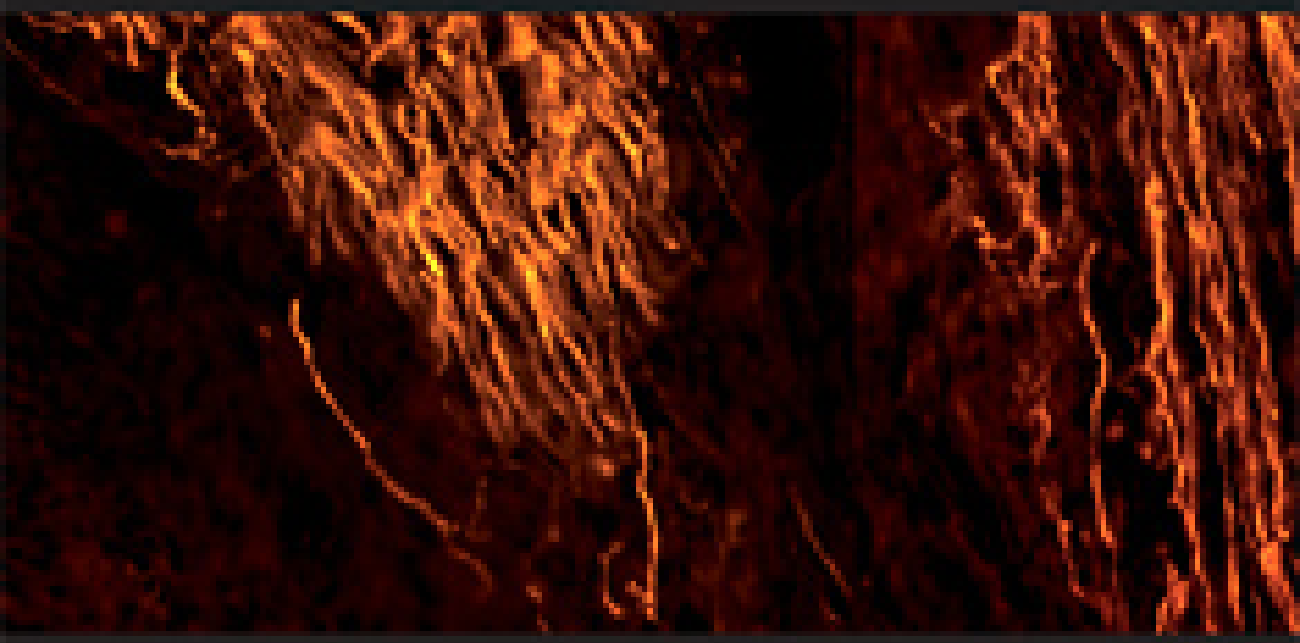

Neurobiology of Peripheral Nerve Regeneration

Douglas W. Zochodne

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Neurobiology of Peripheral Nerve Regeneration

Peripheral nerve disorders are among the most common neurological problems that clinicians face, yet few therapies and interventions are available to arrest or reverse the damage associated with them. Summarizing this important, but neglected, area of neuroscience, Doug Zochodne addresses the peripheral, not central, nervous system and its unique neurobiology. He summarizes current basic ideas about the molecular mechanisms involved in both nerve degeneration and regeneration and what approaches can be used to address it experimentally. Heavily illustrated throughout, and including a 32-page color plate section, this book will serve as a valuable reference for academic researchers and graduate students.

Dr. Douglas Zochodne is a Professor with Tenure and a Consultant Neurologist in the Department of Clinical Neurosciences, Hotchkiss Brain Institute at the University of Calgary. He has recently served (1999–2007) as Editor-in-Chief of the *Canadian Journal of Neurological Sciences*. Dr. Zochodne has run an externally funded research laboratory investigating problems of peripheral nerves since 1989. The work has been funded by the Canadian Institutes of Health Research, Canadian Diabetes Association, Alberta Heritage Foundation for Medical Research, and the Muscular Dystrophy Association of Canada.

Neurobiology of Peripheral Nerve Regeneration

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Dedicated to my wife Barbara and my children Julia and William

Contents

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The Peripheral Nerve Society (PNS) has been an intellectual home for many of us interested in the biology of peripheral nerves and a potent stimulus for me to proceed with this work.

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> "Nature uses only the longest threads to weave her patterns, so each small piece of her fabric reveals the organization of the entire tapestry." (Richard Feynman, *The Character of Physical Law*, 1965)

1

Introduction

This book is about peripheral nerves, their unique biology and how they repair themselves during regeneration. The biology of the peripheral nervous system is not often considered on its own. Much has been learned about the neurosciences of peripheral nerves, specifically during injury and regeneration, but it is my sense that some of this new and exciting information should be consolidated and considered in an overview.

Without nerves, specifically peripheral nerves, there is no movement, no sensation. Peripheral nerves are the essential connections between the body, brain, and spinal cord. The "peripheral nervous system (PNS)" distinguishes itself from the "central nervous system (CNS)" on many levels. Peripheral axons reside in many types of local environments including muscles, connective tissue, skin, and virtually every organ of the body. This reach extends into the meninges that surround the brain, a surprising fact to some. Moreover, peripheral neurons are very different from their CNS counterparts in how they respond to injury or disease, in which cells they partner with and in what axon trees they support. For example, a sensory neuron in the lumbar dorsal root sensory ganglion is required to maintain and support distal axon branches that can extend a meter or more to the skin of the toe. Only a small proportion of CNS neurons have comparable outreach and demands placed upon them.

"Neuropathies," of which there are a large number, are simply disorders of peripheral nerves. A neuropathy might be focal (also known as a mononeuropathy) and involve only a single peripheral nerve, or it might involve peripheral nerves widely (polyneuropathy). Despite being very common problems, comparable in prevalence with stroke and Alzheimer's disease, they are not widely understood by patients, health care providers or neuroscientists! Polyneuropathy can be detected in approximately half of all diabetic subjects, an important issue

to consider in this day of dramatically rising Type 2 diabetes prevalence. Diabetic neuropathy itself, without considering all other forms of neuropathy, has a prevalence of over ten times that of MS.

Consider a few important points. A patient with severe peripheral nerve disease, such as Guillain–Barré syndrome (GBS) can, during the acute phase be completely "locked in," or unable to move a single limb muscle or eye muscle. This patient may require a ventilator to breathe. He may also have lost any sensation to light touch, pain, or temperature. Despite these severe deficits, however, cognition may well be fully retained because the disorder does not involve the brain. It is difficult to conceive of being "locked in" while being fully conscious unless one has suffered from GBS or a comparable disorder of the peripheral nervous system. The reader is referred to books written by patients who have suffered and recovered from GBS [373,374]. GBS is an autoimmune inflammatory polyneuropathy, sometimes triggered by infections or vaccinations that took place 2–3 weeks earlier. Different types of GBS are recognized. Yet it is the unique neurobiology of the nerves damaged during GBS that will most impact how a patient might fare. The most common form of GBS, also known as the classical demyelinating type, involves only the myelin sheath of the peripheral nerve. The underlying axon tree remains intact despite having been rendered nonfunctional by the loss of its myelin sheath. Remyelination of the axons is expected and can be associated with a complete recovery of paralysis and sensation.

Alternatively, a type of GBS recently termed AMSAN (acute motor and sensory axonal neuropathy) primarily attacks the axons and spares the myelin. Recovery is dictated by the rate and likelihood (certainly not guaranteed) that axons will regrow from the injury site to their correct original target, e.g., a small foot muscle, a touch receptor in the finger. The unfortunate result is very limited, delayed, or absent recovery in this severe form of GBS. While axons might be expected to regrow at the rate of about an inch per month in order to reach their targets, this likelihood falls dramatically with time. These limitations will be discussed in subsequent chapters. The tragedy is that, in some instances, severe GBS that primarily attacks axons may not recover at all.

Consider the story of "Nancy B.," a young woman who garnered national attention in Canada because of her peripheral nerve disorder. She developed severe axonal GBS that rendered her "locked in" and ventilator bound without any improvement over 2½ years. While being perfectly lucid about her condition, she made the decision to have her health care workers withdraw her ventilator support. Without this support, she did not survive. The story generated widespread discussion about ethical issues surrounding the maintenance of life support in patients who do not have a chance for recovery. Figure 1.1 is an image of a patient who required intensive care unit hospitalization for 1 year because of GBS.

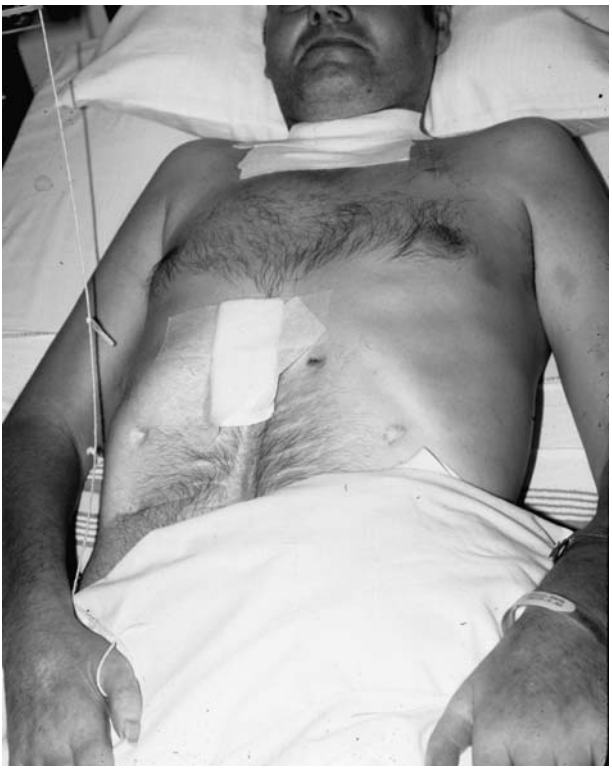

Figure 1.1 A patient with severe Guillain–Barré syndrome with axonal damage and paralysis of all of his limbs. He required intensive care unit support to breathe for a period of 1 year before recovering.

Fortunately, many neuropathies do not render disability as severe as that experienced by "Nancy B." They do, however, impose their own range of disabilities and interference with quality of life. Some are associated with significant loss of function. Consider the diabetic patient seen by the author who developed a focal mononeuropathy of his ulnar nerve at the elbow. This lesion rendered significant, though not complete, hand wasting and weakness. The patient, however, had been a professional tennis player and could not accept his inability to play. The neuropathy compounded underlying depression in this patient and led to suicide. Figure 1.2 is an image of a patient with a focal sciatic nerve injury lesion from a buttock firearm wound, rendering paralysis of muscles in the leg and loss of sensation in the foot.

Loss of sensation is associated with loss of the ability to detect skin and soft tissue injuries. Patients may develop skin ulcers from unrecognized injury to their feet (e.g., stepping on a nail or damaging their skin from overly tight footwear). In some cases these injuries are associated with additional damage, infection,

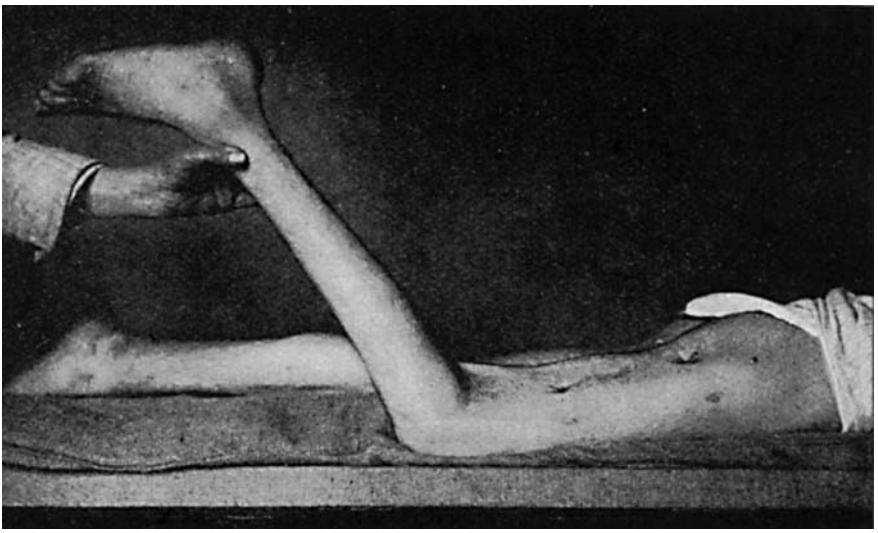

Figure 1.2 A patient with a right buttock bullet wound that damaged his underlying sciatic nerve trunk. The severe axonal disruption caused by the injury resulted in permanent paralysis and atrophy of muscles in the thigh and below the knee with sensory loss in the foot and leg. (From Tinel [681].)

and the need for amputation. Polyneuropathy is a leading cause of lower limb amputation in diabetes. Loss of sensation to position (proprioception) also contributes to falls and injury because it is impossible for a patient to tell where the limbs are in space. Finally, peripheral nerve damage of all types is frequently associated with a severe and debilitating type of pain known as "neuropathic pain." Neuropathic pain can render patients unable to walk, work, sleep, or enjoy life. While this text does not directly address neuropathic pain, full and effective regeneration of the peripheral nervous system usually extinguishes it.

Unlike many other disorders, neuropathies impose a burden of neurological deficit that requires nerve regeneration, irrespective of what caused the damage. Therapy for an active peripheral nerve disease, or microsurgical repair of a transected peripheral nerve trunk (we use the term "trunk" to refer to a peripheral nerve branch containing hundreds to thousands of individual axons and their supporting cells) may address the inciting lesion that caused damage, yet it is regeneration that must ensue to restore proper function. For example, vasculitic neuropathy is a disorder that damages peripheral nerve axons through inflammation of nutrient feeding vessels of the peripheral nerve trunk. It may be "cured" with a course of immunosuppressive therapy, a treatment that arrests the inflammation but does not restore function. This is unsatisfactory to many patients who suffer from neuropathy; previously damaged axons must now regrow to

reverse the deficits that have developed. At the time of this writing, specific therapy designed to coax more complete and effective recovery of nerves is unavailable.

In the neurosciences literature, peripheral neurons have been highlighted as examples of neurons whose axons can regenerate, unlike those of the CNS. Indeed, significant excitement has resulted from findings that injured CNS neurons in the spinal cord can regenerate into and through peripheral nerve grafts. Peripheral neurons have been seeded on substrates of CNS myelin to demonstrate its property to inhibit regrowth. Without diminishing the importance of these findings, however, they do not address the realities of peripheral nerve disease. In neuropathies, the fate of axons regenerating in their own peripheral microenvironment is the important consideration. Recovery is slow, and if the distances to the target tissues are long, regeneration may never occur. Such catastrophic failure occurs despite the fact that axons have a "denervated" distal stump into which to grow. Distal to an axonal injury, axons undergo the process of "Wallerian degeneration" in which disconnected branches are phagocytosed and eventually disappear. They leave behind a denervated distal stump that includes a connective scaffold and supporting Schwann cells. In this text, my intent is to dispel the idea that peripheral neurons serve simply as a model for understanding issues in CNS regeneration. Rather, I seek to convince the reader that PNS disease poses its own unique burdens on a substrate of neurons and supporting cells and has its own, separate but compelling regeneration issues. It deserves an equal and focused place at the neurosciences research table.

Exciting new aspects of peripheral nerve behavior challenge traditional concepts. One such example involves how axons interact with their basement membranes. Extracellular basement membrane constituents of nerve trunks expose specific ligand (e.g., the RGD, or Arg–Gly–Asp tripeptide sequences) moieties that interact with integrin receptors of adjacent axons. Local signaling cascades within axons are triggered by this interaction. These cascades have the capability of altering growth cone behavior and influencing regeneration yet may be completely independent of changes within their cell body. The idea that growth cones and axons *locally* might signal and react is novel. Not only do such signals influence axon behavior but in this scenario they also alter local *protein synthesis*, previously considered the sole purview of the cell body. To coordinate regeneration, the axon and perikarya also sense that there is injury, alter the pattern of nuclear gene expression in the cell body and change the repertoire of proteins they transport down the axon to the injury site. They do this while managing to signal local axons to synthesize regeneration-related molecules. How the whole family of regeneration molecules is coordinated between local synthesis or transport from the cell body is not known at this time. For example, local axon synthesis might act as a "rapid response" program for injured axons,

later supplemented by reinforcements shipped from the cell body. One might imagine that nerve surgeons could one day be capable of implanting regeneration conduits with graded release of signals that would "shore up" such local axonal events.

There are intriguing discoveries in how surrounding and supporting cells of the peripheral nervous system interact with damaged neurons. For example, a remote injury of a sensory neuron axon branch (an "axotomy" is a transection of an axon branch of a neuron) is sufficient to send information to its parent cell body in the dorsal root ganglion (DRG) up to a meter away. In response, perineuronal satellite cells in the ganglion that have not been directly involved in the injury dramatically change their phenotype. Satellite cells are cousins of Schwann cells and both cell types appear in sensory ganglia. Satellite cells, however, are interesting cytoplasmic poor cells that closely surround individual sensory neurons. They exhibit a dynamic form of life and death plasticity with ongoing apoptosis and division within "stable" ganglia. Their plasticity contrasts sharply with the apparent immutability of their neighbor neurons. Within a defined time course, satellite cells enlarge and proliferate around closely associated but axotomized neurons. How neurons communicate with these important and pervasive neighbors is unknown. Communication between neurons and satellite cells is likely to be reciprocal. For example, satellite cells are known to provide trophic molecules such as CNTF (ciliary neurotrophic factor) to support neurons and protect them from injury.

In contrast to sensory neurons, injured motor neurons have cell bodies that are official residents of the CNS, in the anterior horn of the spinal cord. When their remote axons are injured, their dendrites in the gray matter of the spinal cord retract. This accompanies "synaptic stripping" of their connections with other spinal cord neurons. How the loss of these dendritic connections occurs and how they might be restored is uncertain.

The Schwann cell (SC) is a type of glial cell, unique to the peripheral nervous system, that supports all types of axons: sensory, motor, and autonomic. Its roles can be surprisingly multifaceted and differ from those of their CNS myelinating counterpart, the oligodendrocyte. Early after nerve injury, SCs can serve as local inflammatory cells by generating cytokines, inducible nitric oxide synthase (iNOS; an inflammatory enzyme that generates nitric oxide (NO)) and other inflammatory molecules well before macrophages from the bloodstream enter the nerve and assume this function. SCs can interconvert from "stable" myelinating phenotypes to highly plastic proliferating and migrating cells that may direct appropriate and directional axon regrowth after injury. They offer the peripheral nerve a range of trophic molecules but not necessarily simultaneously. In other words, SCs appear to have a sense of timing and coordination

in what they synthesize. While the exact mechanism is unclear, there is evidence that they accurately guage their local microenvironment and respond accordingly. Moreover, like the neuron and its perineuronal satellite cell in the ganglion, there is intimate and bi-directional talk between the axon and the SC. Cross talk is likely critical during regeneration but may also be a feature of normal uninjured nerve trunks.

Axons elaborate neuregulins, potent molecules capable of altering SC protein synthesis, myelin synthesis, and their likelihood to proliferate. Neuregulins, in turn, instruct SCs to synthesize a series of molecules, such as neurotrophins that encourage axon regrowth in a highly directional manner. When peripheral nerve trunks are transected, the tension normally present within them causes the distal and proximal stumps to retract from one another. While such lesions are incompatible with full axon regrowth, the stumps can reconnect by a connective tissue bridge if they are apposed to one another in a graft or conduit. From the proximal stump of the transected nerve, axons then enter the bridge and begin to grow across it. These early events offer fascinating opportunities as to how axons navigate new territories. One interesting finding is that SCs appear to lead axons through complex, three-dimensional trajectories. Their relationship, closely linked with local trails of laminin, is so encompassing and intimate that it might be called the "axon-SC" dance! SC partnership is critical to the success of axon regrowth. Not surprisingly, a group of colleagues interested in SCs call themselves the "Friends of Schwann"!

The purpose of this text is to emphasize the unique structure, plasticity, and challenges of regrowing peripheral nerves. Excepting focal neuropathy associated with direct injury to the nerve, neuropathies are not addressed directly. We refer readers to other comprehensive texts addressing peripheral nerve disorders and peripheral nerve surgery [159,421].

We begin by examining properties of the peripheral nervous system in nerve trunks that house axons, Schwann cells and other tissue components, and ganglia that house cell bodies, or perikarya. Next we address how peripheral nerves are injured by trauma. What are the resulting injuries, their implications, and the barriers to regrowth? We then address experimental approaches to peripheral nerve regeneration. We ask how does nerve regeneration evolve through its early events and later consolidation? Special consideration will be given to the microvascular supply and its impact on regenerative events. Finally, we address important aspects of regrowth: the impact of long-term denervation, the actions of growth factors and molecular barriers of regrowth.

It is my intent that this text might be a project in evolution, consolidating what has been discovered to date, as well as being a catalyst for new ideas and approaches toward resolving the burden of peripheral nerve damage.

2

The intact peripheral nerve tree

A thorough appreciation of the unique anatomy of the peripheral nervous system is essential in understanding how it regenerates. This information, already described in several texts, is nonetheless summarized here to prepare the reader for later chapters. There are many facets to peripheral nervous system anatomy that have a bearing on its response to injury including the multiplicity of neuron subtypes and the qualities of their housing.

Overall structure

The peripheral nervous system (PNS) is complex. The peripheral nerve "trunk" refers to a cable of tissue in which hundreds to thousands of axons may travel. Peripheral nerve trunks form connections from the brain and spinal cord to all skeletal muscles in the body through motor axons. They also connect all sensory organs to the brain and spinal cord through sensory axons (Figure 2.1). Finally, they connect the CNS to smooth muscles, sweat glands, blood vessels, and other structures through axons of the autonomic nervous system. Axons traveling through nerve trunks originate from cell bodies of neurons (perikarya) in the brainstem, spinal cord, and ganglia. Motor neuron cell bodies found in cranial motor nuclei supply the head and neck, and those in the anterior gray matter horn of the spinal cord supply the limbs and trunks. Motor neuron cell bodies have their greatest numbers in the cervical and lumbar enlargements of the spinal cord so that they can supply the large number of muscles in the upper limbs and lower limbs, respectively. Sensory neuron perikarya are found in cranial sensory ganglia and paraspinal ganglia from approximately T1 through to L1 levels. Autonomic neurons are housed in a variety of sites: cranial and cervical ganglia, paraspinal sympathetic ganglia, and a variety of ganglia in the

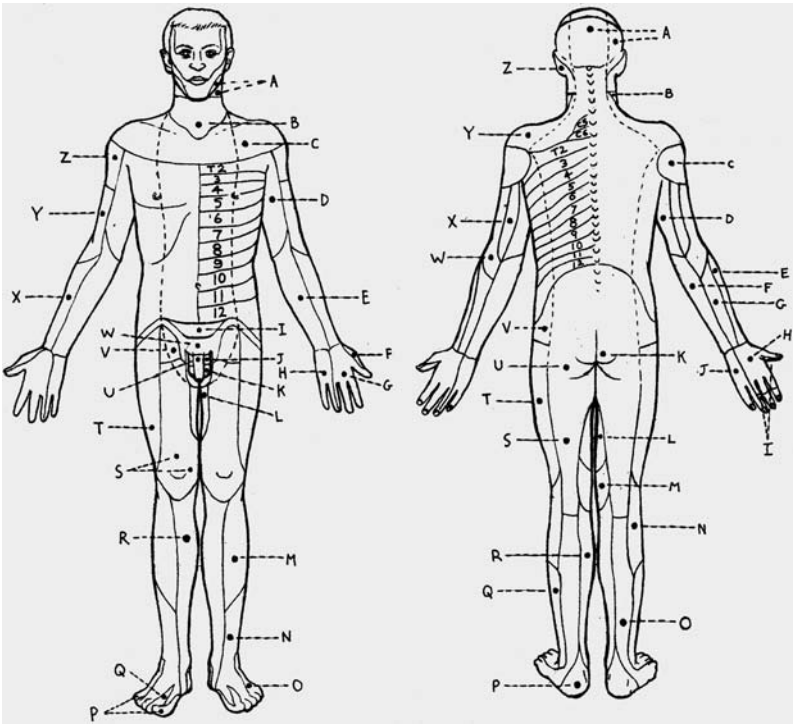

Figure 2.1 An illustration of the peripheral sensory nerve territories of the human body. *Left panel, anterior body:* A – greater auricular nerve; B – anterior cutaneous nerve of neck; C – supraclavicular nerves; D – medial cutaneous nerve or arm and intercostobrachial nerve; E – medial cutaneous nerve of the forearm; F – radial nerve; G – median nerve; H – ulnar nerve; I – iliohypogastric nerve; J – genital branch of genitofemoral nerve; K – scrotal branch of perineal nerve; L – oburator nerve; M – lateral cutaneous nerve of calf; N – superficial peroneal nerve; O – sural nerve; P – medial and lateral plantar nerves; Q – deep peroneal nerve; R – sapenous nerve; S – intermediate and medial cutaneous nerves of the thigh; T – lateral cutaneous nerve of the thigh; U – dorsal nerve of penis; V – femoral branch of genitofemoral nerve; W – ilioinguinal nerve; X – lateral cutaneous nerve of forearm; Y – lateral cutaneous nerve of arm; Z – axilllary nerve. *Right panel, posterior body:* A – greater and lesser occipital nerves; B – anterior cutaneous nerve of neck; C – axillary nerve; D – medial cutaneous nerve of arm and intercostobrachial nerve; E – lateral cutaneous nerve of forearm; F – medial cutaneous nerve of forearm; G – posterior cutaneous nerve of forearm; H – radial nerve; I – median nerves; J – ulnar nerve; K – inferior medial clunical nerve; L – obturator nerve; M – medial cutaneous nerve of thigh; N – lateral cutaneous nerve of calf; O – sural nerve; P – calcaneal branches of sural and tibial nerves; Q – superficial peroneal nerve; R – saphenous nerve; S – posterior cutaneous nerve of thigh; T – lateral cutaneous nerve of thigh; U – inferior lateral clunical nerve; V – iliohypogastric nerve; W – lower lateral cutaneous nerve of arm; X – posterior cutaneous nerve of arm; Y – supraclavicular nerves; Z – greater auricular nerve. (Illustration by Scott Rogers, based on previous illustrations by Haymaker and Woodall [254].)

abdomen that include the celiac, mesenteric, para-aortic, hypogastric, and others. The enteric nervous system includes a large number of neurons within the walls of the gastrointestinal system.

Nerve "trunks" originate through a confluence of branches that supply them. Motor neurons send axons to nerve trunks through the ventral roots of the spinal cord and motor branches of cranial nerves. Sensory neurons have an initial single branch that emerges from the cell body and then divides into two branches, an arrangement called "pseudounipolar" (see below). From the initial single branch, a central branch is directed to the spinal cord entering the dorsal horn and posterior columns and a peripheral branch is sent to the nerve trunks. The peripheral sensory branch in the dorsal root joins motor axons from the ventral roots as they exit the neural foramina of the bony spinal column and together form the mixed spinal nerve. It is at this site that the meningeal sheath (dura and arachnoid) surrounding the spinal cord and its roots forms pockets or sleeves that blend into the epineurial sheath of the peripheral nerve. Mixed spinal nerves then send branches posteriorly to innervate the paraspinal muscles and anteriorly where they form the major nerve trunks of the body. From both the cervical enlargement and the lumbar enlargement, anterior spinal nerve branches merge and intermingle to form the brachial and lumbosacral plexus, respectively. From each plexus, peripheral nerves are then formed from a mixture of motor, sensory, and autonomic axons arising at different root levels. Despite the mixture, most major nerve trunks include axons from only a few spinal root levels. Thus, for example, the human median nerve is composed almost exclusively of axons from the C8 and T1 spinal root level, while the musculocutaneous nerve arises from C5 and C6.

Given this overall arrangement then, most major nerve trunks house a variety of axon types. For example, there are likely no "pure" motor nerve trunks since nerves associated with muscles include both motor axons and a large complement of sensory axons sensitive to muscle pain, or to stretch in muscle spindles. Cutaneous nerves do not include motor axons but do contain both autonomic and sensory axons. In any major peripheral nerve trunk, therefore, there are larger myelinated axons (α motor axons and large Aα sensory axons), small myelinated axons (smaller Aβ and Aδ sensory axons, γ motor axons – see below), and unmyelinated axons (C sensory and autonomic axons). Classical histological approaches do not distinguish whether myelinated axons are motor or sensory or whether unmyelinated axons are sensory or autonomic. Thus, when a transverse section of a peripheral nerve trunk is examined, it is not possible to identify what class a given axon may belong to. In humans the sural nerve is most often harvested for diagnostic purposes and its axons are sensory or autonomic only.

Immunohistochemical labeling does allow axons to be more accurately distinguished. Labeling can be applied for neurotransmitter enzymes or peptides specific for neuron subtypes, e.g., choline acetyltransferase (ChAT) for motor axons, tyrosine hydroxylase (Th) for sympathetic autonomic axons. Substance P (SP), is specific for unmyelinated sensory nociceptive (pain transmitting) axons. Other unmyelinated sensory axons are labeled by a lectin tag known as IB-4 and express receptors sensitive to the growth factor GDNF (glial derived neurotrophic factor). They have also been labeled NGF unresponsive or Trk A negative small afferents.

Large nerve trunks frequently course as "neurovascular bundles" together with blood vessels. Bundles can be identified in humans at the inguinal ligament, popliteal fossa, thoracic inlet, and other areas. This kind of partnership, however, is not invariable and nerve trunks can find their way separately to the muscles and sensory organs they innervate. At periodic intervals along the nerve trunk, they are supplied by feeding branches from adjacent blood vessels forming the vasa nervorum, or the blood supply of the peripheral nerve. Vasa nervorum form into a plexus on the epineurial surface of the nerve that, in turn, regularly penetrates to supply deeper structures. Longitudinal vessels arising from the epineurial plexus also enter the nerve trunk and travel within it. Thus, between penetrating plexus arterioles and longitudinal supplying vessels, portions of peripheral nerves not directly attached to obvious feeding vessels are nonetheless well perfused. For larger peripheral nerve trunks, such as the sciatic nerve in the midportion of the thigh, some portions may be especially vulnerable to interruptions in this supply, or ischemia. These are so-called "watershed" zones of the nerve trunk where, in the absence of adjacent feeding vessels, they instead rely on remote supply from an intact epineurial plexus or a longitudinal vessel.

Structure and characteristics of the nerve trunk

The structure of the peripheral nerve trunk is unique and dramatically differs from that of the brain, the spinal cord, or the optic nerve. Its tensile strength, important for the routine bending and twisting encountered during normal limb movements, is accounted for by its collagen sheath structure. Collagen polymers are oriented longitudinally parallel with the trunk cable and provide resistance to stretch, and protection from compression. When tissue pressure rises within the peripheral nerve trunk, its structure also offers compliance. Compliance means that rises in local pressure can occur without occluding the local vascular supply and that some stretching can occur without severing axons.

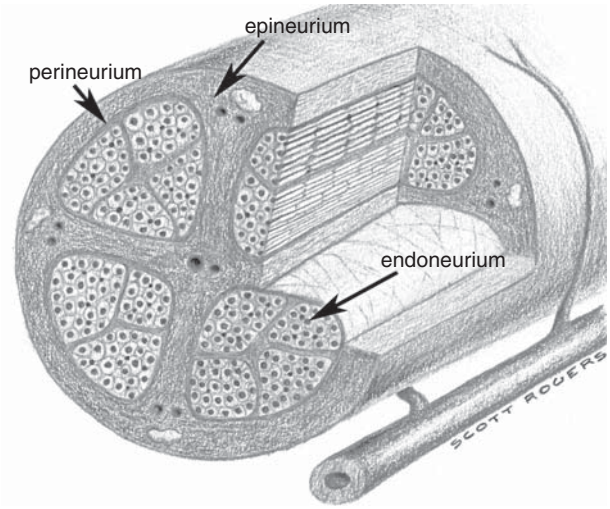

Figure 2.2 An illustration of the overall organization of the peripheral nerve trunk and its compartments. (Illustration by Scott Rogers.) See color plate section.

The epineurium or epineurial sheath is the outer layer of the peripheral nerve trunk and includes the collagen tissue sheath, a plexus of blood vessels, lymphatic vessels, some resident macrophages, fibroblasts, and mast cells. Its thickness can be quite variable and it sometimes includes adipose tissue (Figures 2.2, 2.3). The epineurial sheath has little connection to adjacent tissues allowing nerves to normally slide and move during limb movement [43]. It consists predominantly of Type I collagen but elastic fibers are also present, and they are largely longitudinally oriented. Wavy patterns of the inner epineurial collagen and the axons allow the nerve to be extended or stretched to some degree [43]. The epineurial vasa nervorum of the peripheral nerve trunk lack a specific barrier to blood borne molecules. Thus, for example, classical experiments using intravascular injections of Evans blue albumin noted that it fully permeates out through vessels into the tissues of the epineurial sheath, a permissive state that may be relevant during inflammatory disorders of nerves.

Local blood flow in the epineurium is high, at least two to three times that of the interior, or endoneurium (see below) of the nerve trunk. When peripheral nerves are examined at surgery, the dense plexus of the vasa nervorum can thereby be observed on the surface of the nerve trunk with highly irregular and tortuous contours. Some of this irregularity or redundancy also gives blood vessels flexibility in dealing with stretch or twisting of the nerve trunk without compromising their ability to supply the nerve. It is interesting, for example, that peripheral nerve surgeons can “mobilize” or free significant lengths of peripheral

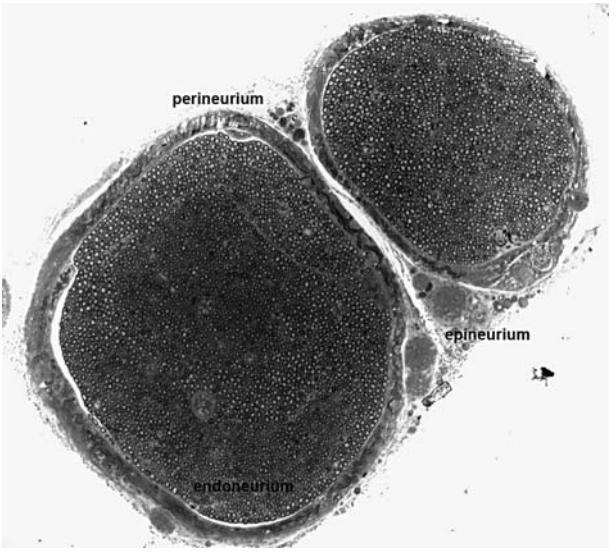

Figure 2.3 A photomicrograph of a sciatic peripheral nerve trunk. Note the presence of two major fascicles with myelinated axons within the endoneurium. The section is a low-power view of a semithin section that was harvested from an adult rat, fixed, embedded in epon and stained with toluidine blue.

nerves from their surrounding tissue without damage. A rich anastomotic supply of blood vessels allows such leeway. Arteriovenous (AV) shunts, direct connections between arterioles and veins, occur in the epineurial plexus.

The epineurial blood supply of the peripheral nerve trunk also has unique physiological qualitities that are discussed in more detail later in Chapter 7. One property is its lack of autoregulation, distinguishing it from cerebral blood flow. Vasa nervorum also possess vasoactive properties. They are richly innervated by both sympathetic adrenergic terminals and peptidergic endings that can influence downstream flow of the blood vessels they innervate. For adrenergic terminals, activation results in vasoconstriction and declines in downstream blood flow. Specific segments of epineurial arterioles probably control flow, for example, at vessel junctions as "precapillary sphincters." Thus arteriolar segments with adrenergic terminals might be critical sites of "control" or "gateways" for downstream blood supply to endoneurial structures. Peptidergic nerve endings on vasa nervorum, containing calcitonin gene-related peptide (CGRP), SP, and others, arise from sensory axons. These have the capacity to dilate epineurial vessels and this may be important during nerve inflammation. Both adrenergic terminals and peptidergic terminals on vasa nervorum provide ongoing "tone" from a basal level of activity. In an otherwise intact epineurial vascular bed then,

interruption of "normal" adrenergic tone results in vasodilatation, whereas blocking peptidergic actions (mainly CGRP as it is the more potent vasodilator) causes vasoconstriction.

Overall then, while epineurial arterioles are controlled by nerve terminals, whether this innervation extends to venules or AV shunts is uncertain. The epineurium is self-innervated. By this, we mean that small unmyelinated axons from the endoneurium of the parent nerve trunk travel outward into the connective tissue of the epineurium. Some, but not all of these axons innervate blood vessels, as discussed above. Termed "nervi nervorum" these small axons have been thought to generate pain in some types of peripheral nerve damage and inflammation [25,53]. Local mast cells that contain histamine and serotonin are often identified as residents near epineurial vessels. Lymph vessels are present in the epineurium, but not in other areas of the nerve [346].

The perineurium is a laminated cylindrical layering of specialized cells that is found deep to the epineurium and that surrounds and protects the endoneurial fascicles (Figures 2.2, 2.3). The perineurial cells are interleaved and interconnected by tight junctions (zona occludens) and gap junctions (zona adherens) forming layers separated by collagen fibrils (Type IV). Perineurial cells also contain pinocytotic vesicles that probably contribute to regulated forms of transcellular transport [43]. They have a basal laminae consisting of collagen, fibronectin, laminin, and glycosaminoglycans. While there has been debate, it is now agreed that perineurial cells are of fibroblast origin [77].

The tight junctions between perineurial cells and the presence of vesicles within them form part of the blood nerve barrier (BNB), a barrier to blood-borne constituents from entry into the endoneurial fascicles. This barrier, like that of the brain (blood brain barrier), helps to protect the endoneurium from potentially toxic serum proteins, micro-organisms, and other constituents. Thus, after the indicator Evans blue albumin is given, tissues within the intact perineurium are unstained because the protein does not penetrate the barrier [534,535]. The BNB is somewhat leakier than its central counterpart, the blood brain barrier, and it protects axons, not neurons. It is more than just a barrier to proteins such as Evans blue albumin but also excludes small molecules and ions. Weerasuryia, Rapoport, and others have described specific quantitative approaches to studying the BNB [575,730,733,734]. The perineurium extends the length of the peripheral nerve and connects with the pia and arachnoid layers of the brain and spinal cord as nerve roots travel toward the CNS. Perineurial cells also extend to help form the inner capsule of the dorsal root ganglia and outer layer of sensory organs such as Pacinian corpuscles [43].

The endoneurium might be considered the most important part of the nerve trunk since it contains axons and their supporting SCs. It also contains mast

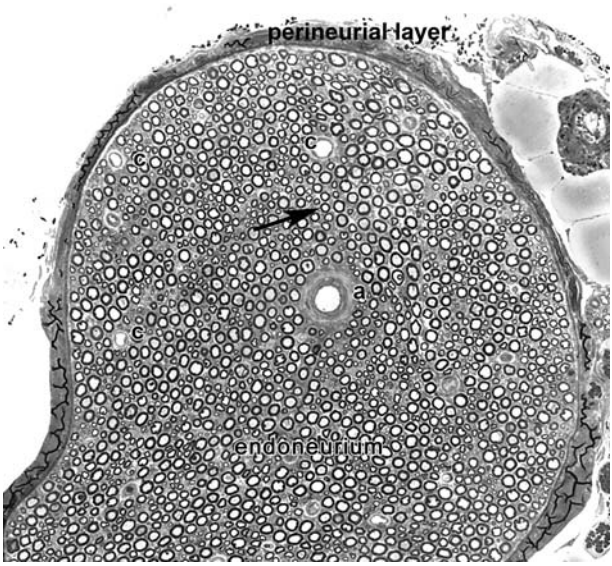

Figure 2.4 A photomicrograph showing the structures within the endoneurium of a nerve trunk fascicle. The arrow points to a myelinated axon. Note that there are populations of larger and smaller myelinated fibers. The section also shows endoneurial capillaries (c) and an arteriole (a). The section is a medium-power view of a semithin section as in Figure 2.3.

cells, resident macrophages, some fibroblasts, and blood vessels (Figures 2.2, 2.3). Types I and III collagens are found within the endoneurium. Endoneurial fluid, in turn, has a unique composition that may be hypertonic based on measures using energy dispersive spectrometry. In 100 picoliter samples of endoneurial fluid its electrolyte composition was calculated as: Na^+ 179, Cl^- 131, K^+ 21 mEq/L [490]. In most peripheral nerve trunks, there may be a variable number of distinct endoneurial compartments, also known as fascicles. Each is surrounded by its own perineurial sheath and the whole nerve trunk is then termed multifascicular. For example, the median nerve at the wrist in humans may contain upwards of ten such individual fascicles. What determines the number of fascicles in a nerve trunk is unknown. During regeneration of nerves "minifascicles" can be formed and they often precede the consolidation and formation of more classical mature nerve trunk structure (see Chapter 6).

Endoneurial blood vessels are largely capillary, although some arterioles are found coursing through the fascicle (Figure 2.4). Capillary endothelial cells may be associated with contractile pericytes. Most endoneurial vessels are not innervated and the endothelial cells are connected by tight junctions. Thus, like the perineurial cells, the endothelium of endoneurial vasa nervorum also contributes to the blood nerve barrier.

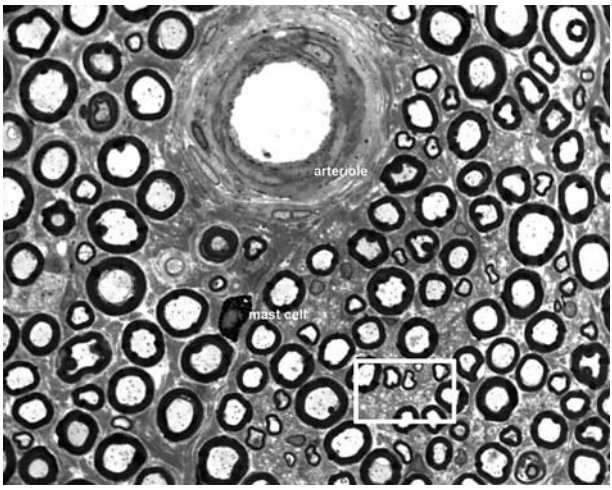

Figure 2.5 A high-power, oil immersion photomicrograph of endoneurial constituents including a mast cell, arteriole, and myelinated axons. Note that some axonal ultrastructural components can be resolved in the large myelinated axons (most likely microtubules and mitochondria) and in the white box, groups of unmyelinated axons can just be resolved. This is a semithin section as in Figure 2.3.

Individual axons may move from one fascicle to another as they travel from proximal to distal. Small "branching" fascicles that contain these mingling axons are commonly observed in transverse sections of peripheral nerves. Axon mingling, however, is incomplete and nerve trunks maintain an overall topographical distribution of axons within them. As a result, axons retain locations within specific fascicles from proximal to distal and there are specific groupings destined for individual muscles. The issue is important because knowledge of this microscopic anatomy of an individual nerve during electrophysiological studies or surgery can help to predict how repair should be approached.

Two major types of axons are identified within the endoneurium (Figures 2.5, 2.6). Myelinated axons are larger in caliber and surrounded by a lamellar lipid myelin sheath. Along the myelinated axon are single Schwann cells (SCs), each forming a length of myelin that form internodes, in turn separated by nodes of Ranvier (Figure 2.7). In a normal human nerve, internodes range from 300–2000 microns in length, depending on the axon size and a very general guide is that the internodal length approximates 100× the axon diameter [194]. Nodes of Ranvier expose a portion of the axon membrane containing sodium channels that allow transmission of action potentials by "saltatory" transmission.

