynaptic Schwann cells (PSCs)
see also Motor unit(s)
components/organization 561, 562*f*
development *see* Neuromuscular junction, development
invertebrates *see* Neuromuscular junction, invertebrate
mature NMJ 564–565
as model synapse
vertebrate 544
plasticity *see* Neuromuscular junction, plasticity
postsynaptic terminals *see* Neuromuscular junction, postsynaptic
presynaptic terminals *see* Neuromuscular junction, presynaptic (motor nerve terminal)
transmission across *see* Neuromuscular transmission
vertebrate
gene transcription at
acetylcholine receptors *see* Neuromuscular junction, acetylcholine receptors
vertebrate
frog 561, 562*f*
tadpoles 563
gene transcription at **544–552**, 545*t*
as model system 545
as model synapse 544
voltage-gated ion channels
$Ca2^+$ channels 533
sodium channels *see* Neuromuscular junction, postsynaptic
Neuromuscular junction, acetylcholine receptors 544
acetylcholine binding 544
aggregation/clustering 544
agrin-dependent *see* Agrin
early accumulation during development 530, 531*f*
fibronectin role 540
motor neuron role 546
redistribution during maturation 531*f*, 533
self-aggregation 531–532, 551–552
visualizing 545
developmental gene expression 532, 534, **544–552**
candidate AChR inducing factors 546
agrin *see* Agrin
ATP 547
αCGRP 547
change in assumptions 544–545
neuregulins *see* Neuregulin(s) (NRGs)
fetal *vs.* adult isoforms 530, 534–535
mRNA accumulation during maturation 535
myocentric model 546, 551
myotubes *vs.* innervated fibers 545
neurocentric model 546, 551
evidence 546
unified model 552
functional fold location 534, 538
subunit composition
adult *vs.* fetal 530, 534–535, 545
timing of switch 545–546
Neuromuscular junction, basal lamina **538–543**, 546
AChR expression/aggregation and *see* Neuromuscular junction, acetylcholine receptors
cell surface and membrane receptors 541
cadherins 542
dystroglycan complex 541
integrins 542
intracellular signaling 542
presynaptic role 542
MuSK *see* Muscle-specific kinase (MuSK)
components 538
acetylcholinesterase 541
collagen IV 538, 539*f*
fibronectin 540
HSPGs 540
laminins 539
nidogens (entactins) 540
functional roles 538
protease regulation 543
matrix metalloproteinases (MMPs) 543
collagen IV cleavage 539
tissue inhibitors of MMPs (TIMPs) 543
Neuromuscular junction, development
important questions of 529
invertebrates 553
Drosophila see Drosophila neuromuscular junction, larval
mammalian *see* Neuromuscular junction development, mammalian
myocentric model 546, 551
neurocentric model 546, 551
Schwann cells and 567–568
unified model 552
Neuromuscular junction, invertebrate
development 553
Drosophila see Drosophila neuromuscular junction, larval
muscle innervation
arthropods
Drosophila larval abdominal muscles 520
neuroactive substances/receptors
retrograde modulation
CaMKII role 527
Drosophila larvae 526, 526*f*
TGFβ superfamily 526–527, 526*f*
Neuromuscular junction, plasticity **561–566**
activity-dependent
Drosophila larval NMJ 526
see also Motor unit, activity-dependent modulation
axonal sprouting
Schwann cells and 564
see also perisynaptic Schwann cells (below)
see also Axon branching
botulin toxins and *see* Botulinum toxin(s)
nerve damage and
Schwann cells and 565
new synapse formation
perisynaptic Schwann cells 563
perisynaptic Schwann cell roles
degeneration 565
regeneration 565
remodeling 564
synaptic maintenance 564
synaptogenesis 563
Neuromuscular junction, postsynaptic
folds 533, 538
acetylcholine receptor location 534, 538
functional implications 532, 535*f*
Na_v1 channel location 534
Na_v1 channels
appearance during development 533
isoform expression during development 535
location 534
mRNA accumulation during development 535
Neuromuscular junction, presynaptic (motor nerve terminal)
capping 561
neuroplasticity and *see* Neuromuscular junction, plasticity
Neuromuscular junction, synapse elimination 530*f*, 532, **553–560**
consequences 553
muscle fiber homogeneity 536
reduction in motor unit size 553
fish 532
humans 532
molecular mechanisms 557
neurotrophic factors 557
proteases and ubiquitin-proteasome system 558

Neuromuscular junction, synapse elimination (*continued*)
muscle fiber type specificity and selective elimination 557
neurodegenerative disease and 558
differences 559
similarities 556, 559
SOD mutant ALS mice and 556, 559
nonneuronal cell types and 558
polyaxonal to monoaxonal (p to m) transition 553
axonal regeneration similarity 555
elimination *vs.* degeneration 555
evidence for
electrophysiological 553–554
histological 554, 554*f*
Jansen, Brown and van Essen's study 554
partial denervation at birth and 554
real-time *in vivo* imaging 555
axosomes 556
fate of losers 556
local competition 555
retraction bulbs 556
transgenic XFP mice 555
synaptic competition and 559–560
activity-dependent 536, 557, 559–560
conclusions about 557
intrinsic MN hierarchies 556
local 554–555
asynchrony 555–556
axonal transport and 556
endplate takeover 555–556
flip–flop behavior 556
in vivo imaging 555
see also Synaptic competition
synaptic size and 533
timing/rates 554
Neuromuscular junction development, mammalian **529–537**
agrin role 509, 531, 535–536
central synapses *vs.* 512–513
early development 530
AChR accumulation 530, 531*f*
aggregation 531
agrin-induced 509, 531
microclusters 530–531
postsynaptic gene expression upregulation 532
see also Neuromuscular junction, acetylcholine receptors
initial muscle-nerve encounters 530
muscle action potential generation 545–546
polyaxonal innervation 530, 530*f*, 553
change to monoaxonal *see* Neuromuscular junction, synapse elimination
evidence for 553
transmission efficacy and 536
Schwann cells 532, 558
timing of muscle innervation 530, 553
motor neuron development 502, 529, 553
ACh release from growth cones 529, 553
axon outgrowth and identity formation 529
birth 529
precision 72, 538
see also Motor neuron development
motor unit maturation 536
firing patterns 536
muscle fiber homogeneity development 536, 557
transmission efficacy 536
myocentric model 546, 551
neurocentric model 546, 551
perisynaptic Schwann cells and
early development 532, 558
synapse elimination 558, 569–570
synaptogenesis 567–568
postsynaptic maturation 532, 533
molecular differentiation 534
AChR expression changes 534
mRNA accumulation 535
muscle activity and 534
Na_v1 expression changes 535
myonuclei accumulation 535
remodeling 533
AChR and AChE redistribution 531*f*, 533
fold formation 533
VGSC (Na_v1) appearance 533, 534*f*
size increases 536
presynaptic maturation 502
nerve terminal 532
broadening 531*f*, 532
Ca^{2+} channel switch 533
factors affecting 532–533
terminal Schwann cells 532
transmitter release and 533
size increases 536
synapse elimination *see* Neuromuscular junction, synapse elimination
skeletal muscle fiber development 529
AChR isoforms 530
delocalized postsynaptic properties 530
myotube formation 529
kinetics 529
primary *vs.* secondary 529
origin of fiber types 530
other muscle types *vs.* 553
Neuromuscular synapse *see* Neuromuscular junction (NMJ)
Neuromuscular transmission
acetylcholine role 544
sites of release
release from developing MNs 529
termination *see* Acetylcholinesterase (AChE)
see also Neuromuscular junction, acetylcholine receptors
adenosine triphosphate (ATP) and *see* Adenosine triphosphate, transmitter role
botulinum toxin and *see* Botulinum toxin(s)
invertebrates
developmental changes, *Drosophila* NMJ 525
see also Neuromuscular junction, invertebrate
miniature endplate potentials *see* Miniature endplate potentials (mEPPs)
NMJ plasticity and *see* Neuromuscular junction, plasticity
retrograde signaling and
invertebrates
Drosophila larvae 526, 526*f*
Neuron(s)
action potentials *see* Action potential(s)
age-related loss 668
see also Age/aging
brain 457–458
cytoskeleton *see* Cytoskeleton
homeostatic regulation *see* Neuronal homeostasis
morphology/structure
axons *see* Axon(s)
brain function 456
cell body (soma) *see* Soma
cytoskeletal filaments *see* Cytoskeleton
dendrites *see* Dendrites/dendritic arbor
production *see* Neurogenesis
progenitors *see* Neural stem/progenitor cells (NSPCs)
survival
FGF-2 638
TGF-β 638
see also Neuroprotection/neuroprotective agents; Neurotrophin(s)
see also entries beginning neuro-/neuronal
Neuronal activity
perisynaptic Schwann cell sprouting 565
Neuronal atrophy *see* Neurodegeneration/neurodegenerative disease
Neuronal degeneration *see* Neurodegeneration/neurodegenerative disease
Neuronal development *see* Neurodevelopment
Neuronal differentiation
corticospinal development 403
REST function 209
spinal cord transcriptional networks 175
see also Cell-type specification
Neuronal-glial interactions *see* Neuron-glia interactions
Neuronal homeostasis 667
Neuronal injury *see* Nervous system injury
Neuronal migration
adult olfactory neurogenesis 649*f*, 650
autonomic nervous system development
gangliogenesis 335
Cdk5 pathways *see* CdK5
cortical development 117, 192–193, 585–586
disorders 192–193, 195–196
lissencephaly *see* Lissencephaly (LIS)
radial migration phase and 194
molecular pathways 194, 195*f*
Arfgef2 role 194
doublecortin (DCX) role 194

filamin A role 194
LIS1 role 194
MAPs and 194
reelins and reelin receptors 194
radial migration 193
tangential migration 193
time lapse imaging studies 193–194
motor neuron soma 189
NMDA receptors 439
Neuronal nitric oxide synthase (nNOS)
dystroglycan complex component 541
enteric
adult expression 632
developmental changes 630–631
PSD 498–499
Neuronal outgrowth
retinal development 257
Neuronal plasticity *see* Neuroplasticity
Neuronal regeneration *see* Neural regeneration/repair
Neuronal spikes *see* Action potential(s)
Neuronal transport
axonal *see* Axonal transport
postsynaptic scaffolding proteins 493
Neuron-glia interactions
axon guidance 394*t*
receptor tyrosine phosphatases 401
Neuronogenesis
definition 662
and stem cells **662–665**
see also Neurogenesis
Neuron-restrictive silencer factor (NRSF) *see* RE1-silencing transcription factor (REST)
Neuropathy
diabetes mellitus and *see* Diabetic neuropathy
therapy
nerve growth factor (NGF) 626–627
Neuropilins
axon growth inhibition 395
Neuropilins 674
Neuroplasticity
autonomic nervous system *see* Autonomic neuroplasticity
definition 333
developmental *see* Developmental neuroplasticity
neuromuscular junctions *see* Neuromuscular junction, plasticity
neuronal homeostasis *see* Neuronal homeostasis
stress effects *see* Stress, neuroplasticity
synaptic *see* Synaptic plasticity
visual development, neural activity in *see* Visual development, neural activity role
see also Behavioral plasticity
Neuropoietic cytokines
CNTF *see* Ciliary neurotrophic factor (CNTF)
ENS development role 629*t*, 630
LIF *see* Leukemia inhibitory factor (LIF)
Neuroprotection/neuroprotective agents
cell death prevention
autophagic, pharmacological prevention 616, 618*t*
neurotrophic factors
in adult and aging gut 632
see also Neurotrophin(s)
Neuropsychiatric disorders *see* Psychiatric disorders
Neuroradiology *see* Neuroimaging
Neuroregeneration *see* Neural regeneration/repair
'Neurosphere' culture, ocular neural stem cells 267
ciliary epithelium derived 269
Neurosteroids
neurogenesis, Alzheimer's disease 664
Neurotransmitter(s)
adult NSC regulation 645
'phenotype switching' 339
postsynaptic differentiation induction 489
release *see* Neurotransmitter release
Neurotransmitter release
developmental changes, NMJ maturation 533
quantal content and 533
readily releasable vesicles and 512
vesicular *see* Synaptic vesicle(s)
see also Neuromuscular transmission
Neurotrophic factors *see* Neurotrophin(s)
Neurotrophic hypothesis
neuronal survival and ANS plasticity 336
programmed cell death 599
Neurotrophin(s) 609, **622–627**, 672
apoptosis and 607, 608*f*
mutant mouse studies 608
receptors 608–609
roles 608
autonomic axonal outgrowth 336
cell survival 624
apoptosis 624
CNS 625
peripheral nervous system 625
developmental roles
cell differentiation/morphology 625
BDNF 625
NGF 625
NT3 625
expression changes 609
guidance cues 672
motor neuron target-derived cues 673
loss of support during development 618
neuronal cell-type specification 216
sexual dimorphism and 351
somatic sensory neurons 420
enteric nervous system *see* Enteric nervous system (ENS)
historical aspects 622
pharmacology 625, 626*t*
Alzheimer's disease 625–626
amyotropic lateral sclerosis 626
availability 626
delivery methods 626
Huntington's disease 626
Parkinson's disease 625–626
spinal cord injury 626
stroke 626
production 622
receptors 622
p75 *see* p75 receptor(s)
specificity 622
Trks *see* Trk receptor(s)
regeneration/repair role
autonomic axonal outgrowth 336
see also Neural regeneration/repair
release 622
intracellular sorting 622
Schwann cells 634
signaling mechanism 672–673, 673*f*
somatic sensory neurons 420
synaptic competition role
NMJ synapse elimination and 557–558
synaptic function 625
BDNF 625
long-term potentiation 625
synaptic plasticity 625
synthesis 622
therapeutic uses
BDNF *see* Brain-derived neurotrophic factor (BDNF)
clinical trials 626
CNTF *see* Ciliary neurotrophic factor (CNTF)
GDNF *see* Glial cell line-derived neurotrophic factor (GDNF)
neurotrophin-3 *see* Neurotrophin-3 (NT-3)
NGF *see* Nerve growth factor (NGF)
programmed cell death 600
see also Growth factor(s)
Neurotrophin-3 (NT-3)
clinical applications 626*t*
developmental role
cell differentiation/morphology 625
enteric nervous system 346, 630
postnatal 631
enteric nervous system 628
development role 630
postnatal 631
gastrointestinal motility 632
IBD role 633
neuroprotective role 632
glial cells 639*t*
astrocytes 635
Müller glial cells 636
oligodendrocytes 635
historical aspects 622
regeneration/repair role
axonal regeneration 678
enteric nervous system 632

Neurotrophin-3 (NT-3) (*continued*)
peripheral nerve regeneration 640
see also clinical applications (above)
satellite cells 634
sympathetic axonal outgrowth 336
synapse formation/maturation 514
Neurotrophin-4 (NT-4)
astrocytes 635
glial cell production 639*t*
historical aspects 622
peripheral nerve regeneration 640
Neurturin (NTN)
apoptotic role 612
autonomic gangliogenesis 335
enteric nervous system
adult expression 631–632
development role
postnatal expression 631
prenatal expression 629
knockouts 629–630
receptor (GFRα2) 629–630
Neurulation **3–9**, 46–47, 80, 271
neural groove closure 3, 6–7
neural tube defects and 8, 9
overview 3
primary 3, 4*f*, 271, 272*f*
cellular mechanisms 7, 7*f*
intrinsic *vs.* extrinsic forces 8
molecular mechanisms 8
neural plate changes *see* Neural plate
stages 3
secondary 3, 271
telencephalon specification 101*f*, 102
NF-κB *see* Nuclear factor kappa B (NF-κB)
NFY (transcription factor), *Hox* gene expression 66–67
NG2 cells 219–220, 243
NGF *see* Nerve growth factor (NGF)
NGL (adhesion molecule)
function 489–490
interactions 489–490
ngn1 gene/protein 151–152, 151*t*
Notch signaling 138
ngn2 gene/protein 151*t*, 152
motor neuron specification 185
LIM factor interaction 189
spinal cord development 175
ngn3 gene/protein 151*t*
Nidogen-2, basal lamina 540
Nidogens (entactins), basal lamina 540
collagen IV binding 539
Niemann-Pick disease
type C (NPC)
autophagic neuronal death 620
Nieuwkoop center, *Xenopus* axis specification 41
Nitric oxide, transmitter role
enteric
developmental changes 630–631
NKCC1 *see* Sodium/potassium/chloride ($Na^+/K^+/Cl^-$) cotransporter
NKCC2 *see* Sodium/potassium/chloride ($Na^+/K^+/Cl^-$) cotransporter
Nkx2.2 transcription factor
DV neural tube expression 31–33, 32*f*
cross-repression 33
oligodendrocyte precursor development 240–241, 240*f*
NKx6.1 transcription factor
DV neural tube expression 32*f*
cross-repression 33
spinal cord dorsoventral patterning 57
NKx6.2 transcription factor, DV neural tube expression 32*f*
cross-repression 33
NKx6 transcription factor(s)
Drosophila motor neuron identity and 522
NMDA receptor(s)
deactivation kinetics 438
developmental *see* NMDA receptors, developmental role
NR1 subunits 438
deficient mice 439–440, 443
deletion 443
expression patterns 438, 439*f*
NR2 subunits 438, 491
expression patterns 438, 439*f*
NR2A 438
deficient mice 446
expression patterns 439*f*
long-term depression 447
NR2B 438, 491
deficient mice 443
deletion 443
expression patterns 439*f*
long-term potentiation 447
NR2C 438
deficient mice 439–440
NR2D 438
expression patterns 439*f*
NR3 subunits
NR3A subunits 438
expression patterns 439*f*
NR3B subunits 438
expression patterns 439*f*
PSD complexes 498
recruitment, neuroligins 491
scaffolding/adaptor proteins 496–497
PSD-95 *see* PSD-95
receptor interactions
GABA receptors
coactivation 573
seizure induction 579–580, 580*f*
signal transduction 448
'silent' synapses 517–518
subunits/structure 438, 457
synaptic plasticity 436
LTD role *see* Long-term depression, glutamate role
LTP role *see* Long-term potentiation, glutamate role
time course 438
NMDA receptors, developmental role **438–448**
complexes 447
guanine exchange factors (GEFs) 448
membrane-associated guanylate kinases (MAGUKs) 447, 448
PSD-95 447–448
SAP-102 447–448
signaling 448
early brain development 438
differentiation 439
gap junction coupling 439–440
migration 439
NR1 knockout animals 439–440
NR2B-deficient animals 439–440
sculpting neural circuits 440
synapse formation 440
long-term depression 447
NR2A complexes 447
long-term potentiation 447
NR2B complexes 447
somatosensory pathways 443
NR1 deletion 443
NR2B deletion 443
rodent whisker-driven afferents 443, 445*f*
spike-timing-dependent plasticity (STDP) 447
subunit expression 438
synaptic development/synaptogenesis 444
axonal arbors 445–446
bidirectional modification 447
blockade experiments 445, 446*f*
cytoskeletal rearrangements 445–446
dendritic spines 444
excitatory 444
pyramidal neurons 444–445
receptive fields 446
'silent' synapses 456–457, 457*f*, 491
somatosensory cortex 444–445
visual pathways 440, 440*f*
β_2-adrenergic receptor deficiency 442
antagonist studies 440–441, 442*f*, 443*f*
AP5 studies 440–441, 442*f*
competitive arbor rearrangement 441–442
cooperative near-neighbor input activity 442
eye-specific segregation 440–441, 441*f*
lateral geniculate nucleus 441
neuroimaging 441–442, 444*f*
ocular dominance column formation 436
superior colliculus 441
N-methyl-D-aspartate receptors *see* NMDA receptor(s)
NMJ *see* Neuromuscular junction (NMJ)
Nodal
mesoderm induction 10

ventral midline patterning 109, 110
HPE associations 110
HPE phenotypes and 110
hypothalamus development 101, 108
retinal development 103
Shh pathway interactions 112*f*, 114
sites and functions 110
Noden, Drew, cranial neural crest work 276–277
Nodes of Ranvier
Schwann cell-axon relationships 296
Noggin
adult NSC regulation 645
holoprosencephaly and 111
neural induction and 45–46, 106
oligodendrocyte precursors 238–239
timing of expression and evidence against default model 11–12
Nogo-A
axon growth inhibition 396, 676, 689
see also Nogo receptor (NgR)
Nogo glycoproteins
axon guidance 396
inhibitors 396, 397*t*
Nogo receptor (NgR)
axon growth inhibition 676
neuroplasticity 437
signaling 676
Nonmammalian vertebrates
optic tectum, behavioral correlates 454
Nonpeptidergic nociceptors, dorsal root ganglia (DRG) *see* Dorsal root ganglia (DRG)
Norrin, Fz/LRP receptor complex interactions 40
Nose
development
olfactory specification and patterning **226–237**
turbinate/septum formation 228–229
olfactory epithelium *see* Olfactory epithelium (OE)
see also entries beginning naso-/nasal
Notch1 receptor
hair cell differentiation 245
signaling *see* Notch signaling pathway
Notch extracellular domain (NECD) 144
DSL endocytosis effects 144–145
Notch intracellular domain (NICD) 140
ankyrin repeats 147
RAM domain 147
Notch signaling pathway 131, 132*f*, 133*f*, **140–149**, 141*f*, 142*f*
activation 131, 140, 145
cell-fate determination
binary cell fate bias 136
cell cycle interactions 597
cortical neurons 587, 588
see also Lateral inhibition
clathrin-mediated endocytosis 142–144
defective
DII1 recycling defects 144
membrane trafficking defects 146
diversity 148–149
Drosophila 140, 144–145
endosomal acidity 146
functional roles 139
glial cells
Schwann cells precursors 287
hair cell differentiation 245
lateral inhibition role *see* Lateral inhibition
ligands
DSL *see* DSL (Delta, Serrate, Lag-2) ligand family
ligand expression
see also DSL (Delta, Serrate, Lag-2) ligand family
regulatory functions 141
retinal progenitor cells (RPCs) 261
mammalian 144–145
oligodendrocyte precursor development 241
differentiation 242
proteins associated 143*t*
regulation 141
endocytosis 145
DSL-independent 146
ligand expression 141
trafficking 143*t*, 145
DSL-independent 146
segmentation
somitogenesis 139
target gene regulation 142*f*, 147
CSL genes/proteins 147
Hes genes regulation 156–157
transcriptional complexes 147
vascular development 148
vertebrate neurogenesis 138
visual system development
retinal development 255–256, 266–267
see also DSL (Delta, Serrate, Lag-2) ligand family
Notochord 47
development 21
neural epithelium signaling 21–22, 23*f*
ventralization 21
neural tube DV patterning and 29, 74
additional signals 33
sonic hedgehog *see* Sonic hedgehog (Shh) signaling
prechordal plate similarity 108
NP2 (Narp) *see* Narp
NR2E1, tectal-tegmental boundary 92
NRG1 (neuregulin 1) gene
AChR induction at the vertebrate NMJ 547
ERBB receptors and 547
neuromuscular development role 547
NRGs *see* Neuregulin(s) (NRGs)
NR1 subunits *see* NMDA receptor(s)
NSFs (*N*-ethylmaleimide-sensitive fusion proteins)
postsynaptic receptor maintenance 491–492
NT-3 *see* Neurotrophin-3 (NT-3)
NTs *see* Neurotrophin(s)
N-type calcium channels *see under* Voltage-gated calcium channels (VGCCs)
Nuclear factor kappa B (NF-κB)
myelination role 308
Nuclear LIM interactor (NLI) 175, 188, 188*f*
Nucleosome(s)
sliding, transcriptional silencing 179–180
structure 179*f*
see also Chromatin; Histone(s)
Nucleus ventromedialis *see* Ventromedial hypothalamic nuclei (VMN)
Numb (and homologs)
as cell-fate determinants 136, 587
cortical neurons 588
retinal progenitor cells (RPCs) 251
Nurr1 transcription factor, dopaminergic neuron formation 222, 224
midbrain 91

O

Oblique-gradient molecules, retina dorsoventral axis development 128
Oct6 protein, Schwann cell development 308, 309
deficiency 308
levels 308
Ocular albinism (OA1)) optic pathway development 431–432
Ocular neural stem cells **266–270**
additional sources 268
ciliary epithelium 267*f*, 268
birds 268–269
mammals 269
intrinsic stem cells 269
Müller glia 269
rod precursors 269
pigmented epithelium 268
ciliary marginal zone 267, 267*f*
amphibians 267–268
birds 268
mammals 268
regenerative capacity 268
teleost fish 267–268
‘neurosphere’ culture 267, 269
regeneration role
ciliary marginal zone 268
RPE role 268
retinal progenitors (normal development) 266, 267*f*
growth factor signaling and 266
neural stem cell characteristics 267
Notch signaling and 266–267
transcription factor expression 266
Oculomotor complex (OMC) 87
midbrain patterning 91
PHOX2A marker 91
Oculomotor nerve (cranial nerve III)
brain development 27

Odorant receptors (ORs) *see* Olfactory (odorant) receptor(s)
Older people *see* Age/aging
Olfaction
  sexually dimorphic nuclei and 356
Olfactory (odorant) receptor(s)
  development
    axon guidance cues 419, 419*f*
    olfactory sensory neuron targeting 418
  as GPCRs 227
  NE *vs.* VNO 227
  pheromone receptors 227
  plasticity/regeneration 648
  sensory mapping 418, 419*f*
Olfactory bulb
  accessory
    VNO input 226
  adult neurogenesis 648
    aging effects 663
  development
    axon growth/guidance
      higher olfactory centers 419
      mitral cells 418, 419–420
      tufted cells 418, 419–420
  sensory mapping 418
Olfactory ensheathing cells (OECs) 226
  post-spinal cord injury regeneration 689
Olfactory epithelium (OE)
  adult neurogenesis *see* Olfactory system, neurogenesis
  development **226–237**, 227*f*
    established neurogenesis 228–229, 230*f*
    primary neurogenesis 228, 228*f*, 229*f*
    see also Olfactory system, neurogenesis
  structure/cell types 226, 227*f*
    glial-like cells
      olfactory ensheathing cells 226
      supporting (sustentacular) cells 226, 228–229
    neuronal cell types 226
      committed progenitors 226–227
      immediate neuronal precursors 226–227
      sensory (receptor) neurons *see* Olfactory sensory neurons (OSNs)
      stem cells 226–227
      see also Olfactory system, neurogenesis
Olfactory marker protein (OMP)
  olfactory sensory neurons 226–227
Olfactory nerve (I) 226
Olfactory receptor neurons (ORNs) *see* Olfactory sensory neurons (OSNs)
Olfactory sensory neurons (OSNs)
  cell bodies 226
  cellular properties 226
  G-protein expression 418–419
  marker proteins
    developing NE 228, 229*f*
    NCAM1 226–227, 229*f*
    olfactory marker protein (OMP) 226–227
  neurogenesis **226–237**
    see also Olfactory system, neurogenesis
  receptors *see* Olfactory (odorant) receptor(s)
Olfactory specific G-protein (G_{olf}) 418–419
  $I7_{DRY}$ mutant 418–419
Olfactory supporting (sustentacular) cells 226
Olfactory system(s)
  adult neurogenesis *see* Olfactory system, neurogenesis
  development
    axon guidance cues 418
    neurogenesis *see* Olfactory system, neurogenesis
  topographic mapping 418
Olfactory system, neurogenesis **226–237**
  adult 228–229, 229–230, 648, 649*f*, 650
    location *see* Subventricular zone (SVZ)
    novelty detection and 651
    olfactory bulb 648
    regulation 650
    see also Neural stem/progenitor cells (NSPCs), adult; Neurogenesis, adult
  extrinsic factors 232, 235–237, 236*f*
    antineurogenic 232
    FGFs 232
      FGF2 234
      FGF8 228, 229*f*, 232–234, 233*f*, 235–237
      FGF8/Sox2 coexpression 230–231, 232–234
      FGF18 232–234
    proneurogenic 232
    TGFβ superfamily 234, 235–237
      BMPs 234
      GDF11 234, 235*f*
  intrinsic factors (transcription factors) 229, 230, 235–237, 236*f*
    Mash1 229*f*, 230*f*, 231
    NeuroD 231–232
    Ngn1 229*f*, 230*f*, 231
    RunX1 231–232
    Sox2 228, 229*f*, 230–231
      Fgf8 coexpression and 230–231, 232–234
  neuronal precursors in NE and VNO 226
    committed progenitors 226–227
      BMP actions on 234
      Mash-1 expression 226–227, 229*f*, 231
    immediate neuronal precursors 226–227
      GDF11 effects on 234–235
      neurogenin1 expression 226–227, 229*f*, 231
    locations 226–227
    mature OSNs 228
      NCAM expression 226–227, 229*f*
    stem cells 226–227
      Sox2 expression 230–231
      Sox2/Fgf8 coexpression 230–231
  phases 228
    established neurogenesis 228, 228*f*, 236*f*
      OE formation 228–229, 230*f*
      VNO formation 229, 230*f*
    primary neurogenesis 228, 228*f*, 236*f*
      cell types present 228, 229*f*
      Mash1 expression 231
      Ngn1 expression 231
      OE formation 228, 228*f*, 229*f*
      Sox2 expression 228, 229*f*
      VNO formation 228
Olig1 gene/protein 151*t*, 152, 240
  knockout mice, myelination defects 240
Olig2 gene/protein 151*t*, 152
  cell fate determination 31
    DV neural tube expression 32*f*
      cross repression 33
    motor neuron specification 185
  oligodendrocytes
    precursor development 240–241
  spinal cord, dorsoventral patterning 57
Olig3 gene/protein 151*t*
Oligodendrocyte(s) 218
  bHLH proteins 152
  cdki control of cell fate 594
  development 218
    differentiation 175, 238
      growth factors 636
      IGF-1 637
      neuregulins 637
      NRG-1α 636
      NRG-1β 636
      PDGF 637
    progenitors *see* Oligodendrocyte progenitor cells (OPCs)
    Sox10 expression 310
    specification **238–244**
    stages 219
  growth factors 635, 639*t*
    BDNF 635
    differentiation and 636
    nerve growth factor 635
    NT-3 635
    transforming growth factor-β 635
  netrin-1 expression, role 478
  synaptogenesis and 567
  visualization *in situ* 238
Oligodendrocyte and type-2 astrocyte precursors (O2A) 219
Oligodendrocyte-myelin glycoprotein (OMgp)
  axon growth inhibition 676, 689
  axon guidance 396
Oligodendrocyte progenitor cells (OPCs)
  adult CNS 242
  differentiation control 242
  embryonic neural tube 238
  generation
    dorsally-derived 239
    forebrain 239

spatial control 238
spinal cord 238
transcription factors 240
ventrally-derived 238, 239*f*
lineage marker expression 219
PDGF receptor expression 241
proliferation control 241, 241*f*
visualization *in situ* 238
markers associated 238
Oligodendroglia *see* Oligodendrocyte(s)
Oncopeltus, morphogen gradients and polarity 17
ON-OFF RGCs *see* Retinal ganglion cells (RGCs)
Ontogenesis (morphogenesis)
GABA, excitatory-to-inhibitory shifts 579
hypothalamus 108
neural fold morphogenesis 5–6, 6*f*
retinal development 251, 252*f*
Optic chiasm
development
axon guidance
midline glial cells 398
optic nerve growth 415
decussation decisions 429
Eph/ephrin 429–430
frog studies 430
hypopigmentation 430
radial glial processes 429–430
Zic2 transient expression 430
sonic hedgehog role 469–470, 469*f*
fiber organization 428, 429*f*
pigment-related mutations 431, 431*f*
retinal fiber reallocation 431, 431*f*
Optic cup
retinal progenitors 251, 266
Optic disk
axon guidance, molecular cues 415
Optic nerve (cranial nerve II)
crush models, axonal regeneration 678
development 429
axon guidance cues 415
dye-labeling studies 429
electron microscopy 429
optic vesicle 429
stalk cells 429
fiber organization 428, 429*f*
Optic pathway **427–432**
competitive arbor rearrangement 441–442
development 429
activity role *see* Visual development, neural activity role
axonal outgrowth 429
chronotropic fiber reordering 429*f*, 430
decussation decision 429
NMDA receptors *see* NMDA receptors, developmental role
retinotopic reordering 430
target innervation 430
fiber organization 428, 429*f*
organization 427
binocular *vs.* lateral position 427, 429*f*
diencephalon 427
mesencephalon 427
partial decussation 427
retinotopic maps 427
stereoscopic depth perception 427, 428*f*
retina *see* Retina
see also Optic chiasm; Optic nerve (cranial nerve II); Optic tract
Optic tectum 87, **449–455**
behavioral correlates 454
fish 454
frogs 454
nonmammalian vertebrates 454
snakes 454–455
characteristics 449
cytoarchitecture 449
afferents (axonal terminals), originating locations 450
efferent fiber groups 450–451
efferents, originating locations 450–451
horizontal neurons 449–450
laminated structure 87, 92, 92*f*, 449, 450*f*
functional 92–93
pyramidal neurons 449–450
stratum album centrale 449–450
stratum marginale 449
stratum opticum 449–450
stratum periventriculare 449–450
definition 449
development 453
acetylcholine role 453
axonal guidance 453
dendritic arbor formation 458
ephrin-A2 453
ephrin-A5 453
FGF-8 453
Gbx-2 453
glutamate 453
Grg-4 452*f*, 453
optic tract development 430–431
Otx-2 453
patterning 92
lineage analysis 92
retinotopy and 92–93
phases 453
reelin (RELN) role 454
retinal ganglion cells and 125
Shh 454
Wnt3 454
functional role 87
mammalian equivalent *see* Superior colliculus (SC)
retinotopic maps 365, 416, 434
EphA/ephrinA expression 416–417, 417*f*
EphB/ephrinB expression 416–417, 417*f*
superior colliculus *see* Superior colliculus (SC)
telencephalization 449
teleost fish 449
projection of retina 451, 451*f*
topographic organization 451
Optic tract
development, retinotopic reordering 430
fiber organization 428, 429*f*
see also Optic chiasm; Optic nerve (cranial nerve II)
Optic vesicle(s) 102, 266
optic nerve development 429
retinal development 103, 266
specification 103
Organic cation transporters (OCTs)
enteric nervous system development 343
Organizers *see* Developmental organizers
Oriented cell division, retinal progenitors *see* Retinal progenitor cells (RPCs)
Orr, H, neuromeres and neural tube segmentation 96, 97*f*
Otic ganglia, Ret signaling 611–612
Otoacoustic emissions (OAEs)
somatic marker of prenatal androgen exposure 352
Otx1 gene/protein
corticospinal development 403
Otx2 gene/protein
AP patterning and 80
isthmic organizer induction 87–88
optic tectum 453
photoreceptor cell specification 263
retina AP axis development 125
Outer nuclear layer (ONL) 259
Outer plexiform layer (OPL) 259
Ovulation
sexual differentiation 354
synaptic plasticity 667–668
Oxytocin
astrocyte-synapse relationship and 571