In a human nerve, such as the sural cutaneous nerve, myelinated axons are distributed into two main size categories (also known as a bimodal distribution

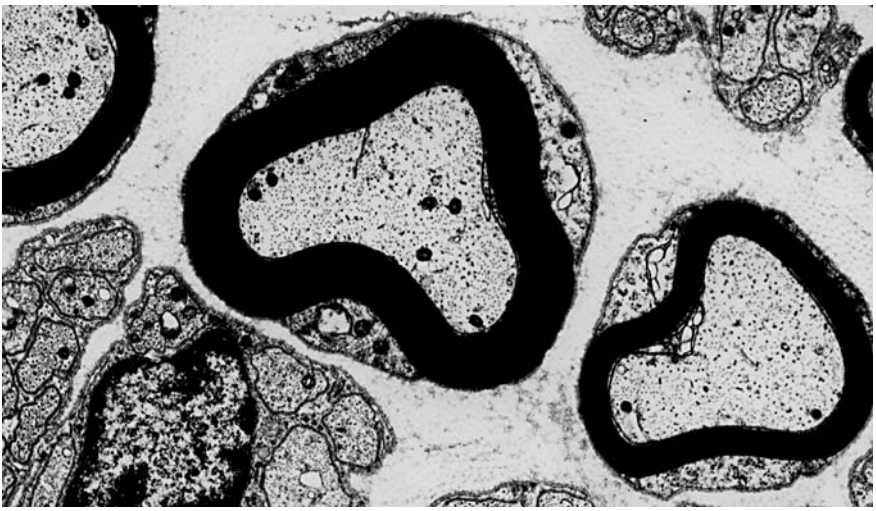

Figure 2.6 An electron micrograph showing two medium caliber myelinated axons with some SC cytoplasm around each. In the lower left part of the image, two clusters of unmyelinated axons (Remak bundle) is seen.

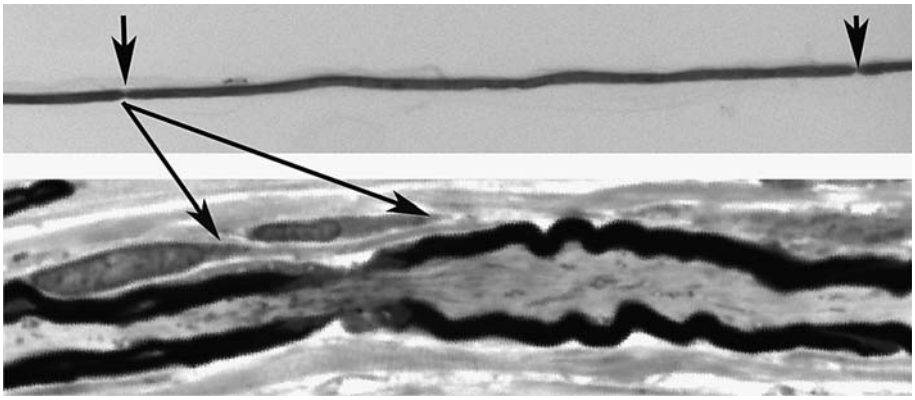

Figure 2.7 A single teased myelinated axon is shown above with the small arrows pointing to nodes of Ranvier (top image). The segment between the arrows is called an internode. Below is a higher power view showing a node. Note the darkened axon in the node region from more closely packed microtubules and neurofilaments. The lower image is a longitudinal semithin toluidine blue section examined under an oil immersion lens.

of fiber sizes). The size distribution can be appreciated by a histogram where the number of axons in given size ranges are plotted (numbers or density of axons on the *y*-axis and size categories on the *x*-axis). Larger myelinated axons, identified by a peak fiber diameter range of 5–12 microns, represent Aα sensory

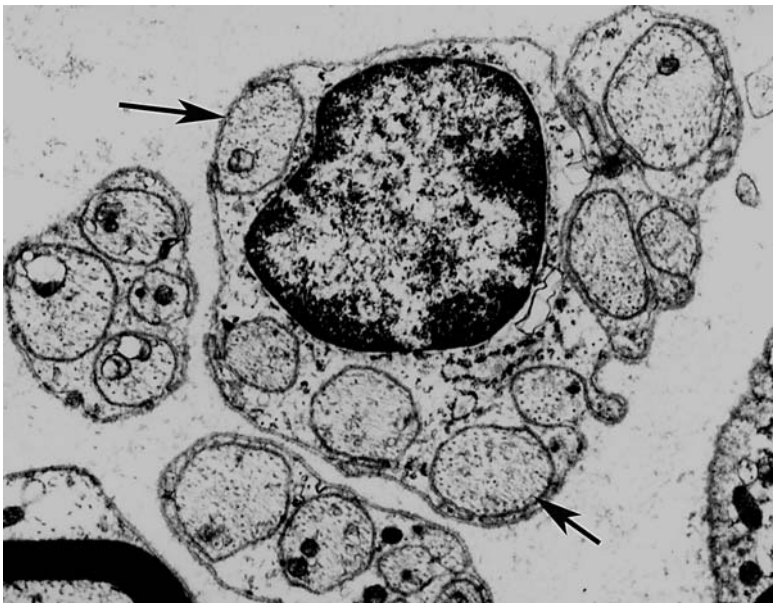

Figure 2.8 An electron micrograph of a Remak bundle of several unmyelinated axons (arrows) associated with a single SC.

axons that serve light touch, vibration, and joint position. Their conduction velocities are in the range of 40–80 m/s or higher. Aβ axons are smaller, intermediate axons with slower conduction velocities. A population of small myelinated axons, also known as Aδ, forms a distinct second peak, and these axons subserve nociception, thermal sensation, and perhaps other functions. Their fiber diameters range from 1–4 microns and their conduction velocities range from 12–30 m/s. In adult humans the mean myelinated fiber density is approximately 12000–15000/mm^2 in the proximal human sural nerve and 7000–10000/mm^2 in its distal portions [156]. For motor fibers, α motor axons are only slightly smaller than Aα sensory axons and γ motor axons are in the mid range.

Unmyelinated axons represent the second major category of axons in the endoneurium. Several of these axons are usually associated with a SC in a unit known as a Remak bundle (Figure 2.8). The number of unmyelinated axons associated with a single SC in a Remak bundle ranges from 1 to 20 or more. The mean number is approximately 6 fibers per SC or up to 36 in a rat, with fewer numbers in man [478]. These SCs differ from those associated with myelinated axons and they form overlapping chains to incorporate axons along their length. A given axon, however, may not accompany the same neighbor axons in SC units along its trajectory. Axons may be resorted along their length into various Remak units. SCs may support different kinds of axons as well. In the

cervical sympathetic trunk of the rat, for instance, the same SC may enwrap both upwardly projecting preganglionic axons and downwardly projecting postganglionic axons [802]. The size distribution of unmyelinated axons is unimodal, or having one peak, with fiber diameters that range from 0.4–1.2 microns and conduction velocities from 0.5–2 m/s. In humans, the mean unmyelinated fiber density is approximately 30000/mm^2 [522,523].

The endoneurium also includes subperineurial Renaut bodies. These are large pale structures, highly variable in number and distribution, that consist of oxytalin fibril connective tissue, components of elastic fibers. Renaut bodies are thought to arise from perineurial cells.

The transitional zone (TZ) refers to the connection between the peripheral and central nervous system [43]. It may include protrusions of CNS material into a PNS root, or insertions of PNS material into the CNS. Astrocytic processes separate axons as they enter through the glial limitans, the outer border of the CNS and basement membranes add barrier properties to segregate the endoneurium from the CNS.

Axon ultrastructure

The intact mature peripheral nerve axon is constructed of a scaffold of proteins consisting of microfilaments, neurofilament intermediate proteins, and microtubules all surrounded by an axolemma (Figure 2.9). Microfilaments are expressed in the subaxolemmal cytoskelton, whereas neurofilaments and microtubules are distributed throughout the axon. Peripheral neurons express five major types of intermediate filament proteins: three neurofilament subunits, peripherin, and α-internexin (for review see [375]). The axolemma is a three-layered cell membrane about 8 nm thick that is anchored to the subjacent axoplasmic cortex by molecules such as ankyrin, fodrin, actin, and A-60 [43]. It therefore also houses several organelles that include mitochondria and polyribosomes but does not include Golgi organs or rough endoplasmic reticulum [43] (van Minnen, personal communication). Mitochondria, identified as flattened tube-like double membrane structures (0.1–0.3 by 0.5–1.0 microns in size), are transported anterogradely and retrogradely and their density is higher in smaller caliber axons. The axoplasmic reticulum is a fine meshwork of interconnected tubules that connect proximally to rough endplasmic reticulum and Golgi organs in the perikaryon or cell body. Dense lamellar bodies and multivesicular bodies are thought to represent lysosomes and residual bodies whereas vesiculotulular profiles likely represent vesicles undergoing rapid anterograde axoplasmic transport. Membranous cisterns can also be identified in axons and they include endosomes.

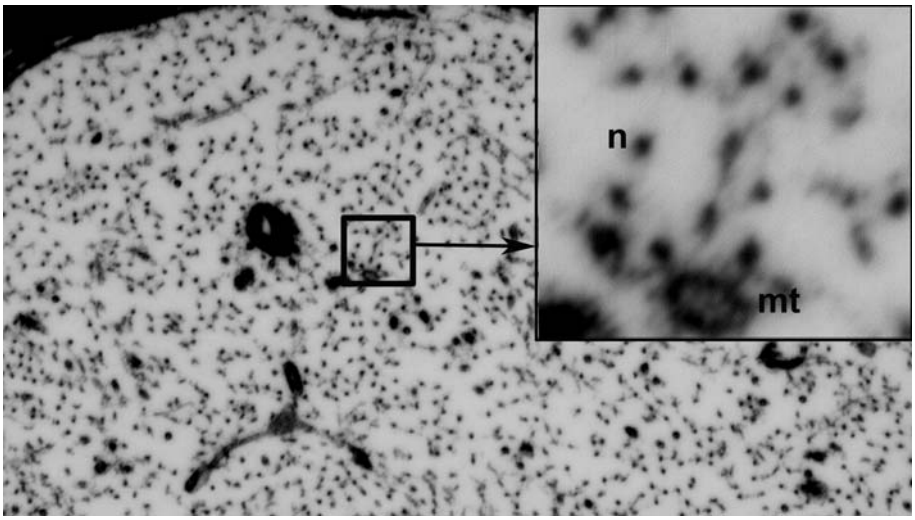

Figure 2.9 An electron micrograph of a myelinated axon illustrating its ultrastructure. The enlarged box identifies neurofilament (n) profiles and a microtubule (mt). Note the faintly resolved sidearms extending from the neurofilaments. The larger structure to the left of the box is a mitochondrion.

Neurofilaments are class IV intermediate filaments. They are stable polymers, 10nM in diameter, that contain three subunit proteins, termed light (68kD, NfL), medium (145kD, NfM), and heavy (200kD, NfH). In transverse sections, neurofilaments are spaced regularly and they provide a scaffold or skeleton that gives the axon caliber or bulk. Each neurofilament subunit consists of a globular "head" region, an α-helical "rod" region, and a globular C-terminal "tail" extension. NfM and NfH have sidearm extensions from their tail zones that help to determine their spacing. Thus, the separation of the polymer from its neighbor influences the overall size of the nerve. Sidearms from NfH and NfM tail regions contain KSP repeat sequences that are subject to phosphorylation, a modification that influences neurofilament spacing and thereby axonal caliber. Moreover, neurofilament polymers in the cell body are relatively poorly phosphorylated, whereas axonal neurofilaments are heavily phosphorylated, a process that occurs after the protein or its subunits are transported from the cell body. At nodes of Ranvier, alterations in neurofilament phosphorylation likely account for the closer spacing and axonal narrowing that normally occurs (Figures 2.7, 2.9). Neurofilaments are also subject to glycosylation [456].

Several animal models have been studied that lack all neurofilaments or one or more neurofilament subunits. Mice generated by Eyer and Peterson [176] completely lacked axonal neurofilaments, but survived normally, and had axon myelination. The model was discovered by serendipity as a result of replacing the

carboxyl terminus of NfH protein with β galactosidase; the fusion protein was sequestrated in perikaryal precipitates as large filamentous aggregates without export of neurofilaments into the axons. Their axons, devoid of neurofilaments, were reduced in caliber but did not degenerate. Microtubules and other axoplasmic contents instead provided the internal lattice-work required to maintain structural integrity. Similar atrophic axons were encountered in neurofilament-deficient quails with a null mutation of NfL [757].

Neurofilaments can be resolved at the ultrastructural level by electron microscopy. Individual triplet polymers are identified as dots regularly spaced through the transverse area of the axon with small sidearms (Figure 2.9). Large myelinated axons may contain hundreds of individual neurofilament profiles and, while small myelinated and unmyelinated axons normally contain some neurofilament profiles, their overall number is much smaller. By immunohistochemistry, using neurofilament antibodies, their expression may be minimal, but it is incorrect to label these axons as lacking neurofilament. The term "neurofilament poor" or "neurofilament lacking," when referring to smaller caliber neurons or axons is therefore technically incorrect.

Prior to the use of immunohistochemical methods, axons were regularly labeled with metal stains (e.g., Bielschowsky or Bodian silver-based stains) that had affinity to neurofilament. When carried out by an experienced technician, a silver-impregnated axon profile can be beautifully resolved. Staining of collagen and other nerve constituents, however, can sometimes make their interpretation difficult.

Peripherin is a Class IIIF intermediate filament found in small sensory and autonomic neurons. The role of α-internexin is less well established. Its mRNA is only expressed at low levels in intact motor neurons [454] and it may not be required for the growth in caliber of axons [387].

Interestingly, neither loss of any of the Nf subunits, peripherin, nor α-internexin is associated with progressive motor neuron degeneration. Most contribute to the radial caliber of axons and there is axon atrophy when they are knocked out but none are essential for axon outgrowth [375]. Mice lacking NF-L or NF-M do have fewer numbers of axons. Alternatively, mice with overexpression or mutations of some intermediate filaments, such as peripherin, may have alterations in stoichiometry that do lead to a progressive degenerative phenotype [375].

Microtubules consist of polymerized tubulin subunits recognized ultrastructurally as 20–26 nM diameter circular profiles (Figure 2.9). They are oriented longitudinally along the axon and, as the axon diameter decreases, their packing density increases. This contrasts with the decline in neurofilament numbers as the axon radial diameter decreases. Most microtubules have approximately 13 subunits in transverse section with a clear center sometimes including

a small central dot. Two tubulin subunits, termed α and β, each approximately 50 kD in size, form a dimer in the soluble form, but link in the polymer to form a spiral lattice of subunits. Subtypes of tubulin subunits are found in nerve. Mice, for example, express α1, αII and βII, βIII, and βIVa [367]. Nerve motor and sensory axons transport βII and βIII tubulin and incorporate them into microtubules [205]. α1 tubulin is also incorporated into regenerating axons [472]. The use of antibodies to βIII tubulin offers a sensitive probe to detect axons *in vitro* or *in vivo*.

Polymerization, or addition of tubulin subunits occurs at the "+" end, or distal end, of the microtubule and depolymerization or removal of subunits occurs at its "−" end. Microtubular assembly, in turn, is facilitated by microtubule-associated proteins (MAPs). In ultrastructure, MAPs can be resolved as "fuzzy"-appearing extensions from the microtubule. Microtubules associate with the MAP kinesin, a "motor" to form the machinery for rapid anterograde axoplasmic transport. Anterograde axoplasmic transport involves movement of vesicles and approximates 400 mm/day. Retrograde axoplasmic transport is approximately 200–300 mm/day and its associated MAP motor is dynein. Slow axoplasmic transport does not rely on microtubules and is timed at approximately 0.2–2.5 mm/day. Slow transport is further divided into types a and b and it transports structural proteins like neurofilaments and tubulin. Other MAPs are considered in Chapter 5 in relationship to growth cone dynamics.

Ribosomal periaxoplasmic plaque domains, identified in rabbit and rat lumbar spinal nerves, are restricted ribosomal domains that may account for localized protein synthesis in axons [352]. These are narrow (2 micron) elongated (10 micron) sites randomly distributed and found variably around the periphery of the axoplasm near the axon–myelin border.

Schwann cell ultrastructure

SCs are recognized *in vivo* at the ultrastructural level by their close association with axons and their near invariable investment of basement membrane. By electron microscopy, their nucleus is described as pale without nuclear condensations or irregularities. Most have pale cytoplasm with a minority (less than 5%) having dark cytoplasm [477] and most do not have prominent organelles. Reich granules are lamellar, one micron long, metachromatic cytoplasmic inclusions, likely lysosomal and are found only in SCs associated with myelinated axons [43]. The basement membrane of SCs is easily recognized under the EM as irregular "fuzzy" material just outside of the plasmalemma. It can be divided into two layers known as lamina densa (dense region) and an inner lamina lucida (clear space) [43] and it is composed of collagen Type IV, laminin, and fibronectin. Single SCs associated with myelinated axons are distinct units.

SCs associated with unmyelinated axons normally ensheath several in a Remak bundle, as previously discussed. A single Remak SC invagination is usually associated with only one, or at most two unmyelinated axons.

Myelin sheaths

Myelin sheaths consist of lipid membrane material, synthesized by SCs in the PNS and oligodendrocytes in the CNS. PNS myelination involves the spiraling of SC membranes around axons to form sheaths of between 60 and 120 layers, or lamellae. The number of lamellae is a function of the size of the axon. The G ratio is the ratio of the axon (alone) diameter to that of the myelinated fiber diameter (including myelin). It is 0.65 in a normal human sural nerve and is independent of axon size. Thus, larger caliber axons have more myelin lamellae and an overall thicker myelin sheath than smaller axons in keeping with a normal G ratio. Axons with thin myelin during remyelination or regeneration have a larger G ratio, approaching 1.0.

When myelin is formed from spiral loops of SCs, the cytoplasm is largely squeezed out between their membrane surfaces. This results in distinct lines within each loop of myelin. The intraperiod line is formed from the apposition of the extracellular surfaces of the SC membrane prior to compaction. The major dense line represents the apposition of their cytoplasmic surfaces. In unfixed tissue, the periodicity of myelin in approximately 18 nm [43]. The inner mesaxon is the portion of retained SC cytoplasm and membranes attached to the myelin sheath found immediately adjacent to the axon. It is separated from the axon proper by the periaxonal space. The outer mesaxon is that portion of the SC cytoplasm and membrane contacting the outer end of the myelin sheath. Schmidt–Lanterman clefts or incisures are separations of the compact myelin lamellae by SC cytoplasm that form elegant spiral connections between the inside and outside of the myelin. They are thought to represent portals for transport between the inner adaxonal SC cytoplasm and the outer abaxonal SC cytoplasm.

The node of Ranvier is a highly specialized structure that constitutes 2%–3% of the length of an internode. The axon is constricted in diameter at nodes of Ranvier with reductions in transverse area of 75%–90% and rises in neurofilament packing density [43] (Figure 2.7). The borders of the node are demarcated by bulbous processes of SC cytoplasm rich in mitochondria, so-called paranodal bulbs that form nodal “collars” [43]. The SC processes are formed from the abaxonal cytoplasm and are associated with basement membranes. Nodal collars send microvilli, forming a brush border within the node. The node itself contains matrix material, one component of which, a chondroitin sulphate proteoglycan

(CSPG; see C10), may serve to inhibit sprouting in stable nonregenerating axons [43]. Paranodes are the segments of the myelinated axon just outside the node of Ranvier. Paranodes thus contain loops of the SC known as terminal cytoplasmic spirals that attach to the axolemma with septate-like junctions [43]. The terminal SC spirals and associated paranodal axon are known as the glial–axon junction. The juxtaparanode is found lateral to each paranodal segment. An additional term used is paranode–node–paranode (PNP) referring to node and both of its neighboring paranodal segments. There is a complex and intimate association between the axon and adaxonal SC in the PNP region with interdigitating processes known as the "axon–SC network" [43].

Overall, myelin is about 70% lipid but also contains an important complement of protein, comprising the remaining 30% [209]. The lipid contains cholelsterol and glycosphingolipids, whereas proteins of the myelin sheath in peripheral nerves have strict localization [661]. P0 (MPZ), the most common myelin protein, is an adhesion molecule that plays a role during developmental myelin compaction and persists in mature compact myelin. PMP 22 (peripheral myelin protein 22) is a membrane protein found in compact myelin. It has complex roles that not only influence myelination but also cellular survival and proliferation. The PMP 22 gene is abnormal in Charcot–Marie–Tooth disease (CMT) Type 1a, an inherited demyelinating polyneuropathy. MBP (myelin basic protein, also called P1) is a cytoplasmic, membrane-associated protein in compact myelin that also aids in compaction during development. P2 protein is found in the major dense line of compact myelin. MAG (myelin-associated glycoprotein) is an adhesion membrane molecule found in noncompacted myelin that helps to maintain myelin and axon integrity. It is also a ligand of the Nogo receptor that collapses growth cones and inhibits regeneration. Cx32 (connexin 32) is a tunnel protein of gap junctions found in noncompacted myelin that is involved in intercellular communication. E-cadherin is a membrane adhesion molecule found in adherens junctions of noncompacted myelin in the mesaxons. Neurofascin 155 is a membrane adhesion molecule of SCs found on the adaxonal side (facing the axon) of paranodal loops. TAG-1 is a SC adhesion molecule found in juxtaparanodal myelin. MAL (myelin and lymphocyte protein) is a tetraspan proteolipid found in compact myelin and with unmyelinated fibers. Periaxin is a SC cytoskeletal-associated protein found on the abaxonal compartment (facing the extracellular matrix) of myelinated fibers.

Molecular structure of myelinated axons

Axonal and SC proteins comprise a complex and highly structured pattern in the myelinated fibers of the peripheral nervous system. A variety

of inherited (e.g., CMT) and acquired (e.g., autoimmune neuropathy with autoantibodies directed against MAG) polyneuropathies involve alterations of proteins required for axon–SC interaction. By disrupting a single element of the interaction, serious consequences (demyelination, axon damage) for the peripheral nerve ensue.

The node of Ranvier is the excitatory nexus of the myelinated peripheral nerve. To convey action potentials through saltatory conduction (not reviewed here), these nodes have a concentration of membrane spanning sodium channels. Specific subtypes of Na^+ channels identified at mammalian nodes of Ranvier are Na_v 1.6, the commonest subtype but also Na_v 1.2, Na_v 1.8 (SNS/PN3), and Na_v 1.9 (PN1). The latter two channels are classified as tetrodotoxin (TTX) resistant because their currents are not blocked by TTX. Na_v 1.7 is present in both sensory and sympathetic neurons [748]. Na_v 1.3 (Type III) is a channel that is re-expressed after injury. All of the subtypes are voltage gated and share a 24-membrane spanning architecture through four distinct α subunits. β subunits are auxiliary components. $Ankyrin_G$ proteins anchor the sodium channels at nodes and interact with the cytoplasmic proteins neurofascin, Nr-CAM, and spectrin. The paranode axonal membrane contains heterodimers of contactin and Caspr proteins and the associated SC membrane contains neurofascin 155, all of which are components of the septate-like junctions mentioned above [604].

Further lateral to the paranode is the juxtaparanode, an area that contains potassium channels, especially Kv1.1 and Kv1.2 linked to Caspr2. The Kv potassium channels, hidden beneath layers of myelin, likely act as rectifiers that can dampen axon excitability. Kv1.5 channels are also found on SCs, in their abaxonal portion where they may serve a buffering function during axonal conduction. Disruption of the normal paranode and juxtaparanode may inappropriately expose axonal channels and interfere with normal myelinated axon conduction. The internode of the axon, located between nodes or more properly between the flanking juxtaparanodes, contains fewer proteins. As may be evident, the complex molecular anatomy of the node of Ranvier is critically important to axon excitability and impulse transmission and to axon–SC interactions. Readers are invited to detailed depictions of the node found elsewhere [23,604].

Motor neurons and the anterior horn of the spinal cord

Motor neurons consist of two populations: larger caliber myelinated α motor axons that signal voluntary control of skeletal or striated muscles and smaller caliber myelinated γ motor axons that innervate muscle spindles (intrafusal muscle fibers). Alpha motor axons have conduction velocities of 40 m/s or higher. By definition, motor neuron perikarya are placed within the Rexed

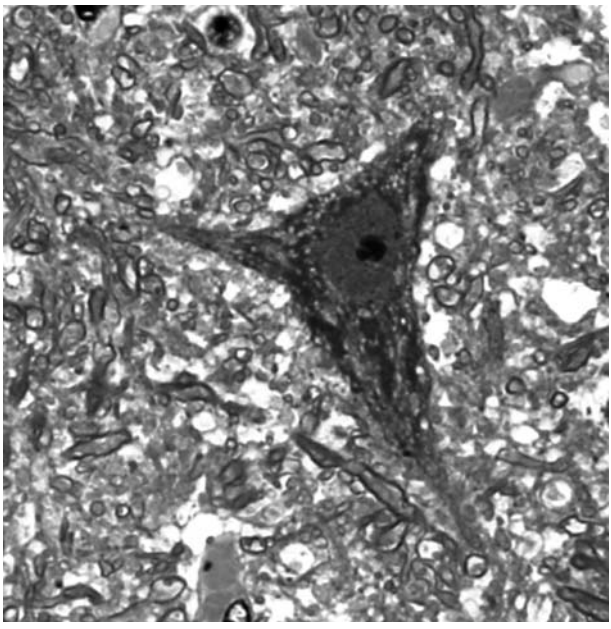

Figure 2.10 An illustration of an anterior horn α motor neuron. Note its triangular shape and its content of dark Nissl bodies. The oil immersion image is from a semithin section, stained with toluidine blue from a mouse spinal cord. (Image taken by Noor Ramji, Zochodne laboratory.)

lamina (laminae that define the cytoarchitecture of the spinal cord) X that is found in the anterior or ventral horn of the gray matter of the spinal cord. Within this lamina, they are intermixed with numerous smaller interneurons. Motor neurons are large, polygonal and contain abundant rough endoplasmic reticululm with extensive dendritic arbours in the the gray matter of their ventral horn (Figure 2.10). Motor neurons innervating appendicular, or limb muscles are found more laterally in the anterior horn, whereas those innervating axial or trunk muscles occupy more medial portions. Since more motor neurons are present in the cervical and lumbar regions of the spinal cord to supply the upper and lower limbs, respectively, there are cervical and lumbar enlargements at these levels. From the anterior horns, axons exit the spinal cord through the ventral roots, then join the dorsal sensory roots to form the mixed spinal nerve. Autonomic axons from the intermediolateral cell columns also exit the spinal cord through the ventral roots.

A motor unit refers to a single motor neuron that has branches to a number of individual muscle fibers. The size of the motor unit can vary substantially among different muscles but once activated, all of its muscle fibers normally fire synchronously. The physiological properties of the muscle fibers within a given

motor unit are dictated by the properties of the axon that innervate them. In some muscles, such as extraocular muscles, motor units may be small innervating only five muscle fibers (also known as their innervation ratio). In others they are large and may innervate 2000 or more muscle fibers, as in the human gastrocnemius muscle [145]. The size of the motor unit has a direct bearing on the precision required of the muscle it innervates. For example, a large limb muscle is required to power activities such as walking that requires synchronized firing of large numbers of muscle fibers. Their motor units are therefore larger. In the eye, however, very small precise movements are required to maintain fixation of the fovea on a visual target. Fine gradations of control and smaller motor units are required.

Sensory neurons and ganglia

Sensory neuron cell bodies, or perikarya, reside in cranial and spinal ganglia. When identified in humans at surgery or autopsy, ganglia are observed in close relationship with nerve roots as an enlargement associated with fat tissue. A distinct pedicle composed of entering and exiting axons can sometimes be identified (Figure 2.11). Ganglia associated with lumbar and cervical roots are larger and contain more neurons than those in the thoracic spine. Surrounded by a specialized capsule, large and small neurons are freely intermixed and each is surrounded by perineuronal satellite cells. Satellite cells have little cytoplasm and their flattened nuclei closely appose neurons. Neuron perikarya and their satellite cells characteristically line the cortex of the ganglia just beneath the capsule, whereas the center of the ganglion includes some neurons but is particularly invested by axons and their associated SCs. Blood vessels supplying dorsal root ganglia arise from adjacent radicular (root) branches supplying the nerve roots and spinal cord and indirectly by connections from the anterior spinal artery [3]. These vessels supply local intrinsic blood flow to ganglia that is higher (30–40 ml/100 g per min) than that of the peripheral nerve trunk and has some degree of autoregulation [792] (see Chapter 7). The blood–ganglion barrier is also less robust than that of the nerve [24]. Thus, proteins access ganglia much more easily than nerves, a feature that may have important implications for neuropathies related to blood borne antibodies and toxins.

Like their axons in the peripheral nerve trunk, neurons in DRGs are distributed into two major size categories. Classically, these are divided into large light neurons with Nissl substance (rough endoplasmic reticulum) separated by neurofilaments and small dark neurons with more concentrated Nissl substance and a lower content of neurofilaments (Figure 2.12). Neurofilaments in the cell body or perikaryon similarly form a lattice network that may anchor the nucleus

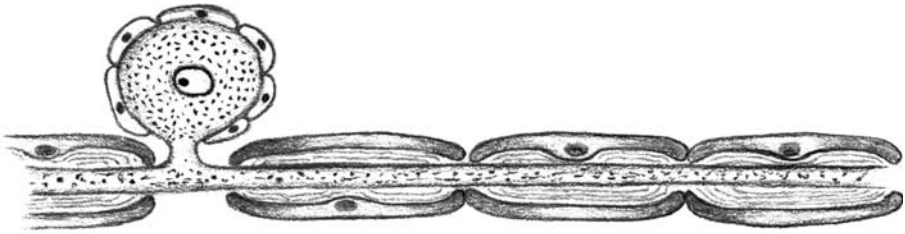

Figure 2.11 An illustration of a primary sensory neuron that resides in a dorsal root ganglion. The neuron is described as "pseudounipolar" with a single branch from the perikaryon (cell body) that then divides into central and peripheral branches. The perikaryon is surrounded by closely apposed perineuronal satellite cells. (Illustration by Scott Rogers.) See color plate section.

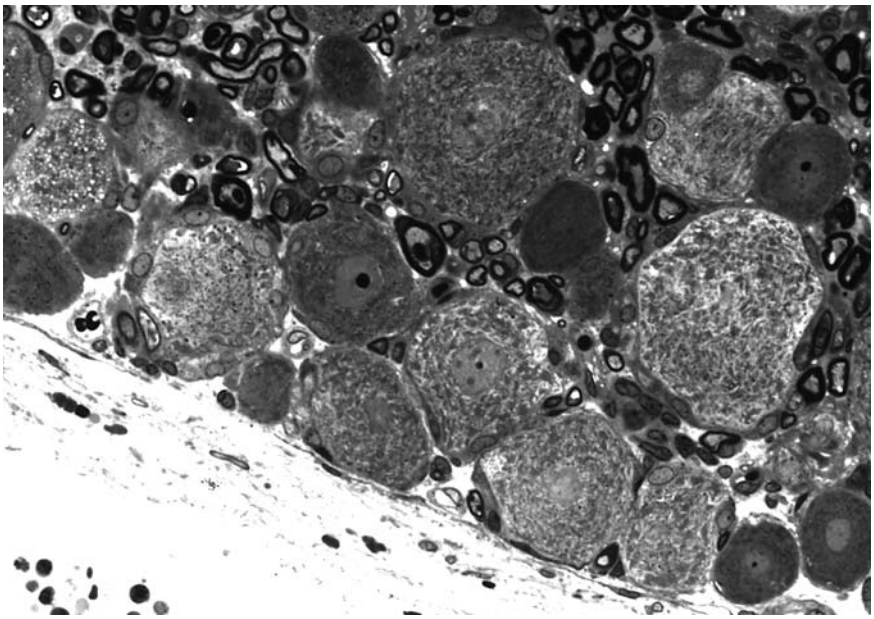

Figure 2.12 A photomicrograph of a portion of a lumbar dorsal root ganglion (DRG) illustrating peripherally placed large and small sensory neurons and more centrally placed myelinated axons. The image is a semithin section from a rat L5 DRG stained with toluidine blue.

into a central position within the cell. Thus when staining DRG neurons by neurofilament immunohistochemistry, the larger neurons may be preferentially stained because of their higher neurofilament content (Figure 2.13). The size of DRG neurons range from 10–100 microns in diameter or a cross-sectional area of under 500μm^2 for small neurons of unmyelinated fibers or Aδ fibers and above this for Aαβ fibers [379].

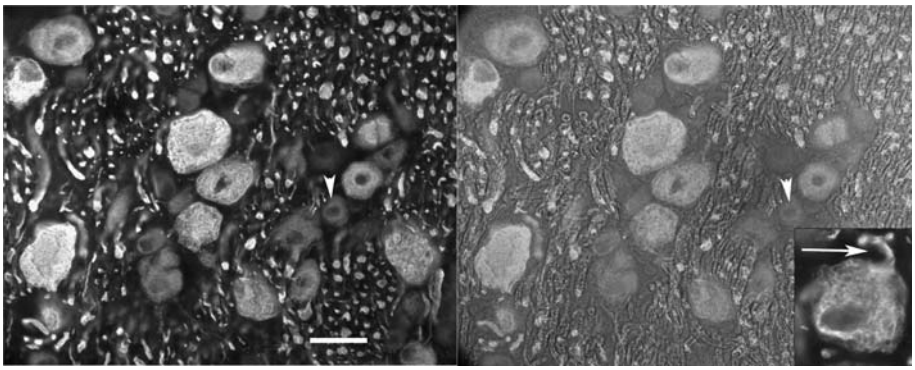

Figure 2.13 An image of DRG sensory neurons labeled by immunohistochemistry with an antibody directed against the heavy subunit of neurofilament (red). The arrowhead points to a smaller neuron with less prominent neurofilament staining (sometimes erroneously called "neurofilament negative"). The right (green) panel is the same section with transmitted light. The inset shows a single large neuron with a "unipolar" process (arrow) emerging from the perikaryon. Note also that the neurofilament label shows a patchy and complex network within the perikaryon that is thought to determine overall neuronal shape (Bar = 20 microns). (Image taken by Chu Cheng, Zochodne laboratory.) See color plate section.

The types of sensory neurons that populate dorsal root ganglia can be classified in several further ways (see review [379]). Some *peptides* are distributed selectively to small neurons associated with C and Aδ axons: ET1 (endothelin-1), galanin, nociceptin, somatostatin, SP (substance P), PACAP (pituitary adenylate cyclase-activating polypeptide), VIP (vasoactive intestinal polypeptide); or to small- and medium-sized neurons: CGRP (calcitonin gene-related peptide) (Figure 2.14). *Receptors* expressed by small neurons include: GLUR1 (glutamate receptor), GLUR5, AMPA, Kainate, B1 (bradykinin receptor), B2, H1 (histamine receptor), EGF receptor (epidermal growth factor receptor), FGF2 (fibroblast growth factor 2 receptor), NK1 (neurokinin-1 receptor), SSTR2a (somatostatin receptor), P2X3 (purinergic receptor), GAL2 (galanin receptor), ETA (endothelin A receptor), GFRα3 (GDNF family receptor); by small and medium neurons: TrkA, p75, GFRα1 (GDNF family receptor), GFRα2, RET (GDNF family coreceptor), MOR (μ opioid receptor), DOR (δ opioid receptor), KOR (κ opioid receptor), ORL1 (opioid-like receptor 1); by large neurons: TRPV2 (transient receptor potential family), CB1 (cannabanoid 1 receptor), GAL1, GLUR2/3, GM1 (GM1 ganglioside receptor), P2Y1 (purinergic receptor), TrkC. *Ion channels* expressed by small neurons include: BK (bradykinin), KCa (calcium-activated potassium channel), L and N type Ca^{2+} channels (calcium channels), Kv1.4 (potassium channel), Nav1.9 (sodium channel), Naβ3, TRPA1, TRPM8, TRPV1, TRPV4, TRPV3; by small and medium neurons: Nav1.7, 1.8,

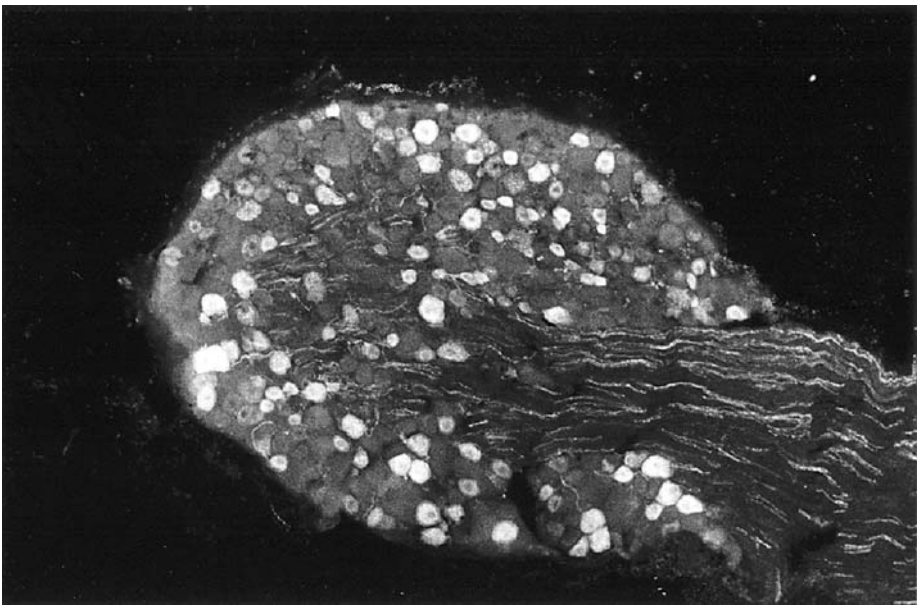

Figure 2.14 An image of a rat lumbar DRG labeled by immunohistochemistry with an antibody directed against the peptide CGRP. Approximately 45% of DRG sensory neurons are medium- and small-sized CGRP positive. Note the emerging axons from the DRG also exhibit CGRP immunoreactivity. (Image taken by Chu Cheng, Zochodne laboratory.)

P2X2/3; by large neurons: Naβ1.1, 2,1, Kv1.1, 1,2, Kvβ2.1, HCN1,2 (hyperpolarization-activated cyclic-nucleotide gated, time- and voltage-dependent nonselective cation channel). This list is not complete, however, as there are cytokines and enzymes also distributed differently among these neurons. Similarly, they can be divided into their sensory target receptive properties and their projections.

The expression of *high affinity receptors for neurotrophin growth factors* [325] is particularly relevant here. Neurons expressing TrkA, the receptor for NGF are preferentially found on small caliber neurons that subserve nociception. These receptors are found on 41% of lumbar DRG neurons. TrkB, the cognate receptor for the BDNF neurotrophin family member is found on 33%, and TrkC, the receptor for NT-3 on 43%. TrkB is found on small- to medium-sized neurons and Trk C on large neurons. P75, formerly known as the low affinity neurotrophin receptor, and capable of binding all family members, is found in 79% of DRG neurons. There is, however, extensive colocalization of Trk receptors and trilocalization in a small percentage. Non Trk bearing neurons are the IB-4 population that express the GDNF receptors GFRα-1 and RET and express the

P2X3 purinoreceptors (ATP sensitive) [59]. The interaction of growth factors with peripheral neurons is addressed in Chapter 9.

Autonomic, enteric ganglia

Peripheral autonomic neurons consist of sympathetic and parasympathetic neurons. The size of a given autonomic ganglion depends on the extent of its target tissue. Sympathetic ganglia are found as chain spinal (also called paravertebral) ganglia or along nerves called satellite ganglia. In humans, the paravertebral ganglia extend from C8 to S5 bilaterally along the spinal column. The ganglia receive preganglionic axons from the spinal cord (also known as white rami communicans (C8-L2, myelinated)) that are branches from the ventral roots. Postganglionic axons, unmyelinated, leave the ganglia as gray rami communicans and rejoin mixed spinal nerves. They eventually innervate blood vessels, sweat glands, pilomotor fibers, or join plexi that innervate most internal organs. The ganglia below L2 do not receive white rami communicans but receive preganglionic fibers from higher levels and send out gray rami. The sacral chains are fused. The C8 and T1,2 roots send preganglionic fibers up the sympathetic chain along the carotid artery to supply the stellate (inferior cervical), middle, and superior cervical ganglia. Postganglionic axons either travel further along the chain or branch off along the carotid arteries as they bifurcate and they supply the head and neck. Other preganglionic axons from lower levels join prevertebral ganglia known as the celiac, superior mesenteric, and inferior mesenteric ganglia.

The parasympathetic fibers are craniosacral in their distribution in humans. This means that they originate in cranial nuclei: third cranial nerve (Edinger–Westphal nucleus), sending preganglionic axons to the ciliary ganglion that, in turn, supply postganglionic axons to the eye; seventh cranial nerve that sends pregangionic axons to the sphenopalatine and submandibular ganglia to supply tearing and salivation; the ninth cranial nerve supplying preganglionic axons to the otic ganglia; the vagus, or tenth cranial nerve sending long preganglionic axons to the pulmonary tree, heart, liver, stomach, intestines, and kidney into ganglia associated with these organs that then project small postganglionic axons to them. The parasympathetic fibers also originate from S2, 3, and 4 sending preganglionic axons in the pelvic splanchnic nerves to supply ganglia associated with the colon and urogenital systems.

Autonomic ganglion neurons have a similar size distribution as sensory neurons, but they are multipolar instead with a number of dendrites and a single axon. Their intertwining dendrites can form “dendritic glomeruli or nests” [363] forming a complex anatomy that includes both axodendritic and

axosomatic synapses. In addition, some sensory neurons are found in autonomic ganglia. Sympathetic neurons express the peptides NPY (neuropeptide Y), somatostatin, VIP, and enkephalin. In parasympathetic ganglia VIP and CGRP are identified. Most sympathetic ganglion neurons are noradrenergic and contain tyrosine hydroxylase (Th, a catecholamine synthesizing enzyme). Most parasympathetic ganglion neurons are cholinergic, and contain choline acetyltransferase (ChAT, an acetylcholine synthesizing enzyme). While this arrangement exists for most sympathetic axons, some that project to the head and neck vessels or to sweat glands of the skin instead are cholinergic. Unmyelinated postganglionic autonomic neurons contact their endorgans through "neuroeffector junctions." Unlike classical synapses, these junctions lack presynaptic concentrations of neurotransmitters, have wider and more variable clefts, and do not have a classical postsynaptic structure.

The enteric nervous system consists of afferent, interneuronal, and motor axons and extends from the pharyngoeseophageal junction to the internal anal spincter in man. It includes the myenteric (Auerbach's) plexus that is largely motor in function, found between the longitudinal and circular smooth muscle layers of the intestine, and the submucous (Meissner's) plexus that has a role in regulation of secretion in the submucosal layer. The enteric nervous system thus has close interactions with sensory, sympathetic, and parasympathetic neurons (see review [616]).

Innervation of the skin

The innervation of the skin involves a system of overlapping sensory transducers placed at varying depths within the epidermis and dermis. Sensations of pressure, light touch, vibration, pain (or nociception), heat, and cold are transmitted to nerves, the spinal cord, and the brain. Pressure and light touch might be more rigorously classified as "cutaneous displacement" [741]. Slowly adapting (SA) units include type SAI that consists of myelinated axons innervating Merkel touch complexes. Merkel cells are recognized by their indented nuclei and cytoplasmic granules and can form into dome-like structures [33]. They are found both in the basal layer of the epidermis of both hairy and hairless (glabrous) skin [152] whereas in rodents, they are also associated with whisker vibrissae [152]. They have small receptor fields. SAII innervate Ruffini endbulbs deeper in the dermis and have wider receptive fields. These structures measure 1 mm by 30 microns and consist of a branched array of terminal myelinated axon branches [33]. Cutaneous velocity detecting units are, in contrast, rapidly adapting and include receptors known as G2 hair follicle and T hair follicle receptors, D hair follicle receptors, F field receptors, and C mechanoreptors.

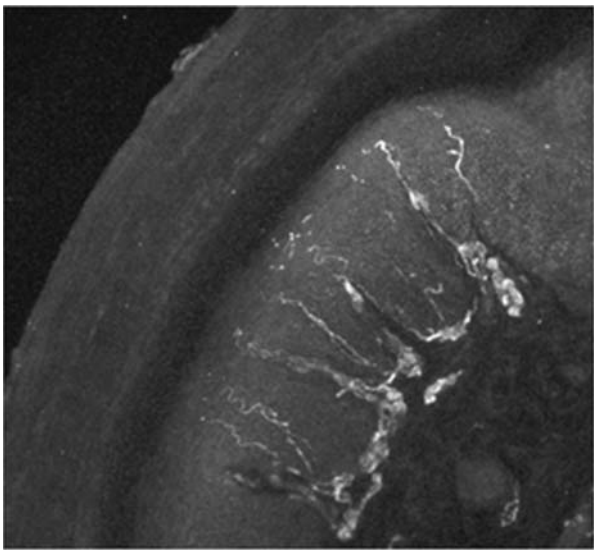

Figure 2.15 An image of the skin of a normal mouse footpad labeled by immunohistochemistry with an antibody directed against the axon marker PGP 9.5 (green). Note the dense ramification of tortuous axons up from the dermal plexus into the epidermis. The epidermis is also covered by thick callus. (Image taken by James Kennedy, Zochodne laboratory and reproduced with permission from [335].) See color plate section.

For example, G2 and T receptors respond to the velocity of hair movements, D are more sensitive to slight movements in smaller, down hairs, and F, field receptors, require movements of groups of hairs. C unmyelinated skin indentation receptors respond to the velocity of slow skin movements. In glabrous (nonhairy) skin, the rapidly adapting (FAI; fast adapting I) receptor with a small receptor field is the Meissner's corpuscle in the dermal ridges. Meissner's corpuscles are 80 by 30 microns in size, oriented perpendicular to the surface of the skin and are in the form of a corpuscle supplied by 3–4 myelinated axons in a knot infiltrated with SCs and a collagenous capsule [33]. There are also transient cutaneous detectors that include G1 hair follicle receptors and Pacinian corpuscles. Pacinian corpuscles are formed at the ending of myelinated fibers that are inserted into layers of flattened cells in an overall ellipsoidal shape of 1 mm by 0.7 mm [33]. Pacinian corpuscles are very sensitive to movement or vibration, especially in the range of 60–300 Hz and are found deeper in the dermis to provide a wide receptor field. Some of these receptors generate ongoing activity at ''rest.''

Free nerve endings of unmyelinated C fibers that end in the epidermis and Aδ fibers in the dermis convey pain (nociception) and thermal sensation (Figure 2.15). Overall, nociceptors have been classified as mechanical nociceptors

(Aδ fibers), polymodal (sensitive to several modes of pain) nociceptors (C fibers), other mechanical nociceptors (C), mechanoheat nociceptors (Aδ), and cold nociceptors (Aδ or C fibers). Chemoreceptors may overlap with these groups. There are also thought to be "silent" nociceptors only activated by inflammation and injury. The biology of sensory transduction, however, has progressed well beyond these classical concepts and the classifications do vary depending on the reference (see review by [419]). I will mention just a few of the new molecules linked to sensory transduction. In particular, the TRP (transient receptor potential) molecules have assumed a central role in this evolving story. Heat transduction is mediated by TRPV1, the classical capsaicin receptor or vanilloid ("V" in TRPV) receptor and also by TRPV2, 3 and 4, depending on the intensity (temperature). In contrast, TRPM8 was discovered as a cold transducer, or menthol ("M" in TRPM) receptor. Another receptor TRPA1 also contributes to cold sensitivity, but it is also important for acute mechanical transduction. TRPN and DEG/ENaC channels may mediate touch and proprioceptive stimuli. It seems likely that cutaneous fibers may express more than one class of TRP in a single fiber indicating a multiplicity of function depending on the exact stimulus.

The skin is also innervated by sympathetic axons that innervate sweat glands (cholinergic) in the dermis and arrector pili muscles (adrenergic) of the dermis (that cause "goose flesh" when activated; also called pilomotor). Sweat glands are divided into eccrine glands, the most widely distributed types in skin, and apocrine glands that are associated with hair follicles. In humans apocrine glands are localized to axillae, perineum, and nipple areolae. Sweat glands are innervated by sympathetic cholinergic axons (receptors called M3). The gland includes a secretory coil where sweat is produced and secretory duct that expels sweat while reabsorbing NaCl. The entire apparatus is contained within a sweat gland capsule.

Innervation of muscle, joints, viscera

Muscles have a rich sensory nerve supply [741]. Muscle spindles are specialized fibers (also called intrafusal) that possess sensory organs supplied by large myelinated axons sensitive to dynamic movements (Group Ia) and by intermediate-sized myelinated axons sensitive to muscle length (static; Group II). Intrafusal muscles (spindles) are also classified into nuclear bag fibers (innervated by Group I primarily) and nuclear chain fibers (innervated by both Group I and II). These muscle fibers also receive input from specialized smaller caliber motor axons known as γ motor neurons. By controlling the activation of the intrafusal muscle fiber, γ motor axons can thus influence its tension (it will also be under stretch because it is connected in parallel with a large number of extrinsic, normal or "extrafusal" muscle fibers) and hence influence the sensitivity of

Table 2.1 *Cutaneous Innervation*[*]

Name	Location	Cutaneous field	Function
Mechanoreceptors			
SAI (Merkel)	Derm/epid jct	Small	Displacement
SAII (Ruffini)	Dermis	Medium-large	Displacement
FAI (Meissner's) and ?Krause endbulb	Dermis	Small	Velocity
Pacinian	Dermis, other areas	Large	Transients, vibration
C	Epidermis and dermis	Unknown	Velocity, displacement
Hair mechanoreceptors			
G1	Large hairs	Single follicle	Transients
G2,T	Large hairs	Single follicle	Velocity
D	Small down hairs	Single follicle	Velocity, low threshold
Field	Hair and ?skin	Multiple hairs, skin	Velocity, displacement
Nociceptors			
Aδ mechanical	Dermis/?epidermis	Small	Damage
C polymodal	Epidermis	Small	Damage, heat, chemical, other
C mechanical	Epidermis	Small	Damage
Aδ mechanoheat	Dermis/?epidermis	Small	Damage, heat
Aδ and C cold	Dermis/?epidermis	Small	Cold

Note:

[*]Adapted and simplified from [741].

the receptors. Golgi tendon organs (innervated by Group Ib afferents) are located in the connective tissue of muscle tendons and they are high threshold receptors to passive muscle stretch. Up to 75% of muscle innervation, however, is from free nerve endings, usually unmyelinated axons that are mainly nociceptive, or sometimes thermal sensitive. There are also Aδ (Group III) fibers in muscle that supply information about mechanical stimulation, pressure, chemical stimulation, stretch, and nociception.

Position sensibility is mediated by muscle and tendon stretch, but also by joint afferents. As in the skin, these include Ruffini slowly adapting joint receptors, transient-sensitive Pacinian corpuscles and joint Aδ and C nociceptors. In the viscera, there is innervation by Pacinian corpuscles in the mesentery and connective tissue, some mechanoreceptors and prominent nociceptors particularly involving the surface of viscera and mucosal linings.

Neuromuscular junction

Neuromuscular junctions are specialized synapses that connect motor axons to muscles. They consist of the motor terminal, the junctional cleft, and the postsynaptic muscle endplate, also known as the motor point (Figure 2.16). Each has a specialized structure. The motor axon terminals lose their myelin sheaths but become closely associated with specialized SCs known as presynaptic SCs. The terminals splay out and become filled with synaptic vesicles approximaly 50 nm in diameter, mitochondria and tubules of smooth endoplasmic reticulum. Finally, they form a synaptic gutter, an indentation into the muscle known as the primary fold. There is also loss of the blood–nerve barrier at this site.

Muscle fibers innervated by a given motor axon are not usually grouped together but intermingle with those of other motor units. The contractile properties of muscle fibers are dictated by the motor axon innervating them, i.e., slow sustained contraction oxidative (Type I) fibers compared with rapid fast twitch glycolytic (Type II) fibers. At the prejunctional terminal, there are P/Q-type voltage gated Ca^{2+} channels (VGCCs) that depolarize the terminal and promote release of acetylcholine from synaptic vesicles. Voltage-gated potassium channels

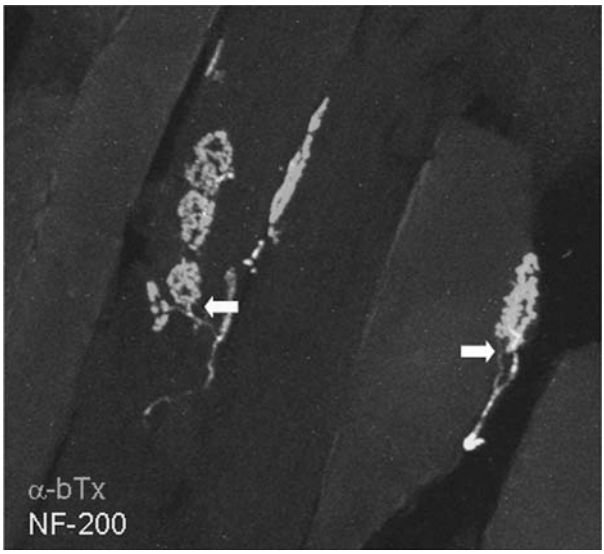

Figure 2.16 Images of neuromuscular junctions from the mouse hindlimb labeled by immunohistochemistry with an antibody directed against neurofilament (NF200, green) identifying terminal motor branches. The junction itself is labeled with α bungarotoxin (α-bTx, red) that binds the presynaptic terminals. (Image taken by Noor Ramji, Zochodne laboratory and reproduced with permission from [571].) See color plate section.

(VGKCs) help to restore the membrane potential of the presynaptic space after depolarization and calcium influx. Choline acetyltransferase (ChAT), the enzyme necessary to synthesize the neurotransmitter acetylcholine (Ach) is found in the presynaptic terminal.

The Ach vesicles interact with electron dense specialized zones of the axonal membrane that are called active zones. By ultrastructure, active release zones resemble a thickened bar associated with parallel arrays of synaptic vesicles that have a close association with the VGCCs. Spontaneous release of single quanta (packets or vesicles) of Ach generate discharges in the muscle endplate zone known as MEPPs, or miniature endplate potentials. An action potential in the presynaptic motor nerve causes influx of calcium, local depolarization, and massive vesicular release of Ach into the synaptic cleft. Ach then diffuses to the postjunctional complex. In the cleft it binds to postjunctional Ach receptors (AchR) or is degraded by acetylcholinesterase found with basement membrane in the cleft. In the muscle endplate, Ach binding of receptors (AchR) generates an endplate potential which, if above threshold, causes a muscle action potential. The neuromuscular junction is approximately 60–100 nm in width and it is lined by a basement membrane that closely follows the junctional folds. The postjunctional complex consists of a folded membrane complex (junctional folds or secondary folds) on the apices of which sit acetylcholine receptors. These consist of two α subunits and one each of δ, β, and γ subunits that form an ion pore. AchR are also closely associated with the proteins MuSK (muscle specific kinase) and rapsyn (receptor aggregating protein at the synapse). When ligated by Ach, the ion pore opens to allow entry of sodium, potassium, and other small ions with subsequent depolarization. Depolarization, in turn, turns on the actin–myosin contractile apparatus that is the basis of muscle contraction.

Summary

Peripheral neurons connect the central nervous system to muscles and sensory organs. In doing so, they traverse diverse territories and incorporate several types of structures. Far from being uniform, peripheral nerve trunks contain a mixture of myelinated motor axons, myelinated and unmyelinated sensory axons, and unmyelinated autonomic axons. Each originates in different parts of the nervous system: ventral horn of spinal cord for motor neurons, ganglia for sensory and autonomic neurons. Each also has separate destinations but while *en route* they share pathways in nerve trunks with unique anatomical and physiological characteristics. An appreciation of the diverse complexity of peripheral neurons is essential to understand how they respond to injury during regeneration.

Suggested reading

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3

Injuries to peripheral nerves

Peripheral nerve injuries are common, disabling and difficult to treat. These injuries arise because nerve trunk anatomical pathways expose them to a variety of injury types, including both blunt and penetrating lesions. The injury type, in turn, has a major bearing on how nerves respond. There are unique and challenging attributes of nerve trauma biology that set the stage for subsequent regenerative activity. These attributes are examined in this chapter.

Overall considerations

The type of injury experienced by a peripheral nerve determines how regeneration might proceed. Several classifications exist and are given here. The simplest classification is provided by Seddon, who grouped injuries into neurapraxia, axonotmesis, and neurotmesis lesions.

Neurapraxia is a lesion that occurs most often from blunt nerve trauma and involves focal demyelination over several internodes at the injury site. By definition, there is no associated axonal injury. Since neurapraxic lesions are identified by the loss of function associated with them, they require that enough internodes be disrupted to block conduction, or function. For example, a common type of neurapraxic lesion is the "Saturday night palsy" of the radial nerve in the arm that develops from prolonged compression of the inner arm by a chair or the head of a partner (and is often associated with alcohol intoxication!). Compression, however, need not be prolonged, since it is highly likely that the mechanical distortion of the nerve is responsible for demyelination. Ochoa and colleagues, in experimental work, described how myelinated axons "intussuscept" from undue mechanical stress [521]. Intussusception is the telescoping together of two hollow tubes, as most often described in infants, where

Figure 3.1 An image of a teased myelinated fiber illustrating segmental demyelination between two nodes of Ranvier (arrows). Demyelination accounts for neurapraxic nerve injuries. See color plate section.

a portion of small intestine is forced into a neighboring segment. For the injured nerve, one internode's myelin sheath is forced up and over the top of its neighbor. The distorted myelin disrupts salatatory conduction, unsheathes inhibitory potassium channels, and triggers myelin phagocytosis. Thus, following the injury, local SCs and resident macrophages, followed by ingressing circulating macrophages, are signaled to remove the abnormal intussuscepted myelin. Segmental demyelination, or loss of myelin strictly in segments between nodes of Ranvier then results (Figure 3.1); there may, of course, also be several adjacent demyelinated internodes. There is acute block of conduction over this segment with loss of distal function. In the case of "Saturday night palsy," the patient awakens with a severe and complete wrist drop from inability to extend the wrist and fingers, normally the work of the intact radial nerve. There is also sensory loss in the territory of the radial nerve. The adjacent SCs are not destroyed by the lesion and begin to proliferate and eventually remyelinate the demyelinated segment. Since several SCs may now be associated with an internode previously myelinated by one SC, the repaired internodes may be shorter in length than the original ones ("shortened internodes") [268,269]. While remyelination, albeit with shortened internodes, alleviates conduction block, there may be persistent long-term mild slowing of conduction velocity through the segment. With the passage of time, internodes likely remodel and probably lengthen back out towards their original proportions [2,268,269]. The precise mechanism by which SCs regulate internodal length is uncertain.

Axonotmesis is a lesion from a blunt mechanical, chemical, or ischemic lesion in which axons are interrupted but the connective tissues and epineurium of the nerve trunk are not (Figure 3.2). Therefore, anatomical continuity between the stump of the nerve proximal to the injury and the distal stump is maintained. Other classifications described below subdivide axonotmesis lesions depending on the extent of associated connective tissue damage. In axonotmesis injuries, it is likely that a very rapid axonal signal is generated by the trauma that initiates irreversible damage to the distal axon. Wallerian degeneration, discussed below, ensues.

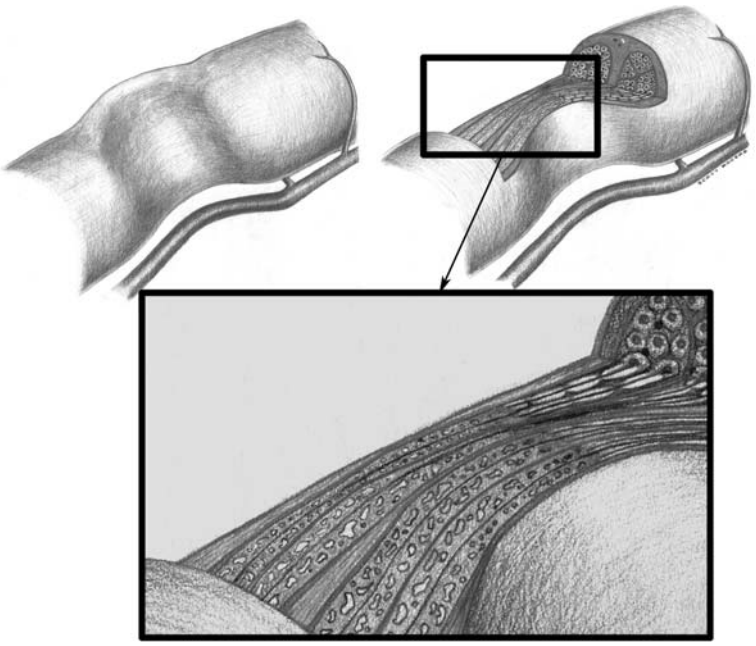

Figure 3.2 An illustration of early events following a peripheral nerve trunk crush (axonotmesis). Distal to the center of the crush zone, Wallerian-like degeneration begins with breakdown of myelin and axons. (Illustration by Scott Rogers.) See color plate section.

Neurotmesis refers to a lesion in which the entire nerve trunk including the connective tissues and epineurium is sundered, thus separating the distal from the proximal stump (Figure 3.3). This lesion develops from a penetrating injury, transection from a nearby fracture bony fragment, or from a surgical mishap. Its severity is heightened because the distal and proximal stumps of the nerve, already under some tension, commonly retract from one another. For regeneration to occur, these bridges must be reconnected by new connective tissue and bridging axons or by surgical apposition. At times, the stumps may retract to the extent that their ends are difficult to identify surgically, especially if there is other associated tissue injury. Associated tissue damage undoubtedly may also play a large role in the repair and regenerative process. For example, compartment syndromes can occur in injured limbs, following trauma in which tissue pressure, confined by fascial planes, rises higher than vascular perfusion pressure. Severe ischemia results. Penetrating injuries also pose a significant risk for superimposed infection, an important cause of failed healing. Surgeons repairing nerves therefore must attend to issues of vascular repair, limb stabilization, nerve decompression, and other critical measures.

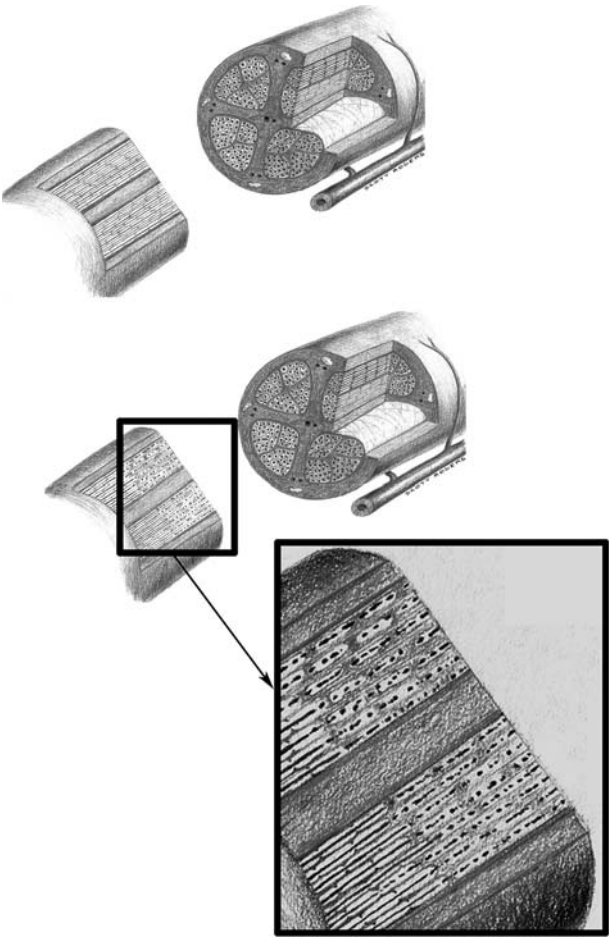

Figure 3.3 An illustration of early events following a peripheral nerve trunk transection (neurotmesis). In the distal stump, Wallerian degeneration begins with breakdown of myelin and axons. (Illustration by Scott Rogers.) See color plate section.

Other forms of nerve injury classification include that of Sunderland [660].

(i) First-degree injury, similar to neurapraxia above, is defined as an injury without structural interruption of the axon or subsequent Wallerian-like degeneration. The lesion is characterized by local demyelination. It is possible that temporary focal "stunning" or focal dysfunction of an axon without demyelination may account for a neurapraxic lesion but no direct evidence for this following trauma has been described. Temporary axon conduction block is described, however, after acute ischemia, discussed in Chapter 7 [314,543].

(ii) Second-degree injury is similar to axonotmesis above. The injury does not disrupt the basal lamina or endoneurial integrity but the axon is damaged and there is distal Wallerian-like degeneration. Recovery occurs through regeneration of axons.

(iii) Third-degree injury involves a loss of axon continuity and subsequent Wallerian-like degeneration but the perineurial sheath remains intact. The general arrangement of the fascicles is preserved but there is endoneurial disruption.

(iv) Fourth-degree injury involves a severe disruption of the peripheral nerve trunk with disorganization of its topography and loss of fascicular arrangement. The nerve trunk is in continuity because the epineurium is not separated.

(v) Fifth-degree injury involves a complete transection or separation of the entire nerve trunk, as described by neurotmesis above.

Mixed types of injuries and partial nerve injuries are common. In human peripheral nerve injuries, a combination of local demyelination and some axonal degeneration is usually the case. The prognosis for recovery depends on the relative proportions of each and they can be estimated with electrophysiological testing. For example, identifying conduction block across an injury segment, with retained excitability of the distal nerve segment identifies local demyelination, and a good prognosis for recovery commensurate with remyelination. If, however, loss of excitability of the distal nerve segment is identified, this indicates Wallerian-like degeneration beyond the injury site and recovery requires much slower axonal regeneration. Similarly, when motor axons undergo degeneration distal to an injury site, the muscle fibers they innervate develop abnormal spontaneous activity. This can be recorded by an electromyography needle electrode: spontaneous fibrillation potentials (abnormal spontaneous action potentials of single denervated muscle fibers) and related potentials termed positive sharp waves. Loss of membrane excitability of the axons in the distal nerve stump and the appearance of fibrillations in the denervated muscle, however, require time to emerge; longer if the lesion is more proximal.

Overall then, the loss of excitability and Wallerian-like degeneration following nerve trunk injury requires a suitable interval to begin (up to 10 days). The relative proportion of damaged axons can then be judged by the amplitude of the compound muscle or nerve action potential (CMAP) recorded distal to the injury site in reference to normal control values or to an intact contralateral nerve (see Chapter 4). Fibrillation potentials are recorded from muscle fibers that have lost their connecting axons. In contrast, distal CMAPs are normal in a demyelinating injury, but there may be complete block of conduction across the injury

site (normal CMAP distal to injury, absent or attenuated CMAP proximal to the injury). No axonal degeneration occurs and no fibrillations appear in the muscle despite paralysis. A detailed description of electrophysiological approaches appears in Chapter 4.

Waller, Wallerian degeneration and Wallerian-like degeneration

Augustus Waller [715] examined the sequence of pathological changes that ensue in peripheral nerves distal to a transection. This complex process is crucial for subsequent regeneration of axons. Waller writes:

> During the four first days, after section of the hypoglossal nerve, no change is observed in its structure. On the fifth day the tubes appear more varicose than usual and the medulla more irregular. About the tenth day the medulla forms disorganized, fusiform masses at intervals and where the white substance of Schwann cannot be detected. These alterations, which are most evident in the single tubules, may be found also in the branches. After twelve or fifteen days many of the single tubules have ceased to be visible, their granular medulla having been removed by absorption.

Without the benefit of staining techniques that were later developed by Golgi, Cajal, and others, Waller described the "fusiform" change of distal nerve segments degenerating into what are now called "myelin ovoids." These ovoids represent globules of degenerative myelin and axon debris coursing along the trajectory of the previously intact axon (Figure 3.4). Strictly speaking, and in keeping with Waller's original description, "Wallerian" degeneration refers to a series of events in axons distal to a sharp nerve trunk transection (Figure 3.5). Local damage of axons from other causes, including axonotmesis or neuropathies, however, releases a nearly identical sequence of degenerative changes as those described by Waller. The similarities have prompted many pathologists and others to refer to all forms of axonal degeneration as "Wallerian degeneration." We will term other forms here as "Wallerian-like" degeneration. There are also important similarities between these processes and those involved in pruning of redundant regenerating axon branches or aberrant branches during development [430].

After an irreversible axon injury (presumably there are reversible types, perhaps from transient depolarization after a blunt injury discussed above), the axon segment distal to the injury site is rapidly signaled to begin degeneration. This is an important concept because formerly, axonal degeneration was thought to ensue from a simple cut off of "nutrients" supporting the distal axon segment. Instead, a specific signal triggers breakdown. The exact signal is unknown but it may involve a calcium transient.

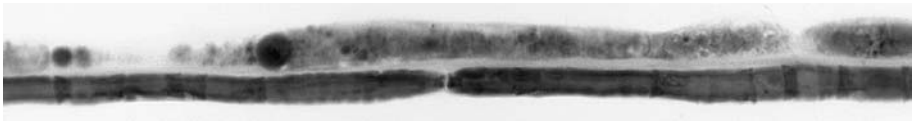

Figure 3.4 An image of teased myelinated fibers illustrating Wallerian-like degeneration (top) with breakdown of the axon and myelin and early formation of digestion chambers. Wallerian-like degeneration occurs after axonotmesis injuries and Wallerian degeneration after neurotmesis. The degenerating fiber sits above an adjacent normal myelinated fiber.

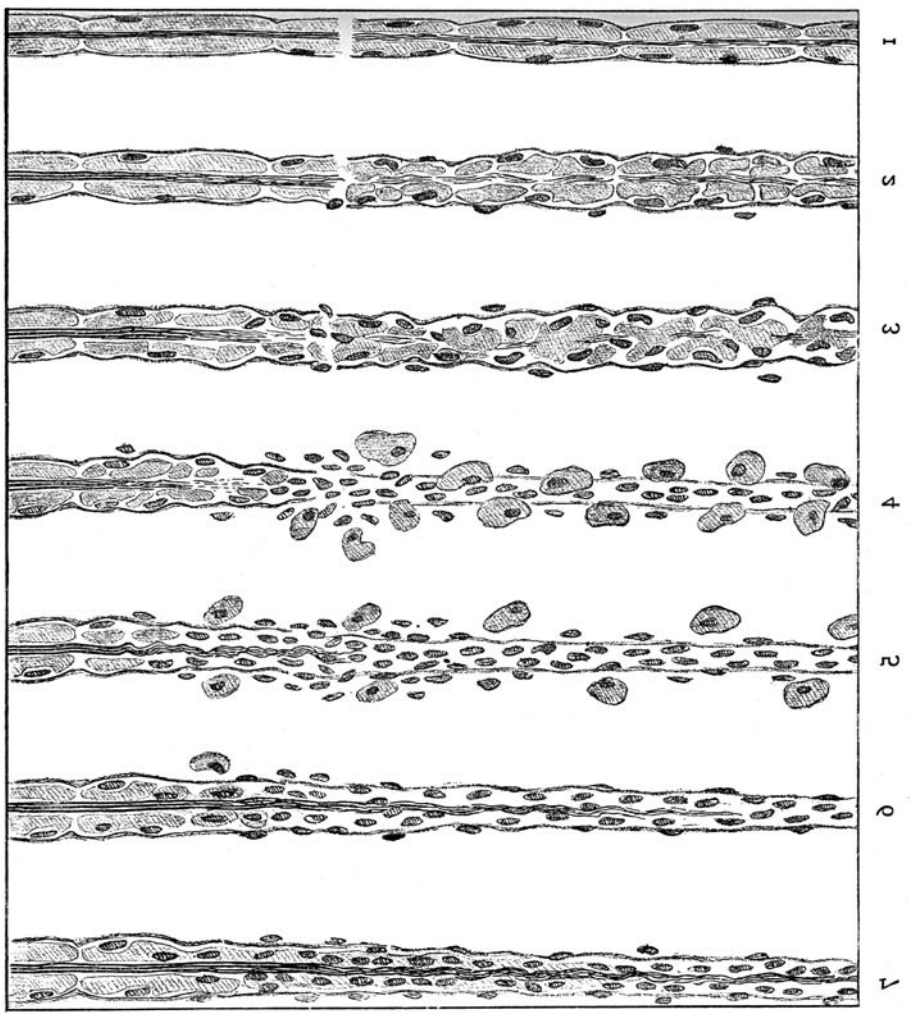

Figure 3.5 An illustration of the sequence, from top to bottom, of Wallerian degeneration and subsequent regenerative sprouting of a transected myelinated axon. Larger rounded hematogenous macrophages invade the injury site in the fourth figure from the top. The illustration is reproduced from the classical monograph by Tinel [681].

Axonal breakdown also involves more persistent influx of calcium. The process of breakdown is then initiated by E3/E4 ubiquitin ligases and proteases including calpain (Figure 3.6). Microtubular dissolution is thought to occur first [728,771], with consequent severe disruption of axonal transport and further cycles of degeneration. Digestion of neurofilament lattices is usually completed over the first 7–10 days and neurofilament material can be recognized immunohistochemically as disorganized fragments that eventually disappear. If examined by semithin LM, the distal nerve stump may, in fact, look normal for a few days depending on the type of nerve injury; intact and uncollapsed myelinated profiles can be seen. Ultrastructural studies resolve this early phase of Wallerian degeneration by identifying loss of normal axoplasm ultrastructure, then atrophy and disappearance of the axon. The sequence of axon degeneration appears to occur first just beyond the site of axonal injury, or proximodistal (centrifugal degeneration) but also at the distal terminals of the injured axons, or distoproximal (centripetal degeneration) [235]. There are also retrograde changes *proximal or above*

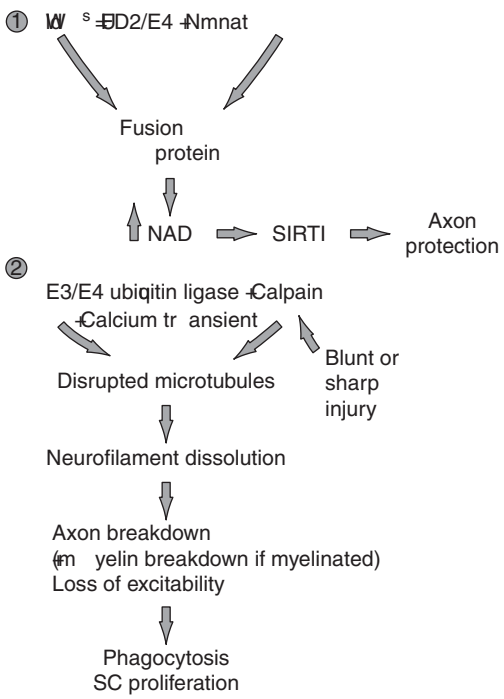

Figure 3.6 A scheme illustrating some of the molecular events thought to occur in active Wallerian degeneration. See the text for details. (Illustration by Scott Rogers.)

the level of the injury itself known as "traumatic degeneration." This can extend to the first or second node of Ranvier of a myelinated fiber of the proximal stump. In more severe instances, traumatic degeneration refers to retrograde loss of the entire neuron, a topic discussed in Chapter 5 [195,572].

Despite the rapid sequence of early events in degenerating axons, it is interesting that they retain their membrane excitability for a period of time after nerve section, mentioned above. If a distal segment of a transected nerve is stimulated, distal nerve action potentials or motor action potentials in motor nerves can be recorded for several days. Excitability gradually declines. In the clinical literature, a rule of thumb is that electrophysiological recordings of injured nerves should be delayed for at least 7–10 days to glean the full extent of their damage. By this time, all permanently damaged axons will have lost excitability and an accurate appraisal can be made.

An early and active role for the ubiquitin–proteasome system (UPS) has also been discovered during Wallerian degeneration. For example, inhibitors of the UPS can delay it [164]. This important finding emerged following identification of a spontaneous mouse mutant known as Wld^S with axons that survive and persist for long periods after they have been transected [433]. In this mouse, the genetic defect involves a dominant triplication overexpression of a ubiquitin regulatory enzyme-related protein known as Wld^S. Wld^S constitutes a fusion protein involving a 70-amino acid portion of UFD2/E4 (a protein involved in protein ubiquination; the 70 amino acid portion, however, does not influence ubiquination) and nicotinamide mononucleotide adenylyltransferase (Nmnat, an enzyme involved in nicotinamide metabolism [430]). Elevated Nmnat activity from overexpression of the fusion protein is then thought to thereby increase levels of nicotinamide adenine dinucleotide (NAD). NAD, in turn, acts as an axonal protectant through a protein deacetylase known as SIRT1 (a member of the Sir family of protein deacetylases and mammalian ortholog of Sir2) [21,598]. All of these players thus orchestrate a balance between retention of axonal integrity and active axonal dissolution (Figure 3.6). The administration of NAD (replicating the impact of overexpresion of Wld^S) or enhancement of Sir2 activity though a specific pharmacological agent, resveratrol, was shown to slow axonal degeneration after transection. Exactly how these interventions act to protect axonal integrity, however, is not completely established.

Wallerian and Wallerian-like degeneration share characteristic morphological features that can be recognized (and counted) in high-quality histological samples (Figures 3.4, 3.7). The techniques used to demonstrate the features include the use of teased nerve axon preparations stained with osmium tetroxide, or semithin transverse or longitudinal sections of nerve that are plastic, or epon embedded (see Chapter 4). Typically, the first change is the disappearance of the

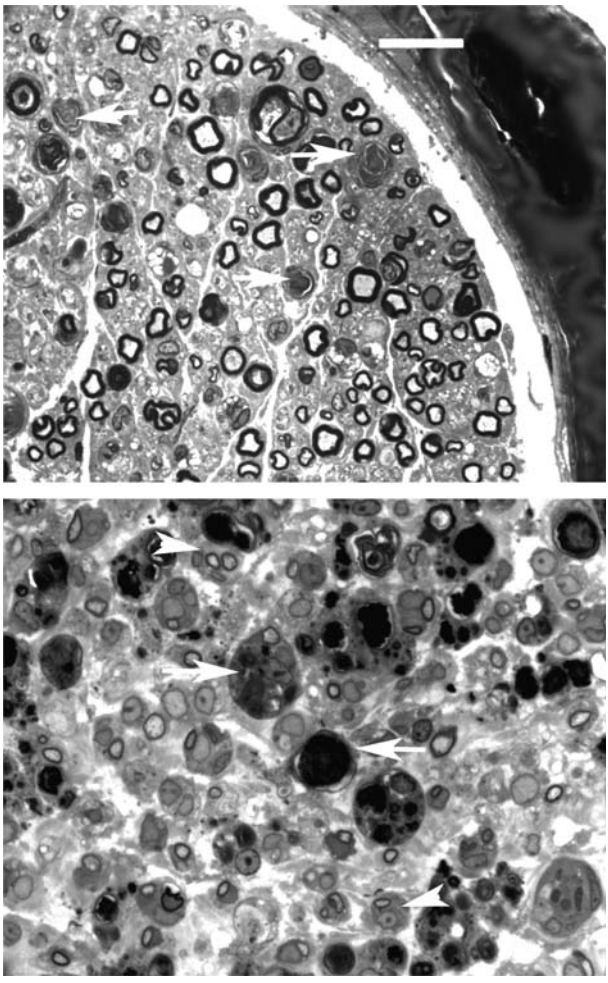

Figure 3.7 Transverse sections of peripheral nerves showing myelinated axon profiles undergoing Wallerian-like degeneration. The white arrows identify degenerating profiles earlier (top) and later (bottom) following injury. In the lower panel, large macrophage profiles containing myelin and axon breakdown products are found amidst newly regenerating axons (arrowheads). The images are from a semithin sections, stained with toluidine blue, from rat sciatic and sural nerves.

fine structure of the axons (neurofilaments, microtubules, see above). Axons become watery in appearance, then swell, or disappear within the confines of the myelin sheath. In transverse section, what might appear to be "normal" myelinated profiles at low power magnification are actually profiles with shrunken or nonexistent axoplasm when examined by EM. Within each internode, axon and myelin contents retract into bulb-like "digestion chambers"

or oblong structures consisting of myelin and axon debris. These digestion chambers are called "E" fibers in the Dyck classification of teased single myelinated axons [159]. Digestion chambers appear along the full length of the severed or otherwise damaged nerve. They likely represent "phagocytosis" by nearby Schwann cells, although later involvement by both resident and invading macrophages play a role.

Morris and colleagues [477] explored the early role of SCs in breakdown of myelin at the ultrastructural level during retrograde degeneration in proximal nerve stumps after section, a process closely related to Wallerian degeneration. The authors suggested that SCs are responsible for digesting their own myelin after injury and sometimes assume a configuration described as "transformed Schwann cells." Early changes included wrinkling and distortion of myelin lamellae, erosion of circumferential lamellae, and myelin fragmentation. Myelin retracted from the nodes and myelin debris was incorporated into vacuoles with membranous material and pale globular inclusions. Hematogenous macrophages invaded nerve injury zones but only within restricted time frames (5–7 days following injury) and only transiently penetrated into sites in the distal degenerating nerve. This process has subsequently been beautifully illustrated using an iron labeling MR technique [38].

In transverse sections, axons and their associated SCs undergoing degeneration are recognized as swollen irregular structures containing myelin debris, i.e., digestion chambers described above. Such profiles may be identified as single degenerating axons, within sectors of the nerve fascicle or may be pervasive, involving all axons of the nerve trunk (e.g., after neurotmesis). At early stages during the removal of myelin debris, SCs and macrophages can be identified heavily laden with cholesterol vacuoles. With time, digestion chambers containing axon and myelin debris atrophy. If longitudinal sections or teased fibers are examined at these later time points, such digestion chambers appear along the length of the original fiber like beads on a string linked by collagen fibrils. These late "E" fibers may persist for years in the nerves of some patients with chronic neuropathies.

The progress of Wallerian degeneration critically depends on the behavior of SCs. SCs alter their relationship with the stable myelin sheaths they maintain and instead initate myelin breakdown. Neuregulins (NRGs) are described in more detail in Chapter 5, but they act as local signaling messengers released from axons during nerve injury and regeneration. In a series of events linked to NRG signaling (discussed further in Chapter 5) through its erbB2 SC receptor, SCs change their phenotype. For example, they undergo dedifferentiation and downregulation of myelin protein synthesis within 2 days of the loss of axon contact [380]. Constitutively, erbB2 is expressed at nodes of Ranvier flanked

by the proteins Ezrin and Caspr and it is thought to signal from the SC microvilli that are in contact with axons. Interestingly, activated erbB2 is also expressed in SCs associated with unmyelinated axons with or without injury. ErbB2 is activated through phosphorylation within minutes of an axonal injury then declines only to again be re-expressed several days later, a time frame that coincides with SC proliferation [242]. ErbB2 activation occurs through the intermediate early gene c-jun. In summary, SCs initiate the self-destruction of their myelin sheaths, followed by changes in behavior that include later migration and proliferation.

Wallerian degeneration also depends on the expression of pro-inflammatory cytokines and chemokines (see review [647]). Expression of anti-inflammatory cytokines can occur concurrently. Macrophage entry is a critical event in the process. After sciatic nerve crush, T lymphocytes and macrophages infiltrate the lesion site within 48h and into the distal nerve stump by day 4 [69,303,304,552,645,646]. There is a biphasic pattern of rises in the expression of several chemokines and cytokines distal to a sciatic nerve transection at day 1 and again at day 14 [551]. The pro-inflammatory chemokines MCP-1 (monocyte chemoattractant protein-1) and MIP-1α (macrophage inflammatory protein 1α) have prominent early rises, especially in the case of MCP-1 just adjacent to the injury site. Anti-inflammatory cytokines, TGF-β1 and IL-10 have similar changes. IL-1β had two types of increase. An early rise occurred close to the injury site, while a later rise developed in segments further from injury. At the mRNA level, similar patterns of expression have been identified [647]: rises in IL-1β, IL-6, and IL-10 peaking 1 day after crush with a more sustained later upregulation of IL-18, IFN-γ, TNF-α, and IL-12p40 out to 14 days. TNF-α, a central player, is expressed in Schwann cells, fibroblasts, endothelial cells, and macrophages [648,713]. Wallerian degeneration is also associated with upregulation of matrix metalloproteinases (MMPs) that act to degrade inhibitor constituents of the extracellular matrix, particularly chondroitin sulphate proteoglycan (CSPG). In peripheral nerve, MMP-2 and MMP-9 rise during Wallerian degeneration and, with laminin, facilitate the later ingrowth of axons [180] (see Chapter 10).

The particular and coordinated pattern of inflammatory molecule expression appears to be intimately related to the success of Wallerian degeneration and the myelin clearance that is part of it. For example, the continuous infusion of neutralizing antibodies to either the combination of MCP-1 and MIP-1α or to IL-1β within the microenvironment of the nerve undergoing Wallerian degeneration was associated with reduced numbers of phagocytic macrophages and preservation of myelin sheaths (but not necessarily axons). Similarly, mice lacking IL-6 had delayed sensory axon regeneration [778]. Mice lacking TNF-α had reduced macrophage influx [404].

Specific macrophage chemoattractants also include MCP-1 discussed above, LIF (leukemia inhibitory factor), and pancreatitis-associated protein III (PAP-III) released from Schwann cells [497,655,682]. Rats with knockdown of PAP-III had slowed macrophage recruitment into injured peripheral nerves and a delay in subsequent regeneration. SCs may be activated by IL-6 in turn synthesizing both LIF and MCP-1 that serve as macrophage attractants. This interesting pathway also involves an autocrine loop whereby LIF induces MCP-1 expression as well [682].

Several additional molecules have important roles in mediating axon breakdown. Nitric oxide, released by rises in the expression of inducible nitric oxide synthase (iNOS) in Schwann cells and macrophages at the site of a peripheral nerve injury [391,799] is one such mediator. Nitric oxide reacts with the superoxide radical to form peroxynitrite, a species capable of lipid myelin peroxidation, an important early step in its eventual clearance during Wallerian degeneration [600,704]. Mice lacking iNOS have delays in the progression of Wallerian-like degeneration and in subsequent reinnervation [392]. Interestingly, erythropoietin (see Chapter 9), generated by SCs, in response to ambient NO production after injury is paradoxically capable of protecting axons and preventing axonal degeneration [338]. NO signals thus have varying roles depending on their timing, intensity, and targets.

RAGE (receptor for advanced glycosylation endproducts) mediates mononuclear phagocyte participation in Wallerian degeneration. Phagocytes lacking proper RAGE signaling had decreased p44/p42 MAP kinase phosphorylation and mice with a dominant negative RAGE had delays in the progression of Wallerian degeneration and in subsequent regeneration [584].

Fewer proteins have been identified that normally dampen Wallerian degeneration. Desert hedgehog protein (Dhh) may be one example: mice lacking it appeared to have accelerated Wallerian degeneration. It is unclear how this signaling pathway integrates with others to regulate the overall rate of degeneration [29].

Neuromas

Neuromas are swollen distorted portions of a nerve trunk that result from severe traumatic disruption. Examples are given in Figure 3.8. “End nerve” neuromas are found at the proximal stump end of a completely interrupted peripheral nerve (neurotmesis). At one time, neuromas were thought to represent enlarged masses of aberrantly sprouting axon profiles misdirected to grow back on themselves. While misdirected axons are a common feature of neuromas, it should be emphasized that they are not simply composed of dense

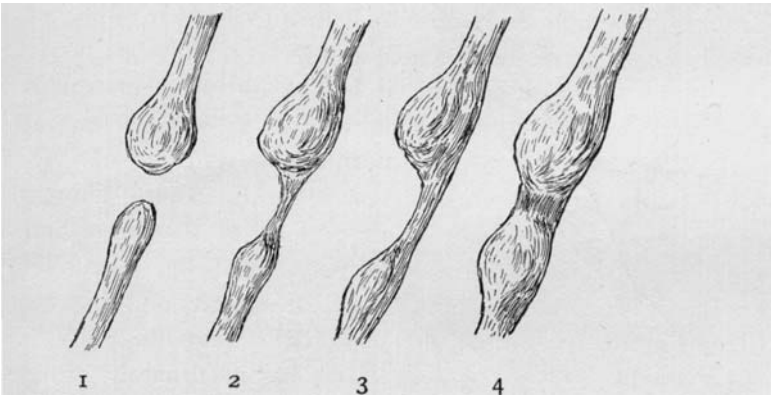

Figure 3.8 Illustrations of several types of chronic nerve trunk injuries from the classical monograph by Tinel. On the left (I) is a neurotmesis injury with an end neuroma on the proximal (top) stump. To the right are examples of progressively more severe axonotmesis injuries with neuromas in continuity. It is possible that injures 3 and 4 have a small number of regenerating axons traversing the injury site [681].

axon bundles. Neuromas are complex structures (Figures 3.9, 3.10). They contain former fascicles, infiltrating macrophages, abundant connective tissue with dense collagen deposition, and avascular zones likely resulting from local ischemia [755,787,807,809]. Angiogenesis also occurs within neuromas, and accompanies a pancellular "burst" of cellular proliferation that develops in their first 5–7 days [805]. Other proliferating cells include mast cells, macrophages, SCs, and fibroblasts. Thus, beyond simple ingrowth, the complex changes that occur in neuromas may involve ischemia, protease activity, local free radical release, and elaboration of growth factors from macrophages and mast cells. They also likely include signals from the extracellular matrix. All of these constituents may play roles in generating neuropathic pain from neuromas.