P

p19^{ink4A}, hair cell differentiation 247
p21 (Cip1), cell-fate determination 594
p27 (Kip1)
cell-fate determination 594
hair cell differentiation 247
p35
Cdk5 complex and cell-fate determination and 595
cortical lamination and 194
p39, Cdk5 complex and cell-fate determination and 595
p53
cell fate/differentiation role 596
p57 (Kip2), cell-fate determination 594

p73, cell-fate determination 596
p75 receptor(s) 622, 676
  apoptosis role 608–609, 609–611, 622–624
  autonomic target innervation and 338
  myelination role 308
  physiological role 622–624
  programmed cell death 600–601
  signaling 622–624, 623*f*
  Trk co-expression 622–624
  see also Trk receptor(s)
PAFAH1B1 gene/protein *see LIS1 (PAFAH1B1)* gene/protein
Paired box gene 6 *see* PAX6 gene/protein
Paleocortex 116
Palmitoylation
  postsynaptic assembly regulation 493
  Wnt protein modifications 39
Pam/Highwire/Regulator of Presynaptic Morphology-1 (RPM-1) *see* PHR (Pam/Highwire/RPM-1)
Paracrine signals
  programmed cell death 600
Paradigms, hypotheses *vs.* 95–96
Parasympathetic nervous system
  apoptosis 611
    CNTF effects 611–612
  development, axon outgrowth 336
  ganglia
    avian ciliary ganglion 329–330
Pard3, retinal development 255
Pard6, retinal development 255
Parents/parenting
  sexual dimorphism and 352–353
PARK1 gene/protein *see* α-Synuclein
Parkinson's disease, pathology/pathogenesis
  autophagy and 619
  glial growth factors 639
  Lewy bodies 619
  see also Dopaminergic neurons/systems
Parkinson's disease, therapy 221, 625–626
  cell replacement therapy 655
  fetal mesencephalic tissue transplants 689
  growth factor/neurotrophin therapy
  stem cells and *see cell replacement therapy (above)*
Parsimony
  models and 95
Partial (bicortical) lissencephaly *see* Subcortical band heterotopias (SBHs)
Partial decussation, optic pathway 427
Parvicellular contralateral red nucleus *see* Red nucleus
Parvicellular red nucleus (RNp) *see* Red nucleus
Patched 1 receptor (Ptc1) 24, 29, 84, 465, 467*f*, 471
  expression 24
  negative feedback loop 34
PAX2 gene/protein
  cochlear development
    hair cell differentiation 246
  isthmic organizer induction 88
  retina AP axis development 125
  retinal ganglion cell axon growth 469–470
PAX3 gene/protein
  adult melanocyte stem cell regulation 319
  melanoblast specification role 314
  neural crest development 272
  Schwann cell development 307
  tectal-tegmental boundary 92
PAX5 gene/protein, isthmic organizer induction 88
PAX6 gene/protein 120
  BMP signaling 46–47
    DV axis inversion 52–53, 53*f*
  DV neural tube expression 31–33, 32*f*
    cross-repression 33
  midbrain patterning
    isthmic organizer induction 88*f*
    midbrain-forebrain boundary 82
  neocortical arealization 120
    expression patterns 120
    regulatory gene interactions 121
  retinal progenitor cell multipotency 259
  spinal cord, dorsoventral patterning 57
  telencephalon specification/development 102
  visual system development
    retina
      amacrine cell generation 262
      anteroposterior axis 125
      horizontal cell specification 263
PAX7 gene/protein
  BMP signaling 46–47
  neural crest 271–272
  tectal-tegmental boundary 92
PAX8 gene/protein, isthmic organizer induction 88
Paxillin, growth cone migration 482
Pbx proteins
  Hox gene expression 67–68
  midbrain-hindbrain boundary formation 84
Pdm/Cator, cell-type specification 590
Pdm gene/protein, cell-type specification 590
PDZ domain(s)
  postsynaptic density proteins
    GRIP 497–498
    PICK1 498
    PSD-95 496
    Shanks 497
Pea3 transcription factor, motor neuron specification
  dendrite patterning 190
Peanut agglutinin (PNA), perisynaptic Schwann cells probes 563
Peripheral nerve(s)
  bridges, spinal cord injury
    injury-cell body distances 689
  cranial *see* Cranial nerve(s)
  degeneration *see* Neuropathy
  development *see* Peripheral nervous system, development
  injury *see* Peripheral nerve injury
  regeneration *see* Peripheral nerve regeneration/repair
  Wallerian degeneration *see* Wallerian degeneration
Peripheral nerve injury
  CNS connectivity changes 688
  gene expression changes 688
  regeneration *see* Peripheral nerve regeneration/repair
  Schwann cell responses *see* Schwann cell(s)
  see also Neuropathy; Peripheral nerve regeneration/repair
Peripheral nerve regeneration/repair **687–690**
  axonal regeneration 485–486
    see also Axonal regeneration
  BDNF 640
  CNTF 640
  GDNF 640
  glial cell growth factors 640
  Schwann cell responses *see* Schwann cell(s)
Peripheral nervous system (PNS)
  glial cells 218
    Schwann cells *see* Schwann cell(s)
  myelination regulation 300
  neuromuscular junctions *see* Neuromuscular junction (NMJ)
  neurotrophins 625
  see also Peripheral nerve(s)
Peripheral nervous system, development 298
  axonal pathfinding, laminin expression 483–484
  diversification/specification, ErbB signaling 303
  sensory organ precursors 132–133
Peripheral neuropathy *see* Neuropathy
Perisynaptic fibroblasts, NMJ synapse elimination and 558
Perisynaptic Schwann cells (PSCs) 562*f*
  ablation effects 563, 564, 571
  axonal sprouting 565
  bridges 565
  characteristics 561
  degeneration 565
  development 532, 567–568
  NMJ plasticity and *see* Neuromuscular junction, plasticity
  probes 561
  regeneration 565
  remodeling 564
  synapse elimination and 558, 569–570
  synaptic maintenance 564
  synaptogenesis 563, 567–568
  terminal 532, 538
  trophic factor expression 564
Periventricular nodular heterotopia (PNH) 192–193
  radial migration phase and 194
Perlecan
  NMJ basal lamina 541

Pexophagy 615
Phagocytosis
  glial cells
    synapse elimination and 569
  programmed cell death 602–603
Phagophores 615
Pharyngula, developmental organizers 81*f*
  see also Developmental organizers
Phenotype switching, autonomic developmental plasticity 339
Pheomelanin 312
Pheromone receptors
  V1R 227
  V2R 227
Phosphacan, axon growth inhibition 401, 676
Phosphatidylinositol 3-kinase (PI3K)
  inhibitors and prevention of autophagic cell death 616
  myelination 301
Phosphoinositide-3 kinase (PI3K) *see* Phosphatidylinositol 3-kinase (PI3K)
Photopigment(s)
  mutations, optic chiasm 431, 431*f*
Photoreceptor(s)
  cones *see* Cone photoreceptor(s)
  development 263
    cell-type specification
      Crx 263
      neural retina leucine zipper (Nrl) 263
      Otx2 263
    patterning 130
    R8, neurogenesis 132–133
  photopigments *see* Photopigment(s)
  retinal pigment epithelium and *see* Retinal pigmented epithelium (RPE)
  rods *see* Rod photoreceptor(s)
Phox2b
  deletion, autonomic nervous system and 326
Phox transcription factors
  autonomic nervous system development 338–339
    gangliogenesis 333–335
  oculomotor complex patterning 91
    congenital cranial dysinnervation disorders 91
PHR (Pam/Highwire/RPM-1)
  invertebrate NMJ
    retrograde signaling and 527
  presynaptic development 510
Piccolo 506–507, 517
  see also Bassoon
Piccolo/Bassoon transport vesicles (PTVs) 506–507
PICK1 (protein interacting with kinase C)
  AMPA receptor localization 497, 498
Pigments, phototransducing *see* Photopigment(s)
PI3K *see* Phosphatidylinositol 3-kinase (PI3K)
Pioneer neurons
  axon guidance 387
Pituitary adenylate cyclase activating polypeptide (PACAP)
  autonomic neurons
    enteric
      developmental changes 630–631
  in development
    enteric neurons 630–631
PITX3 transcription factor
  DA neuron development 222, 223
  midbrain dopaminergic neuron formation 91
PKA *see* Protein kinase A (PKA)
PKC *see* Protein kinase C (PKC)
PKG-Neo cassette, *Olig1* knockout mice 240
Planar cell polarity (PCP) pathway 8
Planaria, regeneration, morphogen gradients and polarity 16
Planar transcytosis
  organizer signals 85–86
  sonic hedgehog signaling 22, 25*f*
Plasticity *see* Neuroplasticity
Platelet-activating factor acetylhydrolase (PAF-AH), LIS1 functions
    196–197
Platelet-derived growth factor (PDGF)
  astrocyte differentiation 636
  glial cell production 639*t*
  glioblastoma 638
  oligodendrocyte differentiation 241, 637
  radial glia differentiation 638
  receptors, oligodendrocyte precursors 241
  Schwann cells 634
PlexinB, *Drosophila* motor neuron target selection 523–524
Plexins 674
Plexitis *see* Enteric ganglionitis (plexitis)
P-NUDEL, neuronal migration 195, 195*f*
Polarity, morphogens 16
Polyaxonal (polyneuronal) muscle innervation
  developmental 530, 530*f*, 553
    bioassays and 557
    change to monoaxonal *see* Neuromuscular junction, synapse
        elimination
    evidence for 553
      electrophysiological 554
      histological 554, 554*f*
    transmission efficacy and 536
Polycomb group (PcG) proteins
  Hox gene expression 66–67
'Polyganglion development,' trunk neural crest defects 273*f*, 279
Polymicrogyria 192–193
porcupine gene, Wnt palmitoylation 39
Positional information, morphogens history 17
Position effect, transcriptional silencing 181
Position effect variegation, transcriptional silencing 181
Posteriorization *see* 'Caudalization' (posteriorization)
Posterior patterning *see* 'Caudalization' (posteriorization)
Postmitotic cell migration, retinal development *see* Retinal
    development
Postnatal development
  corticospinal system 403
    topographic refinement and 406–407
  ENS *see* Enteric nervous system, development
Postsynaptic currents (PSCs)
  GABAergic 575–576
  NMDA receptor-driven activation 577–578
Postsynaptic density (PSD) 487, 495, 502, 512
  assembly/development 500
    kinetics 501
    presynaptic *vs* 500
    scaffold complexes and 500–501
  location opposite active zones 495
  protein composition 495
    adhesion molecules 498
      cadherins 498
      ephrins/Eph receptors 498
      integrins 498
      neuroligin 498
      SynCAM 498
    cytoskeletal proteins 499
      α-actinin 499
      drebrin 499
      IRSp53 499
      αN-catenin 499
      profilin 499
      spinophilin (neurabin II) 492–493, 499
    integral membrane proteins 498
    receptors 498
      adhesion molecules and 498
      glutamate complexes *see* Glutamate receptor(s)
    regulatory/signaling proteins 498
      CaMKII 499
      nNOS 498–499
      Ras GTPases 499
    scaffolding/adaptor proteins 496
      AMPA-receptor-linked 497
      GKAP/SAPAP family 497
      GRIP/ABP 497
      Homer 497
      PDZ domains 496, 497–498
      PICK1 498
      PSD-95 *see* PSD-95
      PSD assembly and 500–501
      Shank *see* Shank
      see also PDZ domain(s)
    study methods 495–496
  rearrangements
    developmental assembly
      cell adhesion molecules 498
  size/structure 495, 496*f*
    molecular organization 496*f*
  structure–function relationship 495
  study methods 495
Postsynaptic density 95 protein *see* PSD-95
Postsynaptic development **487–494**, **495–501**

Postsynaptic development (*continued*)
adhesion molecules 500
assembly regulation 493
lipid modifications 493
PSD-95 phosphorylation 493
ubiquitination 493
differentiation
governing principles 488
induction 489
neurotransmitters 489
neuroligin-1 488
excitatory synapses 487
kinetics 501
morphological view 500, 500*f*
presynaptic development and 495, 502–503, 512–513
PSD *see* Postsynaptic density (PSD)
scaffolding proteins 487, 488*f*
definition 487
F-actin 492
localization 490
neuronal transport 493
organization 490
proteomic approaches 487
synaptic adhesion molecules 490
NMDA receptor clustering 491
signaling pathways 492
stationary *vs.* mobile scaffold complexes 500–501
time course 500
Postsynaptic membrane 512
assembly/development *see* Postsynaptic development
excitatory synapses 495, 496*f*
inhibitory *vs* 495
postsynaptic density *see* Postsynaptic density (PSD)
inhibitory synapses 495, 499
excitatory *vs* 495
see also GABA/GABAergic transmission
receptors *see* Postsynaptic receptors
see also Postsynaptic density (PSD)
Postsynaptic membrane 502
Postsynaptic receptors 491, 498
see also Receptor(s)
Potassium/chloride cotransporter 2 (KCC2)
excitatory-to-inhibitory shift 574–575, 574*f*
activation 575
Pou4f3, hair cell differentiation 249
POU transcription factors
midbrain-hindbrain boundary formation 84
red nucleus development 91
Prechordal plate
brain patterning 27
formation 108
neural epithelium ventralization 21
non-neural organizer of the neural plate 107*f*, 108
holoprosencephaly and 108
hypothalamic morphogenesis role 108
notochord similarity 108
Preoptic and anterior hypothalamus (POAH)
sexually dimorphic nuclei 355
Preoptic area *see* Preoptic and anterior hypothalamus (POAH)
Preplate (PP) 117
Prep/Meis cofactors, *Hox* gene expression 67–68
Prestin
hair cell differentiation, hypothyroid animals 250
Presynapse 512
assembly *see* Presynaptic development
development *see* Presynaptic development
structure 512
active zone *see* Active zone (AZ)
boutons *see* Synaptic bouton(s)
excitatory (glutamatergic) 496*f*
vesicles *see* Synaptic vesicle(s)
see also entries beginning presynaptic; Synaptic vesicle cycle
Presynapse
structure 502
Presynaptic bouton *see* Synaptic bouton(s)
Presynaptic development 500, **502–511**, **512–519**
assembly
vesicular intermediates 488–489
components 502
active zone and cytomatrix 517
criteria for presynaptic site 505
differentiation mechanisms
cellular 506, 508*f*, 517
en route SV release 507
mobile precursors and axonal transport 506, 507, 512–513
Piccolo/Bassoon transport vesicles 506–507
preassembled ‘prototerminals’ 506
SV sources 507
use of pre-existing synapse components 507
molecular 507, 514
agrin 509
cadherins 509, 517
CASK role 515–516
γ-protocadherins 509–510, 517
laminins 509
mechanisms of action 515
N-CAM 509–510
neurexins 509, 515, 516–517
neuroligin 509, 515, 516–517
scaffolding/cytoskeletal molecules 510
SynCAM 509, 515–516, 516*f*
dynamics 503–504, 507–509
initial events 502
axonal growth cones and 502–503, 512–513
en passant synapses (synaptic boutons) 503, 503*f*, 512–513
NMJ formation 502
postsynaptic factors 502–503
secreted molecules and
FGFs 514
glutamate 513–514
neurotrophins 514
Wnts 514
stabilizing effects of 503–504, 507
synaptotrophic hypothesis and 503–504, 504*f*
maturation of nascent sites 504, 512–513
activity role 513, 517
background activity and 518
‘desilencing’ and 517–518
short-term plasticity and 518
functional changes 505, 517
heterogenous populations 505–506
neuronal maturation state and cellular dynamics 505–506
short-term plasticity and 518
silent synapses 506, 517–518
speed of 505
stages 513, 514*f*
structural changes 504
mature synaptic boutons *vs.* 505
structure–function relationship 505
synapse stabilization 503–504, 507, 516
synaptic vesicles
CASK role 516
excitosis and 505, 513–514
numbers of 504
recycling changes 513, 516
related protein expression and 506
model systems/experimental analysis
EM studies 504
imaging studies 503–504, 506, 513
invertebrate systems 509–510
C. elegans 509–510
Drosophila 509–510
vertebrate NMJ 502, 509
Xenopus spinal neuron/muscle co-culture 505, 506
multiple stages **512–519**
orphan release sites 507
postsynaptic development and 500, 502–503, 512–513
prepackaged molecules 500, 513, 515
Prickle molecule, positional information 19–19
Primary afferent neurons *see* Sensory afferent(s)
Primary neurulation 271, 272*f*
Primary visual cortex (V1: striate cortex)
sexual dimorphism 350
Primate(s)
neural circuit development
dendritic spines 578
GABA 576*f*, 578, 578*f*
somatosensory cortex *see* Somatosensory cortex
Primitive streak formation (gastrulation) 56
Prkci, retinal development 255
Probes
perisynaptic Schwann cells 561
Profilin
postsynaptic density 499