At early stages, neuromas exhibit other interesting features that influence their structure and behavior. Transected axons within them form swollen endbulbs or boutons, also discussed in Chapter 5, presumably from accumulated products of anterograde transport and impaired turnaround transport. These endbulbs accumulate biologically active molecules that include neuropeptides, opioid receptors, nitric oxide synthases, sodium channels, and others.

Neuromas "in continuity" arise from partially injured nerves (axonotmesis) in which one or several fascicles do not regenerate (Figure 3.8). Instead, the axons

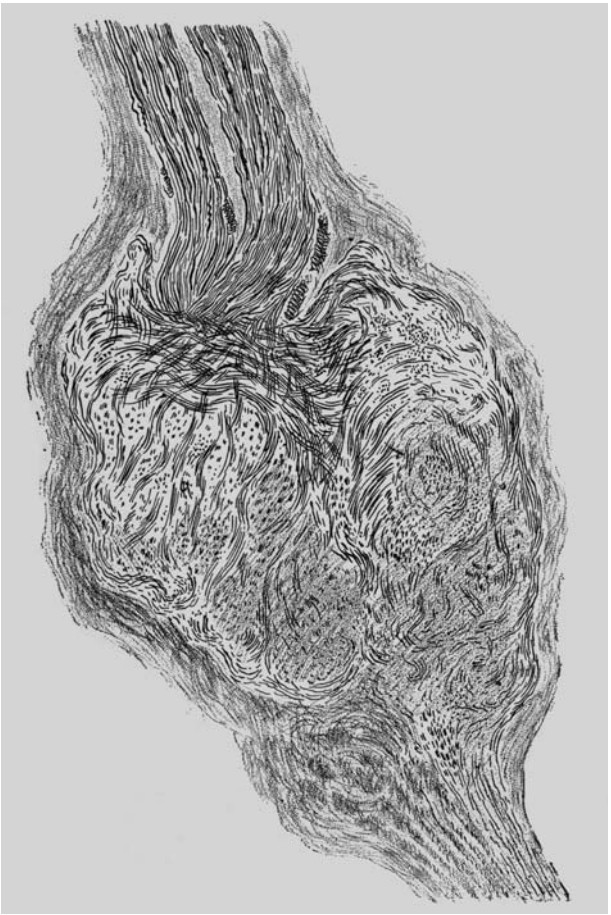

Figure 3.9 Longitudinal section of a neuroma in continuity as illustrated by Tinel. Note the complex disorganization of the neuroma and that most axons from the proximal stump (top) do not reach into the distal stump [681].

and connective tissue of these fascicles form a neuroma as a local enlargement along the nerve trunk. Such lesions provide an important lesson in regeneration neurobiology. This is a scenario in which a nerve trunk remains connected, yet axons interrupted by the injury fail subsequently to grow into the distal portion of the nerve. There is ample guidance by connective tissue to indicate where axons and Schwann cells should travel, yet inexplicably, both form misoriented aberrant neuromas. The lesson is that anatomical reconnection or guidance from connective tissues is insufficient for successful regeneration. The milieu must provide additional signals and guidance to support axonal growth beyond simple continuity.

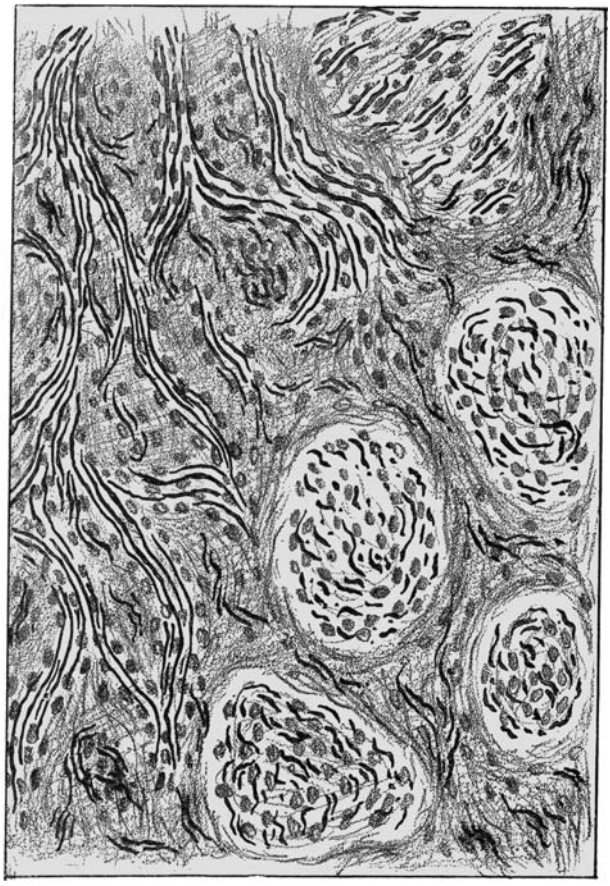

Figure 3.10 Details of the neuroma in continuity from 3.9 by Tinel. Note the presence of minifascicles and the absence of a consistent trajectory direction for axons within this structure [681].

Partial forms of peripheral nerve injury

Chronic constriction injury

There are several partial forms of peripheral nerve trunk injury. The chronic constriction injury (CCI), first described by Bennett and Xie [41], has been widely studied as a model of neuropathic pain. The lesion involves the placement of four loose ligatures around the trunk of the sciatic nerve in rats or mice. The local inflammatory reaction from chromic catgut (dissolvable) sutures causes swelling of the nerve trunk within 2–3 days. The segments of the nerve between the ligatures become ischemic and induce axonal degeneration in a large proportion of the axons. CCI develops more gradually than simple crush or transection. Most likely, an interaction between damaged axons, *en passant* intact

neighbors, and the release of a barrage of inflammatory mediators is responsible for generating neuropathic pain. Once events in the nerve trunk initiate pain, changes in neuron phenotype, their upstream pathways and glia of the ganglion, dorsal horn of the spinal cord and higher then follow [696]. Large myelinated axons are the most susceptible to CCI whereas a proportion of small unmyelinated axons are not injured. Spared smaller caliber axons may allow selective sensory information to traverse the CCI lesion and in turn generate the pain syndrome associated with it. Alternatively, some of the pain behavior may arise from ectopic discharges of injured axons, rather than spared ones. Ectopic or spontaneous activity of large caliber axons, for example, can be detected after CCI [393]. If tested for, behavioral features of neuropathic pain emerge by 48 h and persist over 3 weeks.

Standard measures used to identify pain include the latency of paw withdrawal on the side of the nerve injury to a thermal stimulus. More rapid withdrawal indicates hyperalgesia, whereas delayed withdrawal indicates sensory loss. Allodynia refers to the perception of a normally innocuous stimulus as painful. Mechanical allodynia is tested by determining the degree of withdrawal to a mechanical filament probe of graded bendable force. A greater response than expected to smaller caliber (more easily bent) filaments indicates allodynia (see Chapter 4). At and after 3 weeks following a CCI injury, there is evidence that axon regeneration has begun. Some axons grow around the area of the sutures by penetrating through the epineurium and growing on the outside of the nerve trunk to reach the distal stump. The behavioral measures associated with pain can also be used to track regeneration of sensory axons. Pain behavior diminishes as regeneration proceeds, a time when the local exposure of axons to inflammatory mediators declines.

CCI has been an important model of pain neurobiology and has been widely used to address mechanisms or pharmacological approaches toward treatment. The lesion is partial, probably resembling common types of human injuries. For example, severe neuropathies that occur following trauma in some instances may involve similar types of localized ischemia at the site of injury. Compression may occur from a bone fragment (e.g., radial nerve injuries from humeral fracture), from a hematoma (e.g., sciatic injury from a pelvic fracture), or from localized ischemia during a compartment syndrome.

A related partial nerve injury pain model resembles the CCI model and involves placement of a single tight (in contrast to the loose ligatures of CCI) ligature partway through a portion of the nerve trunk. The model generates crush and inflammation of one portion of the nerve trunk adjacent to an intact and uninjured portion. Finally, the Chung model of neuropathic pain involves the placement of a tight ligature around a single nerve root, L5, supplying the

distal sciatic nerve [115,342]. In this model the intact and injured nerve root axons combine through the nerve plexus as the fibers enter the mixed sciatic nerve trunk. Downstream in the sciatic nerve, therefore, there is an admixture of intact axons from uninjured nerve roots and axons undergoing degeneration from L5. *En passant* interactions of intact axons with degenerating neighbor neurons and inflammatory byproducts of Wallerian-like degeneration may generate ectopic axon discharges associated with pain behavior.

Compression

Compression injuries are common in patients. Forms of chronic compression are known as "entrapment" neuropathies. Sites of frequent involvement in humans include the carpal tunnel at the wrist for the median nerve, the cubital tunnel for the ulnar nerve at the elbow or the exposed fibular head at the knee for the peroneal nerve. Less commonly, a bone fragment, hematoma, or tumor may acutely compress a nerve. There has been a tacit assumption that such lesions are ischemic since they may interrupt the arterial supply or prevent venous drainage. As will be discussed in Chapter 7, however, there is very little actual evidence for significant ischemia in most of these common entrapment neuropathies. Since the vascular plexus of the peripheral nerve trunk is highly redundant from multiple feeding vessels and provides flow in excess of metabolic needs, it is difficult to generate local ischemia from a single pressure point.

Stretch

Stretch injuries are the least studied form of partial nerve injury. The robust tensile properties of longitudinally oriented collagen fibers protect axons from stretch. In addition, axons have redundant curved trajectories within the nerve trunk, allowing them to lengthen substantially before they can be pulled apart. Stretch injuries, unlike those above, may involve a combination of ischemia from disruption of the epineurial plexus, and direct mechanical distortion of axons. They are some of the most common human nerve injuries.

Direct injuries to sensory ganglia

Direct injuries of the dorsal root ganglia (DRG), housing sensory neurons, have not been characterized in the clinical literature. Their importance and prevalence are unknown. It is possible, however, that in the cervical and lumbar regions of humans chronic compression or traumatic injury of DRG can generate forms of spinal or radicular pain [12,410,481]. DRGs are not generally sampled in clinical studies. DRGs, however, may be more vulnerable to ischemia than nerve trunks. Ischemic lesions of the ganglia result in downstream Wallerian-like degeneration of their distal axon branches [753]. Lesions

of peripheral branches of sensory dorsal root ganglia neurons are associated with retrograde cell body reactions and changes in phenotype, discussed in Chapter 5 [403].

Blood nerve and ganglion barriers and injury

Wallerian-like degeneration is associated with loss of integrity of the perineurial component of the blood nerve barrier [731,732]. This may be important to allow inflammatory cells and blood borne trophic molecules access to the injury site and to permit better clearance of axon and myelin debris. The change also has important implications for future therapeutic interventions where penetration into injured and regenerating fascicles is critical.

Summary

Peripheral nerve injuries can vary tremendously in their severity and in their likelihood to recover. While several classifications of nerve injury have been described, the most important consideration is whether myelin alone is disrupted or whether there is additional axon damage. Injuries that only have demyelination, known as neurapraxia, have conduction block across the injury site but can recover relatively rapidly. In contrast, axon damage from either blunt or penetrating damage leads to a series of events known as Wallerian or Wallerian-like degeneration. Subsequent recovery requires axon regrowth that involves a much slower pace of recovery. The Wallerian degenerative process is not passive but an active pathway in which molecular players are only now being identified. Axons undergo a series of structural changes with loss of excitability and eventual phagocytosis while local chemokines, cytokines, and molecules expressed in axon endbulbs have an important influence. The characteristics of injury therefore have a substantial impact on the local milieu of an injured nerve trunk and provide a context for all future regenerative activity, considered in the next few chapters.

Suggested reading

Griffin, J. W., George, E. G., Hsieh, S. T., & Glass, J. D. (1995). Axonal degeneration and disorders of the axonal cytoskeleton. In *The Axon. Structure, Function and Pathophysiology,* Chapter 20. Oxford, UK: Oxford University Press [235].

Luo, L, O'Leary, D. D. M. (2005). Axon retraction and degeneration in development and disease. *Annual Review of Neuroscience*, **28**, 127–156 [430].

Sunderland, S. (1978). *Nerves and Nerve Injuries*. 2nd edn. Edinburgh, UK: Churchill Livingstone [660].

4

Addressing nerve regeneration

The pace of molecular discovery relevant to nerve regeneration has accelerated. New insights into regeneration, however, have not necessarily been partnered with rigorous approaches to measure regeneration. The purpose of this chapter is to engender readers with a healthy appreciation of new findings, based on rigorous approaches, that confirm the complexity and beauty of the regenerative process. Similarly, the reader should be skeptical of approaches that do not live up to that standard. Assays of regeneration should ideally encompass all of the crucial steps involved in the regenerative timetable: early sprouting, axon elongation, regrowth of axon radial caliber or girth, remyelination of larger caliber axons, repopulation of nerve trunks by mature axons, and extension to target tissues. During regeneration axons regain electrophysiological properties that they have lost, features that can be carefully assayed. Finally, it is critical to know whether there has been a resumption of function, addressed through "functional" or behavioral endpoints. This chapter presents a summary of regeneration assays and a discussion of their strengths and limitations.

Structural (histological) approaches

Few other measures can convey the structural beauty of regeneration captured in a high-quality histological snapshot. Histological techniques demand strict attention toward the details of specific protocols and they require optimal handling of specimens that are appropriately sampled. Their exactitude sets a standard of quality that is enormously satisfying. Unfortunately, classical histological approaches are frequently dismissed and substituted with easier or more colorful techniques that have lower resolution. Classical histology continues to be used in clinical laboratories to address disease processes.

In regeneration, use of semithin plastic embedded sections sampled at a fixed distance from an injury site offers an assessment of the numbers and maturity of newly regenerating axons, both myelinated and unmyelinated. They also address the overall architecture of the nerve and can be used to identify mast cells, macrophages, and axons undergoing Wallerian-like degeneration.

I will begin with some very practical issues. The benefits of *careful handling and fixation* cannot be overemphasized. Nerves must be gently manipulated and not pulled or stretched! If specific portions of a nerve are to be harvested or biopsied, forceps should be used only on the very ends of the specimen. Sharp scalpels or microscissors must be used for severing the nerves. Rough handling can be recognized by microscopists. For example, sectors of fascicles have excessively folded and crinkled myelin sheaths, often obscuring the axon within. Some portions of the axon may appear paradoxically denuded of myelin. This appearance results from telescoping of compressed myelinated fibers during harvesting. In some cases, further sections taken deeper into the epon block may save the specimen because they are beyond and bypass the damaged area.

Specimens must be kept moist immediately after resection and before they are fixed. Nerves, particularly those of mice, dry very quickly. The sheaths of myelinated axons swell, lose their contrast and with time completely distort the myelinated axon profile [438] (Figure 4.1). These nerves contain numerous lightly stained donut-like concentric circles without any visible axons visible within them. Our approach to prevent drying, otherwise known as *desiccation artifact*, is to rapidly place specimens in iso-osmolar normal saline and then fixate quickly.

There are a number of classical histological approaches used to study peripheral nerves. While many are appropriate for clinical biopsy laboratories, they are not all helpful for studying regeneration. These methods include paraffin embedding of thick sections and staining with hematoxylin and eosin, myelin stains (Luxol fast blue and others), or silver axon stains such as Bielschowsky or Bodian. The major drawback of paraffin-embedded material is the inability to resolve individual myelinated axons reliably, especially small ones. Silver-stained axon profiles may be confused with collagen profiles. Accurate measures of caliber are not possible. None of the paraffin methods offers the resolution of glutaraldehyde fixed and osmium-stained epon or plastic-embedded *semithin sections* that we clearly favor.

With this acknowledged bias, there are variations in how semithin sections can be made, all with excellent results if carried out meticulously. I will relay the approach of Dyck and colleagues that is used for both clinical and research purposes. The detailed histological protocol is provided in Appendix 4a. It involves fixation in cacodylate-buffered glutaraldehyde fixation, followed by alcohol dehydration, osmium tetroxide staining, and finally epon (plastic) embedding. An ultramicrotome provides LM (light microscopy) semithin sections

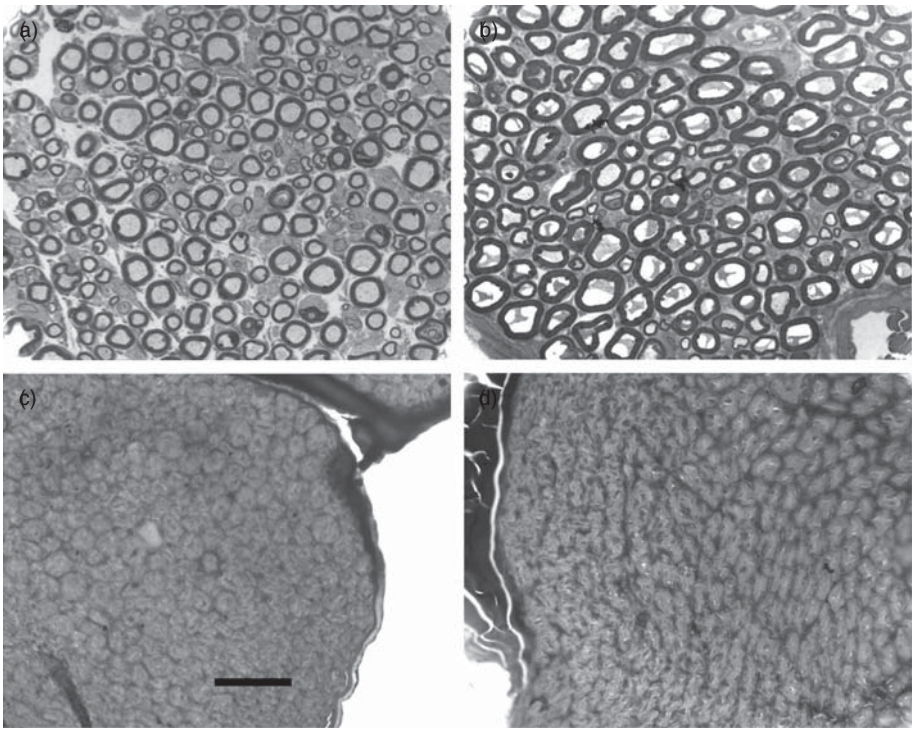

Figure 4.1 Transverse sections of normal sciatic nerves from mice illustrating desiccation (drying) artifact. A is from a properly preserved and fixed nerve. In B, note that the individual axons within myelinated profiles have shrunk and retracted. In C and D, severe desiccation artifact completely distorts the profiles and makes these nerves unsuitable for further analysis. (Reproduced with permission from [438].)

of 0.5 to 1.0 micron diameter that outline the full structure of the nerve trunk in exquisite detail, including internal axonal structure. Transverse LM sections provide measures of *myelinated axon* (*MF*) numbers, axon caliber (area, diameter), and myelin thickness. From these measures, distributions of axon size can be plotted as histograms discussed in Chapter 2. Information about myelin thickness can also be given using the G ratio, the axon diameter divided by the diameter of the whole fiber including myelin sheath (see Chapter 2).

The same epon-embedded samples used for LM can be used to provide ultramicrotome thin sections that are laid on grids for electron microscopy (EM). EMs, in turn, are used to measure the density and caliber of *unmyelinated axons* or fibers (UFs). As with MFs, construction of fiber size histograms of unmyelinated axons generate additional information. Both LM and EM are used to examine blood vessel numbers and structure, basement membrane thickness, macrophage or mast cell numbers, and other features.

Dyck and colleagues also recognized the importance of eliminating *artifacts from fixation of nerve* [527–530]. The use of hyperosmolar fixation in pathology laboratories that emphasize CNS work produces a very common artifact. The fixative shrinks and distorts the axon profiles generating images of highly irregular (but artifactual) myelin sheaths that are collapsed into the axon. The substitution of an iso-osmolar fixation matches the osmolality of the peripheral nerve to that of the fixative and preserves features of the nerve architecture. Despite these long-established protocols, many examples of poorly processed nerves continue to be published.

There are three basic ways to fix nerves: perfusion fixation, *in situ* fixation, and immersion fixation. Perfusion fixation involves the placement of a cannula in the heart (or other great vessel, protocols vary) of an anaesthetized animal and an infusion pump delivers titrated fixative appropriate for the animal size. Blood and fixative are drained from the femoral vein. Many laboratories combine this method with immersion fixation because perfusion may not adequately fix all the tissues. Immersion fixation simply involves placing the specimen (nerves can be loosely tied with nylon to small sticks and a suture placed on the distal end for orientation) in a vial of freshly prepared fixative. Perfusion fixation makes detailed dissection of ganglia and nerves more difficult since the tissue is hardened, discolored, and without blood. Immersion fixation alone may not properly fix larger tissues like human ganglia or rodent spinal cord. This limitation may be circumvented by first dividing the tissues into smaller portions and prolonging the fixation period. *In situ* fixation offers a compromise: the nerve, cord or ganglion is exposed and the bed has fixative applied and left for a period of time (usually 30 minutes). After this fixation is completed, the tissues may be removed and further fixed by immersion [603].

Image analysis programs are available including free software for PCs originally developed by NIH (Scion Image, Scion Corporation, Frederick MD; www.scioncorp.com and Image J, http://rsb.info.nih.gov/ij/). Rigorous calibration for magnification is a requirement of all software approaches. Next for consideration is the extent of the regenerating nerve that should be examined. Some reports have emphasized measurements of the density of remyelinating axons measured distal to an injury. In this approach, selected (arbitrary or random) fields undergo measurement and the behavior of the whole specimen is inferred. Density measurements require less time to complete and consequently are easier. Although density and measurements of total nerve axon numbers may correlate directly with one another, this is not a fixed rule. Indeed, some regenerating nerves may have fields with high densities of regenerating axons but overall have not succeeded in regenerating as many axons. Regenerating nerves sometimes exhibit clusters or minifascicles that demonstrate this disparity.

Small minifascicles may represent sprouts from single parent axons. Thus, a density measure might spuriously suggest high fiber outgrowth but an examination of the entire nerve field would indicate otherwise. In smaller nerve trunks or during early regeneration, measuring the total number of regenerated myelinated axons might provide the most rigorous appraisal of the axon population. In other instances, measuring the density of myelinated axons when large numbers of axons are present may be more feasible. Finally, fiber size histograms can be used to sort out whether there is a subpopulation of axons with interesting behavior. These subpopulations might not be captured or understood by relying only on overall measures of mean axon caliber.

Our approach is to select early time points measuring the numbers of all new axons distal to the injury site and to emphasize distal branches of the sciatic nerve. In this way, the technician might not be overwhelmed by counting large numbers of axons. In the case of unmyelinated axons presented by EM, it is daunting to count entire nerve trunks or fascicles. Density measures in randomized sampling fields are required. The quality of peripheral nerve EMs can be judged by whether individual myelin lamellae of myelinated axons can be resolved and distinguished under higher power.

Transverse sections of the nerve at a fixed distance distal from the injury site (e.g., 5–15mm in a rat sciatic or sural nerve) allow for the estimation of axon outgrowth after injury. As alluded to above, axons identified in this way may arise from a smaller complement of parent axons, i.e., they are multiple regenerative sprouts. These sprouts may be myelinated or unmyelinated axons. Therefore, correlating these kinds of measures with another approach, such as fluorchrome backlabeling (see below) may help to determine the number of actual parent neurons generating new axons.

During early regeneration, myelinated axons are smaller in caliber and have thinner myelin sheaths than mature fibers (higher G ratio). In very early regeneration, none of the axons may have begun to myelinate. The number of regenerative clusters (groups of myelinated axons in minifascicles) may also be measured. Finally, it may be of interest to measure the number of axons undergoing Wallerian-like degeneration. In some conditions, delayed Wallerian-like degeneration can be identified by abnormal persistence of degenerating profiles. Regenerative success, in turn, requires their prompt and efficient clearance.

Immunohistochemical approaches

Immunohistochemistry is likely the most widely used assay of *in vivo* axon regrowth. Its advantages are several. Immunohistochemistry allows labeling using specific molecular markers of axons, SCs, and other peripheral nerve

components. These may label subsets of axons, for example, Substance P (SP) for sensory axons, choline acetyltransferase (ChAT) for motor axons. The caveat is that many molecules are shared by both fiber types, e.g., CGRP. The disadvantages of immunohistochemistry are that some antigens can be capricious to label; they are nicely demonstrated with some fixatives and antibodies, yet elusive to other approaches. Most immunohistochemistry uses immersion fixation but fixatives vary and might include, for example, brief acetone fixation of fresh samples, variations of paraformaldehyde fixation (e.g., Zamboni's fixative), and "antigen retrieval" protocols. These are not described here.

There are two approaches for using an immunohistochemical assay of nerve regeneration. As in the LM approach, transverse sections of nerve at a fixed distance from the injury site can be used to label numbers of regrowing axon profiles. Since antigens are rarely evenly distributed through the diameter of an individual axon, these profiles cannot be used to measure axon diameter or caliber. Specific labeling of myelin proteins, such as MBP can be used to identify myelinated fibers. The second approach is to study longitudinal sections of nerve that may include the injury site. This approach, if used at early time points, can be used to measure the number and outgrowth lengths of axons beyond the injury zone.

There are several important considerations in using longitudinal sections of outgrowing axons. Axons and their endoneurial fascicles bend and twist along the nerve in three dimensions. Given this normal "wavy" trajectory, it may be impossible, for example, to trace the origin of a given regenerating sprout to its parent axon. Axons wind their way through larger distances than even thick 50-micron sections studied by confocal microscopy might sample. Thus, beyond a few individual axons, a comprehensive ascertainment of branching *in vivo* is often not feasible. Also, if only one–two high resolution thin sections are used, it may be incorrect to infer the numbers of axons that are regrowing because many axons may not have been captured in the section through the nerve; several sections through the estimated center of the section are routinely required. If the end of the section cuts through perineurium (because the nerve trunk is curved or embedding is not flat), there may be a false sense that axon growth has ended (i.e., false end of the fascicle). A marker with high resolution (e.g., βIII tubulin; see below) may be associated with such dense labeling that individual profiles cannot be counted easily. Overly dense labeling occurs when analyzing the proximal stump of an injured nerve or the regenerating zone at later time points. Finally, some axon antigens disappear only slowly distal to injury during axonal degeneration. Residual degenerating material might then be counted inaccurately as new axons. In most instances, however, these can be distinguished as punctate nonlinear degenerative profiles.

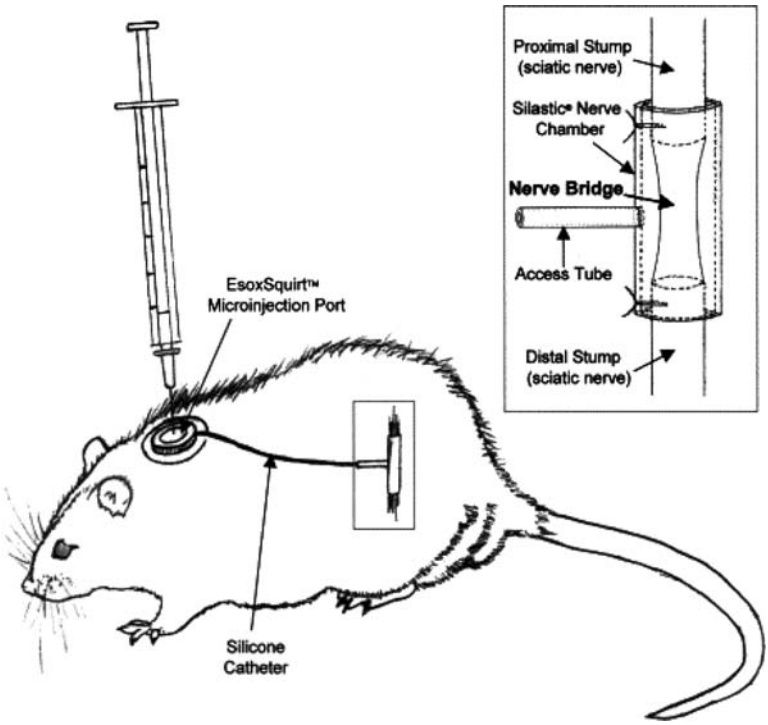

Figure 4.2 Diagram illustrating a setup to analyze early regenerating axons across a peripheral nerve transection gap and to manipulate the regenerative microenvironment with a subcutaneous access port. (Illustration by David McDonald and reproduced with permission from [452].)

Some investigators have attempted to quantitate axon outgrowth by measuring the labeled area from a whole nerve trunk specimen without analyzing individual profiles, e.g., a measure of percent occupied area. While the approach appears deceptively easy, it lacks satisfying resolution and can be distorted by a series of artifacts. These artifacts are variation in sampled area, nonspecific labeling by primary or secondary antibodies, overlap of multiple profiles, and others. We think this approach yields inaccurate results and should be discouraged.

An approach that directs the proximal and distal stump of a transected peripheral nerve (e.g., sciatic nerve of an adult rat) into a regenerative conduit [452] permits the analysis of early nerve regenerative events (Figure 4.2). New axons regenerate by growing along a connective tissue/fibrin bridge connecting the proximal and distal stump. These can be resolved, counted, and measured within the first few days following injury. Moreover, new axon outgrowth occurs independent of the distal nerve stump and products of Wallerian degeneration. At 7 days, for example, outgrowth is not dense enough to obscure individual

profiles: these can be counted at serial distances from the proximal stump to the furthest distance of outgrowth. Both the numbers of outgrowing axons and the longest distance traveled by an individual axon can thereby be estimated.

Some discussion here is warranted of what axon labels are most suitable for addressing nerve regrowth. Neurofilament labels, such as the Nf200-antigen, the heavy unit of neurofilament intermediate protein, are robust and address a large proportion of axons [103,491]. Neurofilaments are heavily represented in larger caliber axons, whereas they are present in much lower densities in small myelinated and unmyelinated axons. Neurofilaments also do not extend to the distal end of the axon and growth cone. Overall, a neurofilament label might therefore underestimate the full extent of axon outgrowth. It may also not label small caliber new axons with enough intensity to be detected. We have estimated that Nf200 labels approximately 25% fewer distal outgrowing axons than a βIII tubulin label. Despite this shortcoming, Nf200 labeling did not substantially underestimate the distance the axons traveled. The distal nerve terminals not labeled by Nf200 were highly variable in their trajectories, perhaps representing unstable new sprouts sampling their microenvironment. Nf200 labels had larger more discrete calibers more amenable to analysis. Overall, Nf200 might be considered a more robust measure of "stable" axon profiles. While products of neurofilament may persist for several days during Wallerian degeneration distal to axon interruption, their profiles can be distinguished from that of viable axons. By 7 days, for example, neurofilament profiles are short, broken, discrete, and segmented in contrast to more continuous profiles of intact regenerating axons.

PGP 9.5 and βIII tubulin are two constituents that can be labeled in all outgrowing axons. PGP 9.5 labels a ubiquitin hydrolase enzyme that is present in all axons. It is the label of choice for addressing epidermal skin innervation (see below). The βIII tubulin antigen is derived from subunits of microtubules. Both molecules extend to the distal axon and growth cone. Their small distal irregular profiles, however, may be difficult to evaluate as individual axons and likely to represent unstable new branches. In longitudinal sections at, and distal to, a crush zone, both markers require several days to disappear during phagocytosis and axonal degeneration. Thus, like neurofilament labels, PGP 9.5 and βIII tubulin must be studied with care at early time points especially after crush. Distinguishing residual material after crush from degenerating axons adjacent to newly ingrowing axons can be challenging.

Several investigators have chosen GAP43/B50 as their label of choice for examining outgrowing axons. Growth cones are preferentially decorated with this growth-associated protein. Disadvantages are that more mature and stable proximal profiles might not be labeled. A detailed comparison of its sensitivity with neurofilament, PGP 9.5 and βIII tubulin has not been published. SCs also

express the antigen and since they elaborate fine processes during regeneration, they may be mistaken for axons. Other markers used to label specific axon subpopulations include CGRP, galanin, IB-4, and other peptides [334,401,691,736].

A novel approach toward evaluating nerve regeneration involves the use of YFP transgenic mice. The neuron specific thy-1 promoter is used to drive the expression of YFP. While not all axons assume fluorescence, the majority do, and the mice have provided elegant approaches toward tracing cutaneous nerve behavior [102] or counting outgrowth of axons beyond injuries [170,744].

Electrophysiological measures of regeneration

There is a range of electrophysiological approaches used to measure peripheral nerve regeneration in animal models or humans. One of the most widely used is *multifiber recording from motor and sensory axons*. The method is also described as a "population recording" from axons within a peripheral nerve trunk. They are carried out under simple anesthesia in rodents or other mammals and without sedation in humans. The recordings are based on the premise that a supramaximal stimulation of a whole nerve trunk will recruit all electrically excitable axons adjacent to the stimulating electrodes. Downstream of the stimulating site, compound nerve action potentials (NAPs) are recorded that represent summated individual axon action potentials. The amplitude (e.g., baseline to peak) has an excellent correlation with the number of excitable axons that have been recruited by the stimulation and that course beneath the site of the recording electrode. Careful measurement of the distance between the stimulating cathode and the proximal pole (G1) of the recording electrodes provides conduction velocity (CV) measurements. By convention, CV = distance/latency (time between stimulation and recording). Latencies are calculated between the onset of the sweep and the first upward deflection of the NAP trace on an oscilloscope. NAPs can be recorded from nerve segments resected and placed on a recording grid, or by using near nerve needle recording and stimulating electrodes. They are routinely recorded by surface (without skin penetration) stimulation and recording from humans in clinical nerve conduction laboratories ("EMG" labs). Cutaneous sensory nerve (e.g., sural nerve at the ankle) NAPs studied using this method are called "SNAPs" (sensory nerve action potentials).

NAP or SNAP amplitudes are influenced by a number of factors beyond axon numbers, a topic beyond the scope of this discussion. For example, demyelinating neuropathies and neuropathies with axonal atrophy are also capable of influencing both amplitudes and conduction velocities. One critical variable that the experimentalist (and human EMG lab) can control is the near nerve temperature during recordings. These should be maintained close to 37°C

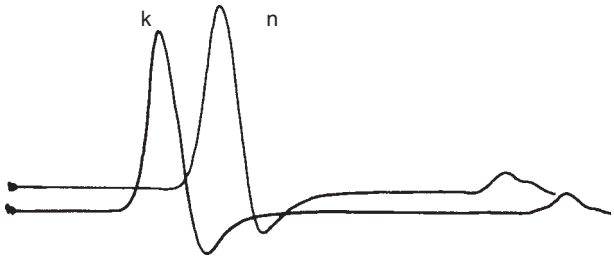

Figure 4.3 Examples of compound muscle action potentials (CMAPs) recorded from the interosseous foot muscles of the rat with stimulation of the sciatic nerve proximally at the sciatic notch (n) or more distally at the knee (k). The smaller potentials after the main peaks at the end of the tracings are F waves. (Reproduced with permission from [789].)

(near nerve) in experimental preparations and greater than 32°C (surface skin temperature) in humans during recordings.

Multifiber techniques are particularly useful in measuring pure motor axon regeneration. The recordings are made over the endplate of a muscle normally supplied by the nerve in question. In this technique, the whole nerve trunk is stimulated with a supramaximal stimulation, but the endplate recording only captures the potentials arising from activated motor axons. The recorded potentials are therefore termed CMAPs (compound muscle action potentials) or "M" waves (Figure 4.3). As with NAPs, there is an excellent correlation between CMAP amplitude and numbers of viable motor axons that have reconnected to the endplate zone of the muscle. As above, however, it is not entirely correct to suggest that only axon numbers influence the CMAP. CMAPs may be dispersed (spread out) when motor nerves are demyelinated. In rats reared in wire bottom grid cages (as opposed to plastic sawdust covered cages) significant disortion of CMAPs is attributed to compression neuropathy of distal hindpaw motor branches, i.e., "wire cage neuropathy" [804]. The use of these cages should be discouraged in rodent studies of regeneration.

CMAPs (or NAPs, SNAPs) recorded after stimulation of a nerve trunk distal to an injury do not immediately disappear despite damage to all axons at the injury site. Whereas stimulation proximal to the injury site, as expected, fails to recruit a response, axons distal to a nerve injury remain excitable for several days. If examined early after injury, these recordings might suggest that the distal axons are normal. The same recordings carried out 7–10 days later, however, may fail to evoke any response. Thus, to assess whether distal fibers have been damaged by a given injury, recordings should therefore be delayed. In a nerve trunk containing thousands of individual axons, it may be that some axons are injured but others are not. To then address how extensive the damage is, a CMAP

recorded from distal nerve stimulation and measured at the appropriate time can estimate the proportion of axons damaged in a partial nerve injury. A 50% reduction in the amplitude of the CMAP from stimulation recorded 7–10 days after injury suggests that 50% of the motor axons have been irreversibly damaged and rendered inexcitable. Finally, the rate of excitability loss after axon injury may be delayed in situations where fundamental processes of axonal degeneration are delayed (e.g., Wld^S mouse discussed in Chapter 3). All of these comments hold true whether CMAPs, NAPs, or SNAPs are considered. There are, however, some special issues concerning CMAPs.

The relationship between the amplitude of a CMAP and the proportion of injured axons changes with time. If denervation and reinnervation are prolonged, collateral sprouting of uninjured distal motor axons occurs and may appear to repair the CMAP. Residual intact motor axons may sprout to individually innervate large numbers of muscle fibers. There may therefore be a rise in the overall amplitude of the CMAP with little new motor axon regrowth.

With the above caveats, multifiber recordings can provide highly sensitive serial indices of nerve recovery. These include the time for reappearance of the first NAP or CMAP after complete injury, the amplitude of the potential at fixed time points after injury, and the conduction velocity of regenerating axons. Conduction velocity is reduced in newly regenerating axons because of their immaturity: their smaller radial caliber, incomplete remyelination, and perhaps their immature nodal apparatus.

The use of nerve conduction recordings in human disease is not the topic of this text and will not be covered further here. In rodents, the sciatic nerve is usually chosen as an accessible, well-characterized site of regeneration studies. Motor recordings can be made by stimulating the sciatic nerve at the sciatic notch and popliteal fossa with recordings of CMAPs over the interosseous muscle endplates of the hindpaw. These are recorded using percutaneous fine platinum electrodes inserted just beneath the dorsal skin of the paw. Near nerve temperatures are maintained by a subcutaneous subdermal thermistor and overhead heating lamp. In normal rats and mice, good-quality CMAPs with G1 (active, over the muscle endplate) and G2 (reference) recording electrodes placed over mid-dorsal paw (the endplate is approximately halfway along the paw) are recognized by low stimulation threshold and an initial negative (upward by North American convention) deflection. An initial downphase instead of upward deflection indicates poor active recording electrode (G1) placement. After sciatic nerve injury and before newly growing axons reconnect to the endplate, CMAPs are unrecordable. Overstimulation at this time, however, can yield artifactual distant direct muscle stimulation volume potentials. These are recognized by their higher stimulation threshold and abnormal shape.

Several approaches to estimating numbers of motor units in humans have been published (motor unit number estimation; MUNE). One approach involves using an incremental rise in stimulation intensity to identify "all or none" steps in the recorded CMAP amplitude. Each step represents the addition of a single motor unit and the amplitude of each step can be averaged. The MUNE is calculated as the total CMAP divided by the averaged amplitude of a single step increment [449]. Other approaches involve the use of multiple intramuscular recording electrodes (macro-EMG) that identify the contribution of individual motor units to the CMAP [68,97,146].

Needle electromyography (EMG), widely used in human laboratories, provides a further direct measure of electrophysiological activity in denervated muscle. It is therefore used to identify regeneration or lack of it. Denervated muscle fibers develop abnormal spontaneous firing because of fiber depolarization and redistribution of acetylcholine receptors (AchRs). Calcium accumulates in sarcoplasmic reticulum luminae. Abnormal spontaneous firing can be easily recorded by fine caliber needle electrodes during electromyography, a technique extensively described in other textbooks. Spontaneous potentials are recognized as "fibrillation potentials" or "positive sharp waves," two waveform configurations likely representing identical physiological information-spontaneous single muscle fiber discharges. In electromyographic parlance, fibrillations recorded with needle electrodes from humans with denervation are sharply contoured biphasic or triphasic potentials, 1–5 ms in duration, 20–200 μV in amplitude, and usually regular (1–30/s) in their firing rate [345] (also see Figure 6.3). Although their amplitude and firing rates may be quite variable, the density with which they are encountered by the recording electrode is correlated with the extent (severity) of denervation. The positive sharp wave, like a fibrillation potential thus represents a similar single muscle fiber discharge. Positive sharp waves are recognized by an abrupt downward (positive by convention) deflection with a gradual sloping recovery. Their altered waveform may arise because of the added impact of the needle electrode on the fiber being recorded from (angle of view, added injury component) [154,360,498,594]. There is a good correlation between the density of these potentials and the degree of denervation (complete, degrees of partial, or none). They disappear early during reinnervation from regeneration. Like CMAPs, fibrillations and positive sharp waves take several days (depending on the distance of the injury to the muscle endplate) to appear fully after an injury. In humans, EMG is also routinely used to assess the number and caliber of voluntary motor unit potentials that have regrown to muscle or have resisted injury. As discussed above, collateral sprouting in partial injuries enlarges the size of these individual motor units. In animal models, recording voluntary activity in an orderly way is obviously challenging.

Other electrophysiological techniques are also used to address regeneration. While more time consuming and requiring greater expertise, rigorous open approaches to motor unit recording combined with high quality motor unit force measures provide exquisite analysis of motor function. This approach, described by the Gordon laboratory [663] accurately identifies numbers of motor units and their degree of sprouting. Unlike CMAP recordings, they are not confounded by collateral sprouting or demyelination but instead accurately address both. The sciatic nerve and tibialis anterior, soleus, medial gastrocnemius, or plantaris muscles are exposed and connected to a force transducer using 2.0 gauge fine-braided silk threads. Twice suprathreshold sciatic nerve stimulation is applied to generate both the maximal evoked muscle twitch force and the tetanic force in response to 1, 5, and 21 pulses at 100 Hz at a repetition rate of 0.5–1.0 Hz. Single motor unit force is generated by stimulation of ventral roots L4 or L5 teased into rootlets of 5–10 motor axons and recordings are made of all or none responses. After sampling approximately 40% of the motor axon population, the motor unit numbers (numbers of motor axons populating the whole nerve) are calculated by dividing the whole muscle twitch force by the mean motor unit twitch force. An excellent correlation exists between motor unit size enlargement measured this way and the number of histologically identified collateral sprouts innervating adjacent denervated endplates after partial denervation.

Some laboratories have published work using F and H wave recordings. These are long loop potentials generated by stimulating a nerve and awaiting a potential that travels to either the axon hillock (F wave) or the spinal cord (H reflex), then back again. They are used to address proximal axon motor and sensory function, respectively, in neuropathy models. An F wave (Figure 4.3) is a motor potential, generated by several motor axons in which an applied stimulus, similar to that used to evoke a CMAP, also travels retrogradely (antidromic conduction) in the motor system. The retrograde discharge re-excites a pool of motor axons at the axon hillock, then travels back to the endplate to be recorded as a late potential. Since F waves represent only a proportion of the excitable motor axon pool, its usefulness for regeneration work is limited. F waves are recognized as potentials that are delayed after the CMAP and are smaller in amplitude.

In many instances, excluding rigorous open electrophysiological approaches, F waves have been mistaken for H reflexes, a potential generated by stimulating sensory axons. The H reflex is a long loop monosynaptic reflex akin to the deep tendon stretch reflex brought out by a neurologist's reflex hammer. It involves the recruitment of large myelinated sensory axons. Once activated, these axons synapse on motor axons in the spinal cord to elicit a response. Specific electrophysiological

characteristics must be present to accurately identify an H reflex: recruitment by low amplitude long duration currents, its appearance prior to that of the maximum CMAP, and its decline with higher stimulus intensities.

Evoked potentials from peripheral nerve stimulation and recording over central structures, like the spine or brain, offer yet another approach toward addressing reinnervation. The amplitudes of these responses are much more variable and do not strictly reflect the number of conducting peripheral axons. This technique is used less frequently.

Target reinnervation

Regeneration of peripheral nerves can be assessed by measuring the onset and extent of target tissue reinnervation. Examples include reinnervation of the epidermis by unmyelinated sensory axons and neuromuscular junction reinnervation.

Consensus guidelines have been developed for processing and interpreting human biopsy samples of the epidermis [377]. Most laboratories have used the axon marker PGP 9.5 in thick (30–50 micron thickness) sections and calculate the number of axons per epidermal length. Density measurements, the number of axons per unit area of the epidermis, can also be calculated. Epidermal axons can be identified by fine axon processes, labeled by PGP 9.5, extending upward 90 degrees to the axis of the skin. The classification of a "single" axon may require that it crosses the dermal epidermal junction. Epidermal axons thus originate from longitudinal axons coursing at, or just beneath, the dermal-epidermal junction. Other measures applied to epidermal axons include: fiber length, numbers of degenerating profiles ("bulbar" degeneration with profiles resembling degenerating axon endbulbs), and dermal measures of sweat gland innervation. Functional reinnervation of sweat glands can be measured by the subcutaneous injection of pilcarpine (5mg/kg) and measuring sweat droplet impressions made in a silicone mold of the hindpaw [499]. One sweat droplet identifies one sweat gland.

For motor axon reinnervation of neuromuscular junctions, immunohistochemistry can be used. For example, it is possible to double label axons (using neurofilament or ChAT) with α bunagarotoxin (or another label) used to label acetylcholine receptors at the endplate (see Figure 2.16). The number of total, innervated, or denervated endplates (those with or without an axon, respectively) can be counted. Sprouting of intact axons to reinnervate adjacent denervated endplates can also be classified, i.e., (i) pre-terminal in which a sprout arises prior to the normal neuromuscular junction of the axon; (ii) ultra-terminal where a sprout arises at the terminal; and (iii) nodal sprouting in which the sprout arises

from the first node of Ranvier proximally in the parent axon. These measures help to characterize the type and extent of the regenerative sprouting [664].

Retrograde, anterograde labeling

Regrograde labeling involves the application of an indicator substance, usually a fluorochrome, to the zone of regenerating axons. The indicator is taken up by axons at the injection site and then is retrogradely transported to the parent cell bodies. The numbers of cell bodies, in turn, reflect the number of neurons that project axons to the injection site. The injection site may be in the target tissue or in the nerve itself at a fixed distance from the injury. The most common indicators are fluorogold (FG), fast blue, mini-ruby, fluororuby (dextran tetramethylrhodamine), fluoro-emerald, diamidino yellow, HRP (horseradish peroxidase), lectins (wheatgerm-agglutinin, concanavalin-A, and others), biocytin/neurobiotin, latex beads, rhodamine-isothiocyanate, carbocyanines such as DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarboncyanine perchlorate), and others [351]. Since the concentrations and approaches vary with each indicator, it is important to refer to published material on their use. FG requires several days (3–4) to be retrogradely transported and label cell bodies. Fast blue (2%), FG (2%), mini ruby (10% solution of dextran, tetramethylrhodamine, and biotin), fluororuby (10% solution of dextran, tetramethylrhodamine) all labeled a full and comparable complement of motor neurons by 1 week when applied to freshly cut adult rat medial gastrocnemius nerve. The labels were applied for 2h in an otherwise sealed (except to the proximal nerve stump) polyethylene tube. Fluoro-emerald (10% solution of dextran, fluorescein) labeled about 10% fewer neurons. By 4 weeks, fast blue and FG labeling persisted, whereas other labels had declines in their expression. Only fast blue stained a near complete majority of motor neurons out to 12 weeks and 24 weeks [511]. For comprehensive reviews of tracing methods see Kobbert *et al.* [351] and Tonge *et al.* [684] (Figure 4.4).

There are additional pitfalls using retrograde labeling. It is important to ensure that the indicator does not spread significantly beyond the injection site where it might label other axons. In some conditions, axons may not take up the label (e.g., FG labeling can be optimized if the nerve trunk is cut and dipped into the source of the indicator). On occasion, uptake might be inexplicably poor and not visualized. Sometimes perineuronal satellite cells acquire the indicator and their labeling might obscure accurate counts of labeled neurons. There are also caveats to performing unbiased three-dimensional neuron counts, not discussed here. Physical or optical dissector counting methods are the techniques of choice [117].

The advantage of retrograde labeling is that it strictly identifies the number of parent neurons that contribute to new growth. This is important because it is

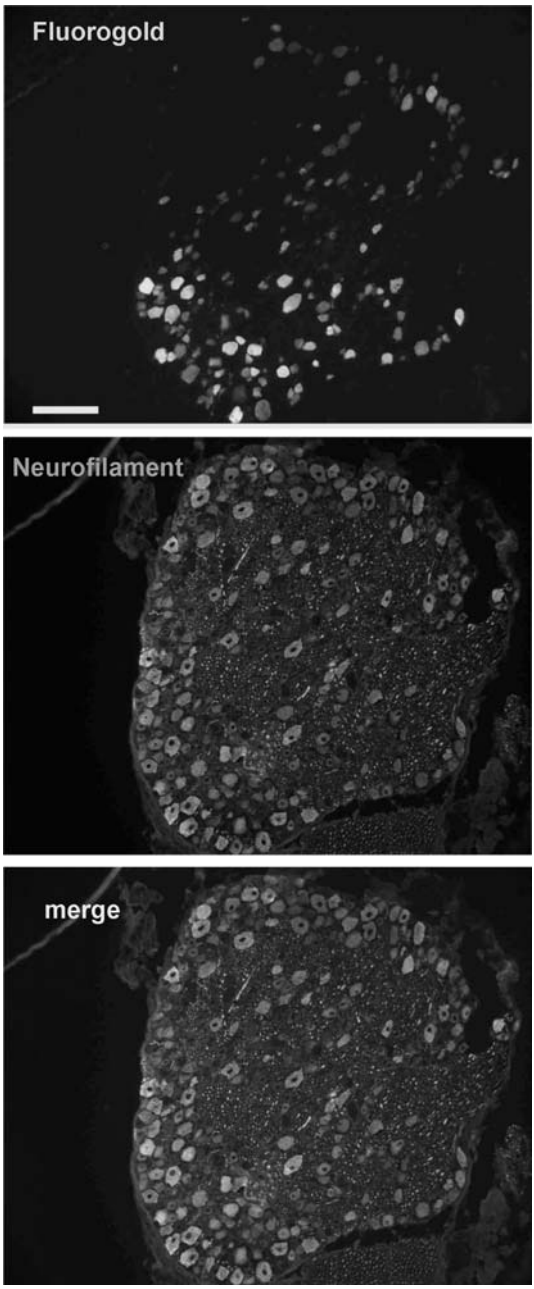

Figure 4.4 Images of DRGs retrogradely labeled with fluorogold (FG, blue) after a sural nerve injury. The sections are colabelled with an antibody against neurofilament (red). (Image taken by X.-Q. Li, Zochodne laboratory.) See color plate section.

possible that a single neuron and parent axon might generate several regrowing axons distal to an injury site. If only sections of nerve distal to the injury site are examined, it might be incorrect to surmise that all the axons observed are from independent parents. Since many injuries are also associated with retrograde loss of parent neurons, tracing approaches can help to identify this kind of loss. Thus, a pool of parent neurons that decreases in number after injury indicates specific retrograde loss of neurons supplying the nerve. It is also possible to examine abnormal innervation patterns by using two retrograde labels that fluoresce at differing wavelengths. For example, motor neurons that inappropriately send axons down incorrect cutaneous branches during reinnervation can be identified by applying a tracer to the incorrect cutaneous nerve and another to the correct motor branch [7]. Incorrectly projecting neurons may be distinguished from those normally reinnervating their appropriate nerve.

Anterograde labeling is also available to examine the distance of nerve regrowth. In one approach, neurobiotin tracer was injected into freshly cut nerve approximately 3 mm proximal to a previous zone of crush carried out 3 days earlier. The samples were harvested for labeling of the distal extent of axon growth 5 hours later (streptavidin cyanine 3 fluorescent marker) [716]. It is also possible to inject [^{35}S] methionine into the area of the cell body (e.g., anterior horn) and to measure radioactivity at specific distances from the injury site [73].

Pinch test

The pinch test is an older and less frequently used technique that examines the distance traveled by outgrowing nerves after a time frame is allotted for regeneration to begin [320,418]. The method involves exposing an injured nerve trunk, for example, in a rodent that is lightly anesthetized. Starting distally, a pair of fine tipped forceps is used to gently squeeze the nerve until it evokes either a twitch from a muscle remote from the injury site (e.g., abdominal muscle) or a vocalization. The distance between this site and the injury site (previously marked with a suture or ink) is measured with sensitive calipers. The resulting measurement provides the distance traveled by the fastest regenerating sensory axons.

While widely used in the past, the pinch test does have significant disadvantages. The site eliciting twitch or vocalization cannot be strictly ascribed as the location of the fastest growing axons. By pinching a nerve, even gently, the distortion of the nerve trunk may be capable of generating abnormal retrograde discharges (basis of the muscle twitch or vocalization response) of axons in a variable zone both proximal and distal to the regenerating axon tips. Abnormal architecture of the nerve after injury might complicate identifying which exact axons are sensitive to the forceps. Theoretically, the pinch test only measures

regrowth of sensory axons. It may also be the case that early regenerating sprouts are less electrically excitable and that only more proximal portions of new axons are capable of generating discharges. Accurate distance measures, particularly after short regenerating intervals, may be difficult to ascertain. Finally, the test deliberately distorts nerve trunks that require gentle handling for high quality histological studies. Pinch testing may thereby ruin histological studies.

Functional sensory recovery

Behavioral tests of sensory recovery provide an important index of peripheral nerve regeneration. All modalities of sensation can be tested in humans by standard neurological examination: sensitivity to light touch, pinprick, cold, vibration, and position. Quantitative approaches (known as QST for quantitative sensory testing) are also available. The most rigorous QST alogarithms, as developed by Dyck and colleagues, involve a blinded (subject) forced choice test approach. Details are reviewed elsewhere [158,161,623].

It is more challenging to assess all functional forms of sensory recovery in rodent models. There are protocols from the experimental pain literature describing testing for thermal sensation (thermal hyperalgesia) and mechanical sensitivity (allodynia or inappropriate pain behavior to a nonpainful stimulus). The recovery of sensation to a thermal stimulus after a peripheral nerve injury is measured by the latency to paw withdrawal from heat. A testing apparatus, designed by Hargreaves, provides a non-noxious heat stimulus and measures the time it takes for the animal to withdraw its paw [253]. Recovery of thermal sensation can be complex after nerve injuries. Early after injury, the withdrawal latency is prolonged or absent indicating sensory loss. If there is no response, the examiner should discontinue the heat stimulus in order to prevent injury. A heat stimulus, however, may not be confined to the nerve of interest. Since a significant volume of the hindpaw is heated, adjacent intact territories may also be stimulated, leading to withdrawal despite ongoing sensory loss in the index nerve. Collateral sprouting can extend the territory supplied by adjacent nerves, into the zone previously innervated by the injured nerve. Thus an apparently recovered thermal withdrawal may arise from collateral branches of intact nerves, not from recovery of the original lesion. Similarly, partial injuries can be associated with hyperalgesia (increased sensitivity) by intact adjacent nerves. In a given injury, therefore, understanding the contributions of all of these confounding factors may be difficult. Serial measurements can help to resolve some of this difficulty. These can identify, for example, an initial period of sensory loss followed by hyperalgesia, then finally a return to normal sensation. Finally, two technical considerations are important. First, animals should

undergo several sessions of testing to accustomize them to the experiment. Second, when testing unilateral injuries, the injured side should ideally be compared to the contralateral intact side and to the pre-injured state.

Recovery of mechanical sensation may be studied by the response of the rat or mouse hindpaw to monofilaments. Known as von Frey hairs, these can be lightly applied to the plantar surface of the hindpaw of a rodent while it is standing over meshed flooring. The animals do not require handling, but should be acclimatized to their housing and unique flooring. Other stress factors should be eliminated and training should be carried out before the experiment. The filament is inserted though the mesh and is pressed lightly against the paw until it begins to bend. Bending forces are calibrated for a range of filaments. For example, in humans a 10 gram monofilament (Semmes–Weinstein monofilament) applied to the surface of the toe is used to quantitate the severity of sensory loss from a neuropathy. In rodents, the withdrawal of the hindpaw to each monofilament can be graded by the examiner. For example, each hindlimb can be tested six times, and the number of times there is foot withdrawal for each stimulus is recorded. Allodynia is an exaggerated response to an innocuous stimulus such as a monofilament with a smaller bending force that does not usually provoke withdrawal. After peripheral nerve injury, allodynia, like thermal hyperalgesia, can develop because of collateral sprouting. Overall, however, monofilaments test a much more discrete localized zone of sensation than heat stimuli. As reinnervation proceeds and sensation returns, withdrawal occurs first to coarse monofilaments then to finer monofilaments.

Galtrey and Fawcett [208] studied median and ulnar nerve injuries (crush or transection) in the forelimb of the rat. Results of sensory testing in normal control limbs and in limbs 15 weeks after nerve injuries were provided. For the forelimb, the authors analyzed electronic von Frey tactile stimuli. Cold sensory testing was assessed with an ice probe made from a 1.5ml microcentrifuge tube placed in freezing water. The mechanical stimuli provided graded force stimuli, whereas the latency to withdrawal from cold was timed.

An alternative test of sensory reinnervation involves antidromic signaling. “Antidromic” refers to the propagation of an impulse along a sensory nerve from proximal to distal, the opposite of normal sensory transmission. The property underlies what is known as “neurogenic inflammation” in which peptides released from sensory axons are released in the periphery and cause both vasodilation and plasma extravasation (plasma leakage from capillaries). This feature can then be exploited to determine whether sensory axons have reinnervated the blood vessels and associated mast cells of the skin. Low frequency stimulation of the proximal sensory nerve thus activates distal sensory axons and causes them to release the neuropeptides (SP and CGRP among others) that mediate

antidromic plasma extravasation. If the animal is concurrently perfused with Evan's blue, blue discoloration will develop at the sites of plasma extravasation in the innervated skin. The technique thereby delineates innervated from denervated skin [45,305,354]. A related approach measures rises in skin blood flow in response to antidromic stimulation to detect reinnervation.

"Metabosensitive" afferent responses are recordings from sensory axons within muscles in response to fatiguing contractions, local infusions of potassium chloride, or infusions of lactic acid. When muscles are reinnervated, recovery of these afferent responses can be recorded in filament bundles of the proximal nerve trunk [9].

Functional motor recovery

There are a number of techniques available to test functional motor recovery in rodent models or humans. In rats and mice, quantitative measures of hindpaw function are widely used. Walking track analysis was described by de Medinaceli and colleagues [131]. The technique involves placement of the rodent hindpaw in photographic developer solution. The gait of the rat is then tested by having them walk on unexposed X-ray films where images of their hindprints will be made. Hindprints can also be made by placing the paw in other indicators such as India ink. Measurements are made from the hindprints to calculate a sciatic functional index (SFI) given as a percentage. The formula for calculating the SFI is: $SFI = [(ETOF - NTOF)/NTOF + (NPL - EPL)/EPL + (ETS - NTS)/NTS + (EIT - NIT)/NIT] \times 220/4$ (all in mm) where the following are measured from the hindpaw imprint:

ETOF = measure from the tip of the experimental foot to the tip of the following contralateral toes

NTOF = distance from the tip of the normal contralateral foot to the tip of the following experimental foot

EPL = experimental (E) paw footprint length

NPL = normal (N) paw footprint length

EIT = experimental paw distance between the center of the second and fouth toes

NIT = as above for the normal paw

ETS = maximum experimental paw toe spread between first and fifth toes

NTS = as above in normal paw

Zero percent (±11%) represents normal function and −100% represents complete nerve interruption. An example of footprint analysis is given in Figure 4.5.

Further reports have described modifications and variations of the SFI, not reviewed here. Their advantages over the original scale may be marginal. For median and ulnar lesions of the forelimb in the rat, Galtrey and Fawcett [208]

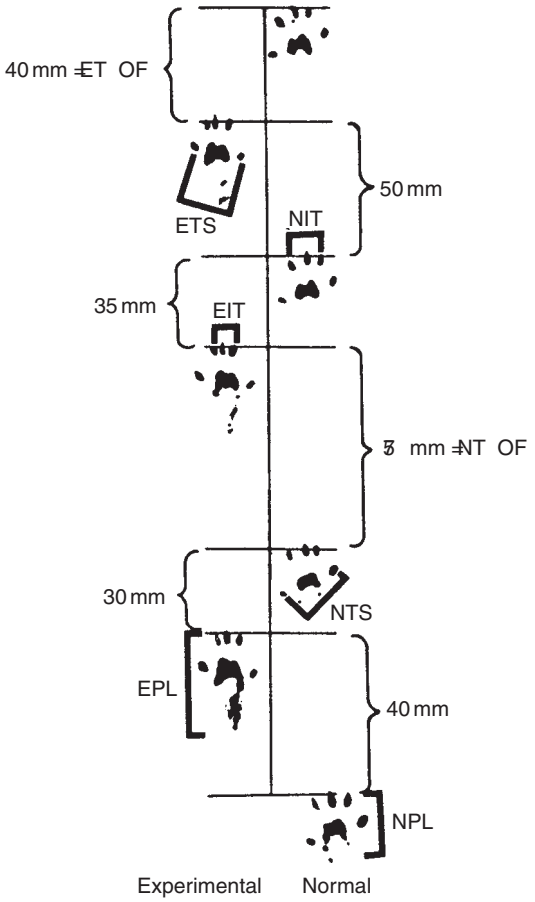

Figure 4.5 Examples of walking prints for analysis from rats with an injured sciatic nerve (left) compared to the contralateral intact nerve (right). (Reproduced with permission from [131].)

used a modification of SFI described by Ozmen *et al.* [540] termed pawprint analysis. Toe spread (intermediate ITS and widest TS) and print length are the critical measurements elicited. Overall, walking track analysis and pawprint analysis both provide crucial indices of recovery from nerve injury. It is important to recognize that it does not simply reflect motor recovery. Gait is a complex behavior that involves coordinated motor output linked to ongoing sensory afferent feedback. Sensory loss has a significant impact on walking. The most suitable tests for evaluating functional recovery after a peripheral nerve injury depend on the site and type of lesion. For example, Eberhardt and colleagues [162] used beam walking trials to test for recovery from femoral nerve injuries that denervate the more proximal quadriceps muscle. Accurate beam walking requires both intact motor and intact sensory function.

Sensitive strain gauges are used to evaluate the resistance of flexed front or hindlimb digits to pulling. The strength of digit flexion is measured as the tension at which the animal is able to hold on to a wire grid before releasing. A caveat is that longstanding denervation of the foot may generate flexion contractures of the paw. This fixed resistance must be controlled for in the measurements. Data for forelimb grip strength in rats are available [208], and the technique can also be applied to mice.

Specific forelimb tests of motor function have also been described to test recovery from median and ulnar injuries in rats [208]. The staircase test is a task that requires climbing and reaching for food pellets. The number of stairs climbed and the amount of pellet retrieval can be quantitated. The horizontal ladder test for rats involves walking along horizontal rungs to measure misses, slips, corrections, and partial placement of the forepaws on the rungs [208,463]. Most of the behavioral tests described rely on a training, acclimitization period before lesions can be studied. Like the SFI, forelimb motor behavior tests also depend on intact sensory feedback.

In vitro measures of nerve regeneration

An analysis of the outgrowth from harvested and cultured neurons can provide critical insights into regeneration. The high resolution provided by *in vitro* studies permits assessment of axon behavior, especially over short periods of time. Growth cone morphology, direction, and speed can be visualized and tracked. Similarly, retraction or withdrawal can be rapidly identified using video recording. Finally, *in vitro* approaches allow new molecular pathways to be rigorously explored before *in vivo* experiments. The disadvantages are obvious. *In vitro* studies do not reflect actual axon growth and navigation in complex multicellular tissues. Removal from a live animal pre-injures neurons and they are subsequently required to grow in artificial conditions. Since embryonic neurons grow at rates much faster than those of adult neurons, interpretation of their behavior should be guarded. Similarly, "neuron-like" cells such as PC12 pheochromocytoma cells that are easily grown allow exploration of early growth cone behavior. Findings in these systems, however, require confirmation in adult neurons prior to extending the approach *in vivo*.

By convention, outgrowing processes from neurons *in vitro* are labeled "neurites" because of the difficulty classifying them as axons or dendrites. Neurons plated at low density allow individual cells and growth cones to be observed. Different types of neurite outgrowth are important to examine. The percentage of neurons with neurites extending from them (process bearing neurons) reflect neurite initiation. Neurite outgrowth may be evaluated by

measuring "total" neurite length, the sum of the length of all neurites. Other measures include the mean neurite length and length of the longest neurite.

Gavazzi *et al.* [212] noted that neurite initiation and the total neurite length were trophic factor dependent but that the length of the longest neurite was not. Facilitated neurite outgrowth can be observed when specific growth factors are combined with basement membrane substrates (e.g., laminin and GDNF for IB-4 neurons [698]). These findings reinforce the caveat that measurements of neurite behavior depend heavily on specific culture conditions, including the substrate and whether specific growth factors have been added to the media.

A number of specific neurite counting approaches have been described. Mearow and colleagues [698] assessed neurite outgrowth at 24h after plating and individual tracings of neurons labeled with βIII tubulin were made using a camera lucida technique. Neurite initiation was defined as the percentage of total neurons having neurites twice the length of their cell body diameter. Only unambiguous neurons were chosen. A technique known as Sholl analysis was used to measure the number of intersection points of outgrowing neurons across 20 micron concentric circles radiating from the cell body. At least 50 neurons were traced for each condition studied. Most investigators also recommend that data sets include cells from several animals and that assays are done on different harvested plates and days.

Compartmented or "Campenot" sympathetic neuron culture dishes were described by Robert Campenot at the University of Alberta [89] (see review [705]). Neurons are harvested and placed in the center compartment of a culture dish. The dish is divided into compartments by a Teflon divider that adheres to its base with silicone grease. Cytosine arabinoside is added to kill nonneuronal cells and NGF is added to the neurons. Neurites grow out from the perikarya in the central compartment, under the silicone grease barriers and enter the side compartments. As a result, the center compartment contains the perikarya and the side chambers contain axons so that the compartments isolate axons from the perikarya. The technique has been a powerful tool to understand the behavior of axons independent of their cell body. Differences in signaling, protein synthesis, or lipid synthesis have been examined (see [344]).

Explants

Explants are resected whole DRG or sympathetic ganglia placed in a media. From these cultured ganglia axons emerge and can be examined. Explants therefore retain the multicellular milieu of a whole ganglia and are easier to examine than dissociated single neurons. The original investigations of Levi-Montalcini and Hamburger leading to the discovery of NGF used sympathetic

ganglia explants to evaluate axon outgrowth. More recently, several innovative explant approaches have been used to study regeneration. Torigoe *et al.* [687] placed transected nerve stumps connected to their ganglia between two sheets of plastic film and examined outgrowth of axons and SCs for up to 4 days later. Axons were identified by silver nitrate impregnation and SCs by S-100 immunohistochemistry. "Reactive" SCs from a previously injured contralateral nerve could be added to enhance outgrowth. In a related approach proximal stumps and DRGs of transected intercostal nerves were placed into shallow gels of Type I collagen [381,685]. Axon outgrowth from the cut end was identified using PGP 9.5 labeling and dark-ground illumination [381].

A frog preparation used resected sciatic nerves and their DRGs placed into a culture system resembling that described by Campenot [685]. The connected ganglionic and axonal components were isolated in different chambers and the distal axon was crushed. To determine later axon outgrowth distance, L-[4,5-^{3}H]leucine was added into the ganglion compartment and axonal transport was allowed to proceed for 24 h. The labeled regenerative front was identified by autoradiography. Advantages were that the preparation could be carried out at room temperature and survived without serum or growth factors for at least 12 days. A similar preparation was described using mice with intercostal nerves or sciatic nerves and their DRGs attached (L4,5 for the sciatic preparation). The mammalian explants, however, were not viable for as long as those of frogs.

Collateral sprouting

Collateral sprouting differs from regenerative sprouting because it requires intact axons to sprout branches into denervated sites. Thus, no injury response of the parent axon is involved. Since many types of nerve damage involve irretrievable loss of the entire neuron tree, collateral sprouting from intact residual neurons can be essential to restore aspects of function. Diamond and colleagues [139,141,149,283,301,508,677] have described an elegant approach toward assessing collateral sprouting in denervated skin. The technique involved preserving a sensory nerve adjacent to denervated skin. Thus, the skin of one side of the dorsum of a rat is denervated by sectioning four dorsal cutaneous nerves rostral and caudal to T13, the lateral branch of T13 and four lateral cutaneous nerves supplying adjacent flank skin. A single nerve territory T13, adjacent to these resections is preserved and mapping of the skin area retaining sensation in its innervation territory can be carried out. This map includes sensation of the skin to pinch (mechanosensitive Aδ fibers), and to a 60°C heat probe (heat nociceptive C fibers). In lightly anesthetized rats, the pinch and heat sensations can be categorized as present or absent depending on a skin twitch of the

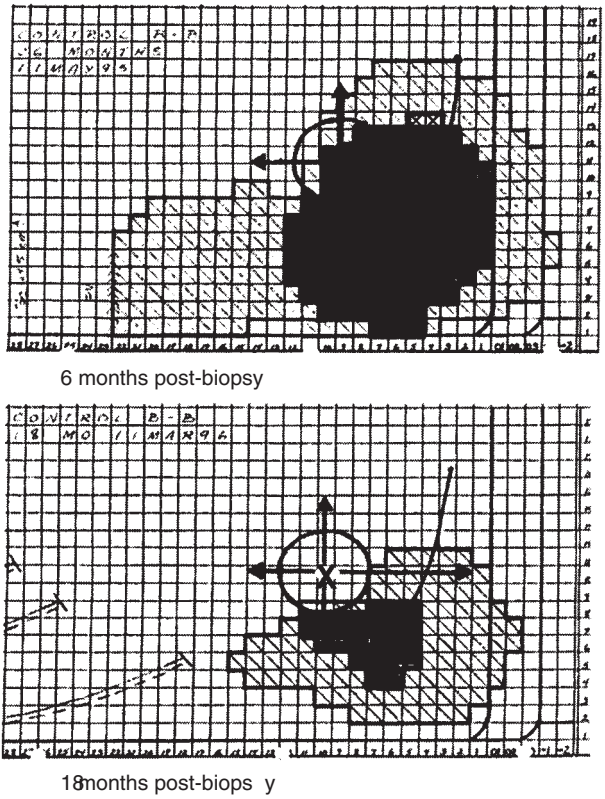

Figure 4.6 Maps of the areas of sensory loss from the side of the foot in a patient that had undergone a diagnostic nerve biopsy with complete resection of the sural nerve at the side of the lower leg. The black areas represent complete loss of sensation to pinprick and the hatched areas outside of this represent partial loss of pinprick sensation. Over time (bottom panel) the area of sensory loss has gradually retracted, accounted for by collateral reinnervation from intact territories adjacent to the denervated skin. (Illustration by M. Theriault and reproduced with permission from [678].)

underlying cutaneous trunci muscle. The map may identify hyperalgesia in some experiments. Sensation to light touch is identified by recording impulses from large myelinated Aβ axons of the dorsal cutaneous nerve in response to brushing the skin with a fine bristle. Collateral reinnervation involves expansion of sensitivity over time from T13 into surrounding areas that have been denervated.

A related approach was used to document the area of sensory loss after sural nerve biopsies in patients. Using light touch and pinprick, the areas of sural nerve sensory loss or sensory attenuation were mapped on the side of the foot and ankle. Serial measurements were used to determine how recovery occurred. Over time, a symmetrical contraction, from the edges in, of the denervated zone was identified indicating collateral reinnervation from adjacent intact nerves [678] (Figure 4.6).

Summary

Rigorous assessment of peripheral nerve regeneration involves unique techniques and interpretation. *In vivo*, morphological assessment of axons at fixed distances beyond an injury zone using epon- or plastic-embedded semithin and thin sections prepared in iso-osmolar fixative is a gold standard approach. It provides information about the number, density, and maturity of outgrowing myelinated axons under light microscopy and unmyelinated axons using electron microscopy. Careful immunohistochemical approaches that analyze individual axons at early time points during regeneration can address the amount and distance of early regrowth. Retrograde techniques are similarly very important in addressing whether retrograde loss of parent neurons or multiple daughter axons have emerged. Electrophysiology allows serial assessment of regeneration of motor or sensory axons. *In vitro* approaches including dissociated neuron cultures or explants allow testing of early molecular determinants of growth cone behavior. Finally, behavioral assays of motor and sensory function are essential in addressing the success of functional recovery. New high resolution imaging techniques capable of identifying the extent of axon outgrowth *in vivo* offer exciting future prospects.

Suggested reading

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Diamond, J., Gloster, A., & Kitchener, P. (1992). Regulation of the sensory innervation of skin: trophic control of collateral sprouting. In *Sensory Neurons: Diversity, Development and Plasticity*, ed. S. A. Scott. Oxford, UK: Oxford University Press, pp. 309–332 [140].

Tonge, D., Edstrom, A., & Ekstrom, P. (1998). Use of explant cultures of peripheral nerves of adult vertebrates to study axonal regeneration *in vitro*. *Program Neurobiology*, **54** (4), 459–480 [685].

Appendix 4a

Protocol for fixation and processing of nerves for semithin and thin sections (adapted from Dyck Laboratory)

Fixation: Place harvested tissue on a stick (e.g., tied at one end gently to a toothpick) and store in small vial (i.e., 1–3 ml) of 2.5% glutaraldehyde buffered by 0.025 M cacodylate (pH 7.4) solution. One end of the stick may be tied with a suture for orientation. Fix for 6 hours to overnight. Wash the nerve for 30 minutes in 6 changes (5 minutes each) of fresh solutions of 0.15 M cacodylate buffer (pH 7.4). Tissues may be cut afterward in smaller pieces prior to osmification (cut distal

angled and proximal straight with a fresh, sharp scapel blade). Tissues in 0.15M cacodylate buffer can be stored for long periods.

Osmification: Prepare osmium by breaking 1 vial of osmium tetroxide into 25ml of ddH_2O. Let osmium dissolve for 3–5 days. Keep cool. Do not allow solution to turn black (should be yellow to light brown). Dissolved osmium may be stored in the refrigerator for approximately 2 weeks or until it darkens (dark brown/black). Osmify samples after the rinse step above. Mix 2% osmium tetroxide in 0.12M cacodylate for 2.5 hours, then wash for 30 minutes (6×5 minutes) in 0.15M cacodylate.

Dehydration: Pipette off storage buffer and rinse with 70% ETOH. Maintain in this for 8 minutes. Pipette off 70% ETOH and rinse with 80% ETOH. Maintain this for 8 minutes. Repeat rinses, each for 8 minutes with 95%, 100% (absolute ethanol), repeat 100%, propylene oxide, and repeat propylene oxide.

Infiltration: Mix epon/propylene oxide solution (50% epon/50% propylene oxide; see below). Add ~1ml of 50/50 mix to each sample and allow to infiltrate over night in a fume hood (~12 hours) and to allow the propylene oxide to evaporate.

Embedding: Two types of epon mixture are used. Mixture A is Epon 812 (Jembed, Canemco, St. Laurent Quebec) 31ml with DDSA (Dodenyl succinic anhydride; Canemco) 50ml. The second, Mixture B, is Epon 812 100ml and NMA (Nadic methyl anhydride; Canemco) 89ml. The solutions are kept for one hour at room temperature for use. All glassware should be dried thoroughly. In a 50ml graduated cylinder 15ml of Mixture A and 35ml of Mixture B is then placed in a beaker. Next 1ml of DMP-30 (Tri-[dimethylaminomethyl] phenol; Canemco) is added, the mixture covered with foil and mixed for 10 minutes with an egg beater. Let epon sit until all bubbles settle out. *Note:* It is only worth preparing mixes A and B in advance when doing large numbers of samples over the space of a week. Otherwise it is simplest to prepare mixes the day of use. The tissue is dipped through two changes of epon and then embedded in a block mold. Epon should be used within 4 hours of mixing to ensure freshness. Fill each mold (e.g., 6 sample mold) with 1mm layer of epon, then place the sample in the narrow end of the mold. The specimen should be oriented in the mold so that the end of interest faces the narrow face where sectioning will occur. The mold should be filled generously.

Baking/Curing: Put filled molds in an oven preheated to 45°C. Check the samples after 12 hours for alignment, then bake for approximately 24 hours. Next increase temperature to 65°C on second day and allow another 24 hours or until blocks are sufficiently hardened. Allow the blocks to cool, then remove from molds. Do not overcook the blocks. Appropriate hardening lock is hard enough when you can lightly mark it with a finger nail by pressing firmly. (For all procedures, technicians should be gloved with appropriate eyeware, clothing protection during preparations and procedures should be carried out in a functioning fumehood.)

Sectioning: Trimmed narrow faces of epon blocks can be sectioned, using an ultramicrotome, at 0.5–1.0 microns in thickness. Sections are placed on glass slides, stained with toluidine blue, and coverslipped.

5

Early regenerative events

In this chapter I discuss the earliest events of peripheral nerve regeneration. What will emerge from this information is a remarkable panoply of cells and molecules that come together for regrowth to occur. Considered separately, the anatomical events, the signaling interactions, and how they influence each other speak to a coordinated beauty. While the anatomical, or cellular features of early nerve regrowth have been described for many years, the molecular cascades involved are only now being considered. Some of this information, such as growth cone signaling and guidance, has not been examined in the specific situation of peripheral nerve regrowth. Nevertheless, there is strong evidence that it eventually will be identified as important in peripheral neurons as in the experimental and central systems studied to date. Thus, some of the material presented relates to the general neurobiology of the growth cone and axon growth, only summarized here. Seen as the forest, rather than the trees, however, it is a critical part of the peripheral nerve regeneration story.

Early axonal events

Immediate responses to injury

When an axon is physically disrupted, a rapid series of molecular events ensues over the next seconds, minutes, and hours. Injured axons undergo rapid depolarization, generating immediate retrogradely propagated "injury potentials." These critical signals, perhaps associated with a calcium wave, from distal axon to proximal portions of the neuron, may be the first to initiate a shift in its properties. That is, the neuron begins to relinquish its role as a stable transmitter to one primed for regeneration.

Transected axons quickly (within hours) seal their ends after transection to prevent further egress of axoplasm. Rapid axon repair requires new membrane synthesis either by local means or from transport along the length of the axon. Not all injuries involve disruption or transection of the axon membrane. Crush or stretch inflict similar types of damage to individual axons without actual physical separation of the axon membrane. Clearly, more than resealing is required to reverse the immediate consequences of injury. This may involve the supporting lattice of the axon, for example, the neurofilament and microtubule networks. Shattered cytoskeletal proteins may disrupt axoplasmic transport and membrane activity that influence recovery. Along these lines, axons from transgenic animals lacking neurofilaments may actually be harder to crush [176]. Whether physical transection or damage from crush or stretch occurs, there is irreversible and irreparable disruption. Downstream axonal Wallerian-like degeneration ensues even if the proximal and distal stumps of the individual axons remain connected. The trigger for axon demise may be transient, such as the calcium wave described, but may be sufficient to activate the machinery for active Wallerian degeneration (see Chapter 3). When peripheral nerve trunks are transected, retrograde degeneration to the first node of Ranvier in the proximal axon also occurs. The retrograde reaction is associated with all of the changes described in distal Wallerian-like degeneration including SC activation and macrophage invasion. Consequently, new axons that grow from proximal stumps are required to make up some lost time to arrive at the injury site!

The distal ends of transected axons undergo ultrastructural reorganization early after injury. In the first few hours after transection, microtubules are first disassembled, then reorganized to form "traps" where vesicles accumulate [171]. As will be discussed below, microtubules are polar longitudinal structures with a + and − end. In intact axons, the microtubules point in one direction with the + end directed distally. Following injury, however, their orientation changes. At 50–150 μm proximal from the resealed axon tip at the injury site, + ends of microtubules reorientate and point to one another instead of pointing in one direction. This is called a positive "trap" and it is associated with collections of arrested anterogradely transported vesicles. These vesicles help to form the growth cone organizing complex (GCOC). Subsequent growth cone formation thus requires that the GCOC fuses anterogradely transported vesicles to provide membrane material. Golgi-derived vesicles are also associated with the GCOC. A separate minus "trap" found more distally in the axon is associated with − ends of misoriented microtubules. Minus "traps" help to segregate "old" membrane material away from the positive "trap."

Table 5.1 *Some regeneration related proteins*

Protein	Alternative name	Role
AIP	Actin interacting protein-1	Helps cofilin to depolymerize F-actin
Akt	Protein kinase B (PKB)	Signaling and outgrowth signal from PI3K
APC	Adenomatous polyposis coli	Tumor suppressor protein, interacts with microtubules
Arp 2/3	Actin-related protein	Promotes actin formation
Cofilin	Actin depolymerizing factor (ADF)	Involved in both polymerization and depolymerization of actin
CDC42		Promotes growth cone extension
CRMPs	Collapsin-response mediator proteins	CRMPs 2,4 are known to inhibit outgrowth
Dhh	Desert hedgehog protein	Involved in nerve fasciculation
GAP43/B50	Growth-associated protein 43/B50	Actin interacting protein
GSK3β	Glycogen synthase kinase beta	Suppresses axon formation
Katinin		Microtubule severing
IL-6	Interleukin-6	Promotes regeneration over myelin inhibitors
LIM kinase		Inactivates cofilin and blocks growth cone advance
mTOR	Mammalian target of rapamycin	?branching (promotes dendrite arborization through PI3K)
NRG	Neuregulin	Signal from axon to SC
NF-κB	Nuclear transcription factor kappa B	Survival transcription factor that is upregulated and translocated to nucleus after axotomy
PAK	P21-activated kinase	Effector of Rac to facilitate growth cone extension
PI3K	Phosphatidylinositol-3 kinase	Survival and outgrowth pathway
PKC	Protein kinase C	Intracellular signaling molecule (multiple roles, subtypes)
Profilin		Influences actin polymerization; complex actions
PIP2		Inhibits cofilin actin binding
PTEN	Phosphatase and tensin homolog deleted on chromosome ten	Suppresses PI3K signaling and increases unphosphorylated GSK3β mediated growth cone collapse
RAC1		Promotes growth cone extension
ROK	RHO kinase	Promotes growth cone collapse
RHOA		Promotes growth cone collapse
Sema3A	Semaphorin 3A	Promotes growth cone collapse
Slingshot		Activates (dephosphorylates) cofilin and promotes outgrowth
ZAS3		Zinc finger transcription factor that competes with NF-κB

Axonal endbulbs and their constituents

Later following axon transection more dramatic changes in the structure of proximal axons develop (Figure 5.1). Within 24–48 hours after injury the proximal ends of damaged axons swell, creating structures known as *axon endbulbs or boutons*. While it is possible that endbulbs develop from plus "traps" they develop later than the microtubular changes described above. First described by Cajal [572] as "sterile clubs" or necrotic profiles, endbulbs are larger structures. They are also to be distinguished from "clubs of growth or buds" that represent new growth cones. Unlike a normal axon terminal, endbulbs exhibit defective "turnaround" of material from antegrade to retrograde transport. Ongoing anterograde axoplasmic transport continues unabated and endbulbs form in swollen axons that contain axoplasmic material, depolymerized neurofilaments, but also apparently functional molecules [153,192,193,393,572,788,800]. Some endbulbs have complex arborizations (helicoids or ring like) of neurofilaments within them that Cajal thought were precursors of outgrowth, and called "structures of *Perroncito*" named after a nineteenth-century anatomist. Axonal endbulbs also persist, especially if regeneration is frustrated, and they can be identified indefinitely after some injuries. For example, in the CNS, they have been identified in brain white matter for years after axonal shear injuries from trauma.

Endbulbs are more than simple pathological curiosities. Besides structural proteins, they also house functional peptides and enzymes. Cajal suggested that endbulbs undergo slow dissolution and that intact viable axons of the proximal stump withdraw contact from them. Once no longer confined to an axon interior, molecules released from the endbulbs are free to influence other cells in the vicinity of the damaged nerve trunk. Thus, the neuropeptide CGRP (calcitonin gene-related peptide) is probably slowly released from endbulbs, and when in the extracellular space behaves as a potent vasodilator of local microvessels [784,788]; it can serve as a mitogen for Schwann cells [111] and it may act synergistically with SP (Substance P) to facilitate local ectopic discharges that initiate neuropathic pain (Figure 5.2). Other peptides have been identified in axonal endbulbs including SP, NPY, and galanin [788]. Furthermore, endbulbs are sites of aberrant sodium channel accumulation, which are also linked to the generation of ectopic axon impulses and pain [135]. Endbulbs, however, also express μ opioid receptors, normally associated with the dorsal horn of the spinal cord. As in the spinal cord, peripheral μ opioid receptors in endbulbs are capable of dampening pain signaling [694] (Figure 5.2).

Axonal endbulbs also accumulate isoforms of nitric oxide synthase (NOS), both eNOS (endothelial NOS) and nNOS (neuronal NOS) [393,394,800]. While nNOS has long been described in neurons and is upregulated after injury, eNOS was

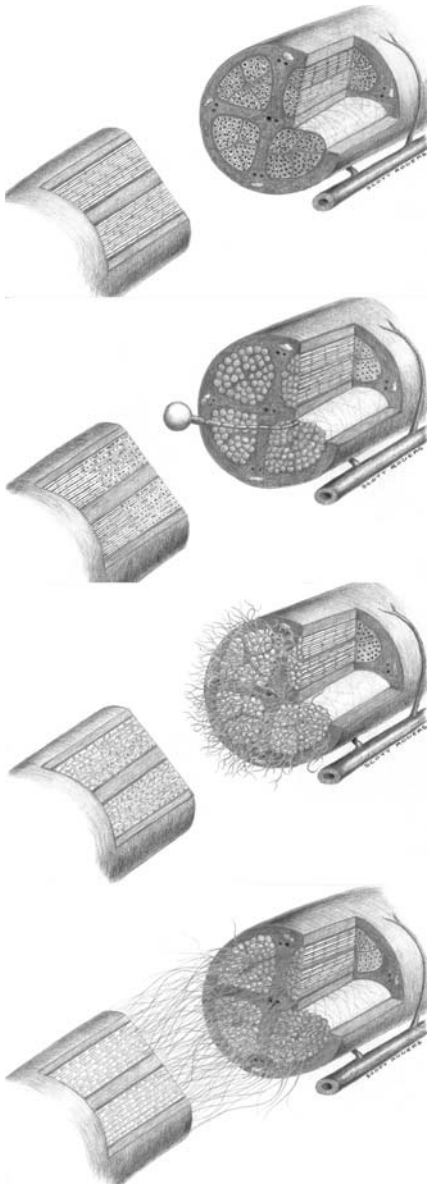

Figure 5.1 Illustration of the sequence of events during early regeneration from a nerve trunk transection injury. The figures from top to bottom show transection, followed by axonal endbulb formation, early regenerative sprouts, and bridging of the transection zone. (Illustration by Scott Rogers.) See color plate section.

once thought to be exclusive to endothelial cells. eNOS has, however, been identified in CNS neurons and PNS sensory neuron perikarya [263,667]. Accumulations of NOS within axonal endbulbs act as a local enzymatic source of nitric oxide (NO), a highly diffusible free radical that can easily spread beyond

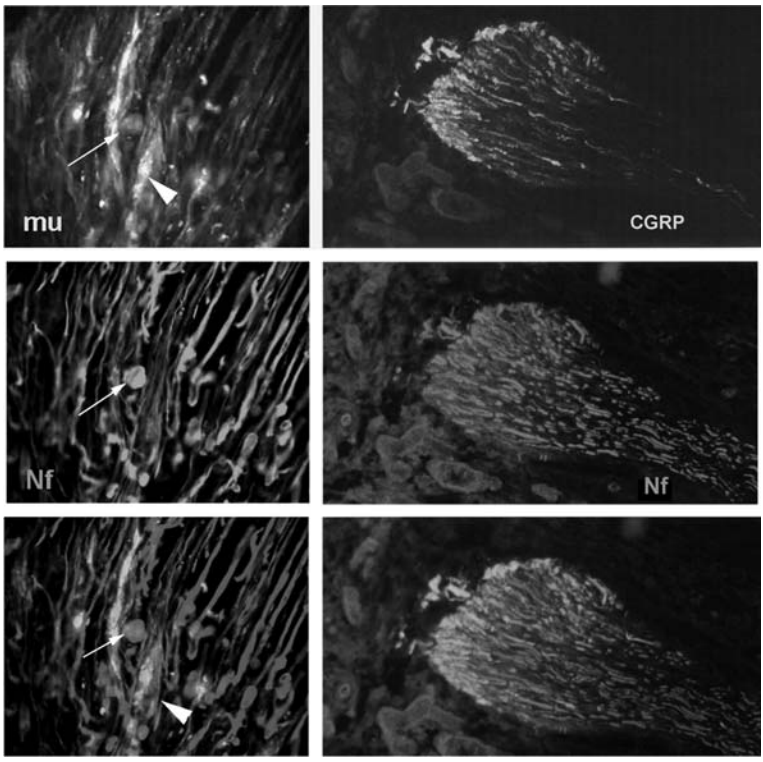

Figure 5.2 Images of axonal endbulbs using immunohistochemistry to label their specific constituents. The panels on the left show discrete (arrow) and irregular (arrowhead) endbulbs proximal to a chronic constriction injury lesion that contain the mu opioid receptor, double labeled with an antibody directed against neurofilament (Nf). The panels on the right show CGRP containing endbulbs proximal to a sciatic nerve transection also double labeled with neurofilament. (Images reproduced with permission from [694] and from [788].) See color plate section.

the confines of the axon. Local NO release may be associated with several actions in injured nerves including the facilitation of ectopic pain discharges, the dilatation of local blood vessels or the alteration of local growth cone behavior. For example, NO is capable of collapsing growth cones [265] but, in some instances (e.g., during development), also signals axon growth through cGMP [611]. Overall, NO may influence several aspects of regeneration depending on the timing and extent of its release. In a preliminary study, systemic administration of a broad spectrum NOS inhibitor improved indices of nerve regeneration in mice [803].