Progenitor cells (neural) *see* Neural stem/progenitor cells (NSPCs)
Progenitor factors, spinal cord development 172, 173*f*
Progesterone
glial development and
Schwann cell differentiation 307–308
Programmed cell death (PCD) **599–606**, 607
apoptosis (type 1) *see* Apoptosis
autonomous *vs.* conditional specification 600
autophagy (type 2) *see* Autophagic cell death
caspase role 602
cell death activation signals 600–601, 602*f*
critical components 604*f*
definition 599
different types of 603
dying cell removal process 602–603
evolution 599
history 599
intracellular regulation 601
genes 604*f*
nervous system 599, 600*t*
neuronal development 601*f*
specific molecule associations 600–601, 603*t*
neurotrophic factors and 600
neurotrophic theory 599
p75 receptors 600–601
Trk receptors 600–601
pathological neuronal death 605
phagocytosis 602–603
sex steroids and 348–349, 351, 357
signaling pathways 600
Proline-rich synapse-associated protein (ProSAP) *see* Shank
Proneural clusters (PNC), Notch-Delta signaling
bearded gene family role 135
cell selection 131
enhancer of split expression 133
gene expression autoregulation 131
genes associated 131
Proneural field(s), neurogenesis 135
Proprioceptive feedback
motor neuron specification 190
Prosencephalon *see* Forebrain
Prosensory patches, hair cell differentiation 245
Prosomeres 95
as model of forebrain development (neuromere model) **95–99**, 103
definition 95
doubts over and columnar model 96–97
historical aspects 96
His's contribution 96, 98*f*
Orr's contribution 96, 97*f*
see also Brain development, models
Prospero
cell cycle regulation 596–597
as cell-fate determinant 587
cell cycle and 593
Prostaglandin(s)
sexual dimorphism and 351
Prostaglandin E_2 (PGE_2)
puberty 639
Proteasome(s)
synaptic elimination at the NMJ 558
Protein kinase(s)
integrin-mediated regulation 542
Protein kinase A (PKA)
axon growth/guidance, olfactory system 418–419
Protein kinase C (PKC)
axon growth/guidance 476–477
PKCα, postsynaptic receptor localization 491–492
Protein synthesis
inhibition
plasticity and 436–437
synaptic plasticity and 436–437
Protein trafficking/transport
retinal development 256
Proteoglycans
axon guidance 400
chondroitin sulfates 398, 400, 401
features 400
heparan sulfates 400
keratan sulfates 400
phosphacan 401
Proteolysis
synaptic elimination at the NMJ 558
Protocadherin(s)
retinal development 257
γ-Protocadherins, presynaptic development 509–510, 517
Protostomes
midbrain patterning 93–94
'Prototerminals' 506
Prox1, horizontal cell specification 263
PSD-95 496
functional roles 497
NMDA receptors and 447–448
clustering 496–497
genes 496
knockout animals 497
localization 496–497, 496*f*
postsynaptic development and 487, 490
function 492
knockout mice 487–488
localization 490, 491
regulation 491–492
signaling pathways 492–493
overexpression 487–488
phosphorylation 493
proteomic studies 487
scaffold role 489–490
signaling pathways 492
splice variants 487
protein interactions
neuroligin 498
protein-binding domains 496
structure 496
protein interaction domain 496
Psychiatric disorders
sex differences 348
see also Sexual dimorphism
Psychological diseases/disorders *see* Psychiatric disorders
Ptf1a 151*t*
GABAergic neurons 154–155
Puberty
glial cell growth factors 639
neuroendocrinology 350
sex differences/sexual dimorphism and
brain structure 350
synaptic sprouting 667
see also Adolescence; Brain development in adolescence
Purpurin 679
Pyramidal neuron(s)
cortical 585
corticospinal 403
layer-specific morphology 585
development, NMDA receptors and 444–445
GABAergic synapses 576
global ischemia and
REST role 208
see also Ischemic stroke (cerebral ischemia)
Pyramidal tract
decussation 404

Q

Quail cells, enteric nervous system development 344, 344*f*
Quail-chick chimera technique 271, 272*f*
cardiac neural crest 277
Quantal content
neuromuscular junction (ACh)
maturation and 533
'Qucks' embryos 275–276, 276*f*

R

Rab3-interacting molecules *see* RIMs
Rab23, sonic hedgehog (Shh) signaling 36
Rab-interacting molecule (RIM) *see* RIMs
Rac1 GTPase
activation 476
Rac GTPases
agrin signaling 550, 551*f*
Rac1 *see* Rac1 GTPase
Radial fibers of Müller *see* Müller cells (glia)
Radial glial cells 592, 648
cell cycle length hypothesis and 592

Radial glial cells (*continued*)
  gene products 238
  Hes genes and nervous system development 158–159
  historical aspects 585
  macroglial lineages 218
  neuronal migration 585–586
  neuronal stem cells/precursors 193, 585–586
  optic chiasm decussation decisions 429–430
  somata 218
Radial migration, cortical development 193
  phases 193–194
Radial sorting, Schwann cells 289
RALDH1
  DA neuron development 223
  retina dorsoventral axis development 128
RALDH3, retina dorsoventral axis development 128
Ramón y Cajal, Santiago
  axonal growth cones 426
  netrins, discovery 388, 389, 472
Rap1, transcriptional silencing 181
Raphe nuclei (RN)
  development 91
Rapsyn, AChR aggregation at the NMJ 531, 533
Ras GTPase(s)
  PSD proteins 499
Rb tumor suppressor *see* Retinoblastoma (Rb) gene/protein
RE1-silencing transcription factor (REST)
  activator role 211
    L1 cell adhesion molecule 211
  adults 208
    global ischemia 208
    isoforms 208
    kainate-induced upregulation 208
  cancer 209
    overexpression 209–210
  developmental role 207
    de-repression 208
    gain-of-function experiments 208
    'master neuronal regulator hypothesis' 207–208
  expression 206–207
  gene organization 206
    exons 206
    splice variants 206
      REST4 206, 209
  historical aspects 206
  localization 209
  protein structure 206
    DNA binding element 206, 207*f*
    repressor domains 206, 207*f*
  regulation 208
    neuronal differentiation 209
  targets 208
  terminal differentiation **206–212**
    cellular environments 210
    chromatin modifications 210–211, 210*f*
    coREST 211
    downregulation 210
    epigenetic markers 211
    protein degradation 211
  transcriptional repression role 182, 207*f*, 210
Reaching
  corticospinal refinement and 410*f*, 411
  neural basis
    corticospinal tract 411
Reactive oxygen species (ROS)
  neuropathologies associated
    autonomic dysfunction
      enteric dysfunction, neurotrophin
          neuroprotection 632
Readily releasable vesicle pools (RRPs) 512
  probability of release and 512
Receptive field(s) (RF)
  development, NMDA receptors 446
  retinal ganglion cells *see* Retinal ganglion cells (RGCs)
Receptor(s)
  postsynaptic 498
    adhesion molecules and 498
    glutamate complexes *see* Glutamate receptor(s)
    see also Postsynaptic membrane
  presynaptic *see* Presynapse
Receptor tyrosine kinase(s) (RTKs)
  Hox gene expression regulation 64–65
Receptor tyrosine phosphatase(s) (RTPs)
  axon guidance 401
    neuron-glial interactions 401
  Drosophila motor neuron defasciculation 523
Red nucleus 87
  midbrain patterning 91
Reelin(s)
  neuronal migration
    cortical lamination and 194, 195, 195*f*
  receptors
    apolipoprotein E receptor 2 (ApoER2) 194
    VLDL receptor 194
Regeneration *see* Neural regeneration/repair
Regeneration-associated genes (RAGs) 678–679, 688
Rehabilitation
  corticospinal development and 413
Related to receptor tyrosine kinases (RYKs)
  retinal ganglion cell effects 416–417
  Wnt signaling
    expression 463
    topographical mapping 369
    Wnt5 signaling 463
Remak bundles, myelination 300–301
Repair *see* Neural regeneration/repair
Reperfusion *see under* Ischemic stroke (cerebral ischemia)
Repression (gene) *see* Transcriptional repressors/repression
Reproduction
  behavior and *see* Reproductive behavior(s)
  synaptic plasticity 667
Reproductive behavior(s)
  sexual differentiation 354
  see also Sexual behavior
Repulsive guidance molecules (RGMs) *see* Axonal guidance cues
RET gene/protein
  apoptosis
    GDNF-related ligands 608–609
    glycosylphosphatidylinositol-linked cell surface
        receptors 608*f*, 611
    knockout mice 611
    sympathetic nervous system 611
  autonomic gangliogenesis 335
  enteric nervous system 628
    adult expression 632
    development and 345
    Hirschsprung disease 628, 630
    prenatal expression 630
  GDNF family ligands (GFLs)
    apoptosis 608–609
  GDNF receptor complex 628, 629
  parasympathetic axon outgrowth 336
Retina
  anatomy/physiology 259, 260*f*
    amacrine cells *see* Amacrine cells
    bipolar cells *see* Bipolar cells (retina)
    ganglion cell layer 259
    ganglion cells *see* Retinal ganglion cells (RGCs)
    horizontal cells *see* Horizontal cells (retina)
    inner region
      inner nuclear layer 259
      inner plexiform layer *see* Inner plexiform layer (IPL)
    melanocytes 312
    neuronal cell types *see* Retinal neurons
    organization 251, 252*f*
      laminar 251
    outer region
      outer nuclear layer 259
      outer plexiform layer 259
    projection onto optic tectum (teleosts) 451, 451*f*
  development *see* Retinal development
  LGN topographic maps and 435
    see also Retinotopic maps
  neurons *see* Retinal neurons
  regeneration
    partial retinal ablations and 452*f*
    stem cells *see* Ocular neural stem cells
  stem cells/progenitors *see* Ocular neural stem cells
  topographic maps and *see* Retinotopic maps
  see also entries beginning retino-/retinal
Retinal axon guidance (RAG) molecule 395
Retinal bipolar cells *see* Bipolar cells (retina)
Retinaldehyde
  oxidation 55

Retinaldehyde dehydrogenase gene(s) *(Raldh)* 55
Raldh2 55, 56–57
knockout embryos 57
somite formation 58–59
spinal cord motor neuron differentiation 57
retinoic acid synthesis 55
Retinal development **251–258**, 266, 267*f*
anteroposterior (A-P) axis 125
DNA binding-dependent mechanisms 126
DNA binding-independent mechanisms 126
EphA4 gene/protein 126
EphA5 gene/protein 126
EphA6 gene/protein 126
EphA7 gene/protein 126
ephrin A5 gene/protein 126
fate mapping 125
fibroblast growth factor 8 125
FoxD1 gene/protein 125–126
FoxG1 gene/protein 125, 126
Gbx2 gene/protein 125
Gh6 gene/protein 126
Irx3 gene/protein 125
Otx2 gene/protein 125
Pax2/En1 gene/protein 125
Pax6 gene/protein 125
Six3 gene/protein 125
SOHo1 gene/protein 126
cell cycle and 594
cdki effects 595
cyclin effects 595
prox1 593
Rb protein and 595–596
TGFβ 597
cell differentiation regulation 125, 255
cell adhesion 257
cell cycle exit waves 255–256
cell type-specific differentiation 256
chromatin remodeling 256
neuronal process outgrowth 257
protein trafficking 256
SOC3 protein 255–256
STAT signaling pathway 255–256
synapse partnering 257
synaptogenesis 257
cell fate/proliferation coordination 259
asymmetric divisions 263, 264*f*
bHLH 261
cell cycle withdrawal 259–260
environmental factors 260
homeodomain transcription factors 261
intrinsic factors 261
Zebrafish models 263–264
cell type specification 253–254, 256, **259–265**, 261*f*
Mash1 262
competency model 253, 254*f*
atoh7 254
cell cycle exit 253–254
cell fate decisions 253–254
cell type 253–254
'clock-like' mechanisms 254
GDF11 254
dorsoventral (D-V) axis 125
BMP2 gene/protein 128
BMP4 gene/protein 127–128
bone morphogenic protein (BMP) 127
cVax gene/protein 127–128
Cyp26 family 128
early stages 127, 127*f*
Emx homeobox gene 127
EphB2 gene/protein 127–128
EphB3 gene/protein 127–128
ephrinA2 gene/protein 128
ephrinB1 gene/protein 127–128
ephrinB2 gene/protein 127–128
FoxG1 gene/protein 128
late stages 128
oblique-gradient molecules 128
RALDH1 128
RALDH3 128
retinoic acid 128
Shh gene/protein 127
Tbx5 gene/protein 127–128
Vax2 gene/protein 128–129
ventropin 127–128
extrinsic signaling factors 266
histogenesis
mammalian 267
nonmammalian 267–268
inhibitory (repulsive) guidance cues
ephrins/Eph receptors 125
anteroposterior (A-P) axis 126
dorsoventral (D-V) axis 127–128
initiation 253
lineage/birth date relation 253, 253*f*, 254*f*
ontogenesis 251, 252*f*
optic cups 266
optic vesicles 103, 266
postmitotic cell migration 254
apical-basal axis 255
basement membrane 255
bidirectional movements 255
cell polarity 255
guided cell migration 254–255, 255*f*
migration modes 254
modes 255*f*
neuroepithelium apical cell junctions 255
somal translocations 254–255, 255*f*
tangential displacement 255
unconstrained migration 254–255, 255*f*
retinal patterning 129
A-P axis *see anteroposterior (A-P) axis (above)*
D-V axis *see dorsoventral (D-V) axis (above)*
melanopsin-containing retinal ganglion cells 129–130
photoreceptor cells 130
retinal waves 433
visual response coordination 433
signaling pathways
Hedgehog signalling 103
Nodal signalling 103
Notch signaling and 266–267
spatial control 253
fibroblast growth factor 253
transcription factor expression 103, 266, 593, 594
ventrotemporal (VT) retina
EphB1 gene/protein 126
FoxD1 gene/protein 126
Zic2 gene/protein 126
see also Retinal progenitor cells (RPCs)
Retinal fiber reallocation, optic chiasm 431, 431*f*
Retinal ganglion cells (RGCs)
axons
growth/guidance 415
lateral geniculate nucleus 417
optic chiasm crossing 415–416
optic disk 415, 416*f*
Pax2 gene 469–470
retinal projections 415
sonic hedgehog role 469, 469*f*
see also Retinal development
topographic maps 373
cyclin-dependent kinase inhibitors 259–260
development 125
astrocytes and synapse formation/maturation 514, 567, 569*f*
axon guidance *see axons (above)*
differentiation
Brn3b transcription factor 262
Math5 262
optic tectum 125
superior colliculus 125
see also Retinal development
ganglion cell layer (GCL) 259
generation 259, 261*f*, 427
feedback inhibition 260
green fluorescent protein studies 260
growth differentiation factor 11 (GDF11) 260–261
retina as target 434
retinal waves 433
Sonic Hedgehog (Shh) 260–261
melanopsin-expressing *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
photoperiodic timing *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
photosensitive *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)

Retinal ganglion cells (RGCs) (*continued*)
retinotopic maps *see* Retinotopic maps
specification 262
superior colliculus 434
topographical mapping 365, 366–368, 367*f*, 415, 416, 417*f*
branching 366–368
gradient-determined repulsion 368
opposing gradients 368
projection specificity 365–366
zebra fish, optic nerve disruption effects 415
Retinal ganglion cells, melanopsin-expressing *see* Intrinsically photoreceptive retinal ganglion cells (ipRGCs)
Retinal neurons 251, 252*f*
bipolar cells *see* Bipolar cells (retina)
ganglion cells *see* Retinal ganglion cells (RGCs)
horizontal cells *see* Horizontal cells (retina)
precursors 266, 594
specification *see* Retinal development
Retinal pigmented epithelium (RPE)
ocular neural stem cells 268
Retinal progenitor cells (RPCs)
interkinetic nuclear migration 251
definition 251, 252*f*
optic cup neuroepithelial cells 251
multipotency 259
competence changes 259
Pax6 259
tracer experiments 259, 260*f*
oriented cell division 251, 252*f*
Numb protein 251
symmetric *vs.* asymmetric 251
proliferation 251
proliferation regulation 252
cyclin inhibitors 252–253
cyclin kinases 252–253
cyclins 252–253
fibroblast growth factor (FGF) 252–253
Hedgehog growth factor 252–253
Notch ligands 261
transcription factors 252–253
Wnt family 252–253
zebrafish models
asymmetric division 263–264
imaging studies 263–264
'Retinal stem cell' *see* Ocular neural stem cells
Retinal waves
retinal development *see* Retinal development
retinal ganglion cell generation 433
Retina/optic tectum/superior colliculus map, axonal growth and guidance models 385
Retinitis pigmentosa 263
Retinoblastoma 595–596
Retinoblastoma (Rb) gene/protein
cell fate/differentiation role 595
cell cycle independent 596
knockout mice 596
photoreceptor specification 263
MyoD interactions 596–597
phosphorylation
cell cycle control 594, 595
as tumor suppressor 595–596
Retinoic acid (RA)
developmental role **55–60**, 56*f*
dopamine neurons 223
hair cell differentiation 249
hindbrain AP patterning 56
Hox gene expression
motor neuron specification 75
regulation 64, 65
regulation via signaling gradients 65
response diversity 64
response specificity 64
motor neuron specification 74
Hox genes 75
neural tube DV patterning 33–34
posterior central nervous system 55
paracrine action 59
posterior patterning and *Fgf8* expression 58
retina DV axis development 128
somite pair formation 58–59
spatiotemporal aspects 55
spinal cord DV patterning 57
synthesis 55
enzyme degradation 55, 56–57
isomers 55
Cellular retinoic acid (RA) binding protein II *(Crabp2)* 57
Retinoic acid nuclear α receptors (RARs)
expression patterns 55
Hox gene expression 64
Retinoic acid response elements (RAREs), *Hox* gene expression 56–57, 64
direct repeats 64, 68
transgenic mice 64
Retinoid X receptor(s) (RXRs)
neural patterning 55
Retinol
oxidation 55
Retino-tectal projection, ephrin guidance cue model 673–674
Retinotectal system
topography development 366–368
Retinotopic maps
comparative biology
fish 434
frogs 434
mammals 434
pharmacological blocking 434
degree of 434
development
activity-dependent
optic tectum 93
optic tectum 92–93
refinement 370
lateral geniculate nucleus 417
midbrain
optic tectum 87
nicotinic receptor knockouts and 434
optic pathway 427
optic tectum 416, 434
retinal ganglion cells 415, 434
targeting 434
target overshooting 434
retinotopic *see* Retinotopic maps
superior colliculus 416
visual cortex 417
Retinotopic reordering, optic pathway development 430
Retraction bulbs, NMJ synapse elimination 556
Retrograde signaling
transynaptic influences
synapse formation
laminins and 509
neuroligin and 509
scaffolding proteins 510
SynCam and 509
see also Presynaptic development
Retrovirus(es)
adult NSC identification 643
Ret signaling *see RET* gene/protein
Rett syndrome 478
Rhodnius, morphogen gradients and polarity 17
Rho GTPase(s)
agrin signaling 550, 551*f*
axonal development
growth gradient guidance models 384, 426
Rac1 *see* Rac1 GTPase
synaptic muscle nuclei clustering and 550
Rhombencephalon *see* Hindbrain
Rhombomeres 95
hindbrain patterning 56, 396–397
axon guidance 396
retinoic acid role 56–57
neural crest cell migratory pathways 275, 275*f*
Rho-ROCK pathway signaling
inhibitory guidance cues 677
see also Rho GTPase(s)
RILP protein, RE1-silencing transcription factor localization 209
RIM1α
presynaptic membrane role 517
RIMs
synaptic transmission role 517
RNAi *see* RNA interference (RNAi)
RNA-induced initiation of transcriptional silencing (RITS) 182
RNA interference (RNAi)
autophagic cell death 617
RNA silencing 182

Robo proteins
  axon guidance 398–399
  midline crossing 405
  trunk neural crest cells 279
Robo receptors, axon guidance 389
  fasciculation 389
Robust nucleus of the archistriatum, sex steroid effects on axonal growth/guidance 357–359
Rod cells *see* Rod photoreceptor(s)
Rodent(s)
  brain
    hippocampus, excitatory-to-inhibitory shift 575
  oligodendrocytes 238
Rodent whisker-driven afferents, NMDA receptors 443, 445*f*
Rod photoreceptor(s)
  anatomy/organization
    intrinsic stem cell precursors 269
    location 259
  generation 259, 261*f*
Rohon-Beard sensory neurons (zebrafish)
  autophagic cell death 618
  Notch signaling 138
Roof plate
  forebrain midline development role 109
    dorsal midline induction 107, 110
    epidermal ectoderm role 107
    FGF8 regulation 112, 112*f*
    MIH holoprosencephaly and 110
    Wnts/BMPs and 109
    Zic2–roof plate pathway in dorsal midline 112*f*, 113
  induction
    forebrain 109
    midbrain 89
  spinal cord dorsoventral patterning 172–173
Rostral migratory stream (RMS)
  adult neural stem cell migration 641, 642*f*, 649*f*, 650
Rostrocaudal (RC) patterning *see* Anteroposterior (AP) patterning
Roundabout (Robo) receptors 381
  slit binding 674–675
Roux, Wilhelm 16
RPM-1 *see* PHR (Pam/Highwire/RPM-1)
R-spondin(s), Fz/LRP receptor complex interactions 40
RunX1 transcription factor, olfactory neurogenesis 231–232
Rx transcription factor(s)
  eye development role 103
  retinal development 266
RYKs *see* Related to receptor tyrosine kinases (RYKs)

S

Sacral spinal cord, secondary neurulation 3
sad-1, presynaptic development 510
Saint-Hilaire, Geoffroy, DV axis inversion model 53*f*
St.-Hilaire, Geoffroy, DV axis inversion model 52–53, 53*f*
Salivary gland, development 330*f*
Sanpodo, cell fate decisions 136
SAP-102, NMDA receptors 447–448
Sarcoglycan(s)
  dystroglycan complex components 541
Satellite cells (glial) 306
  growth factors 634
    nerve growth factor 634
    neurotrophin-3 634
    transforming growth factor-α 634
    transforming growth factor-β 634
    vascular endothelial growth factor 634
Scaffold proteins
  glutamate receptors and
    AMPA receptors 497
    NMDA receptors 496–497
      PSD-95 *see* PSD-95
      PSD clustering 491
  postsynaptic density *see* Postsynaptic density (PSD)
  postsynaptic development 487
    definition 487
    F-actin 492
    localization 490
    neuronal transport 493
    organization 490
    proteomic approaches 487
    synaptic adhesion molecules 490
      NMDA receptor clustering 491
  presynaptic development 510
    RIMs *see* RIMs
Schizencephaly 192–193
Schwann cell(s) 298
  axonal relationships, motor neurons and 532
  axon relationships **293–297**
    axonal regeneration and 485–486
    communication between 293
    dependence on 296
    differentiation
      growth factors affecting 294*t*
      to immature cells 293
      transcription factors affecting 294*t*
    embryological origin 293
    myelination *see below*
    node of Ranvier 296
    precursor sustainment 293
    regeneration 297
    terminal Schwann cells 297
  development *see* Schwann cell development
  functions
    morphogenesis 289
    trophic support, developing neurons 288
  growth factors/receptors 634, 639*t*
    ciliary neurotrophic factor 634
    cytokines 634
    development and *see above*
    epidermal growth factors 634
    glial cell line-derived growth factor 634–635
    leukemia inhibiting factor 634
    nerve growth factor 634
    neurotrophins 634
    platelet-derived growth factor 634
    transforming growth factor-α 635
    transforming growth factor-β 634
    tumor necrosis factor-α 634
  injury response
    perisynaptic Schwann cells 565
    Wallerian degeneration and 687–688
  multiple sclerosis 634
  myelination and 290, 294, 295*f*, 298, 307–308, 309
    multiple sclerosis 634
    negative regulation 291
    β-neuregulin-1 role 290
    regulation 300, 308, 309
    related transcription factors 290
  neuromuscular junction (NMJ) *see* Perisynaptic Schwann cells (PSCs)
  nonmyelinating 307–308
  synaptogenesis and 567
  terminal cells 297
  transcriptional control **306–311**
    basic helix-loop-helix proteins 310
    immature cell development 307
    immature cell maintenance 307
    neuregulins 306
    terminal differentiation 307
    transcription factors 306, 307, 308
  Wallerian degeneration 687–688
Schwann cell development **282–292**
  gliogenesis from neural crest 284
    β-neuregulin-1 284
    SOX10 role in PNS 284
  growth factors and differentiation 636
    FGF-2 637
    GGF-1 637
    NRG-1α 637–638
    NRG-1β 637–638
    TGF-β1 637
  immature cells 288
    autocrine survival mechanisms 288
    cell numbers, control 290
    myelination 289
    radial sorting 289
  lineage 282, 284*f*
    embryonic stage 285*f*
    immature cells 282
    major steps 282
    neural crest 282, 283*f*
    plasticity 282
    precursors 282
  lineage regulation 299
  markers 282

Schwann cell development (*continued*)
activator protein 2α 282–283
cadherin 19 282–283
categories 282–283, 285*f*
sex determining region Y (SRY) 282–283
myelination, onset of 290
myelin-related transcription factors 290
negative regulation 291
β-neuregulin-1 role 290
neural crest lineage *see* Neural crest
precursors *see* Schwann cell precursors (SCPs)
spinal roots 288
KROX20 (EGR2) 288
Schwann cell precursors (SCPs) 286, 306
embryonic phase of lineage 282
functions 288
endoneurial fibroblasts 289
morphogenesis 289
trophic support, developing neurons 288
β-neuregulin-1 286, 287*f*
Notch 287
transition to immature cells
control of cell numbers 290
radial sorting 289
Schwannoma(s)
ErbB2 role 303–304
Sclerosteosis-associated SOST/sclerosin family protein, Wnt signaling 40
S-cone photoreceptors *see* Cone photoreceptor(s)
Scube 2, neural patterning role 24
Sculpting neural circuits, NMDA receptors 440
sec15 gene/protein
Notch signaling 144
Secondary (local) organizers (developmental) *see* Developmental organizers
γ-Secretase
Notch signaling 146
Sef repressor, FGF negative feedback regulation 83–84
Segmentation (developmental)
AP somite polarity and *see* Segmentation, AP somite patterning
boundary formation
cell affinity role
ephrins/Eph receptors and *see* Ephrins/Eph receptors
Hox genes, function 61–62
neuromere model and 95
organizers 81*f*
see also Developmental organizers
Segmentation, AP somite patterning
spinal cord 96–97
Seizure(s)
GABA role 579
NMDA synergy 579–580, 580*f*
receptor antagonists 575–576, 576*f*
hippocampus 579
unprovoked
see also Epilepsy
see also Epilepsy
Seizure disorder *see* Epilepsy
Semaphorin(s) 674
classification 381, 674
inhibitory (repulsive) guidance cues 381, 395, 674, 675*f*
adult CNS 677
signaling
receptors 425, 674
somatic sensory neuron development 420
structural features 395–396
Semaphorin 2A (Sema2A), *Drosophila* motor neuron target selection 523–524
Semaphorin 3A (Sema3A) 485
somite expression 279
Semaphorin 5A (Sema5A), growth cone inhibition 677
Senescence
see also Age/aging
Senile plaques
see also Amyloid beta (Aβ)
Sensory afferent(s)
columnar model of brain development and 96–97
development
Hox genes/proteins and specification 78–79
somatic *see* Somatic sensory neurons
Sensory cortex
representations in *see* Topographic maps/representations
Sensory neuron(s) *see* Sensory afferent(s)
Sensory organ precursors (SOPs) 132–133
autoregulatory enhancer 135–136
cell fates 136, 137*f*
Sensory organs
precursors *see* Sensory organ precursors (SOPs)
Septal area
AVP fibers, sexual dimorphism 350
D-Serine
as astrocytic gliotransmitter
NMDA-mediated LTP and 570
Serotonergic receptor(s) *see* Serotonin (5-HT) receptor(s)
Serotonin *see* Serotonin (5-HT)/serotonergic neurons
Serotonin (5-HT) receptor(s)
5-HT_3
antagonists
enteric nervous system effects 343
5-HT_4
antagonists
enteric nervous system effects 343–344
Serotonin (5-HT)/serotonergic neurons
adult NSC regulation 645
autonomic nervous system
enteric nervous system role 629
development 342
gastrointestinal
enteric nervous system and 629
Serotonin transporter (5-HTT/SERT)
enteric nervous system development 343
backup transporter effects 343
Serrate ligand *see* DSL (Delta, Serrate, Lag-2) ligand family
SERT *see* Serotonin transporter (5-HTT/SERT)
Set point, neuronal homeostasis *see* Neuronal homeostasis
Seven-pass (Flamingo-like) transmembrane cadherins *see* Cadherin(s)
Sex chromosome(s)
see also Sex determination; X-chromosome(s); Y-chromosome
Sex determination 348, 354
see also Sexual dimorphism
Sex determining region Y (SRY), Schwann cells 282–283
Sex development
determination *see* Sex determination
differentiation *see* Sexual differentiation
Sex differences *see* Sexual dimorphism
Sex hormone(s) *see* Gonadal hormone(s)
Sex steroid(s) *see* Gonadal hormone(s)
Sexual behavior
androgens and 352
see also Reproductive behavior(s)
Sexual differentiation **348–353, 354–361**
in adolescence 350
in adulthood 350
bipotential brain 354
chromosomes and the developing gonad 348, 354, 356
critical periods 348, 354–355
hormonal effects on behavior 354, 355*f*
human brain and behavior 351
genetic disorders 352
somatic markers 352
major principles/model systems 354
neurobiology 348, 357
estrogens and
avian song systems 356
axon guidance and 357–359
dendrite morphology effects 359
gene expression regulation 354–355
neurogenesis and 357
receptors 359, 360*f*
signaling pathways 359–360
testosterone regulation 354–355
genetic signals 356
sex steroids and connectivity patterns 354–355, 357
axon guidance 357, 358*f*
birdsong systems 357–359
synaptogenesis 359
sex steroids and programmed cell death 351, 357
sexually dimorphic nuclei *see* Sexually dimorphic nuclei
sites of action and downstream regulation 351
social context and 351–352
testosterone and 348
CAH and 352
CAIS and 352
estrogen control of 354–355
homosexuality in women 352
neurotrophic factors and 351