In summary, axonal endbulbs and their constituents can alter the nerve trunk microenvironment within days of an injury. The early axon–microenvironment interactions associated with endbulbs may in turn influence cardinal properties of nerve repair: microvascular control, local cellular proliferation, and the induction of pain.

Sprouting

Early axon growth cone sprouting can occur within hours after injury, even when the axons are no longer connected to their cell body. Membrane protrusions from neurons form the earliest outgrowths. The term "neurite" refers to early outgrowths that are either axons or dendrites, indistinguishable at this stage. While the full repertoire of molecules involved in early neurite formation remains to be defined, one protein molecule that promotes directional neurite outgrowth is protrudin. By interacting with other membrane proteins such as Rab 11, protrudin helps to determine the direction of membrane sorting and trafficking [620].

How axons acquire new lipids to manufacture membrane is important in determining the rate of outgrowth (see review [705]). Lipids may be transported in vesicles by anterograde fast axonal transport (microtubule dependent, 400 mm/day) or may be transported on cholesterol- and spingolipid-rich lipid rafts. Not all constituents of the outgrowing axon and growth cone need to be manufactured at the cell body. For instance, phosphatidylcholine, the major membrane phospholipid is locally synthesized in axons in a process that is essential for axon outgrowth [705]. Lipid may be added all along the length of the neuron from cell body, axon to the growth cone. In contrast, cholesterol synthesis may be confined to the perikarya, or cell body. Apolipoprotein E may facilitate lipid delivery [705].

After an injury *in vivo*, 1 to 2 days is required before axon extension from an injury site is detected. This form of outgrowth does not occur without a connection to the cell body [586]. Most sprouts from myelinated axons originate within the retracted first node of Ranvier, and some from the second [195]. Friede and Bischhausen performed a detailed subserial ultrastructural analysis of five myelinated axons transected 72 hours earlier. Sprouts were detected within the first day. Three of five axons had extensive sprouting, whereas the remaining two had large axonal endbulbs without sprouts (Figure 5.3). This uneven response to injury is an important finding. Additional findings included an orderly microtubular proliferation (tenfold rise in numbers while retaining their axial orientation) in the proximal axon, and rises in the density of axoplasmic organelles such as mitochondria and smooth endoplasmic reticulum.

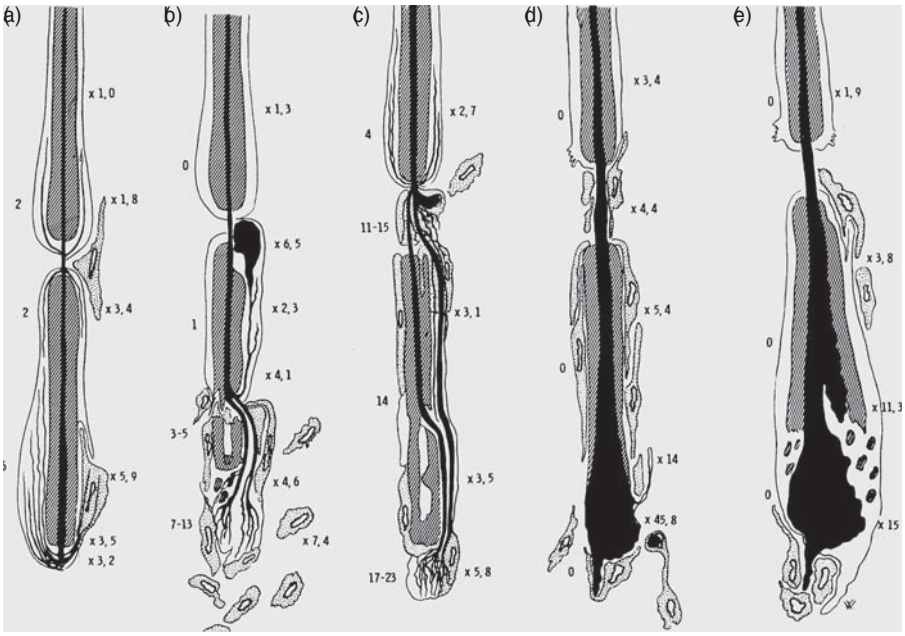

Figure 5.3 Responses of axons proximal to a rat transection injury (72h earlier) site based on detailed ultrastructural observations by Friede and Bischhausen. The parent axon is highlighted in black. Note the variation in growth patterns with some axons forming multiple sprouts at either distal sites or from proximal nodes of Ranvier. The two fibers on the right have formed very few sprouts but demonstrate prominent endbulb formation. (Reproduced with permission from [195].)

Specific regenerative responses to injuries

Crush injuries regenerate much more successfully than transection because new axon sprouts can easily regenerate into basement membrane tubes beyond the injury site (residual of previous fibers) (Figure 5.4). These tubes are associated with proliferating SCs in the distal stump and are otherwise known as *Bands of Bungner* [47,153,425]. Alternatively, transection requires that sprouting axons make their way across a gap (the nerve is under tension and a gap results from the injury) to find an appropriate landing site on the distal nerve stump. As a common clinical problem, transection is a severe nerve injury that poses major limitations on full nerve regrowth. The severe hesitancy or restraint exhibited by early ingrowth of axons into regenerative bridges across nerve transections is in itself remarkable. Very few axons, in comparison with the large population residing in the proximal nerve stump, seek to enter initial

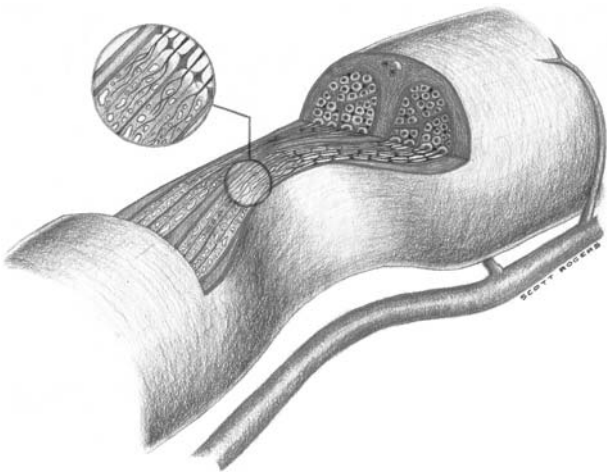

Figure 5.4 Illustration of early axon sprouting following a nerve trunk crush injury. Note that each parent axon can send multiple daughter sprouts. (Illustration by Scott Rogers.) See color plate section.

connective tissue bridges or cables that link proximal and distal stumps. The explanation for the meager outgrowing axon population in this scenario is unknown. Certainly, a suprising and substantial hostility to peripheral axon regeneration exists.

When a peripheral nerve transection injury has been directly or primarily sutured by a surgeon, regenerating axons do not automatically identify their appropriate and previous pathway. Microscopic re-anastamosis for nerves by surgeons regrettably has not solved this problem. Witzel and colleagues [744] describe an "evolving menu of molecular cues upon which to base pathway decisions." Their work describes how such axons fare when they express a green fluorescent protein (thy1-YFP) to highlight their regeneration trajectories. Axons must decide which distal stump Bands of Bungner to enter. They arborize, travel laterally across the stump face and can have a choice of up to 100 Bands (for a single regenerating axon) to choose from (Figure 5.5). Thus, local, rather than distal target cues determine their fate and axons make "wrong-way" decisions often to inappropriate fascicles and targets. Some axons grow retrogradely. Other axons can be observed to undergo Wallerian-like degeneration, illustrating the process of "pruning." Not all axons emerge from the proximal stump at the same time. A small subset of early axons begin to enter the distal stump, later followed by others, indicating "staggered" regrowth.

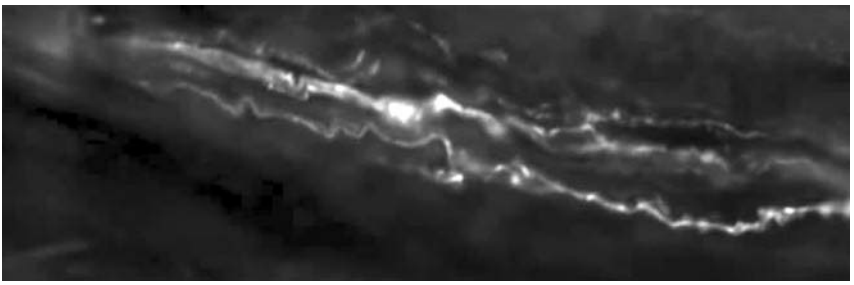

Figure 5.5 Images of axonal sprouts after a crush injury of the rat sural nerve using immunohistochemistry to label CGRP. CGRP is displayed prominently in early regrowing axons. (Image taken by X.-Q. Li, Zochodne laboratory.) See color plate section.

The growth of axons after crush injury is usually given as 1–3 mm/day, a value linked to the rate of slow axoplasmic transport [200]. New axon sprouts emerge from the proximal border between viable axon and degenerating axon, often, as mentioned, at a node of Ranvier [195,572,610]. After nerve transections, rates of regeneration are much slower. For example, after nerve transection and primary repair with sutures, the fastest axons grew at rates of only 0.84 mm/day [744]. Regeneration, however, is uneven and the *average* rate of all axon growth after these more severe injuries is yet much slower than the rates given above. As gleaned from the above discussion then, a number of important criteria determine whether regrowth will match ideal levels. Many additional barriers exist during later regrowth.

Staggered outgrowth

One might predict that regrowth of thousands of new axons within a peripheral nerve trunk would take place in a uniform style when they are injured simultaneously. This is not the case. "Staggered" outgrowth refers to the property of axons to grow with different onsets and rates. This property was first described in motor axons, regrowing through a zone of transection and resuture [7]. It has also been observed when axons enter into a regenerative bridge connecting transected nerves. Ingrowth is slow and hesitant, with only a small proportion of axons entering the bridge at any given time. In the nerve trunk distal to the injury site, regenerating axons can illustrate great variability in their maturity because of their nonsychronized regrowth. Axons of different levels of maturation, some newly myelinating, others unmyelinated are observed [450] (Figure 5.6). Why "staggered" outgrowth occurs is uncertain. It has been suggested that "pioneer" axons lead the way through zones devoid of obvious guidance posts encouraging new axons to follow.

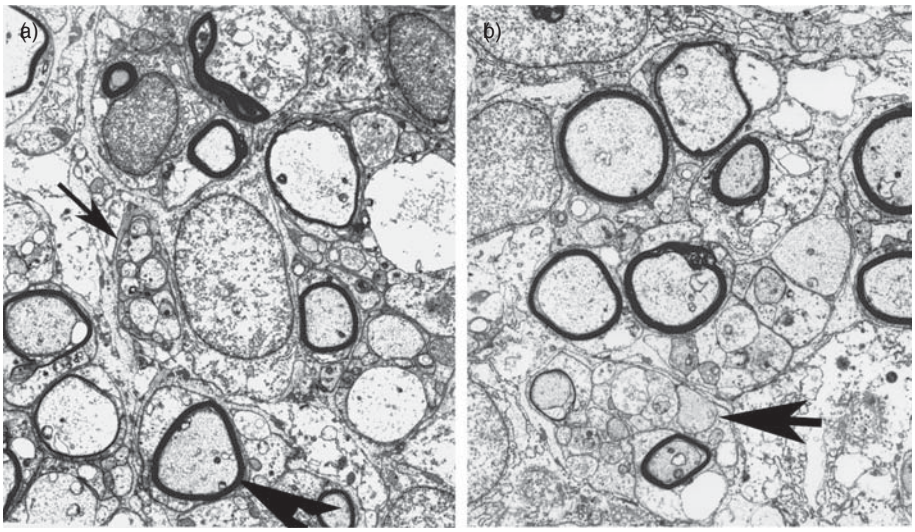

Figure 5.6 Electron micrographs of regenerative bridges traversing a conduit after a rat sciatic nerve transection injury. The micrographs illustrate wide variation in the maturity of the outgrowing axons because of "staggered" regeneration from the proximal nerve stump. On the left panel, the arrow points to a cluster of unmyelinated axons surrounded by SC cytoplasm and basement membrane. Contrast this appearance of closely packed axons with the Remak bundle from Figure 2.8. The arrowhead on the left shows a thinly myelinated regenerating axon sharing its SC processes with at least two umyelinated axons, described as a Type I regenerating unit by Morris *et al*. (see text). On the right panel, the short arrow points to a regenerative cluster containing two small myelinated axons and a number of unmyelinated axons. (Images taken by D. McDonald and reproduced in part with permission from [450].)

Growth cones and peripheral neurons

An overview of growth cones

Growth cones are the exploratory hands of axons of the regenerating peripheral nervous system (Figures 5.7, 5.8). Their properties are not the sole determinants of regeneration *in vivo*, but do have a major influence on how subsequent regeneration will fare. In complex tissues, growth cones continually sample their environment through extension of filopodia, retraction, and turning. Detailed considerations of growth cone neurobiology are found elsewhere [228]. In this section, we will briefly cover some relevant pathways and molecular players. Classical growth cones consist of motile finger-like filopodia largely built around actin filaments that emerge from web-like lamellipodia

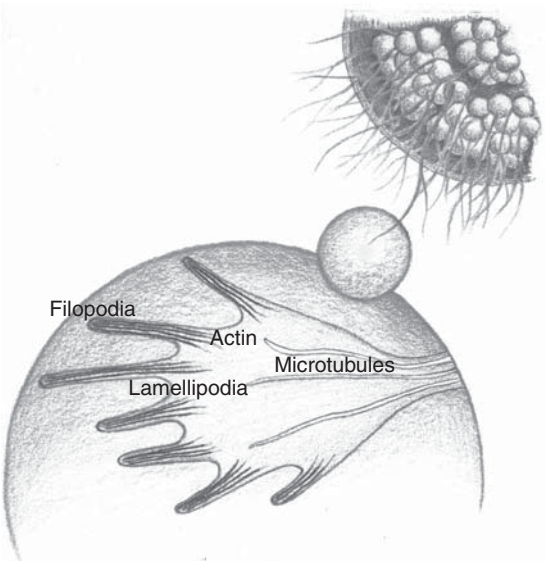

Figure 5.7 Illustration of axon sprouts and a growth cone with its main components labeled. (Illustration by Scott Rogers.) See color plate section.

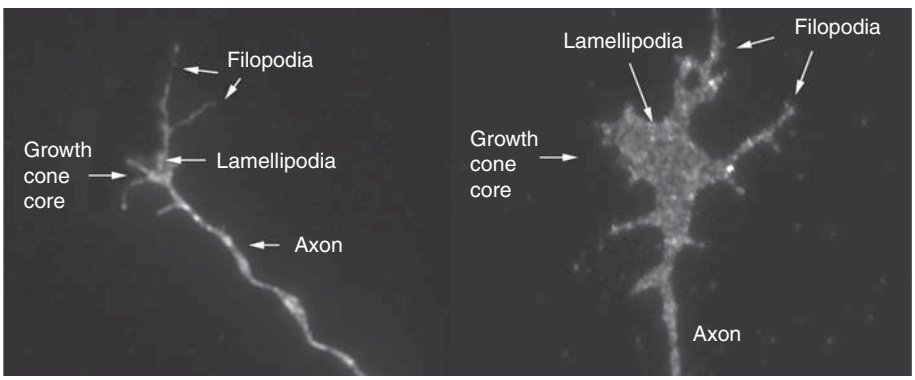

Figure 5.8 Images of growth cones from adult sensory neurons harvested and grown *in vitro*. (Images taken by C. Webber, Zochodne laboratory.)

extending from a central area or "palm" of a "hand" where microtubules and axoplasm terminate. The central domain (C) of the growth cone contains splayed endings of microtubules that probe distally into what are called the peripheral transition areas. In these zones, myosin motors are involved in the dynamics, assembly, and depolymerization of microtubules, thereby influencing

growth cone behavior. The transitional zone thus found at the peripheral edge of the C domain acts as a dynamic interface between microtubules and actins that form filamentous (F actin) polymers. The most distal, peripheral domain (P) of the growth cone with its web-like lamellipodia and finger-like filopodia is the motile exploring part of the growth cone. Its properties center on F-actin assembly and disassembly. The overall size and shape of growth cones are therefore highly variable, thought to be larger and more complex during pauses in outgrowth and smaller and streamlined during rapid forward growth [54,94,98,221,442,688]. Temporally and spatially restricted calcium transients help to determine how a growth cone turns and moves [51]. These, in turn, signal calcium-dependent enzymes such as calmodulin dependent protein kinase II (CaMKII; see below). A key central signaling mechanism involves the phosphatidylinositol-3 kinase (PI3K) pathway, in turn capable of regulating growth cone behavior. PI3K signaling is essential to axon branch formation and axon turning [206,473]. Molecules involved in growth cone advance or retraction and signaled by PI3K include the RHO family GTPases discussed below, and actin and microtubule interacting proteins (see review [779]). Extrinsic growth factors promote outgrowth (advance), while other signals cause growth cone retraction (stop, or collapse, and retreat) depending on the specific signaling cascades generated [506]. In addition to variations in forward growth, growth cones also turn toward (attraction) or away from (repulsion) chemotactic cues, such as gradients of neurotrophins or netrin (see below).

Actin

Actin is assembled into filaments (F-actin) from monomers (G-actin) at the leading tips of filopodia (+, or barbed end), a process that requires nucleation, polymerization, and annealing. The polymerizing, +, end is associated with ATP binding. Next, retrograde translocation of F-actin to the C domain by myosin motors occurs, a process that helps to propel the growth cone forward. Finally, there is depolymerization (− end or proximal part of the actin filament bundle) in the transitional zone that is essential to recycling of actin. At the −, depolymerized end, ATP associated with actin becomes dephosphorylated to ADP (see review by [133]). The entire F-actin cytoskeleton is thought to turn over rapidly, and there are changes in actin localization detectable within minutes [226]. A series of proteins that interact with, and regulate, actin dynamics, essential for growth cone behavior, is discussed below. In general, the assembly and disassembly rates of F-actin are criticial for growth cone stability and outgrowth or collapse, respectively. These rates also influence turning: asymmetric F-actin assembly or disassembly in the growth cone result in changes of direction. The motile exploratory behavior of growth cones thus depends on

polymerization and depolymerization of F-actin filaments in filopodia with concurrent plasticity of microtubules inserted into the transitional domain of the growth cone, discussed next [207].

Microtubules

Microtubule plasticity has a major influence on growth cone dynamics and behavior. Microtubules form parallel networks within intact axons but splay apart as they reach the central domain of the growth cone, sometimes forming loops [133]. Most microtubules only rarely extend more distally. Like actin, there are distal + ends where tubulin dimers are added: α-tubulin and β-tubulin. Depolymerization of the mictotubule occurs at the − end. The polymerizing zone or + end has dynamic instability with properties that include shrinkage or "catastrophe," and growth, termed "rescue." These properties are critical for growth cone exploratory behavior and they are regulated by a series of proteins known as MAPs (microtubule associated proteins) or MBPs (microtubule binding proteins). "Structural" MAPs that stabilize microtubules include MAP1B, MAP2, and tau (the latter is important in CNS neurodegenerative disorders), whereas others act as motors for axoplasmic transport (dyneins, kinesins). Further MAPs are called + end tracking proteins (+TIPs) that are thought to influence microtubule dynamics (see [133] for an extensive list of MAPs). Analogous to ATP-actin polymerization, β-tubulin is also associated with GTP during polymerization. As the microtubule polymer "ages," GTP-β-tubulin is then converted to GDP-β-tubulin prior to depolymerization. "Aging" occurs from the + end toward the − end of the microtubule, or distal to proximal. During subsequent depolymerization, β-tubulin and α-tubulin dimers are thus released at the − end of the microtubule. α-tubulin is also tyrosinated at the + end of the microtubule during polymerization and detyrosinated at the − end during depolymerization [133]. Acetylation of tubulin also influences growth cone stability.

Microtubule severing, like that of actin above, has an essential role in axon outgrowth. Katanin is a heterodimeric protein severing enzyme that creates multiple short microtubules and allows greater microtubule plasticity. A fine balance between excessive severing and inadequate severing is critical in optimizing microtubular dynamics [255,322].

Molecules that influence growth cone behavior

Growth cone receptors include Trks (tropomyosin-related kinase; high affinity neurotrophin receptors) that are selective for specific neurotrophins (e.g., NGF for TrkA, BDNF for TrkB) but also p75, expressed widely on axons

(formerly called the "low affinity nerve growth factor receptor"). Recent work has also suggested that proneurotrophin molecules, precursor molecules of classical neurotrophins, specifically bind p75 receptors and induce widespread actions on axons [293,383]. p75 activation alters the behavior of the *Ras superfamily GTPases*. These molecules operate as molecular switches in altering cell polarity and symmetry and are localized in growth cones [175]. Overall, GTPase behavior involves translocation to the growth cone membrane, a property that can be identified using newer imaging techniques. Three members, RHOA, RAC1, and CDC42 have substantial but differing influences on neuronal growth cone dynamics [217,769]. Therefore p75, when occupied by a ligand (e.g., a proneurotrophin), inhibits RHOA activation, an action that facilitates outgrowth [175,213,217,758]. In contrast, when unoccupied or unligated, p75 may instead activate RHOA [213] to promote growth cone collapse. This is one of many examples of plastic function among growth-related molecules, depending on the context and type of interaction.

RHOA, the most extensively studied of the Ras GTPases in growth cones, is a growth cone brake. It acts through RHO kinase (ROK) that enhances myosin II phosphorylation and subsequent actin mediated growth cone retraction [217,506]. RHOA may accomplish retraction in other ways as well, such as inhibiting of myosin phosphatase activity; this action leads to increased myosin ATPase activity and growth cone retraction [429]. The net result of ROK activation responsible for retraction are increases in actin "arc" formation and central actin bundle contractility and stability. RHOA localizes not only to growth cones but also to the leading edges of migrating cells where it can promote its actions [553] (Figure 5.9).

In the CNS, RHOA activation is associated with impaired regeneration of spinal cord axons [122,134]. Specific CNS mediators of RHOA-mediated regenerative failure include the myelin-associated molecule Nogo-66, myelin-associated glycoprotein, semaphorins, ephrins, oligodendrocyte myelin glycoprotein, and chondroitin sulphate proteoglycans [5,44,52,185,188,302]. Most of these molecules generate complex interactions with the Nogo receptor (NgR), p75, LINGO-1, TAJ/TROY, calcium, and the epidermal growth factor receptor (EGFR) [183,468,615], and eventually RHOA. In the PNS, SC-derived growth factors are capable of disrupting these kinds of interactions through regulated intramembranous proteolysis of molecules involved in the cascade, extracellular interruption of NgR binding, and intracellular disruption of NgR p75 interactions [5,6]. Thus, they interrupt RHOA activation. mDia is an additional downstream RHO effector thought to influence axon lengthening [48]. Transfection with a adeno-associated virus containing the inhibitor C3 ADP-ribosyltransferase that inactivates RHOA in retinal ganglion cells [185], or use of a cell permeable

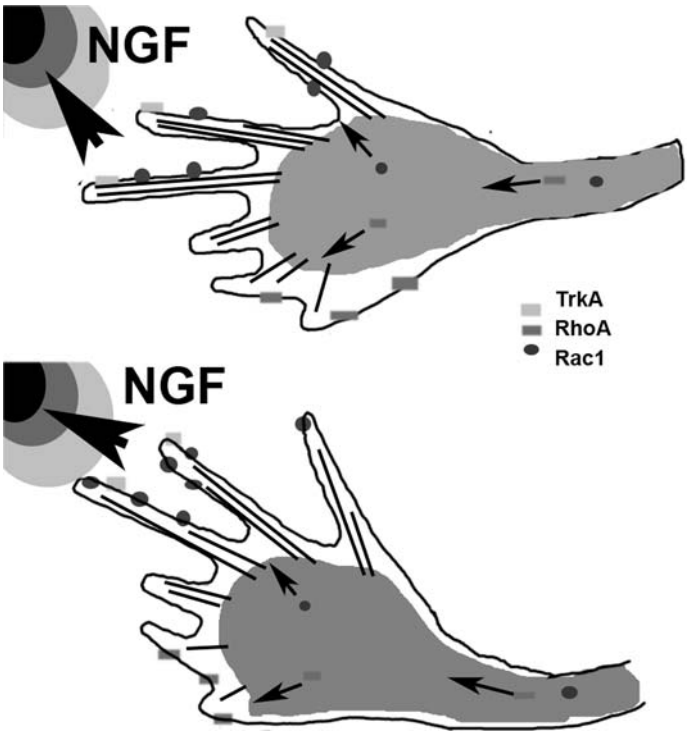

Figure 5.9 Simplified drawing illustrating how growth cones change the direction of their trajectory in response to localized gradients of growth factors such as NGF acting on their TrkA receptors (green). RHOA GTPase (red) is thought to mediate active repulsion and may be localized to growth cone membranes that are collapsing and not advancing whereas RAC1 facilitates outgrowth and may be localized to the membranes of advancing parts of the growth cone. See color plate section.

modified version of C3 ADP-ribosyltransferase, increased regeneration in CNS neurons [44,185].

RHO GTPases play key roles in growth cone turning, a determinant of whether misdirected axons compromise regeneration [217,769]. Turning behavior is dependent on asymmetric release of intracellular calcium within the growth cone [311]. In keeping with this mechanism, RHOA levels decline in growth cones that are undergoing attractive turning in response to a calcium-mediated signal generated by ligation of BDNF or netrin. This allows one portion of the growth cone to expand. In other areas of the growth cone, concurrent RHOA upregulation may mediate active repulsion [311]. Thus, rises and falls in local RHOA molecules associated with the growth cone membrane determine whether it actively promotes repulsion or allows attraction, respectively.

RHO and ROK are mediators of *actin regulation*. RHOA-ROK activates LIM kinase, a pathway that in turn phosphorylates *cofilin*, also known as actin depolymerizing factor (ADF). When cofilin is phosphorylated, it fails to bind actin and to participate in normal actin turnover. Altered phosphorylated cofilin function therefore interferes with growth cone advance [214,505]. To exert its effects "normally", active nonphosphorylated cofilin binds to filamentous actin (F-actin) that is bound in turn to ADP. The proximal end (–) of the actin filament in lamellopodia undergoes depolymerization when bound. The units of cofilin then disassociate from released units of actin, now in the form of G-actin (globular depolymerized). Actin-interacting protein 1 works with cofilin to disassemble actin filaments by capping actin filaments that have been severed. Capped actin is not available to reassemble and depolymerization is thereby promoted. "Slingshot" is yet another protein that acts as a cofilin phosphatase, dephosphosphorylating it (activating it) and thereby enhancing actin turnover [168,507,723]. Further proteins that inhibit actin binding include phosphatidylinositol 4,5-*bis*-phosphate (PI(2)P) and tropomyosin.

Cofilin has complex roles. We have just discussed its activity during actin depolymerization, but it is also capable of promoting active actin polymerization. At the + barbed (forward) assembly end of the actin filament, G-actin interacts with cofilin and ATP to begin polymerization. Overall, active cofilin therefore has a number of roles that induce cell protrusion and set the direction of cell migration [214]. How the complete repertoire of proteins (including those that interact with cofilin) is critical to growth cone dynamics has only been recently clarified (for review, see Ono [536]).

The proteins that regulate actin filament dynamics may have considerable influence on the fate of peripheral nerve regeneration. A more complete list of their roles can be found elsewhere (see reviews [133,465]). Many are localized with F-actin at the leading edges of growth cones [226]. Arp2/3 is a complex of seven proteins (Arp2, Arp3, p41Arc, p34Arc, p21 Arc, p20Arc, and p16Arc) that nucleates new actin branches from existing actin for outgrowth of the growth cone. N-WASP, cortactin, and Scar/WAVE are activators of ARP2/3. Ena/Mena/VASP proteins block capping of the barbed (+) ends of actin, thereby allowing longer filopodia to form [226]. Barbed end actin capping proteins include Cap Z and actin monomer binding proteins (thymosin β4 and profilin) that are expected to reduce growth cone advance [34,493,702]. Profilin, however, also sequesters G-actin monomers to facilitate actin polymerization, thus enhancing filopodia formation. Proteins of the Ena/Mena/VASP family also enhance polymerization rates by recruiting profilin to sites of actin assembly [226]. Thus, profilin is another example of a molecule with apparent roles in both growth cone advance and collapse [343,761].

CDC42 and RAC, also members of the Ras GTPase family, facilitate growth cone advance. Their behavior thus differs substantially from that of RHOA. CDC42 promotes filopodia and lamellipodia extension. Rac influences actin filament behavior, possibly by interacting with cofilin. It also stabilizes adherent contacts, an important step in promoting outgrowth [217,429]. RAC1 signals through the kinase PAK (p21-activated kinase) that activates NFκB and MAPK and increases neurite outgrowth [127,196,197].

Despite all of this critical information, the significance of RHO family GTPase function in the peripheral nervous system is largely unexplored. Peripheral neurons can be coaxed *in vitro* to grow across inhibitory CNS substrates by inhibiting RHO pathways [5,52,302]. Wu *et al.* [749] recently demonstrated that embryonic dorsal root ganglion neurons had RHOA synthesis localized to developing axons and growth cones. At this site, semaphorin 3A, a molecule that repels axons by collapsing growth cones (see below under GSK3β), signaled growth cone axons to synthesize RHOA protein. Despite these findings, however, we do not know how adult peripheral axons might be influenced by RHOA in peripheral nerve trunks. RHOA is expressed and functional in the peripheral nervous system, and there is preliminary evidence that it has actions in the PNS similar to those in the CNS. For example, Terashima and colleagues [673] identified RHOA, RAC1, CDC42, and PAK in intact peripheral nerve trunks and ganglia. They were localized to sensory neuron plasmalemmae but were also localized in axons and SCs further out in the nerve trunk. Hiraga and colleagues [270] noted that fasudil (HA-1077), a pharmacological ROK antagonist given systemically in mice, increased the recovery of motor reinnervation (CMAPs) and the caliber of their regenerating axons. Activated RHOA was identified in the portion of the spinal cord where motor neurons reside, but not in distal stumps of the injured nerves. In separate work, fasudil increased the neurite outgrowth of adult sensory neurons even when grown on noninhibitory substrates *in vitro.* It also increased the growth of axons through regeneration conduits spanning nerve transections in rats [105]. There are important inhibitory cues in peripheral nerves that may activate RHOA such as myelin-associated glycoprotein (MAG) and chondroitin sulphate proteoglycans.

RHOA-ROK and other Ras GTPase family members also influence microtubular organization [774]. RAC captures and stabilizes microtubules through molecules called IQGAP1, CLASP, and Clip-170 [779]. CDC42 acting through the protein Par3/6-aPKC inactivates GSK3β, discussed below, and thereby promotes microtubule assembly through a molecule called APC (adenomatous polyposis coli tumor suppressor protein) [779].

cAMP is a critical mediator and facilitator of growth cone behavior, especially during guidance [412,474,636]. By increasing the content of cAMP, or "priming"

in sensory neurons, for instance, their outgrowth inhibition to MAG can be overcome [84,564]. Interestingly, cAMP also facilitates how SCs support outgrowth, discussed below.

Glycogen synthase kinase 3β (GSK3β) is multifunctional serine/threonine kinase, localized to the leading edges of growth cones. In its constitutively active nonphosphorylated state, it suppresses growth cone extension and axon formation [165]. When it is phosphorylated through the PI3K-Akt (PKB) signaling pathway, GSK3β activity is shut down, allowing growth to occur. This pathway was originally linked to neuroprotection and survival, providing one further example of how axon outgrowth and neuronal survival signaling pathways are closely linked [113,143]. GSK3β inhibits axon outgrowth in part by targeting APC mentioned above [780]. GSK3β can also be inactivated by NGF through PI3K, by dishevelled proteins of the Wnt signaling pathway, and also by preconditioning of neurons subsequently grown on laminin to facilitate integrin binding [779]. Integrins are the receptors on cells for extracellular basement membrane components. As suggested, basement membrane–integrin interactions also involve signaling properties beyond simple adhesion (see Chapter 10) [780].

PI3K (phosphatidylinositol 3-kinase)-Akt [70] is a critical downstream survival pathway that mediates trophic factor support of neurons and blocks apoptosis. Loss of neurons through apoptosis is an important consideration during regeneration. For example, retrograde apoptotic loss of neurons after axotomy, more prominent in neonates, reduces the pool of parent neurons available to send out regenerative sprouts. Within individual neurons, however, it may be that PI3K-Akt activation serves roles that promote growth and differentiation (for review see [189]). PI3K increases the conversion of PI-4,5-P2 (PI(2)P; phosphatidylinositol 4,5-*bis*-phosphate) to PI-3,4,5-P3 [PI(3)P] that in turn phosphorylates and activates the downstream molecule Akt (also known as PKB). *PTEN* (phosphatase and tensin homolog deleted on chromosome ten) is a phosphatase that inhibits the conversion to PI(3)P. Predictably, molecules that suppress PI3K activity such as PTEN facilitate growth cone collapse [95]. Like GSK3β, PTEN is also inactivated by phosphorylation, including one pathway involving NGF acting on p75 receptors independently of TrkA. A second pathway involves a kinase known as CK2 (casein kinase II) [22] that also phosphorylates and inactivates PTEN. Alternatively, growth inhibitor molecules may activate PTEN. Thus, several pathways can converge on PTEN to block it and promote axon outgrowth.

CRMPs (*collapsin-response mediator proteins*) are also involved in growth cone collapse. This family, including members CRMP 1 through 5, are cytosolic phosphoproteins that influence the formation of microtubules through tubulin

binding [229]. When CRMP 2 is phosphorylated by active GSK3β, axon formation is inhibited (for review see Li[399]). CRMPs interact with *semaphorins*, a family of proteins both secreted and membrane bound, that influence axon guidance. Through their neuropilin receptors (NP-1 and NP-2) and their interactions with CRMPs, they are also critical mediators of growth cone collapse [544,596]. Class 3 semaphorins, or Sema3s are secreted repulsive molecules and among them Sema3A has had the most attention. Semaphorin-mediated growth cone collapse has been specifically linked to CRMP2, in turn inhibiting microtubule assembly [201,602]. There are further series of associated protein interactions that can be initiated by Sema3 to collapse growth cones. These include not only phosphorylated CRMP 2, but also α2-chimaerin, CDK-5/p35, and RAC [67]. The participation of RAC in the collapse of growth cones is surprising, considering our discussion above of its facilitatory role. RAC may, in fact, shuttle between dual roles promoting either outgrowth or collapse depending on the multimolecular scaffold it interacts with. CDK-5 may also phosphorylate CRMP 2 together with GSK3β [120]. Sema3A operates through PTEN to reduce PI(3)P levels, reduce Akt signaling, and to prevent GSK3β inactivation. These steps lead to growth cone collapse. To allow these interactions to occur, close localization is important. Thus, both Sema3A and PTEN are localized to growth cone membranes [95]. These interactions assume relevance in peripheral nerve regeneration because Sema3A is expressed during Wallerian degeneration.

Sema3A is expressed in cranial and spinal motor neurons, and its levels in these neurons decline after peripheral axotomy [544]. It is also expressed in terminal SCs of neuromuscular junctions (see Chapter 6) and may help influence the reinnervation of specific fiber types. Moreover, other cell types express Sema3s, and its presence in the extracellular matrix allows growth cones to be exposed to gradients of the molecule [544]. All of these forms of expression indicate that Semas play a central role in both ligand signaling to growth cones, and also in subsequent signal transduction within neurons.

CRMP4, in particular CRMP4b, binds to RHOA within growth cones to inhibit neurite outgrowth [8,585]. Knockdown of CRMP4b or binding competition using a portion of its N terminus (known as C4RIP, or *CRMP4b-RHOA inhibitory peptide*) allows sensory neurons to grow on inhibitory substrates such as myelin or aggrecan, a chondroitin sulphate proteoglycan.

Protein kinase C (PKC) and GAP43/B50

PKC subtypes (both calcium-dependent α, β, and γ subtypes and calcium-independent δ, ε, and ζ) are localized to regenerating growth cones *in vivo*, especially their plasma membranes [329,531]. This is an important finding in

metabolically active growth cones, indicating their role as a substrate for signal transduction, regulation of ion channel function, growth, and modulation of local protein production. PKC modulates how *GAP43/B50* (growth-associated protein 43 or B50), another central signaling molecule, is upregulated by injury (see below) and how it alters growth cone behavior. GAP43/B50, originally discovered by Zwiers, Ostreicher, and colleagues [10,119,525,526], and later by Skene and colleagues [630] is associated with the cytoplasmic surface of the growth cone membranes. It alters growth cone plasticity when membrane bound and may operate to amplify extracellular signals [296]. In a hypothesis developed by Ostreicher, Gispen, Zwiers, and others [119,524,709], calcium entry into axon terminals causes the release of GAP43/B50 from binding with calmodulin. GAP43/B50 then is phosphorylated by PKC and can interact with F-actin at growth cone membranes. It is unclear whether the phosphorylation of GAP43/B50 influences its membrane localization, but when it undergoes ADP ribosylation it is released from plasma membranes into the cytosol [524]. CAP-23, a related molecule also upregulated after injury, may offer similar actions [443]. In growth cones, calmodulin that is released from its association with GAP43/B50 also has diverse actions, including membrane recycling, excytotic/endocytotic activity, and polymerization of microtubules and actin. It also phosphorylates GAP43/B50. A dominant inhibitor of calmodulin stalled the growth of CNS pioneer axons [343].

More recent work has updated how PKC, GAP43/B50,CAP-23, and a related molecule known as MARCKS (myristoylated alanine-rich C kinase substrate) influence growth cones GAP43/B50, CAP-23, and MARCKS are expressed in membrane raft microdomains and control PI-4,5-P2 availability at the growth cone. PI-4,5-P2, GAP43/B50, and CAP23 are all capable of altering actin dynamics in concert with actin binding proteins. From above, we also know that PI-4,5-P2 is converted to PI-3,4,5-P3 by phosphatidylinositol 3,4,5-triphosphate (PI(3)K) to facilitate axon outgrowth [191,378]. As an aside, both are also expressed in SCs [443,556]. PKC may play a role in laminin–integrin interactions, and local neurotrophin signaling [676]. These are aspects of regeneration mentioned earlier but considered in more detail in later chapters. CaMKI (calmodulin kinase I) is expressed throughout the neuron including growth cones where it acts as a positive transducer of growth cone motility and axonal outgrowth [729]. It has not been studied in peripheral neuron growth cones.

In summary, localized calcium transients signal calmodulin and CAMKI or II, calpain, PKC, as well as the inhibitor phosphatase calcineurin. An eventual outcome is a change in the phosphorylation status and localization of GAP43/B50, features that influence its role as an actin capping factor [51].

In vivo *growth cones*

Few descriptions of *in vivo* growth cone morphology are available. They can be difficult to accurately identify in histological preparations. Ultrastructural studies provide some descriptions [740]: enlarged axonal-like profiles with increased cytoplasmic electron density, and accumulations of smooth endoplasmic reticulum. Scanning electron microscopy depicts them as enlarged (3–5μm in diameter) axon structures that are highly variable in configuration but have relatively few filopodia or F actin bundles. They have a close and almost invariable relationship to SCs and their basement membrane [364,532]. *In vivo* growth cones also include heterogenous vesicles, mitochondria, and peripheral accumulations of electron dense filamentous material. Neurofilaments are not usually identified within them since the neurofilament protein polymer lattice instead ends proximal to the site of growth cone formation. Using immunohistochemistry, the distal portions of axons with growth cones can be labeled with PGP 9.5, βIII tubulin or GAP43/B50. All of these markers highlight the complexity of *in vivo* growth cones [450] (Figure 5.10). Some proteins have been localized using immuno-EM. For example, there is diffuse cytoplasmic expression of synaptophysin, an important component of the synaptic vesicle membrane [532]. This protein regulates the exocytosis of vesicles and is prominent in early regenerating sprouts arising from nodes of Ranvier.

Early SC behavior

In the earliest phases of regeneration, there are dramatic changes in SC phenotype and behavior that have important impacts on axon regrowth (Figure 5.11). By day 5–7 after a peripheral nerve injury, there is an initial wave of Schwann cell (SC) proliferation in both the proximal and distal stumps, as well as proliferation of other cellular constituents including mast cells, endothelial cells (angiogenesis), and fibroblasts [234,787,805,807]. The classical view of SC behavior suggests that SCs first alter their phenotype secondary to loss of contact with the axon. This behavior is most prominent in those SCs associated with myelinated axons where the integrity of the myelin sheath critically depends on the presence of an adjacent axon. Once that contact is interrupted, SCs become "reactive." They dissolve their own myelin, downregulate their synthesis of myelin protein mRNAs, and begin to proliferate, forming Bands of Bungner as guideposts for new axons. Proteins rapidly upregulated by SCs following interruption of axon contact include p75, the low affinity nerve growth factor receptor discussed above (and also expressed on SCs) and GFAP (glial fibrillary acidic protein) [106,307,475]. Transgenic mice lacking GFAP develop normally,

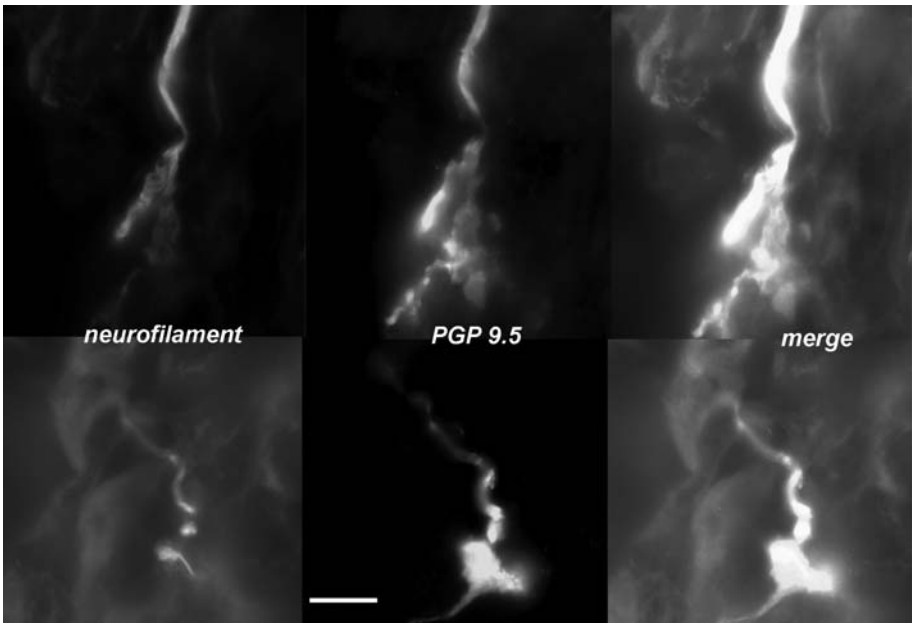

Figure 5.10 Examples of complex *in vivo* growth cones found at the proximal stump of a transected rat sciatic nerve and labeled using immunohistochemistry for neurofilament (red) or PGP 9.5 (green). Note that the appearance is substantially different from growth cones studied *in vitro*. The PGP 9.5 labels the full extent of the structure, whereas neurofilament is only found in portions of the growth cone and in its proximal stem. (Bar = 20 microns) (Reproduced with permission from [450].) See color plate section.