organizational action on CNS 354
programmed cell death and 348–349, 351, 357
receptors 359, 360*f*
SDN-POA development 348–349, 349*f*
sex determination 348, 354
SNB development 356
target-dependent control 351, 356
Sexual dimorphism **354–361**
age-related changes 353
sexual differentiation in adolescence 350
sexual differentiation in adulthood 350
behavioral consequences 354
balancing/minimizing behavioral differences 352–353
birdsong 356
human brain and behavior 351
parenting and 352–353
sex steroids and 354, 355*f*
social context and 351–352
see also Reproductive behavior(s)
cognition and 348
developmental aspects *see* Sexual differentiation
major principles/model systems 354
pathophysiology and
psychiatric disorders 348
sensory systems/chemosensation
sexually dimorphic nuclei and olfaction 356
structural differences 348, 352
adolescent brain development and 350
anterior commissure 352
avian song control system 356
AVP fiber innervation of the septal area 350
brain weight 352
functional consequences 357
hemispheric asymmetry/specialization
callosal 352
sexually dimorphic nuclei *see* Sexually dimorphic nuclei
thalamus 352
Sexually dimorphic nuclei
adolescence changes 350
adult changes 350
amygdala 350, 356
avian song system 356
connectivity and 357–359
differentiation 348, 355
estrogen actions during development 359
male dominance 355
sex steroid effects on axon guidance 357–359, 358*f*
testosterone actions during development 354–355, 356
programmed cell death and 348–349, 351, 357
hypothalamus 352, 355, 357–359, 358*f*
dendritic morphology 359
olfaction and 350, 356
spinal cord 356, 356*f*
thalamus 352
Sexually dimorphic nucleus of the preoptic area (SDN-POA) 355
mechanism of sexual differentiation 348–349, 349*f*, 351
PGE_2 effects 351
Sexual orientation
prenatal androgen exposure and homosexuality in women 352
Shank
protein interactions 497
glutamate receptor binding 497
protein-binding domains 497
PSD-95 interactions 490, 492–493
structure 497
PDZ domains 497
SHH (Shh) see Sonic hedgehog (Shh) signaling
shibire mutant
DSL ligand regulation 141–142
Short gastrulation (Sog) protein
cell fate specification 48–49, 50*f*
gradients 48–49
DV axis establishment, spiders 51
expression 45–46
Short-term synaptic plasticity
presynaptic maturation and 518
Shroom, neural tube defects 9
Sidekick cell adhesion molecules
retinal development 257
Signaling centers (developmental) *see* Developmental organizers
Signaling pathways
apoptosis *see* Apoptosis
cell death associations 603*t*
developmental 10
AP patterning *see* Anteroposterior (AP) patterning
DV patterning *see* Dorsoventral (DV) patterning
signal spreading and functional range 85, 85*f*
see also Developmental organizers
GDNF family ligands (GFLs) *see* GDNF family ligands (GFLs)
GPCRs *see* G-protein-coupled receptor(s) (GPCRs)
NMDA receptors *see* NMDA receptor(s)
PSD and 498
Silencing mediator of retinoid and thyroid hormone receptors (SMRT),
Notch signaling 147
Silent synapses 491
activity-dependent maturation 456–457, 457*f*
'desilencing' 517–518
see also AMPA receptor(s)
glial cell thrombospondin-induced synapses 568
NMDA receptor expression 517–518
presynaptic development and 506, 517–518
Sip1, neural induction in chicks and cell fate specification 14
Six3 gene/protein
amacrine cell generation 262
horizontal cell specification 263
retina anteroposterior axis development 125
six family genes/proteins
neural plate regionalization 100
Six3 and midline development
hypothalamic specification/development 102
Shh-FGF8 signaling regulation 111, 113
Skeletal muscle
cadherin expression 542
development
cadherin expression 542
early appearance of delocalized postsynaptic properties 530
myotube formation 529
kinetics 529
primary *vs.* secondary 529
NMJ formation *see* Neuromuscular junction development, mammalian
origin of fiber types 530, 557
function 538
innervation
autonomic
neuroeffector junction *see* Autonomic neuroeffector junction
Skilled movements
corticospinal system 403
motor maps and 412
development, corticospinal control 410
see also Corticospinal development
Slit protein(s) 674
adult CNS 677
axonal growth and guidance 381
DA neuron development 223–224
inhibitory (repulsive) guidance cues 389
defasiculation 415
expression 389
mitral/tufted cell repulsion 420
retinal ganglion cell repulsion 415, 416*f*
thalamocortical axon repulsion 417
midline crossing 405
receptors 381
trunk neural crest cells 279
Slug, neural crest expression 272–273
Smads (transcription factor)
Hox gene expression 68
Notch signaling 148
'Small brain' *see* Cerebellum
Small eye mutant mouse, neocortical arealization 120
Smell *see* Olfaction
'Smooth brain' *see* Lissencephaly
Smoothened (Smo) 24, 29, 36, 84, 460–461, 465, 467*f*, 469, 471
inhibitors 27
mutants 460–461, 462
SMRT (silencing mediator of retinoid and thyroid hormone receptors),
Notch signaling 147
Snail-1, neural crest expression 272–273
Snail-2, neural crest expression 272–273
Snake(s)
optic tectum 454–455
SNARE complex
interactions
active zone proteins 517
SOC3 protein, retinal development 255–256

Social factors
sexual dimorphism and 351–352
Sodium/potassium/chloride (Na+/K+/Cl-) cotransporter NKCC1 574–575
SOHo1 gene/protein, retina anteroposterior axis development 126
Soluble Frizzled-related proteins (SFRPs) 40
Soma
translocations, retinal development 254–255, 255*f*
Somatic motor neurons, specification 187
axon pathfinding and 190
soma migration and 189–190
Somatic sensory neurons
cell bodies 420
growth
branching 420, 420*f*
fasciculation 420–421
neurotrophins 420
peripheral sensory axons 420–421
semaphorins 420
projection
neurotrophins 420
semaphorins 420
Somatosensation *see* Somatosensory perception/processing
Somatosensory cortex
development, NMDA receptors 444–445
Somatosensory perception/processing
midbrain processing 87
Somatosensory system
development
axon guidance cues 420
neuron growth 420
neuron projection 420
physical stimuli 420
see also Somatosensory perception/processing
Somitic mesoderm
preganglionic axon guidance and 336
Somitogenesis
Notch signaling 139
retinoic acid role 58–59
'clock and wavefront' model 58–59
Raldh2 knockout embryos 59
Song systems
sexual dimorphism 356
sex steroid effects on axonal growth/guidance 357–359
Sonic hedgehog (Shh) signaling
adult NSC regulation 645
anteroposterior (AP) patterning
FGF8 feedback loop in rostral (anterior) midline 109, 112, 112*f*
ZLI signaling and forebrain boundaries 81–82, 84, 104
brain 27
chemoattractant role 460, **465–471**, 466*f*, 671
activity blocking 466
adults 677–678
axon guidance 388, 460, 465, 468
Boc, receptor function 467
gradients 469
molecular mechanism 470
retinal ganglion cell axon growth 469, 469*f*
Smo role 460–461, 466
studies 466–467
chemorepellant signaling 461, 470
developmental functions **21–28**, 671
dorsoventral (DV) patterning 29, 172
cell specification regulation 172, 173, 173*f*
concentration gradient-dependent mechanism 30, 31*f*
formation and maintenance of gradient 34
Hh-binding proteins and negative feedback loop 34, 35*f*
posttranslational lipidation 34
intracellular mediators 36
Gli proteins and 36, 37*f*
issue to be resolved 38
long-range signaling 30–31
loss-of function mutants 29–30
motor neuron specification 74, 74*f*, 184–185
neuroectoderm signaling 47
retina development 127
spinal cord 57
transcription factor code and cell fate 31, 32*f*, 185
class I genes/proteins 31
class II genes/proteins 31
cross-repression between classes 32*f*, 33
evidence for graded Shh control 31, 32*f*
model of graded Shh control 33
ventral midline formation 109, 110
genetic associations with HPE 110–111
HPE phenotypes and 111
hypothalamic development and 108
sites and functions 111
expression pattern 29
holoprosencephaly and 110
posttranslational modification 110–111
as long-range signal ('at-a-distance' mode of action) 30–31, 90–91
mesencephalic dopaminergic neuron development 221, 224
midbrain patterning 89, 89*f*
midbrain arcs/interarc formation and 90–91
midbrain-forebrain boundary 81–82, 84
tectal-tegmental boundary and 92
midline development
FGF8 feedback loop in 109, 112, 112*f*
evidence for 112
eye field signaling and 113
negative regulation by BMPs 113
positive regulation by Six3 111, 113
rostral (anterior) midline patterning 110
ventral midline formation 109, 110
genetic associations with HPE 110–111
HPE phenotypes and 111
hypothalamic development 101, 108
sites and functions 111
morphogen gradients and positional information 18, 21, 27
gradient shaping 23, 24*f*, 25
neural epithelium 21–22, 23*f*
concentration gradients 21–22, 23*f*
ventralization 21
neural tube 25
N-terminal peptide 22
oligodendrocyte precursors 238–239
OLIG2 gene expression 240
optic tectum 454
planar transcytosis 22, 25*f*
posttranslational modification 34
holoprosencephaly and 110–111
proteins associated 23, 26*f*
proteolysis 22
Ptc1 role 24, 26*f*
receptors 239
responses 24
retinal ganglion cell generation 260–261
secretion 465
signal transduction 36
cyclic AMP inhibition 671
intracellular mediators 36, 84
Gli proteins 36, 84
inhibitors 27
Rab23 36
Smoothened 24, 26*f*, 27, 29, 36, 84
Suppressor of Fused 36
receptor *see* Patched 1 receptor (Ptc1)
spinal cord development 21
commissural axon guidance 388, 466*f*, 468
neuroectoderm 52
trafficking 24
SOX (Sox) genes/proteins **165–171**, 166*t*
chick development 169–170
misexpression effects 168
classification 165
HMG domains 165
mouse development 170
neural competence and 165
neural progenitor cells
central nervous system 166
mouse central nervous system 167*f*
mouse peripheral nervous system 169*f*
peripheral nervous system 168
posttranslational modification 165
SRY gene 165
Xenopus 165–166
SOX1 gene/protein, neural induction 56
SOX2 gene/protein 165
aging and neurogenesis 663
hair cell differentiation 246
interaction effects 165
neural crest development 272

neural induction 56
neural progenitor cells 167–168
olfactory neurogenesis
Fgf8 coexpression and VNO formation 230–231, 232–234
potential stem cell marker 230–231
primary 228, 229*f*, 230–231
potential stem cell marker
adult NSC identification 643–644
olfactory neurogenesis 230–231
Schwann cell development 307
SOX3 gene/protein
ectodermal cells 165–166
induction by FGF8 during chick neural induction 13–14
SOX8 gene/protein
peripheral neural progenitor cells 170
SOX9 gene/protein
neural crest cells 169–170
peripheral neural progenitor cells 169–170
SOX10 gene/protein
adult melanocyte stem cell regulation 319
enteric nervous system development 345
melanocyte development role
melanoblast differentiation 318
melanoblast specification 314
neural crest, PNS gliogenesis 284
oligodendrocytes 310
precursor development 240–241
peripheral neural progenitor cells 168
Schwann cell development 306, 308–309
immature cell maintenance 307
SOX11 gene/protein, CNS neural progenitor cells 168
SOXB1 gene/protein
ectodermal cells 165–166
neural competence 165–166
neural progenitor cells
of central nervous system 166–167
of peripheral nervous system 170
Sp1 transcription factor, *Hox* gene expression 66–67, 68
Sp8 transcription factor 120
neocortical arealization 120
expression patterns 120
FGF8 interactions 120–121
regulatory gene interactions 121
Spatial colinearity, Hox genes/proteins 75
Spatial-sensing strategy, axonal growth gradient guidance models 383–384
Spemann organizer
neural induction 45–46
see also Developmental organizers
Sperry, Roger Wolcott
chemoaffinity hypothesis 365
topographic maps 372–373
Sperry hypothesis, growth inhibition 395
Sphenopalatine (pterygopalatine) ganglion
Ret signaling 611–612
Sphingomyelinase, deficiency *see* Niemann-Pick disease
Spider(s)
DV axis establishment 51
Sog expression 51
Spike(s) *see* Action potential(s)
Spike timing-dependent plasticity (STDP)
NMDA receptors 447
Spinal cord
astrocytes
generation 219
corticospinal system *see* Corticospinal system (CS)
development *see* Spinal cord development
disorders/dysfunction
ALS *see* Amyotrophic lateral sclerosis (ALS)
injury-induced *see* Spinal cord injury (SCI)
SMA *see* Spinal muscular atrophy (SMA)
ventral horn
motor neurons *see* Motor neuron(s) (MNs)
OPC generation 238
progenitor cell domains 172, 238, 239*f*
see also entries beginning spino-/spinal
dorsal horn
commissural axon growth 461*f*, 462
OPC generation 238
injury *see* Spinal cord injury (SCI)
regeneration *see* Spinal cord regeneration/repair
sexually dimorphic nuclei 356, 356*f*
Spinal cord development 172
anteroposterior (AP) patterning and segmentation
motor neuron specification 184
Hox gene expression *see* Hox genes/proteins
motor pools/columns **72–79**
subclass specification 184
see also Motor neuron specification (vertebrate)
zebrafish embryos
see also Zebrafish development
see also Motor neuron specification (vertebrate)
caudal neural tube origin 3
see also Neurulation
cell-type specification 29, 30*f*
interneurons 29
motor neurons *see* Motor neuron specification (vertebrate)
columnar models 96–97
cortical arealization *vs.* 123
corticospinal system *see* Corticospinal development
dorsoventral (DV) patterning **29–38**
motor neuron specification 30*f*, 184–185
notochord and mesoderm-secreted signals 29
additional signals 31–33
sonic hedgehog *see* Sonic hedgehog (Shh) signaling
retinoic acid 57
see also Motor neuron specification (vertebrate)
locomotor circuitry 190
Hox genes and 78–79
precision 72
mesoderm-secreted signals
anteroposterior (AP) patterning 184
dorsoventral (DV) patterning 29, 30*f*
motor neurons *see* Motor neuron development
oligodendrocyte generation 219
precursors 238
sonic hedgehog signaling *see* Sonic hedgehog (Shh) signaling
transcriptional networks **172–177**
bordered patterns of expression 123
cascade stages 177
cell specification factors 174
dorsoventral patterning 172
Hox genes 61–62, 176
MN development *see* Hox genes and motor neuron specification
see also Hox genes/proteins
LIM networks, post-mitotic neurons 175
motor pools 176
negative gene regulation 173
cross-repression 173
derepression 173
neurogenesis 175
retinoic acid role 55
rostrocaudal patterning 176
ventral-medial axis 21
Spinal cord injury (SCI)
management 626
see also Spinal cord regeneration/repair
stem cells
and CNS repair 655
neural stem cells 655–656
Spinal cord regeneration/repair **681–686**
aim 681
axon regeneration 682
axon growth ability 682
glial scar 682
myelin inhibitory molecules 682
treatments to promote regeneration 683, 685*f*
mechanisms affecting axon growth ability 683
neutralizing myelin inhibitory molecules 683
overcoming glial scar inhibition 683
permissive bridging material 684
transplantation of permissive cells 683
clinical trials 686
CNS plasticity 684
decline and aging 684
enhancement treatments 684
rehabilitation treatments 685
plasticity as a target 681
potential treatments 681
prosthetic devices 685
protection (from additional damage) 681
see also Neuroprotection/neuroprotective agents
research directions 686
strategies 681

Spinal muscular atrophy (SMA)
  animal models
    NMJ synapse elimination and 559
  gene mutations 558–559
  type 1 (Werdnig-Hoffman disease)
    gene mutations 558–559
Spinal nucleus of the bulbocavernosus (SNB), sexual differentiation 356, 356*f*
  adulthood 350–351
  neurotrophic factors and 351
  testosterone and cell death regulation 349–350, 349*f*, 351, 357
Spindle fibers *see* Microtubule(s)
Spinophilin (neurabin II)
  postsynaptic density 499
  postsynaptic development 492–493
'Spongioblasts' *see* Radial glial cells
Sprouting, synaptic plasticity *see* Synaptic plasticity
Spry repressor, FGF negative feedback regulation 83–84
Squeeze (sqz) gene, Ap neuron clusters 213–214
Squint (strabismus), ocular dominance column formation 436
Src kinase(s)
  integrin-mediated regulation 542
  postsynaptic development 492
Sry (sex determining region of Y) gene 348, 354
Sry HMG-box genes, neural crest expression 272–273
S-SCAM/MAGI-2 (PDZ protein), postsynaptic development role 492
Stalk cells, optic nerve development 429
Starburst (amacrine) cells *see under* Amacrine cells
stargazin *(Stg)*
  function 491
STATs
  retinal development 255–256
Steel factor *see* Stem cell factor (SCF)
Stem cell(s)
  adult melanocyte stem cells 318
  definition 318–319
  embryonic *see* Embryonic stem cells (ESCs)
  mesenchymal, CNS repair 660
  neural *see* Neural stem/progenitor cells (NSPCs)
  'niches' 318–319
  repertoire
    chaperone effects 654
    environmental cues 654
    as vectors for gene therapy 654
  therapeutic use *see* Cell replacement therapy
Stem cell factor (SCF)
  melanoblast specification 314
    MITF feedback loop 314
  melanocyte development 280
Stereopsis *see* Stereoscopic vision
Stereoscopic depth
  optic pathway 427, 428*f*
Stereoscopic vision
  RGC projections 415–416
Stern, Curt 18
Sterol regulatory element binding proteins (SREBPs) 309
Strabismus (squint)
  ocular dominance column formation 436
Stratum griseum centrale (SGC) 449–450, 450*f*
Stratum griseum et fibrosum superficiale (SGFS) 450*f*, 452–453
Stratum marginal 449
Stratum opticum
  goldfish 449–450, 450*f*
  horizontal neurons 449–450
  pyramidal neurons 449–450
Stratum periventriculare 449–450
  optic tectum (goldfish) 450*f*
  pyriform neuronal signaling 452–453
Stress
  adult NSC effects 644, 664
Stress, neuroplasticity and
  adult neurogenesis and 644, 648–649
    aging and 644, 648–649
    hippocampal 648–649
      see also Hippocampal damage, stress-related
    negative effects of corticosteroids 648–649
  damage/dysfunction due to
    see also Hippocampal damage, stress-related
Stress activated protein kinases (SAPKs) *see* c-Jun N-terminal kinase (JNK) signaling pathway
Stretch reflex
  development, Hox genes and 78–79
Striate cortex *see* Primary visual cortex (V1: striate cortex)
Stroke
  ischemic *see* Ischemic stroke
  management 626
    stem cell repair 655, 658*f*
  pathogenesis/pathophysiology
    autophagic neuronal death 618–619
    excitotoxicity *see* Excitotoxicity
    glial responses
      see also Ischemic stroke
    glutamate
      excitotoxicity *see* Excitotoxicity
  recovery/rehabilitation
    adaptive plasticity
      see also Motor maps
Structural plasticity 667
  see also Synaptic plasticity
Subcortical band heterotopias (SBHs) 192–193, 195–196
  genetic basis 196
    doublecortin *see* Doublecortin (DCX)
  radial migration phase and 194
  see also Lissencephaly
Subgranular zone (SGZ)
  adult neural stem cells 641, 649
    cell types 649
    neurogenesis of 642*f*
    neurotransmitter regulation 650
    SVZ *vs.* 646
  stem cells and CNS repair 653–654
Subplate neurons, axon guidance cues 390, 417–418
  ablation 390
Substantia nigra (SN) 87
  development, dopaminergic neurons 223, 224
Subventricular zone (SVZ)
  adult neural stem cells 641, 649*f*, 650
    aging/stress effects 644
    ependymal cells and 650
    historical studies 641
    identification 643
      cell division markers 643
      genetic methods 644
      molecular markers 643
    migration 641, 642*f*, 649*f*, 650
    neurogenesis of 642*f*
    regulation
      extracellular 644
      intracellular 645
    SGZ *vs.* 646
    types 642*f*, 643
  corticogenesis and 117
    Cux2 589–590
    glial cell production 588–589
    identity of upper-layer neurons 588
    Svet1 589–590
    SVZ and VZ progenitor segregation and 588
  definition 588
  evolution 588–589
  stem cells and CNS repair 653–654, 654–655
'Suicide bag' hypothesis, autophagic cell death 615–616
Superior cervical ganglion (SCG)
  apoptosis 607
  Trk expression 609
    BDNF deficient mice 609–611
    NGF deficient mice 609
    NT-3 role 609
    TrkA deficient mice 609–611
    TrkC deficient mice 609
Superior colliculus (SC)
  anatomy/physiology
    retinal ganglion cells connection 434
    retinotopic maps 434
  visual pathway development 441
  visual system topographic mapping 416
Superoxide dismutase 1 (SOD1)
  amyotrophic lateral sclerosis
    mouse model
      programmed cell death 605
  mutants/mutations
    familial ALS and 558–559
    see also Amyotrophic lateral sclerosis (ALS)
Suppressor of Fused (SuFu), sonic hedgehog (Shh) signaling 36
Suppressor of Hairless (Su(H)), Notch-Delta signaling 133–135

Suprachiasmatic nucleus (SCN)
anatomy/physiology
sexual dimorphism 352
Svet1 gene/protein
cortical neuronal cell fate 589–590
SWI/SNF
retinal development 256
Syg2, presynaptic development 509–510
SymAtlas, molecular anatomy of mammalian brain 202
Sympathetic nervous system (SNS)
anatomy/physiology
postganglionic neurons *see* Sympathetic postganglionic neurons
preganglionic neurons *see* Sympathetic postganglionic neurons
apoptosis *see* Apoptosis, autonomic nervous system
development
axonal outgrowth 336
NGF role 336–337
neonatal 607
nerve growth factor effects
development 336–337
plasticity and 333
Sympathetic noradrenergic system *see* Sympathetic postganglionic neurons
Sympathetic postganglionic neurons
location 326
neuroeffector junction *see* Autonomic neuroeffector junction
Synapse(s)
cadherins *see* Cadherin(s)
chemical signalling 512
definition 495
development *see* Synaptogenesis
elimination *see* Synaptic pruning/elimination
excitatory *see* Excitatory synapses
features
mature 456
nascent 456
formation *see* Synaptogenesis
glial cells and *see* Glial cells, synaptic functions
inhibitory *see* Inhibitory synapses
maturation 456
steps 456, 457
ultrastructural studies 457
neural cell adhesion molecules *see* Neural cell adhesion molecules (NCAMs)
neuromuscular *see* Neuromuscular junction (NMJ)
other cell–cell junction *vs.* 512
plasticity *see* Synaptic plasticity
pruning *see* Synaptic pruning/elimination
retina
partnering development 257
retrograde signaling *see* Retrograde signaling
silent (mute) *see* Silent synapses
stabilization 516
glial cells and *see* Glial cells, synaptic functions
structure/morphology 502, 513*f*
adhesion molecules and 512, 514
asymmetry 512
central synapses 512
changes and long-term plasticity
see also Synaptic plasticity
postsynaptic *see* Postsynaptic membrane
postsynaptic structures *see* Postsynaptic membrane
presynaptic *see* Presynapse
presynaptic structures *see* Presynapse
vesicles *see* Synaptic vesicle(s)
turnover 667
see also entries beginning synapto-/synaptic
Synapsin I
presynaptic development and 506
Synaptic adhesion molecules
postsynaptic development 490
Synaptic bouton(s)
development
en passant boutons 503, 503*f*, 512–513
functional changes 505
initial events 503, 503*f*
structural changes 504
nascent sites *vs.* mature boutons 505
structure–function relationship 505
distribution 503
origins 503
Synaptic cell adhesion-like molecule (SALM), postsynaptic receptors 491
Synaptic cell adhesion molecules (SynCAM) *see* SynCAM
Synaptic cleft
laminin 540
Synaptic competition
activity-dependent
mechanisms
trophic factor hypothesis 557–558
see also Synaptic pruning/elimination
neuromuscular junction *see* Neuromuscular junction, synapse elimination
see also Synaptic pruning/elimination
Synaptic elimination *see* Synaptic pruning/elimination
Synaptic formation *see* Synaptogenesis
Synaptic fragmentation, NMJ synapse elimination 556
Synaptic membrane(s)
postsynaptic *see* Postsynaptic membrane
presynaptic *see* Presynapse
Synaptic memory
see also Synaptic plasticity
Synaptic plasticity
aging and
sprouting 668
development
dendritic spines
morphological change 666
turnover 666
glutamate receptors *see* Glutamate receptor(s)
see also Long-term depression (LTD); Long-term potentiation (LTP)
enhanced transmission
potentiation
long-term *see* Long-term potentiation (LTP)
fear conditioning
molecular mechanism
see also Synaptic plasticity, molecular mechanisms
genes associated
see also Synaptic plasticity, molecular mechanisms
glial cell contribution *see* Glial cells, neurotransmission modulation
molecular mechanisms *see* Synaptic plasticity, molecular mechanisms
neuronogenesis and stem cells **662–665**
see also Neural stem/progenitor cells (NSPCs); Neurogenesis
sprouting and **666–670**
aging 668
definition 666
environmental cues 668
exercise effects 668
normal adult plasticity 666
experience-dependent learning 666
self-optimization 666
puberty 667
reproduction 667
sex steroids 667
Synaptic plasticity, molecular mechanisms 436
dynamics 666
neurotrophins and 625
signaling pathways
BNDF/TrkB receptors
see also Brain-derived neurotrophic factor (BDNF)
calcium-mediated
CaMKs *see* Calcium/calmodulin-dependent kinase(s) (CaMKs)
glutamate receptor-mediated
LTP role *see* Long-term potentiation (LTP)
NMDA receptor role *see* NMDA receptor(s), synaptic plasticity
see also Glutamate receptor(s)
transcription and gene expression
transcription factors
CREBs *see* CREB (cyclic AMP response element-binding protein)
Synaptic pruning/elimination
glial cells 558, 569
see also Glial cells, synaptic functions
NMJ development *see* Neuromuscular junction, synapse elimination
synaptogenesis balance 512–513
Synaptic vesicle(s) 512
cycling *see* Synaptic vesicle cycle
'pools' *see* Synaptic vesicle pools
presynaptic development and
active zone proteins 517
CASK role 516
mobile SVs and axonal transport 506, 507
Piccolo/Bassoon transport vesicles (PTVs) 506–507
recycling changes 513, 516
related protein expression 506
SV exocytosis 505, 513–514
SV numbers 504

Synaptic vesicle(s) (*continued*)
SV sources 507
protein folding/chaperone proteins and 512
quantal content
NMJ vesicles
maturation and 533
Synaptic vesicle cycle
docking 502
membrane fusion/release (exocytosis) 502
active zone proteins and 517
see also Neurotransmitter release
refiling/recycling 502
intrasynaptic Ca^{2+} and 512
synaptic response rate and 512
regulation
protein scaffolds as spatial regulators 517
Synaptic vesicle exocytosis *see* Synaptic vesicle cycle
Synaptic vesicle pools
readily releasable *see* Readily releasable vesicle pools (RRPs)
reserve pool 502, 512
see also Endocytosis; Neurotransmitter release
Synaptogenesis 456, 458–459, **512–519**
activity-dependent
presynaptic maturation and 517
see also Synaptic competition; Synaptic pruning/elimination
adhesion molecules and 514, 516*f*
postsynaptic development 498
presynaptic development 507, 509
variety 514–515
autapses 514–515
dendritic spine filopodia and 512–513
error prone nature 516–517
filopodia *see* Filopodia
GABAergic *vs.* glutamatergic 575
glial role *see* Glial cells, synaptic functions
inherent neuronal ability 514–515
injury-induced 514–515
multiple stages 512
neurexin-neuroligin complex *see* Neurexin-neuroligin complex
neurexins *see* Neurexins
NMDA receptors 440
see also NMDA receptors, developmental role
NMJ
central synapses *vs.* 512–513
developmental *see* Neuromuscular junction, development
perisynaptic Schwann cells and
ErbB signaling 563, 564
role 563
Xenopus 563
paracrinic action 579
postsynaptic specializations **495–501**
PSD development 500
see also Postsynaptic membrane
presynaptic development *see* Presynaptic development
promiscuity 514–515
retinal development 257
sex steroids and sexual differentiation 359
stabilization and 516
steps associated 456
synapse elimination relationship 512–513
see also Synaptic pruning/elimination
synapsin 1 expression 506
timing 512–513
Wnt signaling 41–42
Synaptotropic hypothesis
presynaptic development and 503–504, 504*f*
SynCAM
postsynaptic differentiation 489
synapse formation and 515
CASK as downstream target 515–516
postsynaptic density and 498
presynaptic development 509, 515–516, 516*f*
SynGAP
postsynaptic development 492
Syntrophin(s)
dystroglycan complex components 541
NMJ development and 533
α-Synuclein
dopamine neuron development 222
gene (*α-synuclein: Park1*)
mutation and disease *see* α-Synucleinopathies
neurodegeneration/neurodegenerative disease *see* α-Synucleinopathies
α-synuclein (PARK1) gene
mutation and disease *see* α-Synucleinopathies
α-Synucleinopathies
Parkinson's disease 619

T

Tadpole(s)
neuromuscular junctions 563
Tail bud, secondary neurulation 3
Talin, growth cone migration 482
Tangential displacement, retinal development 255
Tangential migration, cortical development 193
Target innervation, optic pathway development 430
Target recognition, axon guidance 365
see also Axonal guidance cues
Taste pathways *see* Gustatory system
Tay-Sachs disease (TSD)
autophagic neuronal death 620
Tbx5 gene/protein, retina dorsoventral axis development 127–128
TCF/LEF protein family, β-catenin nuclear import 41
Tectal-tegmental boundary, patterning 92
Tectofugal projections, cadherin expression 485
Telencephalization, optic tectum 449
Telencephalon
adult diversity 102
olfactory bulb *see* Olfactory bulb
specification/development 101*f*, 102, 116
ANB/ANR specification and 109
FGF expression 102
Wnt antagonism 102
cortical progenitors 193
dorsal midline specification 110
early subdivisions 102
evolutionary conservation 102
neurulation 102
Pax6/Emx2 and 102
Teleost fish
ocular stem cells
ciliary marginal zone 267–268
regenerative capacity 268
Müller glia 269
rod precursors 269
optic tectum 449
Telomerase, Terc, adult NSC regulation 645
Temporal lobe epilepsy *see* Epilepsy
Tenascin(s)
axon guidance 399
extracellular matrix component 399
isoforms 399
Tenascin-C (TN-C) 399
axon growth inhibition 676
structural organization 399
trunk neural crest 279
Tenascin-R (TN-R)
axon growth inhibition 676
Tenascin receptors 399–400
TeraGenomics 202
Terc, adult NSC regulation 645
Terminal differentiation
cell cycle and *see* Cell cycle, terminal differentiation role
cell-fate *see* Cell-type specification
melanoblasts 318
REST *see* RE1-silencing transcription factor (REST)
Terminal Schwann cells 532
Testosterone 348
masculinization 354
receptors *see* Androgen receptors
sexual differentiation *see* Sexual differentiation
Tetraspanin(s), activity-dependent development of *Drosophila* NMJ 524
Tetrodotoxin (TTX)
LGN retinotopic map studies 434–435
see also Voltage-gated sodium channels (VGSCs)
Tgif, holoprosencephaly and 110, 114–115
Thalamocortical (TC) pathways
afferents *see* Thalamocortical afferents (TCAs)
visual system, internal capsule projections 417
Thalamocortical afferents (TCAs) 116–117
development
cortical functional specialization and 119
mitogenic activity 119
pathfinding 119

neocortical connections 116–117, 119
arealization during development 119
Thalamus
anatomy/physiology
ascending (cortical) projections
thalamocortical afferents 116–117
see also Thalamocortical (TC) pathways
dorsal nuclei 116–117
sexual dimorphism 352
development 116
lineage restriction boundary 83
zona limitans intrathalamica (ZLI) signaling 80–81
sexual dimorphism 352
see also entries beginning thalamo-/thalamic
Third interstitial nucleus of the anterior thalamus (INAH3), sexual dimorphism 352
Thoracic spinal cord
motor neuron types, specification 187
soma migration 189–190
Thrombospondin(s)
glial-mediated synaptogenesis 514, 568
knockouts 568
programmed cell death 602–603
receptors *see* Integrins
Thymidine analogues, adult NSC identification 643
Thyroid hormone (TH)
actions
oligodendrocyte differentiation 242
Schwann cell development
differentiation 307–308
signaling role, in hair cell differentiation 249
TIMP2, NMJ basal lamina 543
Tissue inhibitors of metalloproteinases (TIMPs)
NMJ basal lamina 543
Tissue oxygen pressure, DA neuron development 224
Tissue patterning, hedgehog family proteins 465
see also Hedgehog (Hh) signaling pathway
Tissue plasminogen activator (tPA)
neuroplasticity 437
Tlc, AP patterning and posteriorization 80
rostral midline 109
TNF-α *see* Tumor necrosis factor α (TNFα)
Tolloid (Tld) protein 46
Topographic maps/representations **372–380**
activity-dependent refinement 372, 378
NMDA receptors, post-synaptic effects 379
RGC firing, correlated activity 378
axon guidance cues 372, 373
ephrins 373
Ephs 373
classes 373
retinotectal projection
dorsal-ventral mapping 376
historical prospective 373
nasal-temporal mapping 373, 374*f*
stripe assay 373, 375*f*
thalamocortical projections 377, 378*f*
definition 365, 433
development/maturation
anterior-posterior mapping 368
gradient-determined repulsion 368
opposing gradients 368, 369*f*
axonal growth and guidance models *see* Axonal growth/guidance, models
chemoaffinity hypothesis *see* Chemoaffinity hypothesis
motor maps and 403, 408*f*, 409*f*, 412, 413*f*
retinotopic maps *see* Retinotopic maps
historical perspective 372
'chemoaffinity hypothesis' 372–373
olfactory 418
see also Olfactory bulb
purpose 372
visual system *see* Retinotopic maps
see also Motor maps
Torus semicircularis (TS)
amphibians 87
Tracers
retinal progenitor cell multipotency 259, 260*f*
Transbilayer cholesterol distribution (TCB) *see* Cholesterol
Transcriptional repressors/repression
negative feedback regulation, FGF signaling 83–84
spinal cord transcriptional networks 173
cross-repression 173
derepression 173
Transcriptional silencing **178–183**
chromatin 178
active form (euchromatin) 178, 179*f*
histone replacement 179–180
nucleosome sliding 179–180
repressed form (heterochromatin) 178, 179*f*
DNA methylation 179
CpG islands 179
methyl-CpG-binding protein 179
higher order packaging 180
euchromatic *vs.* heterochromatic DNA 180
nuclear organization and 180
interphase DNA 180
histone modification 178
chromatin remodeling 178
reversible posttranslational modification 178
mechanisms 178
nervous system 182
mature neurons 182
neural development 182
chromatin remodeling evidence 182
neuron-restrictive silencing factor 182
nuclear organization 180, 180*f*
purpose 178
triggering events 180
DNA integration 181
DNA rearrangement 181
position effect 181
position effect variegation 181
endogenous signaling pathways 180
Ca2+/calmodulin-activated protein kinases 180–181
cyclic AMP response element-binding protein 180–181
neuroactive drug effects 181
invasive DNA 181
repetitive DNA sequences 181
RNA silencing 182
Transcription factors (TFs)
developmental expression
autonomic nervous system 338–339
gangliogenesis 333–335, 334*f*, 335*t*
cell fate specification and
DV patterning of spinal cord 31
melanoblast specification 314, 315*t*
motor neurons *see* Motor neuron specification (vertebrate)
Hox gene regulation 65
neocortical areas *see* Neocortical arealization (area differentiation)
olfactory neurogenesis 229
oligodendrocyte precursor generation 240
retina 266
progenitor regulation 252–253
spinal cord *see* Spinal cord development
oligodendrocytes *see* Oligodendrocyte(s)
REST *see* RE1-silencing transcription factor (REST)
Schwann cells *see* Schwann cell(s)
Transducers, retina *see* Cone photoreceptor(s); Rod photoreceptor(s)
Transforming growth factor α (TGFα)
astrocytes 635
dopaminergic neuron development 223
glial cell production 639*t*
puberty 639
satellite cells 634
Schwann cells 635
Transforming growth factor β (TGFβ) superfamily
age-related changes 639
astrocytes 635
motor neuron specification 75
neural crest development 271–272
neuronal survival 638
olfactory neurogenesis 234
BMPs 234
GDF11 234
oligodendrocytes 635
retrograde signaling, invertebrate NMJ 526–527, 526*f*
satellite cells 634
Schwann cells 634
spinal cord dorsoventral patterning 172–173
Transforming growth factor β1 (TGFβ1)
Alzheimer's disease 639
glial cell production 639*t*
Schwann cell differentiation 637