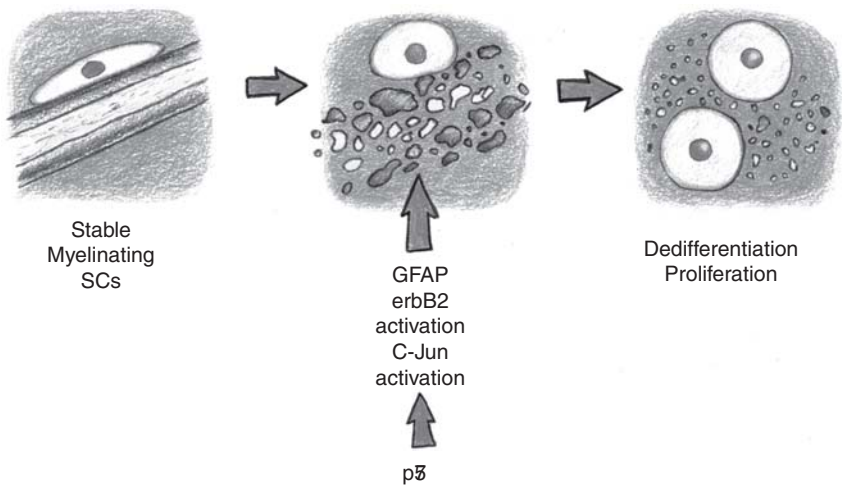

Figure 5.11 Illustration of changes in SC phenotype after nerve injury. (Illustration by Scott Rogers.) See color plate section.

but lack appropriate plasticity after injury and regenerate more slowly [693]. At somewhat later time points, SCs upregulate GAP43/B50, already discussed above in regenerating axons [126]. It is likely that there is a continuum of SC plasticity that responds to changes in the local microenvironment. Like the immune system, sequential and coordinated changes in behavior and protein expression of this class of cells illustrate a surprising beauty of planning and action.

The first signals for this new behavior of previously "quiescent" SCs are likely to be more subtle and interesting than a simple lack of contact. Indeed, some of the protein markers that are rapidly upregulated in stable SCs ensheathing myelinated axons are expressed in "intact" SCs comprising the Remak bundle of unmyelinated axons: GFAP, N-CAM, A5E3, Ran-2, and p75 [308]. This supports the idea that Remak SCs are much more plastic and dynamic than expected [126]. While GFAP is typically expressed in Remak SCs and not in the SCs of myelinated axons, the latter cells begin to express it after a nerve injury, even if they are remote from the injury site [108]. A rapid signal capable of facilitating dynamic SC behavior may be at play, possibly heralded by axon atrophy and distortion of the inner mesaxon of the myelin sheath. As will be discussed below, the molecule NRG (neuregulin) may be partially responsible for changing SC behavior [172]. In their subserial ultrastructural study of transected myelinated axons, Friede and Bishhausen [195] described asymmetric hypertrophy and additional cytoplasmic changes of SCs near the injury site and as far proximal as two nodes of Ranvier. These changes were found in portions of the SC found closest to the site of axon interruption. They included microtubular profliferation, increased rough endoplasmic reticulum, and formation of SC processes or tongues resembling those of Bands of Bungner. These findings indicate a remarkable alteration of properties that increases, even within portions of a single SC cell, near an injury site in a proximal stump.

An interesting and parallel form of behavior involves the perineuronal satellite cells, cousins of SCs, that normally surround and support neurons in dorsal root ganglia. Normally these cytoplasmic-poor cells can be barely resolved as they intimately wrap around the perikarya of sensory neurons. After injury, they become hypertrophic and upregulate their expression of GFAP, forming ring-like profiles around each sensory neuron [103] (Figure 5.12).

The molecules and pathways that are important for SC proliferation partake in a complex synergy between *cAMP* and growth factors. For DNA synthesis in SCs to occur, as required for proliferation, a rise in SC cAMP levels is a necessary first step, priming subsequent actions by NRG, PDGF, or bFGF [643]. Experimentally, cAMP levels can be boosted by activating adenyl cyclase through pharmacological approaches. Other SC mitogens include CGRP [111], insulin, insulin-like

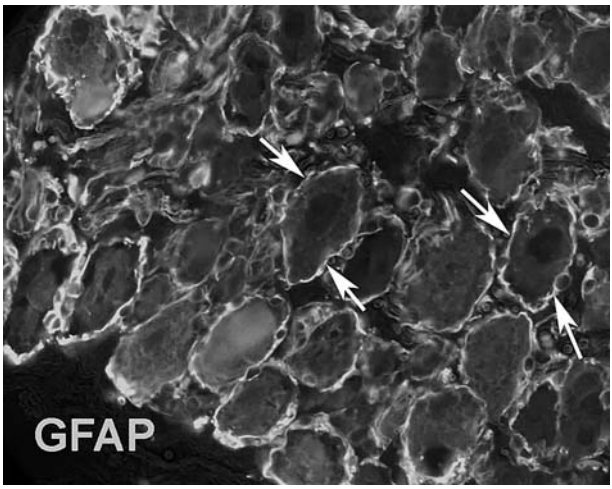

Figure 5.12 Immunohistochemical images of a DRG proximal to a sciatic nerve injury carried out 5 days earlier labeled with GFAP (glial fibrillary acidic protein). GFAP labels glial cells including perineuronal satellite cells. These cells, closely apposed to and surrounding sensory neurons, become hypertrophic and upregulate their content of GFAP after an injury (arrows). (Images taken by Chu Cheng, Zochodne laboratory.) See color plate section.

growth factors I and II (IGFI and II) [633], and others. GFAP, discussed above, is also important in signaling SCs to proliferate. It does this through an interaction with integrin αvβ8 involving ERK phosphorylation [693]. Parathyroid hormone-related peptide (PTHrP), synthesized by SCs, is important in promoting SC migration and alignment or bundling with axons and collagen fibers [432]. It may signal SCs by the phosphorylation of CREB.

There are a large number of additional molecules that have an important influence on SC behavior. For example, p21 and p16 within SC nuclei act to shut down their proliferation [26]. IL-6 and LIF can act to activate SCs to produce further LIF or MCP-1, a macrophage chemoattractant [682]. When axons contact SCs, however, SC LIF mRNA levels are turned back down [447]. The low-density lipoprotein receptor-related protein (LRP-1) is upregulated in SCs after injury [86]. This transmembrane protein, a member of the low-density lipoprotein receptor family, is widely expressed and is acted on by over 40 ligands. LRP-1 possesses cytoplasmic signaling motifs, some of which influence SC survival through upregulation of phosphorylated Akt. Its upregulation in SCs may be driven by TNF-α. Thus, LRP-1 ligands, like PDGF, NT-3 and IGF-II above, may serve as autocrine SC survival factors that are important during regenerative dedifferentiation, proliferation, and migration [462].

Local axon–SC interactions and early outgrowth

The axon–SC relationship

SCs are essential partners in axon guidance and in the elaboration of growth factors and adhesion molecules for regrowth (Figure 5.13). Thus, SC support extends beyond simple provision of laminin basement membrane scaffolds (Figure 5.14). As we have discussed, the SC phenotype can change rapidly after injury, even preceding frank Wallerian degeneration, indicating SCs that are closely "attuned" to axon events [108]. These aspects of partnership are described next.

The support offered by SCs has been illustrated by recent work transplanting cells into nerve transection gaps. Stem cells (e.g., from hair follicles) that are capable of differentiating into SCs, or transplanted syngeneic SCs, when implanted into sciatic nerve transection gaps substantially improve axonal regeneration [13,241,455,482]. Using another approach, axon outgrowth onto bioartificial films was studied. If reactive SCs were seeded onto the film substrate in advance to partner with new axons, there was enhanced outgrowth [687]. Conversely, irradiation of regenerating nerve segments severely impaired nerve regeneration by preventing SC proliferation [601]. Similarly, the inhibition of SC mitosis using mitomycin prevented axon and SC ingrowth into acellular nerve grafts [247]. While axons therefore depend on SCs for outgrowth, SCs may not need axons to grow out across nerve transection gaps. Without axons, however, they do so with substantially less directional specificity. "Pure" SC outgrowths lacking axons can produce "pseudo-nerves," macroscopic tissue bridges that resemble normal nerve bridges [776]. Overall, these observations highlight the essential partnership or "dance" between the axon and SC during early regeneration.

The close, almost invariable relationship between axon and SC outgrowth has been confirmed in several studies [103,426,450]. Proliferating and migrating SCs send long processes that appear to lead and guide axons in an outward direction from their transection site [103] (Figure 5.15). Most regenerating axons follow SC processes in exquisite detail through three dimensions of space; invariably diving and turning together. In previous studies, Ramon y Cajal [572] and others [295] suggested that early outgrowing axons are often "naked," traveling alone without a SC partner. While examples of this exist, double label immunohistochemistry, confocal microscopy, and electron microscopy have more recently indicated that they are the exception. Immunohistochemical markers such as βIII tubulin identify fine and irregular axon growth patterns at their furthest limits, probably indentifying growth cones "sampling" distal

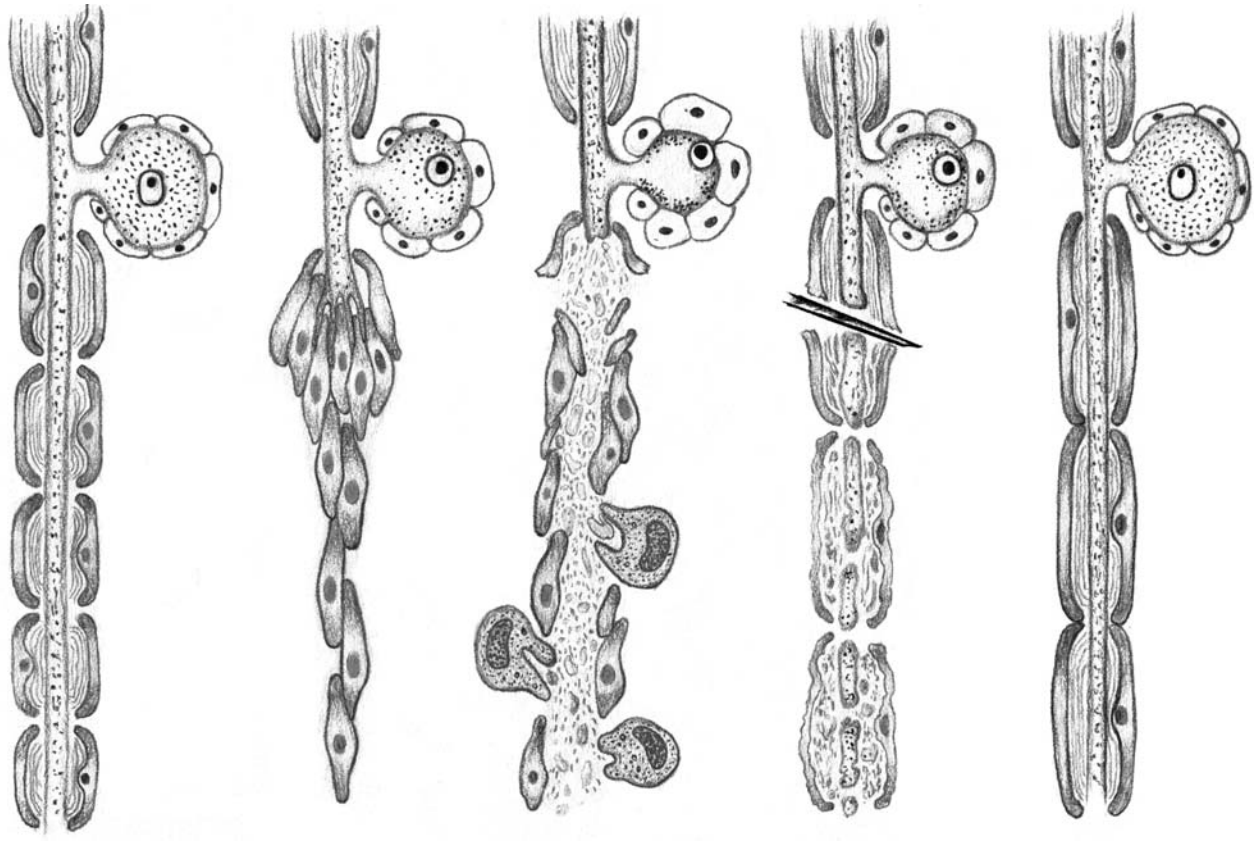

Figure 5.13 Illustration showing the sequence of changes in sensory neurons and SCs after a transection injury. Note the development of hypertrophic perineuronal satellite cells. Neurons have peripheral displacement of their Nissl substance and their nuclei. Invasion of hematogenous macrophages accompanies proliferation and dedifferentiation of SCs preparing the way for new axon outgrowth. (Illustration by Scott Rogers.) See color plate section.

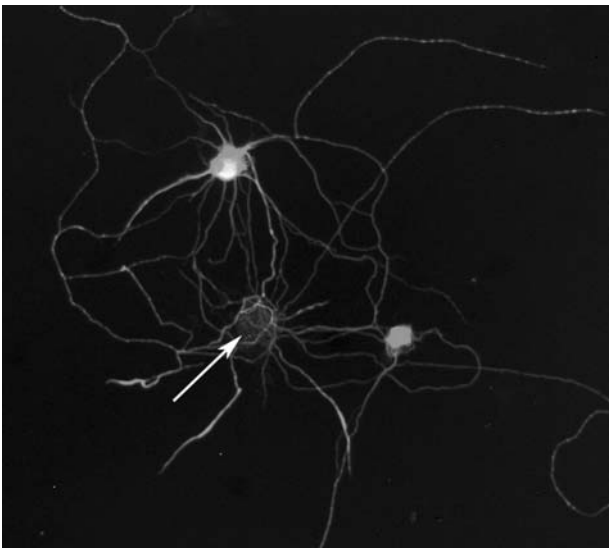

Figure 5.14 Adult rat peripheral sensory neurons *in vitro* showing extensive neurite formation. The neurons underwent a preharvesting preconditioning injury and are examined at day 3. The arrow points to a collection of glial cells in the culture that have attracted neurites to grow towards it. The image illustrates the tropic properties of supporting cells during axon outgrowth. (Culture prepared by Sophie Dong, Zochodne laboratory.) See color plate section.

terrain. Most growth cones, however, appear to routinely accompany SC processes. Their behavior replicates developmental growth where Schwann cell precursors are close and consistent cellular companions of growth cones as they approach targets [725]. During development, one analysis suggested that SC precursor cytoplasmic processes covered over 80% of the growth cone.

Regenerative units

Morris and colleagues [478] describe two types of regenerative *axon-SC units* that begin to emerge from the proximal stumps of transected peripheral nerves: Type I units consist of an apparently mature myelinated axon sharing a single SC with one or more unmyelinated axons. The unmyelinated axons are applied to the SC surface beneath the basement membrane (BM) and the unit thereby forms one axon–SC–BM complex. Type II units consist of nonmyelinated axons only. These axons, unlike normal Remak bundles, grow in clusters and are not always separated by SC cytoplasmic leaves. Complex and sometimes reduplicated BM surrounds the units. Axon sizes are also more variable in a regenerative unit than in a normal Remak bundle and a single SC might be associated with

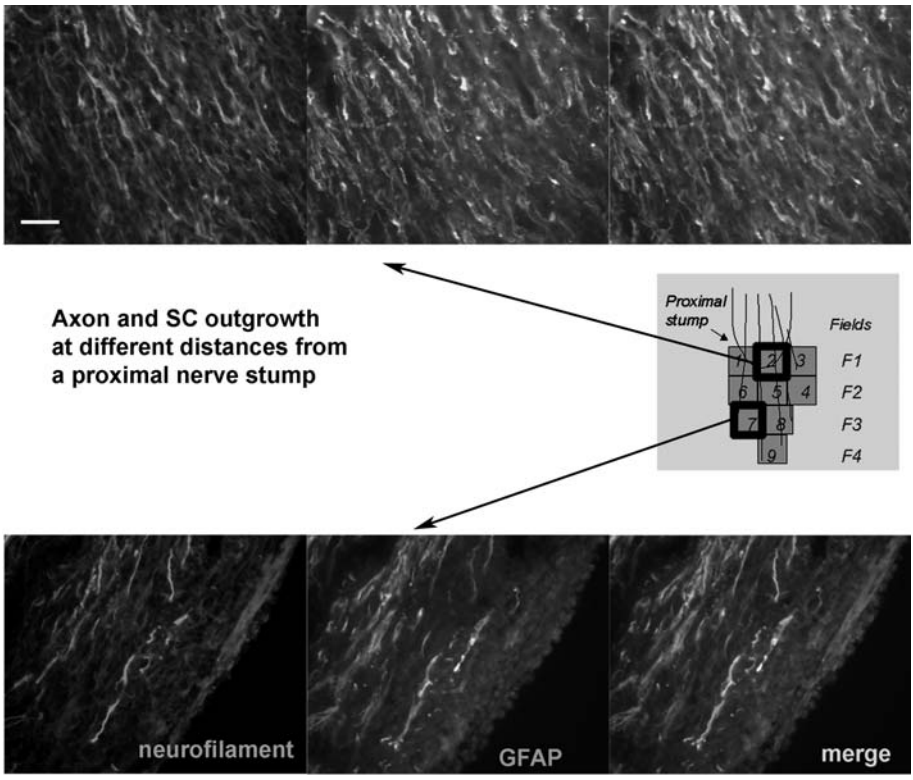

Figure 5.15 Immunohistochemical images of axon (labeled with an antibody directed against neurofilament) and SC (labeled with an antibody to GFAP) outgrowth at day 7 from the proximal stumps of transected rat sciatic nerves. The images in the top panel illustrate extensive outgrowth close to the proximal stump and the images in the bottom panel are further distally. Note the close relationship between axons and SCs. (Images taken by Y. Y. Chen, Zochodne laboratory.) See color plate section.

up to 80 axons. Both Type I and II units probably arise from single parent axons. Overall, regenerating units are also associated with "halos" of small caliber (30 nM) collagen fibrils. Regenerative units are illustrated in Figure 5.16.

In the original ultrastructural descriptions, some additional features of regenerating units were identified. For example, with time, one or more axons in Type II units became myelinated. Near the injury zone, Morris and colleagues [477,478] reported that frequent SCs contained membranous debris from phagocytosis, while at the same time associating with axons. This is an interesting finding indicating that individual SCs can have multiple concurrent roles that include phagocytosis while actively supporting new regenerating axons. Other morphological changes of "reactive" SCs included alterations in their endoplasmic reticulum and even the presence of cilia.

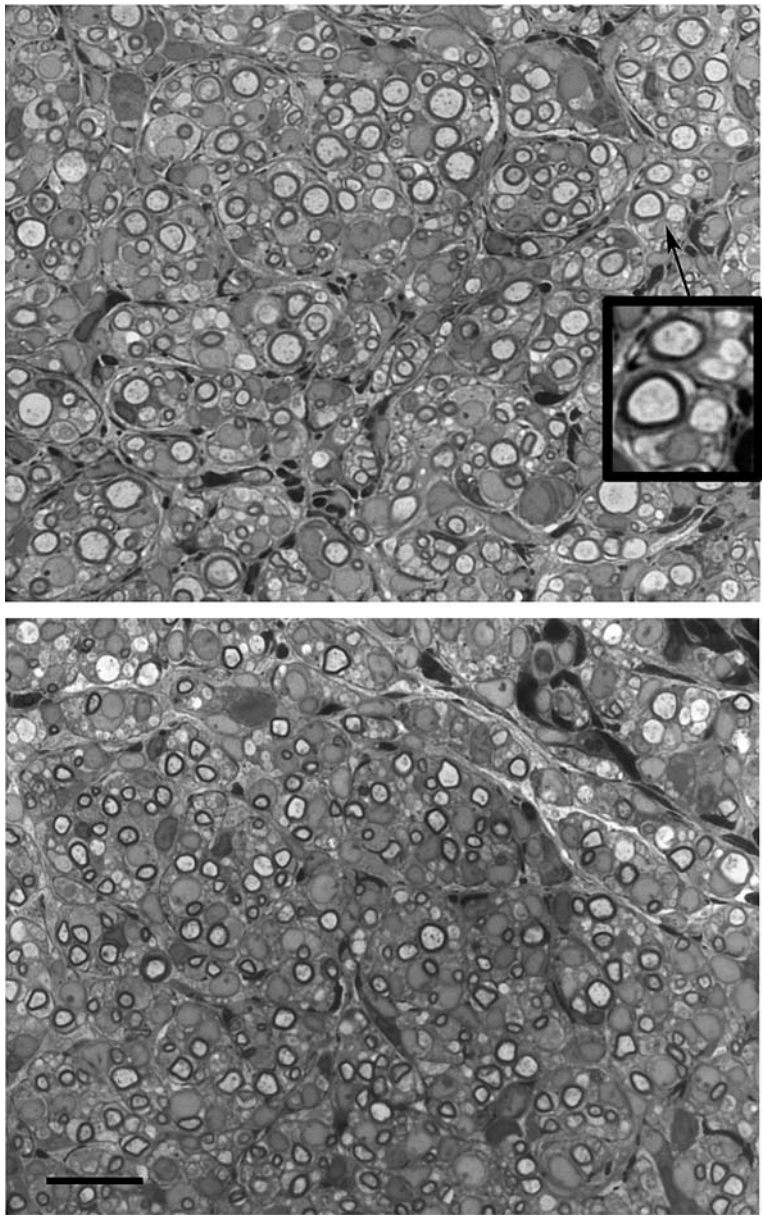

Figure 5.16 Examples of early myelinated axon repopulation of regenerative bridges growing through a conduit connecting the proximal and distal stump of a transected rat sciatic nerve at day 21. Note that the axons are often in clumps resembling minifascicles. The inset shows a higher power view of two small clusters of myelinated and unmyelinated axons. The images are from semithin sections, epon embedded and stained with toluidine blue. (Bar = 20 microns)

Local axon–SC interactions during early outgrowth can be further studied using a regeneration conduit connecting the proximal and distal stumps of a severed nerve. Conduits allow an analysis of early axon and SC outgrowth that is free from adjacent products of Wallerian degeneration found in the distal stump proper. Surgical literature on the use of conduits, largely emphasizing variations in approach or design that might enhance regeneration, is available. Their use has become widespread. Biodegradable conduits have been used surgically [320,423,428,775] while experimentally they have been used to instill growth factors [320], genetically engineered SCs or SC precursors. Immunohistochemistry to label axons (neurofilament, βIII tubulin, PGP9.5), SCs (GFAP), and other constituents can be examined during early regrowth after transection and conduit placement. For example, within the first week following transection, the approach can evaluate the number of outgrowing axons, their directionality, their association with SCs, and the furthest distance they have regrown. Regenerative bridges can be examined for the caliber (maturity), myelination, and number of axons by LM or EM somewhat later (e.g., 2–3 weeks). If each stump is placed in a conduit, a coaxial fibrin matrix cable connects the proximal and distal stump by 1 week. By 2 weeks, there is migration of Schwann cells, fibroblasts, and endothelial vascular cells from both stumps, all of which precede later ingrowth of axons from the proximal stump. The initial ingrowing axons appear in clusters. They are unmyelinated, and similar to the Type II regenerating units described above. As axons mature, they are joined by other new axons, collectively forming compartments or minifascicles.

Directionality

Complex patterns of regrowth are typically a consequence of peripheral nerve injury [740]. How do axons know which direction to grow? Classical experiments by Lundborg and colleagues demonstrate that axons prefer to grow toward their own distal stump [426,492]. Transection injuries place much greater demands on direction finding from peripheral nerve axons of the proximal stump. After nerve transection, there is retraction of both the distal and proximal nerve away from the transection site (e.g., by 3–5mm in the mid-sciatic nerve of rats) because the nerve is usually under some degree of tension. The upper limit for successful growth across nerve gaps in rats generally is 10–15mm. If placed in a conduit connecting the proximal and distal stump, axons from the proximal stump forge ahead more successfully if a distal nerve stump instead of a portion of unrelated tissue is placed at the far end of the conduit. Thus there is an important *tropic* effect of distal nerve tissue on regrowth of the nerve. In many instances, both SC and axon also turn together but

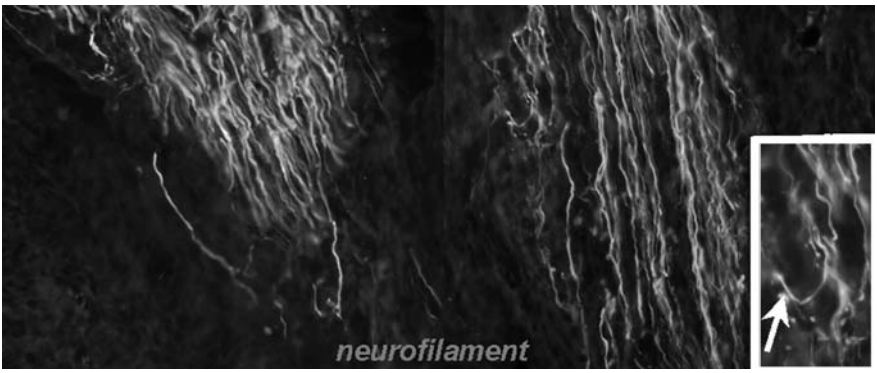

Figure 5.17 Immunohistochemical images of axons emerging from the proximal stump of a transected rat sciatic nerve (connected by a conduit) at day 7 labeled with an antibody to neurofilament. Note the rapid fall off in axon numbers as they penetrate the regenerative bridge. The inset and arrow shows an axon in a misdirected, or "wrong way" trajectory. (Reproduced with permission from [450].) See color plate section.

incorrectly; they can even extend backwards into the epineurium of the proximal nerve stump. We have called these "wrong way axons" (Figures 5.17, 5.18). SCs whose proliferation was arrested, for example, by exposure to local mitomycin, a mitosis inhibitor, fail to properly support regrowth. Axon regeneration was severely attenuated and misdirected [103].

Laminin and other components of SCs

Leading SCs may elaborate trails of laminin for detailed guidance of following axons. Laminin receptors on axons, known as integrins (see Chapter 10), may then allow axons to adhere to these trails and closely follow trajectories of SCs. It may also be that laminin from more than one source could help axons to navigate. For example, vascular laminin is elaborated and is associated with ingrowth of endothelial cells into new nerve trunks. Axons growing into regenerative bridges in conduits, however, did not appear to track vascular laminin. Nonetheless, simple infusion, of soluble laminin into regeneration conduits improved axon growth, directionality, and repopulation [103] by being incorporated into the regenerative bridge. Laminin infusion, therefore, can partly compensate for a failure of SC support. This is not surprising since *in vitro* growth of neonatal sensory axons is facilitated by laminin-coated filaments in the absence of SCs [573]. Thus, laminin, elaborated by SCs may be a key link that confers directional specificity and outgrowth enhancement to following axons.

In addition to laminin and other basement membrane extracellular molecules (including tenascin C, fibronectin, and heparan sulphate proteoglycan),

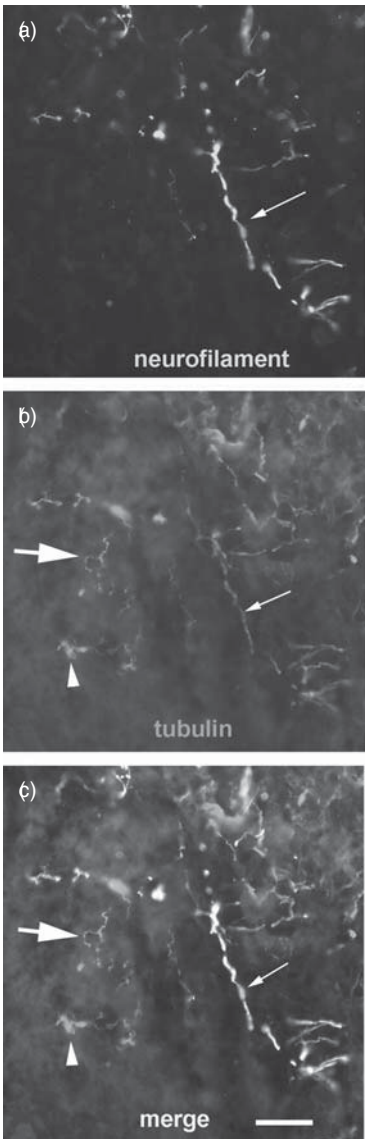

Figure 5.18 Immunohistochemical images of fine axons at the most distal end of regenerative outgrowth by day 7 following transection of a rat sciatic nerve (connected by a conduit). The axons are labeled by antibodies to βIII tubulin and neurofilament. The small arrow shows a double labeled profile. The arrowhead and large arrow show a tubulin, but not neurofilament labeled axon profile that is highly irregular and partly misdirected. The images illustrate that very fine distal axon outgrowth at the regenerative front may not label with a neurofilament antibody. (Bar = 50 microns) (Image taken by Chu Cheng, Zochodne laboratory and reproduced with permission from [450].) See color plate section.

SCs also synthesize additional molecules likely to influence peripheral nerve regeneration [200]. These molecules can support either the SCs themselves, known as autocrine support, or axon partners. Specific examples include the classical neurotrophins (NGF, BDNF, NT-3, NT-4/5), proneurotrophin precursors, neuropoietic cytokines (including CNTF, IL-6, oncostatin, and LIF), other neurotrophic factors (including insulin, IGF-1, and GDNF), fibroblast growth factors (FGFs), and cell adhesion molecules (including NCAM, L1, and N-cadherin). Examined in more detail in Chapter 9, growth factors in particular can signal both locally and centrally following retrograde transport, and they appear capable of supporting both neuron survival and axon outgrowth [100,466,489,566]. One of the most important consequences of SC growth factor synthesis is to trigger their *neuregulin* release from axons, discussed next.

Neuregulins

Neuregulins (NRGs) are intimate signals from axons to SCs. They are important during development, during SC maintenance, and at several stages of regeneration. NRGs arise from alternative splicing of the NRG1 gene and belong to a family of growth and differentiation factors. These splice products have included previously identified proteins called neu differentiation factor (NDF), heregulin, acetylcholine receptor inducing activity (ARIA), and glial growth factor (GGF). All have an EGF-like domain that activates erbB tyrosine kinase receptors, and several have extracellular heparin-binding, immunoglobulin-like domains. ProNRG, expressed as a transmembrane protein is cleaved, released, and binds to heparan-sulphate proteoglycans in the extracellular matrix.

NRG is a "bridge" that links altered behavior in SCs to subtle axon injury or frank axonal retraction. Thus NRG is released by axons from its proNRG axon transmembrane precursor as a result of exposure to neurotrophins released by SCs. PKC activation within the axon is involved in the release process [172]. Moreover, the sensitivity of axons to growth factor-NRG release may depend on the type of axon and the type of growth factor. For example, NGF and GDNF trigger NRG release from sensory neurons, whereas GDNF and BDNF trigger its release from motor neurons. NRG accomplishes its actions using receptors erbB2, erbB3, and erbB4 [101,211,464,480,582,745]. In SCs, erbB3, or erbB4 NRG receptors are usually complexed to erbB2 as heterodimers. In fact, erbB2 does not directly bind to NRG, but it signals NRG ligation through its association with the other receptor isoforms. These involve activation of both the MAPK and PI3K growth and survival pathways to facilitate subsequent SC survival, proliferation, and later myelination [80,173,242]. NRGs also play roles in early Wallerian or Wallerian-like degeneration (see Chapter 3) to break down myelin and they later provide information about axon size to SCs during decisions about

remyelination [470]. Once an axon attains a given caliber, initiating myelination by its SC is a function of NRG signaling, in conjunction with other local factors such as BM–integrin signaling. Questions about the role of erbB2, however, do persist. For example, erbB2 null mice do not have deficits in SC proliferation after nerve injury and their myelinated axon structure is preserved [27]. It seems likely, however, that erbB2 signaling does overlap with that of other molecules, and that these alternative pathways may subsume similar tasks in its absence.

Other SC proteins influence NRG signaling. For example, caveolin-1 is a structural protein of lipid caveolae raft domains that acts as a cholesterol binding and shuttling protein, and inhibits erbB2 signaling. Caveolin-1, in turn may be upregulated by NGF binding to p75 in SCs, a complex pathway capable of modifying SC behavior [665].

Retrograde loss of neurons

Following transection or axotomy of a peripheral nerve, retrograde loss of motor, sensory, and autonomic neurons occurs. While such loss is less pronounced in adults than neonates, it nonetheless reduces the pool of parent neurons and axons available for reinnervation. When sensory neurons degenerate and disappear in ganglia, they leave behind a distinctive collection of perineuronal cells that appear to proliferate around and within the site formerly occupied by the neuron. These tombstones of sensory neurons are known as "nests of Nageotte." For motor neurons, loss is associated with local microglial activation.

Retrograde loss is thought to occur because an axotomy deprives the neuron of retrogradely transported, target-derived growth factors. This idea is the main tenet of the "neurotrophic hypothesis." A large number of growth factors including the NGF-related neurotrophin family members [365], but also many others (see Chapter 9), are capable of rescuing retrogade loss.

There has been controversy over the presence and extent of retrograde loss of peripheral neurons after axotomy. Identifying *true* retrograde loss of sensory neurons requires rigorous methods for counting, such as the *physical or optical dissector* [117]. Complicating this issue is that some retrograde loss may occur early after axotomy [118], but there may be more extensive ongoing retrograde loss over time. There is less information available about chronic neuronal survival after axotomy [239]. Kuo *et al.* did not observe retrograde loss of L4 and L5 sensory neurons ipsilateral to a sciatic transection in adult rats at 2 weeks but there was 10%–15% loss by 4 weeks from apoptosis [239,365]. Moreover, the apparent loss in numbers later "disappeared," a phenomenon attributed to neuronal replacement. Coggeshall *et al.* [118] noted no loss of dorsal root

myelinated axons, reflecting the number of parent sensory neurons in the ganglia, out to 8 months after axotomy from cut or crush. Loss of unmyelinated axons was noted, however, suggesting an interesting selective process of retrograde degeneration. Higher proportions of retrograde neuron loss have been noted with more proximal lesions, perhaps from interrupting a greater proportion of axons entering a given ganglia [132,764]. In the case of motor neurons [58], retrograde loss is generally uncommon in adults, but death can occur after root avulsion and after axotomy of cranial motor neurons.

Thus, true retrograde loss of parent neurons after axonal damage is not extensive in adult neurons. Their lesser retrograde loss indicates less reliance on target tissue support. Instead, they rely on growth factors synthesized by themselves or by neighbor neurons (autocrine) or by supporting cells in the ganglia (paracrine). Another mechanism of adult neuron survival may be intrinsic resistance to apoptosis. Heat shock protein 27 (HSP27) is a mediator of resistance to retrograde neuron loss and it is upregulated after adult axon injury [123]. After activation by phosphorylation, it operates as a molecular chaperone that protects neurons by refolding altered proteins. HSP27 inhibits cytochrome c-mediated apoptosis by associating with Akt, stabilizes mRNA, and stabilizes the neuron cytoskeleton, actions that rescue neurons from diverse insults [39,123,376,485,618]. It is also expressed in growth cones.

A number of other molecules help to protect neurons from retrograde loss. Members of the p53 tumor suppressor family, in particular ΔNp73, protect adult sensory neurons by acting downstream of JNK proapoptotic pathway activation [719]. PI3K and PKC signaling mediate neurotrophin-independent survival of adult sensory neurons [143]. Overall then, while frank retrograde loss of injured adult peripheral neurons can occur, it is frequently circumvented. Surviving neurons, however, undergo alterations in their phenotype and behavior in response to injury, and these changes are considered next.

Regeneration and perikaryal phenotype

A cascade of cell body changes is associated with axon injury and regeneration, first molecular and then morphological [181,403,707]. These changes, in turn, have a profound influence on the later behavior of regrowing axons and are discussed next. In both CNS and PNS neurons, a large menu of changes in transcription and protein synthesis represents their conversion from a stable transmission status to a dynamic and plastic regenerative phenotype. For example, sensory neurons upregulate genes that express tubulin, actin, and the growth-associated protein B50 (GAP43). The genes that are altered in response to injury during regeneration are known as *regeneration associated genes* (*RAGs*).

In many instances they synthesize proteins that may be expressed within or just proximal to growth cones and that participate in axon growth [707]. RAGs are substantially influenced by growth factors, a topic discussed in Chapter 9.

The cell body reaction

There are early morphological changes, known as the "*cell body reaction*," of the perikarya of neurons that have undergone axotomy. Lieberman's classical work describes these structural changes in detail [403]. Briefly summarized, the most prominent include changes in cell volume with variable early swelling and late (if no reinnervation occurs) atrophy, an early rise in nucleolar volume, disintegration of Nissl bodies, and later displacement of the nucleus from its usual central position to an eccentric one near the cell membrane. Other changes include: increases in smooth endoplasmic reticulum, variable changes in the Golgi apparatus, loss or contrary increases of neurofilaments, and mitochondrial hypertrophy. The changes in mitochondria are distinguished from artifactual swelling or vacuolation that can occur as a result of harvesting and fixation [400].

The classical retrograde axotomy reaction, known as "*central chromatolysis*," describes dissolution and peripheral displacement within the cell body of dark basophilic Nissl substance. Nissl substance is a histological term that describes rough endoplasmic reticulum containing RNA and that stains dark blue and punctuate on hematoxylin and eosin stains. "Nissl bodies" are normally prominent in the center of the neuron and near the axon hillock. Hence the term "central" chromatolysis after axotomy refers to its peripheral redistribution. Central chromatolysis is thought to peak between 1 and 3 weeks following axotomy and usually recovers with reinnervation. In some instances it persists for long periods of time following an axotomy injury that has not recovered. When recovery does occur, there may be a hyperchromatic phase with closely packed numerous Nissl bodies suggesting upregulaton of RNA content. In the case of motor neurons, axotomy also results in loss of its dendrite arbor and its synaptic connections [62–65,610].

Many of the morphological changes following axotomy may simply reflect the impact of altered protein synthesis. For example, the downregulation of neurofilament that maintains the internal lattice of the perikarya may allow the nucleus to move to a peripheral rather than central cellular location. Despite the displacement of RNA containing rough endoplasmic reticulum, overall neuronal RNA content and trafficking are thought to increase after axotomy [403]. There is increased amino acid uptake for new protein synthesis.

There are other interesting aspects of the axotomy reaction. For instance, after axotomy of the central branch of sensory neurons, the cell body reaction

is attenuated or absent [403]. This may indicate that the local microenvironment that axons occupy plays a very important role in determining how the entire neuron tree responds to an injury. Both central and peripheral branches are essential to sensory transmission, but their differing and segregated behavior indicates unique molecular features. Another important feature is that neurons contralateral to an injury develop changes in neuron morphology and gene expression. In the case of mRNAs, these may be quite striking. The existence of contralateral change is interesting and may operate through spinal cord connections or through injury signals that circulate by the spinal fluid. From an experimental point of view, contralateral effects are very important to control for when examining the impact of nerve injury.

Signals, RAGs

Neuronal survival, perikaryal support of distal regeneration, and RAG expression are closely linked. The perikarya has the capacity to recognize, often very quickly, when their peripheral axonal branches are injured or impaired. How an injury to a distal axon tells the central cell body, up to a meter away, to change a vast repertoire of genetic programs is unclear. Some of the candidates for inducing this change include a retrogradely transported protein, a kinase, and a calpain cleavage product of an intermediate filament [405,550]. Initial upregulation of *intermediate early genes* may be the trigger for the subsequent change in protein expression of injured neurons. The intermediate early gene c-Jun is a major component of the AP-1 transcription factor complex. Its activation by JNKs occurs in response to injury and was observed to rise in facial motor neurons of adult mice after transection. Mice lacking c-Jun had defective target tissue reinnervation, axonal sprouting, and attenuated expression of other regeneration molecules (CD44, galanin, and α7β1 integrin) [569].

Following axotomy or sectioning of their distal branches, dorsal root ganglia sensory neurons upregulate mRNAs of a number of molecules that include: GAP43/B50 (growth-associated protein 43), CAP-23 (cytoskeleton-associated protein-23), tα tubulin, ATF3 (activating transcription factor 3), PKCβ (protein kinase C β), MAPK, NPY (neuropeptide Y), SCG10 (superior cervical ganglia neural-specific 10 protein), HSP 27 (heat shock protein 27), and neuronal NOS (nitric oxide synthase). In contrast, they downregulate TrkA, the high affinity NGF receptor, TrkC, the NT-3 receptor, p75, the low affinity NGF receptor, NfM, the medium subunit of neurofilament, and CGRP (calcitonin gene-related peptide) [28,123,179,443,581,685,707]. Galanin, normally expressed in 2%–3% of intact ganglia sensory neurons, is observed in 40%–50% of neurons after distal axotomy. Recent evidence suggests that this upregulation plays a significant role during regeneration. For example, mice lacking galanin have an accelerated

developmental apoptosis of sensory neurons, whereas adult knockout mice exhibit a 35% reduction in regenerative activity [282]. Specific neuron subtypes within dorsal root ganglia, of course, may preferentially upregulate one RAG but not another. The exact time course for changes of gene expression during regeneration vary.

In facial motor neurons, there is a rise in the expression of actin and tubulin proteins after axotomy and a decline in the NfM and NfL neurofilament subunits [675]. Additional molecules downregulated by axotomized motor neurons, summarized by Boyd and Gordon [58] include: choline acetyltransferase (ChAT), acetylcholinesterase (AchE), vesicle-associated membrane protein-1 (VAMP-1), NT-3, NT-4/5, and TrkC. Upregulation takes place for: BDNF, TrkB, p75, RET, GFR-α1, IL-6, LIFR, CGRP, VAMP-2, GAP-43, and HSP27 [123]. Among all of these changes in both motor and sensory neurons, a critical theme is the upregulation of tubulin, a key component of the microtubule and growth cone, and downregulation of neurofilament subunits, structural proteins of stable axons.

Overall then, changes in axotomized neurons reflect the shift from a stable maintenance phenotype to a regenerating one. Many more changes are likely to be discovered. Some of the alterations may have significant signaling implications. The upregulation of the stress-activated transcription factor ATF3, for example, may alter expression of other genes by binding together and acting with other leucine zipper transcription factors as heterodimers. Neurons with overexpression of ATF3 have enhanced neurite outgrowth [612]. The relevance of other alterations, such as those involving intermediate filament proteins is less clear. α Internexin, an intermediate protein that has scaffolding roles and is discussed in Chapter 2, has only low level expression in intact motor neurons, but dramatically rises following axotomy [454]. Peripherin, a related Class III intermediate filament protein, has a rise in its expression in peripheral sensory neurons after injury [519]. Another interesting molecule that appears to rise in the nucleus of injured motor neurons is EXT2, a heparin sulphate-glycosaminoglycan synthesis enzyme. Its rise correlates with increases in the heparin sulphate proteoglycans glypican-1 and syndecan-1 [484]. EXT2 may have a role as a coreceptor for FGF-2 growth factor signaling (see Chapter 9).