- Transforming growth factor β2 (TGFβ2)
  - glial cell production 639*t*
  - retinal development 266
    - cell cycle and cell fate 597
- Transforming growth factor β3 (TGFβ3), glial cell production 639*t*
- Transforming growth factor-β/bone morphogenic protein (TGF-β/BMP), neuronal cell type specification 216
- Transgenic animals
  - adult NSC identification 643
  - LGN retinotopic maps 435
  - mice
    - XFP mice 555
  - oligodendrocytes *see* Oligodendrocyte(s)
  - SOD1 mice (ALS model) *see* Amyotrophic lateral sclerosis (ALS)
  - synapse elimination at the NMJ 555, 556
  - *see also* Knockout animal(s)
- Transneuronal labeling studies, ocular dominance column formation 435–436
- Transport, axonal *see* Axonal transport
- Transsynaptic adhesion 489
- Transsynaptic influences, retrograde signaling *see* Retrograde signaling
- Traumatic brain injury (TBI)
  - autophagic neuronal death 618–619, 620*f*
  - excitotoxicity
    - *see also* Excitotoxicity
  - management
    - neuroprotective agents
      - *see also* Neuroprotection/neuroprotective agents
- 3,5,3′-Triiodothyronine (T3)
  - oligodendrocyte precursor differentiation 242
- Trimeric GTP-binding proteins (G proteins) *see* G-protein(s)
- ‘Tripartite synapse’ concept 567, 568*f*
- Trithorax group (TrxG) proteins, *Hox* gene expression 66–67
- Trk receptor(s) 622, 623*f*
  - ligands
    - sympathetic nervous system apoptosis 609
    - *see also* Neurotrophin(s)
  - p75NT receptor co-expression 622–624
  - programmed cell death 600–601
  - signaling 624, 624*f*
  - sympathetic nervous system
    - apoptosis 609
    - BDNF deficient mice 609–611
    - expression 609
    - NGF deficient mice 609
    - NT-3 role 609
    - TrkA deficient mice 609–611
    - TrkC deficient mice 609
- TrkA receptor(s)
  - enteric expression
    - adult gut 632
    - postnatal 631
  - NGF binding 336–337
  - *see also* p75 receptor(s); TrkB receptor(s)
- TrkB receptor(s)
  - enteric expression
    - adult gut 632
    - postnatal 631
- TrkC receptor(s)
  - differential splicing 622
  - ENS development
    - postnatal expression 631
    - prenatal expression 630
- Trochlear nuclei
  - development 91
- Tropomyosin-related kinase (Trk) receptors *see* Trk receptor(s)
- Tubingen genetic screen, topographic mapping 370
- *Tubularia,* regeneration, morphogen gradients and polarity 16
- Tufted cells
  - axon guidance 418, 419–420
- Tumor necrosis factor α (TNFα)
  - Müller glial cells 636
  - production
    - Schwann cells 634
  - synaptic modulation and glial release 570
- Tumor suppressor genes (TSGs)
  - Rb 595–596
- Turing, Alan 18
- Tv neurons *(Drosophila)* 213, 214*f*
- Twisted gastrulation (Tsg) 46
- 2A12 monoclonal, perisynaptic Schwann cells probes 563, 564
- Type 2 cell death *see* Autophagic cell death
- Tyrosine hydroxylase (TH)
  - dopaminergic neuron development 222, 223
- Tyrosine kinase(s)
  - growth cone migration 482
  - receptor-linked *see* Receptor tyrosine kinase(s) (RTKs)
- Tyrosine-negative albinism 431

U

- Ubiquitination
  - DSL ligands 142–144
  - postsynaptic assembly regulation 493
- Ubiquitin ligases (E3)
  - cell cycle control *see* Anaphase-promoting complex (APC)
  - Notch signaling 146
- Ubiquitin-proteasome system
  - neurodegenerative disease and
    - ALS 558
  - pathway
    - ubiquitin conjugation/enzymes
      - E3 enzymes *see* Ubiquitin ligases (E3)
  - synaptic elimination at the NMJ 558
- Ulcerative colitis
  - neurotrophin effects 633
- Unc-5 genes/proteins
  - axonal growth and guidance 381, 476
    - expression 476, 478
    - misexpression 476
  - protein interactions 476
  - ZU5 domain 476
- Unc-6 gene/protein, axon guidance 461*f*, 463, 472
  - mutations 474
- Unc-40 gene/protein, axon guidance, mutations 474
- Unconstrained migration, retinal development 254–255, 255*f*
- Utrophin(s)
  - dystroglycan complex binding 541–542
  - NMJ development and 533, 550

V

- V1 cortex *see* Primary visual cortex (V1: striate cortex)
- Vagal afferents
  - enteric nervous system
    - development 341
- VanGogh/Strabismus molecule, morphogens, positional information 19–19
- Vascular development, Notch signal transduction 148
- Vascular endothelial growth factor (VEGF)
  - adult neurogenesis and 650
  - adult NSC regulation 645
  - Müller glial cells 636
  - satellite cells 634
- Vasoactive intestinal peptide (VIP)
  - autonomic nervous system
    - enteric nervous system
      - developmental changes 630–631
  - enteric nervous system development 342
- *Vax2* gene/protein, retina dorsoventral axis development 128–129
- Ventral horn *see* Spinal cord
- Ventral nerve cord (VNC)
  - *Drosophila* 213, 214*f*
  - organization 213
- Ventral patterning *see* Dorsoventral (DV) patterning
- Ventral spinal cord *see under* Spinal cord
- Ventral tegmental area (VTA)
  - dopaminergic system
    - neuronal development 223, 224
- Ventricular zone (VZ)
  - corticogenesis and 117, 193, 585–586
    - neuronal fate-restriction 589–590
    - SVZ and VZ progenitor segregation and 588
  - midbrain patterning and arcuate territories 90
  - motor neuron progenitors (pMN region) 185
  - neural tube 218
  - neuroepithelium 218
- Ventromedial hypothalamic nuclei (VMN)
  - sex steroids and sexual differentiation 359
- Ventropin, retina dorsoventral axis development 127–128
- Ventrotegmental area *see* Ventral tegmental area (VTA)
- Ventrotemporal (VT) retina development *see* Retinal development

Versican
optic tectum retinotopy development 92–93
Vertebrate(s)
neurogenesis, Notch-Delta signaling 138
nonmammalian *see* Nonmammalian vertebrates
Very low-density lipoprotein (VLDL) receptor
cortical lamination and 194
reelin and 194
Vesicle pools *see* Synaptic vesicle pools
Vestibular system
structure 245
vHnf gene, retinoic acid-mediated expression 56–57
Vinculin
growth cone migration 482
Visceral motor neurons, specification 187
soma migration 189–190
Visceral motor neurons, specification, soma migration and 189–190
Visual cortex
anatomy/organization
functional organization 390
retinotopic maps 417
V1 (primary: striate) *see* Primary visual cortex (V1: striate cortex)
development
axon guidance cues 417
innervation 417
subplate neurons 390, 417–418
thalamocortical projections 417
V1 (primary: striate) *see* Primary visual cortex (V1: striate cortex)
Visual deprivation
corticospinal refinement and 413–414
monocular 436
Visual development, neural activity role **433–437**
fine-tuning 433
instructive 433
ocular dominance column formation 435
definition 435–436
NMDA receptors 436
plasticity 436
strabismus 436
transneuronal labeling studies 435–436
permissive 433
plasticity 435
Bienenstock–Cooper–Munro (BCM) plasticity model 437
binocularity studies 436
brain-derived neurotrophic factor 437
Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) 436
calcineurin 436
CREB transcription factor 436–437
dark-reared animals 433
diazepam studies 437
extracellular signal-related kinase (ERK) transcription factor 436–437
GABAergic basket cells 437
molecular mechanisms 436
monocular deprivation 436
NMDA receptors 436
NogoR 437
ocular dominance column formation 436
protein synthesis inhibition 436–437
tissue plasminogen activator 437
visual deprivation experiments *see* Visual deprivation
sources 433
internal generation 433
local circuits 434
visual deprivation effects *see* Visual deprivation
see also Visual system development
Visual field maps *see* Retinotopic maps
Visual pathway *see* Optic pathway
Visual response coordination, retina development 433
Visual system development
autophagic cell death 618
axon guidance cues 415
lateral geniculate nucleus 417
optic chiasm, optic nerve growth 415
optic disk 415
optic nerve formation 415
visual cortex 417
innervation 417
thalamocortical projections 417
eyes *see* Eye development
Vitamin A-deficient (VAD) embryos, *Hox* gene expression 64
vnd gene/protein, neuroectoderm signaling 49
associated gene repression 49–50, 49*f*
Voltage-gated calcium channels (VGCCs)
neuromuscular junction
maturation and type switching 533
N-type 533
P/Q type in adult NMJ 533
N-type channels ($Ca_v2.2$)
immature NMJ 533
P/Q-type channels ($Ca_v2.1$)
adult NMJ 533
Voltage-gated sodium channels (VGSCs)
Na_v1 subfamily
NMJ development 533, 534*f*, 535
mRNA accumulation 535
neuromuscular junction
postsynaptic maturation 533
Na_v1 expression 533, 534*f*, 535
Voltage-operated calcium channels (VOCCs) *see* Voltage-gated calcium channels (VGCCs)
Vomeronasal organ (VNO)
adult neurogenesis 229–230
see also Olfactory system, neurogenesis
development **226–237**, 227*f*
established neurogenesis 229, 230*f*
primary neurogenesis 228, 228*f*
stem cell markers 230–231
see also Olfactory system, neurogenesis
pheromone receptors
V1R 227
V2R 227
see also Olfactory (odorant) receptor(s)
structure/cell types 226, 227*f*
neuronal cell types 226
immediate neuronal precursors 226–227
progenitors 226
sensory (receptor) neurons *see* Olfactory sensory neurons (OSNs)
stem cells 226, 230–231
see also Olfactory system, neurogenesis
Voxelation, molecular anatomy of mammalian brain 200

W

Waardenburg syndromes, melanocyte depletion and 312
Wallerian degeneration 687–688
morphological changes/pathogenesis
Schwann cells 687–688
synapse elimination *vs.* 555
Werdnig-Hoffman disease (SMA type 1), gene mutations 558–559
Wide-field ganglion cell (alpha cells) *see* Retinal ganglion cells (RGCs)
wingless gene/protein 39
antagonists 41
anterograde signaling in *Drosophila* NMJ development 527
expression 42
morphogenic function 39
as neuron guidance cue 672
organizer functional range and 85–86
overexpression 39
posttranslational modifications 39
secretion 39, 42–43
signaling pathways 672
synapse formation/maturation in 514
transport 39
see also Wnt signaling
wingless related factors, spinal cord dorsoventral patterning 172–173
wishful thinking (wit), retrograde signaling and the invertebrate NMJ 526–527
Wnt1, midbrain-hindbrain boundary 82
Wnt3
axon guidance
growth cones 42
retinal ganglion cell repulsion 416–417, 417*f*
synapse formation/maturation 514
Wnt4, axon guidance 464, 469
Wnt5
axon guidance, neuron-glial interactions 393
derailed receptor expression 462–463
Wnt5a, expression pattern 463
Wnt7a
axonal branching 42
synapse formation/maturation 514
Wnt7b, dendritic complexity 42, 43*f*
Wnt8, midbrain-hindbrain boundary 80

Wnt signaling 672
adult NSC regulation 644–645
antagonism and AP patterning 80, 100, 109
anterior neural border role 107, 109
forebrain boundaries 80–81, 103–104
hindbrain boundaries 80
hypothalamic development 101–102
roof plate expression and dorsal patterning 109
telencephalon development and 102
anterograde signaling in *Drosophila* NMJ development 527
Caenorhabditis elegans 463
canonical pathway 40*f*, 42*f*
cytosolic events 40
eye field specification 102–103
ligands, alternative 39
nuclear events 41
REST regulation 208–209
Wnt receptors 39
Xenopus axis specification 41
cell cycle interactions in cell fate determination 597
dorsal-ventral mapping 369
functional roles 462
as guidance cue 672
chemoattractive 464
Drosophila 464
spinal cord explant studies 461*f*, 464
chemorepulsive 462
Drosophila 462
holoprosencephaly and 114
Hox gene expression 65
mammalian 463
melanoblast specification 314–316
midbrain patterning 82
isthmic organizer induction 88, 88*f*
midbrain arcs/interarc formation 90
midbrain-forebrain boundary 80–81, 82–83
midbrain-hindbrain boundary 80, 82
optic tectum retinotopy development 93
roof plate induction 89
midline specification/patterning
anterior neural border induction 107, 109
dorsal patterning 109
eye development 102–103
hypothalamic development and 101–102
telencephalic development and 102
unanswered questions 114
neocortical arealization 121
neural crest induction 271–272, 312, 314–316
neural patterning **39–44**
calcium pathway 43, 43*f*
synapse development 41
neural plate shaping and 8–9
noncanonical pathway, planar cell polarity pathway 8–9
protein secretion 39
retinal progenitor cell regulation 252–253
synapse formation/maturation 514
Wortmannin, prevention of autophagic cell death 616

X

Xath 5, retinal cell-fate determination 594
Xbra, positional information 19
X-chromosome(s) 348, 354
Xenopus laevis
development *see Xenopus laevis* development
primary neuron distribution 138
proneural basic helix-loop-helix proteins 151–152
retina
cell specification 261
Xenopus laevis development
axon guidance experiments
ephrinB expression 415–416, 416*f*
spinal 466–467
cell-fate determination, Cdkis and 595
morphogens, positional information 19
neural inducers 45–46
synaptogenesis
perisynaptic Schwann cells 563
Wnt signaling, axis specification 41
Xic1, cell-fate determination 595
Xolloid (Xld) 46

Y

Y-chromosome 348, 354
Yeast
genetics, autophagic cell death 617
YY1 (transcription factor), *Hox* gene expression 66–67

Z

Zebrafish
development *see* Zebrafish development
primary neuron distribution 138
retina
retinal ganglion cells
optic nerve disruption 415
retinal progenitors *see* Retinal progenitor cells (RPCs)
Zebrafish development
axon guidance, sonic hedgehog role 468
Notch signaling 138–139
oligodendrocyte precursors 241
retinal 263–264
progenitors *see* Retinal progenitor cells (RPCs)
time-lapse experiments 257, 257*f*
spinal cord
Gli proteins and DV patterning 38
Zellweger syndrome (ZS) 192–193
Zic2 gene/protein
optic chiasm decussation decisions 430
ventrotemporal (VT) retina development 126
Zic2-roof plate pathway in dorsal midline 112*f*, 113
Zic3, neural crest development 272
Zic transcription factors
roof plate induction 89, 112*f*, 113
ZLI *see* Zona limitans intrathalamica (ZLI)
Zona limitans intrathalamica (ZLI) 100
diencephalon specification/development 101*f*, 103
function 82–83
signaling
FGFs 82–83
hedgehog proteins 81–82, 82–83, 84, 104
iroquois (Irx) transcription factors 80–81
tissue competence and signal integration 84
Wnts 80–81, 82–83, 103–104
transcription factor boundaries 103–104
Fez/Fezl transcription factors 80–81