In the case of GAP43/B50, its rise in synthesis by DRG sensory neurons after a distant axon injury is accompanied by a rise in the levels of HuD [14]. HuD is a neuron specific RNA-binding protein that interacts with the U rich regulatory element of the 3′ untranslated region (3′UTR) of the GAP-43/B50 mRNA and delays its degradation. HuD protein and GAP43/B50 mRNA expression increased together in DRG neurons peaking at 7 days after a nerve crush and lasted 3 weeks. Moreover, both were colocalized to granules containing ribosomal RNA in the cytoplasm of neurons. By interacting with specific mRNAs, HuD and related

proteins may offer protection and a longer half-life of important regeneration related molecules like GAP43/B50. They may also offer protected transport into axons to effect local later translation of protein.

PI3K-Akt [70] is discussed earlier as a survival pathway that mediates trophic factor support of neurons and blocks apoptosis. Within individual neurons, however, it may be that PI3K-Akt activation also serves roles that promote growth and differentiation (for review see [189]).

In convincing perikarya to change (e.g., upregulating PI3K or RAC1) in ways that facilitate axon regrowth, perineuronal glial cells may play a particularly intriguing role. Perineuronal satellite cells in DRG elaborate GDNF and other growth factors for use by adjacent neurons [250].

Local axon signaling in regeneration

Axonal protein synthesis

At one time, all protein synthesis was considered to arise from perikarya with subsequent transfer down axons by axoplasmic transport. Over the past decade, however, several investigators have established that axons are capable of their own protein synthesis locally [124,703]. Moreover, axon protein synthesis may be influenced by signals from SCs [124].

That local protein synthesis may help direct events in growth cones and regenerating axons is important news in regeneration. Local approaches to bolster synthesis of proteins and facilitate growth cones advance could eventually be applied therapeutically. In axons separated from their cell bodies, protein synthesis may sustain "normal" axon and growth cone behavior for several hours before Wallerian degeneration ensues [710]. Eventually these isolated axons fail, however, and undergo degeneration.

Proteins with critical localized roles in axon protein synthesis include p38 and TOR (target of rapamycin), caspase-3 (apparently independent of its role in apoptosis), ribosomal-P0, and eIF-4E (eukaryotic translation initiation factor-4E). Similarly, proteasome-mediated protein degradative machinery can also be identified: 20s proteasome core, ubiquitin, and ubiquitin–protein conjugates. Local axon synthesis may be important during preconditioning. Preconditioning refers to an enhanced regenerative response to injury in axons having undergone a prior injury. Both synthetic and degradative protein machinery, locally synthesized, are increased in preconditioned axons to prepare them for their augmented response.

Yet other examples of locally synthesized proteins by mRNA in axons are: β actin, peripherin, vimentin, γtropomyosin 3, cofilin 1, heat shock proteins (HSP 27, 60, 70, grp75, αβ crystallin), endoplasmic reticulum proteins

(calreticulin, grp78/BiP, ERp29), ubiquitin C-terminal hyrolase L1, rat ortholog of human DJ-1/Park7, γ-synuclein, superoxide dismutase 1, peroxiredoxins 1 and 6, PGK1, α enolase, aldolase C/ZebrinII [742]. NGF and BDNF increased β–actin, peripherin, and vimentin mRNA transport into axons to facilitate local protein production.

Signaling that relies on local protein production influences growth cone behavior. Several examples have emerged recently. For example, the role of exclusive local axon signaling has been elegantly demonstrated using fluorescent conjugates of growth factors. Cy3-NGF induces rapid changes in chick embryonic sensory growth cones within 1 minute, while still confined to either the membrane or the peripheral cytoplasm of the growth cone [669]. Similarly, as a result of local signaling, the proteins synthesized at injury sites may be those that shut down growth cones and restrain regeneration. Recently, Wu and colleagues [749] have demonstrated that RHOA GTPase is synthesized, localized, and functional in growth cones where it facilitates collapse in response to inhibitory cues. Several other locally synthesized proteins are likely crucial for growth cone behavior. For example, β-thymosin, a molecule that binds and regulates G actin polymerization into F actin, is locally synthesized in Lymnae (freshwater snail) axons where it constitutively inhibits neurite outgrowth [702]. How coordination of regenerative events occurs between local sites and the cell body is uncertain. At this time, the evidence would suggest that both local axon synthesis, local signaling, but also anterograde transport of new proteins together orchestrate the response of growth cones. The relevant site of signaling may also depend on the molecule being considered. Insulin, a potent neuron growth factor with receptors on both axons and perikarya, is capable of promoting distal regrowth, and phenotypic changes of axons when delivered centrally (intrathecally) to access perikarya. Similar doses are ineffective when given locally at the injury site [689].

While yet unproven, it is possible that growth cones are primarily responsible for a rapid local response that depends on local cues. The central cell body is brought into play depending on the extent and severity of the injury. Lesions closer to the cell body generate more rapid RAG changes. Transection with prolonged disconnection from a target involves a much more prolonged loss of target contact than crush and may require much more extensive perikaryal input. Perikarya thus receive retrograde signals from the injured axons that serve as indicators of the type and nature of the lesion. Other retrograde messengers such as growth factors seem to serve a general alerting role. The perikaryon is then prompted to shift its priorities for synthesis. To direct local machinery for protein synthesis, regulatory proteins are transported anterogradely to the injury site. They influence the repertoire and activity of synthesis.

Central perikarya not only respond to events in the axons, but interact locally with each other and with adjacent glia. Thus there is an overall instructive dialog at several levels to help direct the fine tuning of an injury response.

An importin–vimentin–pErk retrograde signal

Recently, a complex retrograde kinase signaling mechanism involving an injury-induced upregulation of importin proteins in axons has been described. Importins interact with dynein, the retrograde transport motor of axons, and act as soluble transport proteins. They "normally" mediate translocation through the nuclear pore complex of a cell but have roles in axons as well where they have been identified. Regeneration-modulating proteins may employ a nuclear localization signal (NLS) that, in turn, binds to importins for retrograde transport [252]. Thus, specific critical proteins, when complexed with importin and dynein are capable of modulating cell body and nuclear function. A related cascade of events involves vimentin, an intermediate filament protein that is upregulated in sciatic axons after injury. Vimentin, in turn, links pErk (phosphorylated and activated Erk1 and Erk2), members of the MAPK signaling family, to importin β in a signaling complex that is retrogradely transported [549]. This intricate story continues. Local axonal injury may promote association of pErk to vimentin through a calcium signal. When bound to the vimentin scaffold, pErk is protected from deactivation by local phosphatases and is transported retrogradely with importin to signal within the nucleus.

Once at the cell body, pErk is released from vimentin and signals widely by directly phosphorylating transcription factors. pErk may inhibit cAMP phosphodiesterase and allow sustained rises in cAMP levels in the perikarya. Rises in cAMP, in turn, mimic the action of a conditioning lesion [503,564]. This overall mechanism also could explain why lesions of axons closer to the perikarya induce more rapid changes in gene expression after axotomy. Proximal lesions may facilitate more rapid and substantial retrograde signaling to the cell body and nucleus.

Given the central signaling role of vimentin, it is not suprising that adult sensory neurons in mice lacking vimentin had impaired regeneration. Regeneration was restored by delivering calpain-treated recombinant vimentin, a procedure that disrupts its polymerization into intermediate filaments. In this specific series of interactions, therfore, it is "normally" polymerized vimentin that is inactive during the cascade, and the depolymerized protein is necessary to convey a signal or act as a transport scaffold. Interestingly, it is a high local calcium intra-axonal environment that inhibits polymerization of vimentin, in turn permitting its participation in retrograde signaling.

Vimentin intermediate filament is expressed only at negligible levels in intact axons. Overall, the findings indicate an unexpected nonstructural second role for an intermediate filament protein vimentin that is independent of its role in the cytoskeleton. They also illustrate a remarkable conservation of proteins for several tasks in the peripheral nerve. Vimentin and other intermediate filaments may therefore act as motile scaffolds in a variety of circumstances [549].

Roles of inflammatory cells and mediators

The context of axon regrowth is critical to its success and is determined by neighboring cells and their actions. By day 3–5 following a peripheral nerve injury, there is local *hematogenous macrophage* invasion into the proximal and distal stumps of an injured nerve [236,580]. Brisk and abundant influx of inflammatory cells appears critical to the success of peripheral nerve regeneration. As discussed in Chapter 3, a major role for inflammatory cells is to accelerate Wallerian degeneration and allow regeneration to follow.

There are also important direct interactions between regenerating neurons and specific inflammatory molecules [417,580]. Examples include interleukin 6, interleukin 1β, TNFα, RAGE, and leukemia inhibitory factor (LIF) [267,287, 489,781]. Interleukin 6 (IL-6) helps to prevent retrograde neuron loss. Its synthesis is upregulated in injured sensory and motor neurons [488,489], in turn triggered by a signal from mast cells. IL-6 is also capable of promoting axon outgrowth over myelin inhibitors similar to that of a conditioning lesion [92]. BDNF may participate in IL-6 actions [487]. Interleukin 1β, acting through non-neuronal cells in ganglia, enhances axon outgrowth [287]. RAGE also influences neurite outgrowth. Thus, for example, sensory neurons grown on amphoterin (a RAGE ligand) with a dominant negative RAGE had attenuated neurite outgrowth [583]. Phosphorylation of STAT3 is involved in RAGE regenerative signaling [583].

Both RAGE and TNFα are also triggers for NF-κB activation, a transcription factor. Thus NF-κB rises in perikarya of peripheral sensory neurons and translocates to their nucleus during activation after a peripheral axotomy injury. Moreover, the TNFα induction of NF-κB appears important for cell survival after injury [182]. ZAS3 is a zinc finger transcription factor protein that competes for sites on target genes with NF-κB and thus can modulate its activity. In contrast to NF-κB, ZAS3 declines after axonal injury, a response that allows enhanced NF-κB action [750].

Galectin-1 is a member of the β-galactoside-binding lectins that is expressed in motor and sensory neurons. When oxidized, it binds receptors on macrophages

that in turn stimulate SC migration and axon outgrowth [285,286]. Oncomodulin is a macrophage-derived calcium binding factor capable of promoting regeneration in retinal ganglion cells [766]. Many additional inflammatory molecules relevant to peripheral nerve regeneration likely exist, but studies have been limited or have emphasized CNS actions [184].

Later clearance of macrophages from the regenerative milieu is also important since some of their products, such as NO and other free radicals, if overexpressed, might collapse regrowing axons. Fry and colleagues [198] demonstrated that the myelin of regenerating axons signal the Nogo receptors NgR1 and NgR2 on macrophages to stimulate their efflux from injured nerves. Nogo signaling is better understood during inhibition of axon outgrowth and activation of RHOA, but this work demonstrates another role for these molecules in the complex regenerative milieu. While macrophages are essential to Wallerian degeneration and subsequent axon growth, prolonging their presence after they have accomplished these tasks may be detrimental.

Collateral and regenerative sprouting and pruning

Axon "sprouts" can be of different types. Regenerative sprouts form when an axon is damaged by transection or crush. Sprouts can form either at the injured end of the axon, or more proximally. In the case of myelinated axons, sprouts tend to form at the next most proximal intact node of Ranvier. Sprouts can also develop from intact axons when they are adjacent to target tissues that have been denervated. Branches from intact axons grow out to reinnervate all, or part, of the denervated territory, a process termed "collateral sprouting." Diamond and colleagues identified important differences in the requirements of NGF for collateral, but not regenerative, regrowth of cutaneous sensory nerves [139–141]. Despite these intriguing findings, less overall attention has been directed toward additional mechanisms that favor collateral sprouting in the peripheral nervous system. It is possible that branching behavior in CNS cortical neurons employs molecular mechanisms similar to peripheral collateral sprouting. In the CNS, for example, netrin-1 induced axon branching by generating calcium transients. These, in turn, activated calcium/calmodulin-dependent protein kinase II (CaMKII) and mitogen-activated protein kinase (MAPK) to accelerate the growth of sprouts from otherwise intact neurons [668]. Arborization of dendrites is promoted by PI3K-Akt-mTOR ("mammalian target of rapamycin") signaling [306].

Since cultured neurons undergo injury on harvesting, the mechanisms of collateral sprouting are difficult to study *in vitro*. This is unfortunate since collateral sprouting has profound importance in understanding recovery from

nerve lesions in humans. For example, after human diagnostic sural nerve biopsies, a procedure that involves resection of a length of the nerve trunk, there is a significant area of sensory loss on the lateral side of the ankle [678]. If followed over time, however, the area of sensory loss gradually contracts from its outside edges inward (centripetal recovery) (see Figure 4.6). Recovery most likely occurs from collateral reinnervation by intact neighboring sensory axons growing into the denervated skin. In other instances, collateral sprouting does not seem to help clinical injuries, an observation that indicates unforeseen barriers to recovery.

Collateral sprouting also reinnervates sweat glands in the skin. Navarro and Kennedy [499] investigated its time course from sural or saphenous axons in mice. Adjacent skin next to either nerve was denervated and the function of individual sweat glands was assessed by measuring sweat droplet imprints formed in response to an injection of pilocarpine. Collateral reinnervation of sweat glands, identified as sweating outside of the normal territory of these nerves, began approximately 28 days after denervation. Young mice had accelerated reinnervation, whereas in older mice reinnervation was slowed.

Collateral sprouting is important in the response to partial motor axon loss discussed in the next chapter [568]. In disorders with chronic motor neuron dropout, remaining motor units undergo considerable remodeling and enlargement from collateral sprouts. Plastic motor unit remodeling helps to maintain more muscle function than might otherwise be possible.

Collateral sprouting explains why injury to a mixed (containing motor and sensory axons) peripheral nerve can result in axons being sent to inappropriate branches. For example, a motor neuron may send branches back down the correct motor branch after an injury but may also project collateral sprouts to an inappropriate sensory branch being sent to the skin (cutaneous). Brushart and colleagues [71] have shown that motor axons preferentially travel to motor branches despite the growth of collateral sprouts, a property called preferential motor reinnervation (PMR; see Chapter 6). PMR increases over time as the projecting motor axons reach their correct muscle targets and inappropriate cutaneous axons are pruned away. The degree of persistent arborization may depend on the accuracy of the pathfinding [578].

Removal of redundant regenerative sprouts, or pruning of improperly projecting branches, helps to recapitulate the matching of parent axons to targets that were present before injury. This activity involves active breakdown of excessive branches rather than a simple process of withdrawal or retraction. Remodeling by breakdown of redundant collateral branches has been observed, for example, during development of the CNS in *Drosophila* mushroom bodies [728]. Pruning has been difficult to document during peripheral nerve regeneration

because most models examine axons penetrating into distal stumps undergoing Wallerian-like degeneration. Distal stumps, for example, after a crush injury, contain an admixture of new regenerating axons and damaged axons undergoing degeneration. In this milieu, it may not be possible to differentiate new axons undergoing degenerative pruning from those degenerating as a result of the original injury. An exception involves axons regenerating across nerve transection gaps early after injury. New axons may be accompanied by profiles undergoing apparent degeneration from pruning before they have reached the distal stump [450].

Collateral reinnervation, pruning, and sprouting have implications for chronic degenerative disorders of the peripheral nervous system and in severe disorders where the inciting disease process (e.g., Guillain–Barré syndrome) has ceased or been arrested. Functional regeneration is unlikely or even impossible in disorders with severe proximal (or perikaryal) damage. Remarkable neurological improvement can occur, however, if residual axons capable of providing axon sprouts are present. This has been demonstrated experimentally in long-term studies of mice rendered diabetic with streptozotocin (STZ), a toxin that destroys insulin-producing pancreatic β cells [335]. The diabetic mice develop neuropathy with eventual drop-out of sensory neurons and loss of skin innervation. If their insulin producing pancreatic β cells spontaneously recover from STZ toxicity, their diabetes resolves but repopulation of lost sensory neurons does not occur. The denervated skin, however, can be reinnervated by residual sensory axons through collateral reinnervation. There are many chronic neuropathies associated with irreversible neuron loss but some degree of recovery might be available through collateral sprouting. Alternatively, inappropriate pruning may limit this kind of recovery.

Nerve trunk architecture

One of the most striking changes in overall nerve trunk architecture after injury is compartmentation, the formation of *minifascicles*, complete with their own multilaminate perineurium [479]. These changes occur in the distal stump during regeneration as axons elongate within them and mature. Minifascicles are also found retrogradely in the proximal stump and in experimental neuromas [755]. Why injured nerves form such minifascicles is intriguing. For example, they never develop without axons within them. Morris *et al.* [479] originally suggested that they are a response of axons in an injury zone struggling to preserve some modicum of protection within their immediate microenvironment. Like the intact endoneurium, minifascicles may offer protection through a blood nerve barrier.

The full repertoire of molecular signals critical to fascicular organization in peripheral nerves is undefined. One relevant pathway is that of *Desert Hedgehog (Dhh) protein*, a member of the hedgehog family of secreted ligands identified during morphological development. Dhh null mice have peripheral nerves that develop abnormally and have persistent minifascicle formation [541]. Dhh signals through a complex that includes the transmembrane receptor components, Patched (Ptc) and Smoothened (Smo). Ptc2 appears to be expressed in SCs. Both it and Dhh are downregulated in distal nerve stumps following injury, subsequently rising during regeneration [29]. Despite the unusual nerve trunks in Dhh null mice, the exact relationship between Dhh ligation, axons, and SCs during injury and repair is unknown. Additional molecules such as members of the semaphorin guidance protein family (Sema3F and Sema3A) and their receptors [602] are thought to regulate fascicle formation during nerve repair.

Fibroblasts are important participants in nerve injury and repair. While the role of "endoneurial fibrosis" is probably overstated (a terminology that does not truly distinguish between SC proliferation, fibroblast population, or involvement of other cell types), these cells nonetheless have an important role in remodeling the nerve architecture. They are distinguished as stellate or fusiform cells with little cytoplasm but prominent rough endoplasmic reticulum and a variable Golgi apparatus. The nucleus is ovoid with only minimal peripheral nuclear condensations. Fibroblasts are not surrounded by the basal laminae that SCs possess, a feature that is evident by EM and can be used to distinguish them. Some have cilia and some have coated pits. Like SCs, fibroblasts can develop alterations of their rough endoplasmic reticulum with deposits of dense inclusion material in cisternae. Overall, their numbers increase after an axonal injury [479].

The *perineurium* undergoes plastic changes following nerve trunk injury. The first is separation of its layers [479]. Degenerative changes may occur later including a reduction in the number of layers, in tandem with compartmentation described above. Axons, SCs, and epineurial-like collagen fibrils can be found between perineurial layers, a feature absent in normal intact nerve trunks. Some perineurial cells participate in phagocytosis.

Mast cells are the tissue cousins of circulating basophilic leukocytes. Some mast cells reside in intact peripheral nerve trunks specifically in the endoneurium and perineurium. After injury, there is a rise in the numbers of mast cells and increased mast cell degranulation within the peripheral nerve. While their overall importance during regeneration is uncertain, their constituents include histamine, serotonin, growth factors, and proteases. Degranulation of mast cells during nerve injury likely contributes to rises in local blood flow and breakdown of the blood nerve barrier [169,533,787,807,809].

Summary

A symphony of interactions characterizes the early regenerative response of the peripheral nerve. The major elements involve organizing membrane formation for new sprouts, reprogramming of the gene expression of the entire neuron, local axonal synthesis, dynamic growth cone behavior, and phenotypic changes of SCs that facilitate axons in outgrowth and guidance. All of these elements must come together in a coordinated fashion for significant regeneration to occur. New molecules and signaling ideas, understood at the level of single growth cones, have become part of the orchestra. In particular, the control of polymerization and depolymerization of actin and microtubules and their relationship to Ras family GTPases have profound effects on growth cone behavior. "Survival" pathways such as PI3K–Akt are also crucial to the story. Collateral sprouting of axon branches from intact neurons differs from regenerative sprouting and presents itself as a key alternative for self-repair of the damaged peripheral nervous system.

Suggested reading

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6

Consolidation and maturation of regeneration

Regeneration is an ongoing process. Once axons form growth cones that cross injury zones, navigate distal stumps and approach former targets, they encounter additional challenges. The interaction of new axons with their targets involves a new series of events with new molecular requirements. Similarly axons are not fully effective the moment they reach their target. They must grow in caliber and may need to myelinate. This chapter will address these events.

Forming new nerve trunks after transection

After the transection of a nerve stump, the proximal and distal stump retract apart because they are normally under some degree of tension. Retraction leaves a gap. In some cases, the gap can be repaired by suturing nerve stumps together, but this is not always possible. Early studies successfully connected the stumps with conduits, tubes, or in one report, synovium propped open by a stainless steel spiral. Various surgical innovations are reviewed in more detail in other texts and sources [421,422,660,775]. They include nerve grafts, silicone tubes, vein–muscle conduits, fibronectin mats, veins, synthetic longitudinal filaments, collagen tubes, polymer biodegradable tubes, and others. With these strategies, eventual complete reconstitution of a severed new nerve trunk from the proximal to the distal stump can occur. This was initially described by Lundborg and colleagues [424–427].

When proximal and distal stumps are connected by tubes, an eventual connection therefore does develop as discussed in the previous chapter [610,740]. In the first week, extracellular exudative fluid and a fibrin matrix clot form within the tubes. The clot retracts from the inner walls of the tube and

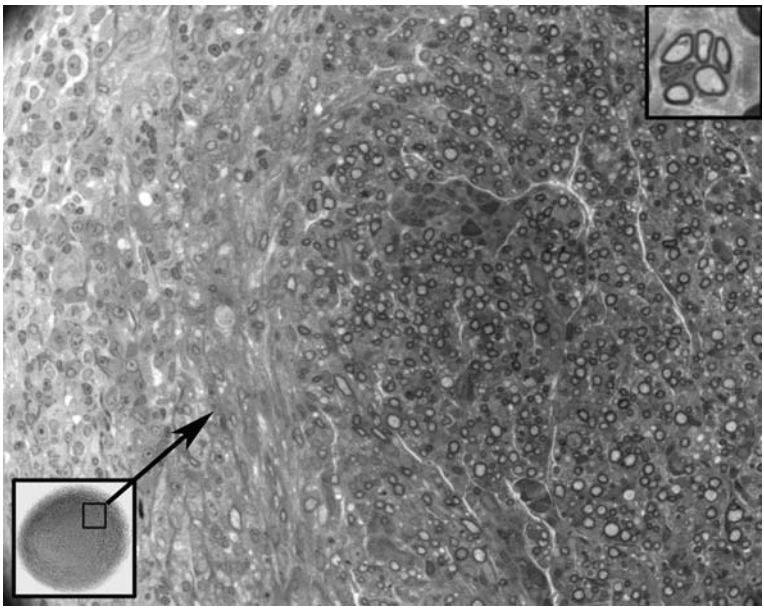

Figure 6.1 Images of reforming peripheral nerve trunks growing through a conduit connecting a transected rat sciatic nerve at 21 days. There is a central core of myelinated axons surrounded by layers of connective tissue. The insets show a macroscopic view of the bridge (lower left) and a higher power view of a regenerative unit with myelinated axons. The images are from semithin sections, epon embedded and stained with toluidine blue.

forms a bridge across which axons and SCs navigate toward the distal stump. In successful bridges, later consolidation of regrowth includes the formation of small "minifascicles" that begin to consolidate in the center of bridge material. With time (months), gaps can be reconstituted with axons and resemble their original nerve trunks (Figure 6.1).

While axons seem to eventually find their way across transection gaps, SCs have difficulty in identifying the correct route. This is illustrated by using conduits with a choice of which direction to grow. Y-shaped conduits were constructed and attached to the proximal stump offering axons or SCs two possible destinations. When one of the Y ends contained the distal stump and the other contained an unrelated tissue, axons preferred to grow in the direction of the distal stump. In contrast, SCs exhibited no such preference and migrated into both ends. Axons crossing excessive gap lengths, however, require support from additional growth factors or other interventions to arrive at the distal stump [428,610]. In rats, this distance limit is approximately 10–12mm. All of these findings illustrate the requirement that axons and SCs closely partner to provide properly directed growth.

Reinnervation of muscle

Reinnervation of muscle involves reconnection of α motor neurons to their endplates, reconnection of γ motor neurons to spindles but also regrowth of sensory axons into muscle. The latter comprise several types of axons as well, including unmyelinated nociceptive axons and large myelinated axons that reinnervate muscle spindles. When regenerating motor axons approach their target muscles, they identify previous endplates in order to re-establish neuromuscular junctions. Terminal SCs, also known as perisynaptic SCs, first cluster at denervated endplates to facilitate reconnection. Once this occurs, regenerating motor axon terminals are then guided to denervated endplates initially by growing along old "SC tubes" from terminal SCs. Despite this form of support, reinnervation rarely fully recapitulates the preinjured state. Remodeled motor units and fiber type grouping, discussed below, become permanent fixtures of reinnervated muscles.

In collusion with terminal SCs, reinnervating motor axon terminals "escape" after innervating their original endplate to branch further and innervate adjacent neuromuscular junction endplates. When reinnervation begins after a partial injury, first, terminal hypertrophic SCs send out processes over a distance of several microns to link adjacent denervated junctions. By next fasciculating with terminal SCs of adjacent junctions, they form a linked network of neuromuscular junctions. As this process unfolds, new reinnervating motor axons follow these SC processes so that any given muscle fiber may now be innervated by several sprouts, a condition known as polyneuronal innervation. During later maturation, as axon reconnections are made, polyneuronal innervation is eventually pruned away.

Terminal SCs have other interesting features. They appear to "sense" synaptic transmission and have rises in their internal Ca^{2+} levels during motor nerve stimulation. They also have muscarinic and purinergic receptors and, if synaptic transmission is interrupted, changes in their gene expression occur. Blockade of motor terminals through botulinum toxin can induce sprouting of terminal SC processes followed by sprouting motor axons. This property has led to the hypothesis that motor axon activity, rather than the presence of axons alone, plays a critical role in whether axons (following terminal SC processes) sprout. Since terminal SC processes engulf or "cap" axons at neuromuscular junctions, they are also well placed to have other roles, such as buffering extracellular potassium. For reviews of the properties of terminal SCs, see papers from the Thompson and Gordon laboratories [319,632,664].

Sprouts into partly denervated muscle therefore can arise from any of the intact residual motor axons innervating a given muscle. Tam and Gordon [664]

described several forms of motor axon sprouts in the vicinity of neuromuscular junctions. Sprouts originating within the original junction area are called "ultraterminal" while those arising from the motor axon just before the junction are called "preterminal." Others arise from more proximal nodes of Ranvier. In some instances, combinations of these patterns and quite complex arborizations may be identified. The unique behavior of terminal SCs can therefore explain several features of reinnervation in the context of partial denervation. First, it illustrates the influence of SC processes in guiding fine motor axon sprout behavior. As discussed in Chapter 5, axons seem to accomplish very little if they are "naked"; rather, they are most influential when they are tightly controlled and shielded by adjacent SC processes. If denervation is prolonged, however, and an axon has not yet arrived at an endplate, the terminal SCs may eventually abandon them (see Chapter 8). Without terminal SCs, axons that arrive later fail to recognize or reinnervate endplates [319].

The behavior of perisynaptic SCs and motor terminals also explains how reinnervating motor units enlarge and render distinctive histological changes within target muscles. In normal muscles, fibers innervated by axons originating from a single motor unit are dispersed through the muscle and are admixed with fibers innervated by other motor units. After reinnervation, this pattern changes. Fiber type grouping refers to the tendency for muscle fibers of a given motor unit to cluster together, indicating the reinnervation of denervated neighbors by sprouts. Clusters, or "groups" from a single enlarged motor unit develop because of the close collaboration between terminal SCs and axons that migrate to nearby denervated neighbors.

Since axons dictate the contractile properties of individual muscle fibers (i.e., slow or fast twitch), all fibers in one motor unit develop identical physiological properties. These properties can also be predicted by histological techniques. Certain types of staining identify specific muscle fiber types: slow twitch fibers, also called Type 1 fibers, contain higher levels of aerobic–oxidative proteins, while Type 2 fibers emphasize anaerobic–glycolytic metabolism and are known as fast twitch. Further subdivisions, not examined here include Types 2A, 2B, and 2C. The ATPase stain can be used to discriminate these fiber types. At high pH values (9.4), the stain is dark (strong) in Type 2 fibers but not in Type 1. At low pH levels (4.3), Type 1 fibers are dark and Type 2 fibers light. With a given histochemical fiber type stain then, fiber type grouping can be easily recognized. Muscle fibers belonging to one reinnervating motor axon unit are clustered together and have identical histological characteristics. In contrast, normal muscles have a scattered, or "checkerboard" pattern of fiber types indicating an admixture of motor units.

Glycogen depletion is another classical method used to demonstrate fiber type grouping during reinnervation. This is based on the idea that high frequency

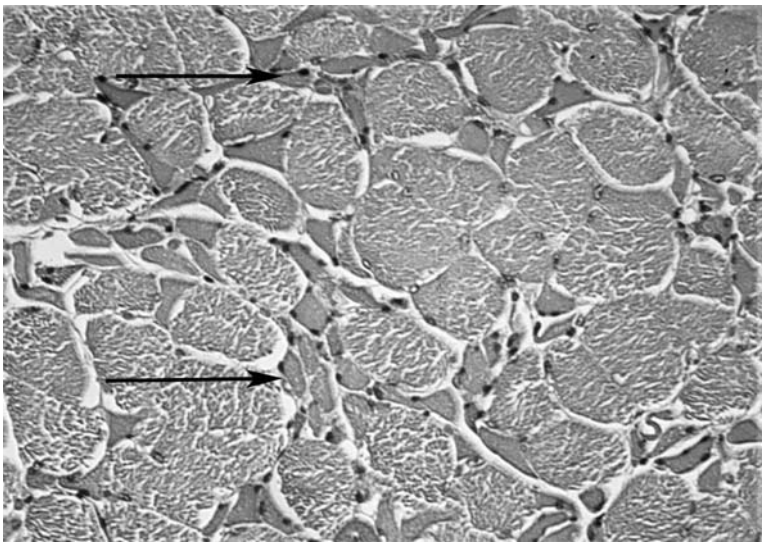

Figure 6.2 A photomicrograph of a sample of ileopsoas muscle from a patient with a severe progressive axonal polyneuropathy showing grouped fiber atrophy (arrows). (Reproduced with permission from [785].) See color plate section.

stimulation of a single motor unit metabolically activates the muscle fibers it supplies, forcing the muscle fibers to consume their glycogen supplies to sustain excitation–contraction. If muscles are rapidly harvested and then frozen and stained for glycogen after stimulation, the fibers innervated by the stimulated motor unit are identified by their loss of glycogen staining; muscle fibers of a given motor unit are normally scattered throughout the muscle. A reinnervated "group" can be recognized because adjacent fibers from the same unit are depleted of glycogen after stimulation.

In a slowly progressive disorder involving loss of motor axons and denervation, there may be ongoing loss of motor axons, while remaining axons sprout in order to compensate for them. Motor neuron disease is an example. Thus, remaining motor units enlarge, but eventually they too may succumb from the disorder. When an enlarged motor unit that has been compensating for denervation in turn disappears, the consequences may be catastrophic. Weakness once characterized as mild may rapidly become severe. If large remodeled motor units drop out during a progressive motor disorder, the change can be detected histologically as "grouped fiber atrophy." Atrophy, a response of muscle fibers to denervation, may occur in groups of adjacent fibers if they had been reinnervated together at an earlier stage of the disorder. Thus, grouped fiber atrophy (Figure 6.2) is a signpost of progressive loss of motor axons. Grouped fiber atrophy contrasts with an acute denervating process where atrophic denervated

fibers are dispersed or scattered throughout the muscle because sprouting and grouping had not occurred. Atrophy of single muscle fibers from denervation can be severe and can result in gross muscle wasting. In the past, neurodegenerative disorders with muscle atrophy were called "wasting diseases."

Reinnervation of muscles can be mapped by one or more electrophysiological approaches described in Chapter 4. These include measures of CMAPs, force measurements, motor unit counts (motor unit number estimates (MUNE)), and motor conduction velocities. In addition, the disappearance of spontaneous electrical discharges (fibrillations) of denervated muscle fibers detected by needle electromyography indicates reinnervation.

CMAP amplitude correlates with both the numbers of innervated muscle fibers and the number of motor axons innervating the endplate. During reinnervation, there is a gradual rise in the amplitude of the CMAP. In serial studies, both the times when the CMAP reappears (the initial potential will be small, reflecting reconnection of the first motor unit), reflecting the first connections of regenerating motor axons to the endplate, and its growth in amplitude, are of interest. However, because of sprouting, recovery of CMAPs may not accurately reflect the numbers of reinnervated axons. Measurements of overall muscle force, rather than that of single motor units, however, have the same caveats that apply to CMAPs. An apparent rapid recovery in force may occur because of sprouting rather than the addition of new motor axons. MUNE estimates may help to solve this difficulty by strictly identifying how many motor units are present.

Motor axon conduction velocities slowly rise after contact and reconnection of regenerating axons with the endplate. The rises reflect axon regrowth in radial diameter, myelination, and maturation of nodal properties. During further maturation, increases in myelin thickness and internodal length also take place.

More mature neuromuscular junctions acquire more "stable" neuromuscular transmission as both the presynaptic terminals and endplates reform. This is described as a greater "safety factor." Unstable transmission can be detected by protocols that are routinely used to detect human disorders of the neuromusucular junction such as myasthenia gravis (Figure 6.3). Early partial motor denervation or reinnervation, like these disorders, also exhibits physiological properties indicative of immature and unstable neuromuscular junctions. Repetitive stimulation is a technique that involves supramaximal stimulation, between 2 and 50 Hz, of motor axons innervating a given muscle. Trains of 6–10 CMAPs are recorded and the amplitudes between initial and subsequent CMAPs are compared. Normally, this form of repetitive stimulation does not impair neuromuscular transmission and CMAP amplitudes are maintained throughout

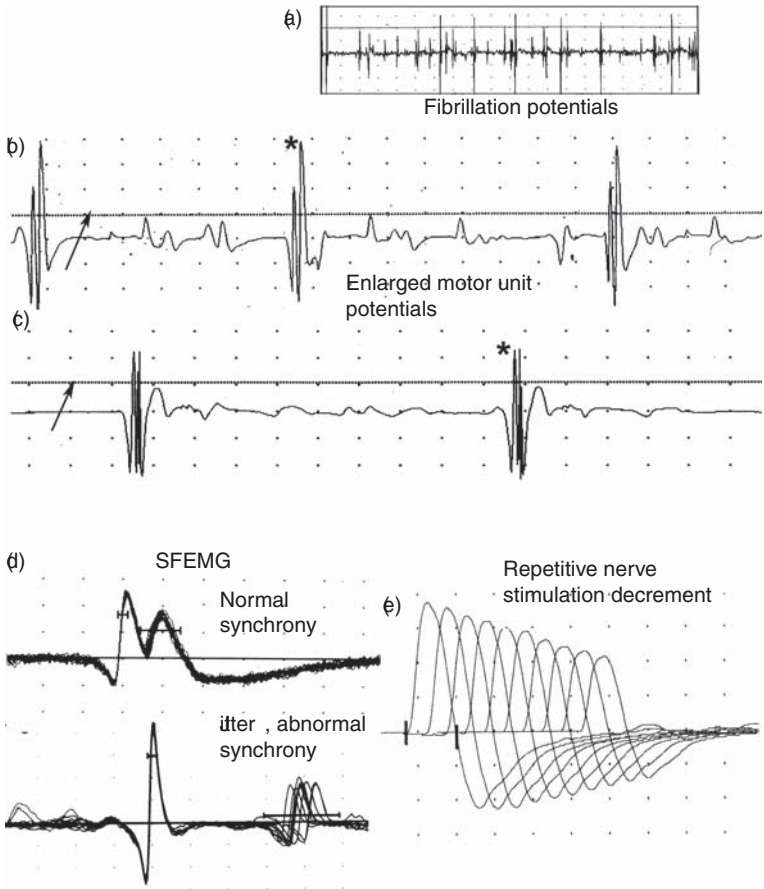

Figure 6.3 Electrophysiological changes associated with denervation and reinnervation in humans. Runs of fibrillation potentials, recorded by electromyography (EMG), arising from individual denervated muscle fibers are shown in (a) (scale is 50 μv/d; 10 ms/d). Enlarged voluntary recruited motor unit potentials, recorded by EMG, arising because of collateral sprouting during partial denervation, are shown in (b), (c) (scale, trigger line and arrow are at 1 mv/d and time scale is 10 ms/d; *indicates a motor unit potential). Unstable neuromuscular transmission is shown by SFEMG in (d) and repetitive nerve stimulation in (e). SFEMG records the synchrony of two adjacent muscle fibers from the same motor unit. Jitter refers to loss of synchrony or variation in the timing of the second potential, shown in the lower trace. The upper trace is normal with near complete synchrony between the discharges (scale is 100 μv/d; 0.5 ms/d). With repetitive nerve stimulation, CMAPs decline in amplitude because of progressive failure of neuromuscular junction transmission by motor units (scale 2 mv/d; 5 ms/d).

the stimulation train. In neuromuscular junctions with a reduced "safety factor," however, the CMAPs progressively decline, since a proportion of the stimulated units begin to fail. This is known as an electrodecremental response, and declines in the CMAP of over 10% are considered abnormal. Single fiber electromyography (SFEMG) is another approach used to examine neuromuscular junction instability. In this technique, popularized by Stalberg and colleagues [638], a needle electrode with a small, active recording site on the side of the needle shaft is introduced into a muscle. Voluntary potentials can be identified as arising from individual muscle fibers by filtering out activity from most neighboring fibers. By careful electrode placement and slight voluntary activation, potentials from only two adjacent muscle fibers from the same motor unit can then be isolated and their firing synchrony examined. Unstable neuromuscular junctions (reduced "safety factor") have increased "jitter," referring to an excessive variation in the synchrony of firing. Thus, the adjacent muscle fiber potentials have variability in their ability to fire simultaneously. Blocking, a phenomenon in which one potential fires but the adjacent potential fails, also occurs. A variation of the SFEMG technique involves stimulating the nerve rather than using voluntary recruitment, an approach applicable to animal models. Overall then, electrophysiological properties can be used to indicate that neuromuscular transmission is unstable, with a reduced "safety factor" reflecting immaturity of sprouted innervating axons or unstable axons undergoing degeneration.

EMG recordings can also be used to gauge reinnervation. During reinnervation, there is repair of abnormal spontaneous electrical activity. Fibrillations disappear first. Next, functional reconnection occurs, and single or multiple new motor units can be activated by slight, voluntary contractions. Early during reinnervation, motor units may be quite small with multiple peaks (polyphasia). Motor units then gradually enlarge. If axon reconnection to the muscle endplates is complete, motor units regain their normal sizes. If, however, there is persistent partial loss of motor units, residual motor unit sizes can exceed normal; this is due to sprouting. In conditions with long-standing partial but permanent denervation, such as previous poliomyelitis, they may enlarge up to 5–10 times the normal size, and form "giant motor units" (Figure 6.3).

Reinnervation of the skin

We do not understand the guidance cues and trophic factors that allow precise reinnervation of the skin, or what barriers are overcome in doing so. It is assumed that, once axons reach the skin, their full ramification throughout the dermis and their invasion of the epidermis becomes possible. Nonetheless, some

curious barriers to full reinnervation can be identified. Reinnervation may never be complete after nerve transections and abnormal function may persist [152]. For example, patients note considerable fluctuation in their sensation early during recovery from neuropathies. Paraesthesiae, or tingling asleep sensations, may be prominent, a development that may arise from abnormal ectopic impules generated by immature axons. There may be variations in sensation depending on limb temperature and there may be misrepresentation of sensation. Innocuous sensations of heat or pressure that instead evoke pain are termed allodynia. In patients with neuropathy, cold sensations may be misinterpreted as heat [767].

Polydefkis and colleagues examined the time course for skin reinnervation in human subjects given local topical treatments with the neurotoxin capsaicin (0.1% cream over an area of 35×50 mm) [559]. Capsaicin activates TRPV1 channels and can eliminate unmyelinated axons in the skin within 48 hours. The mean rate of epidermal nerve fiber reinnervation was approximately 0.18 fibers/mm per day. The onset of regeneration, indicated by the reappearance of epidermal axons, was apparent by about 1 month after treatment. Sustained regeneration, however, was slow, over 100 days and up to 350 days in some subjects. It is uncertain to what distance or depth capsaicin neurotoxicity extends. The success of reinnervation in this study may have thus depended on how capsaicin dissipates after delivery and whether deep parent axons were damaged. Overall, these and subsequent studies indicate that skin reinnervation and remodeling can be prolonged.

Wiberg and colleagues [739] examined skin reinnervation in humans after hand reimplantation. Eight patients were studied at intervals of 26–81 months after surgery to coapt and repair the median and ulnar nerves, whereas the radial nerves were not repaired. Multiple approaches were used to examine sensory reinnervation of the hands. Four patients of the eight had a return of two point discrimination, six to light touch, one to pain, five to cold, and six to sweating. SNAP amplitude recovery was less than 40% of values recorded from their intact contralateral hands, and sensory conduction velocities ranged from 50%–90% of the intact values. Mechanical thresholds to von Frey hairs were variable with most ranging over 150% of the intact values, indicating that a greater stimulus was required to evoke a response. Temperature thresholds to heat, cold, and heat-pain were also variable, but in the range of 150%–200% of the intact side, again indicating impaired sensory recovery (higher threshold than normal). While recovery of PGP 9.5 labeled axons innervating the epidermis occurred in two patients, the remaining patients had only approximately 50% of the expected numbers of epidermal axons. Axons labeled with other markers, such as CGRP and VIP, were also reduced in most patients to varying extents.

Interestingly, the radial nerve territories, where repair had not been carried out, were nonetheless found to contain axons labeled for PGP 9.5, CGRP, or VIP. All of these axons, however, were severely reduced in number. Their presence suggested robust collateral reinnervation by the adjacent reconnected median and ulnar nerves since spontaneous reconnection of the unrepaired radial nerve was unlikely. Taken together, these results in humans illustrate the substantial delays in reinnervation of the skin and the inability of collateral reinnervation to adequately compensate for these losses.

Analagous to the terminal SCs associated with the neuromuscular junction, terminal SCs help to guide regenerating sensory axons toward their original targets [152]. These SCs have different characteristics from the SCs of peripheral nerve trunks. They do not proliferate after denervation, and they also have somewhat different biochemical properties when denervated. While the significance of these differences is uncertain, these SCs upregulate acid phosphatase while downregulating alkaline phosphatase, ATPases, and oxidoreductases. Terminal SCs also express p75 NGF receptors and L1 basement membrane protein. For sensory axons transducing touch/pressure, there are additional guidance cues to aid reinnervation. Merkel cells, or touch domes that mediate slowly, adapting small receptor field touch/pressure sensations may be one of these cues. Thus, a proportion (40%) of Merkel cells survive denervation to guide sensory axons back to their original sites. It is also thought, however, that newly regrowing sensory axons can form new Merkel cells [152].

There may also be differences in the regeneration rate of different types of axons. C and Aδ axons may reinnervate more effectively than large caliber sensory axons. Pacinian corpuscles can be redundantly reinnervated by multiple sensory axons, and Meissner's corpuscles reappear deeper in the dermal papillae than they were originally placed [483,770]. Pacinian corpuscles, Meissner's corpuscles, Merkel touch domes, and the pilineural complex associated with guard hairs may all display persistent structural abnormalities despite reinnervation. Why different types of axons should regenerate differently is unclear, but it is likely there are different guidance cues and differing distributions of growth factors available to them. There may be unique physical constraints to the types of targets that different kinds of axons must reach and what basement membrane they need to associate with. During development, presumptive proprioceptive neurons *in vitro* extend more robustly on laminin and fibronectin and preferentially express α3–5β1 integrin receptors, whereas cutaneous neurons prefer laminin and express higher levels of the integrin α7β1 [240]. The regrowth of sensory axons may be more prone to error than that of motor axons [284,609,720]. There is also the possibility that sensory neurons have differing intrinsic regenerative capabilities. For example, IB-4 non-TrkA GDNF sensitive

sensory neurons *in vitro* have less regenerative potential. Their tardy behavior was not corrected even if they were grown on laminin or were forcibly transfected with α7 integrin and GAP43/B50 [381].

Navarro and colleagues [501] examined reinnervation of the footpad skin after sciatic nerve lesions in mice. After a crush lesion, PGP 9.5 labeled axons reappeared in dermal nerve trunks by 14 days with rapid subsequent reinnervation of the epidermis, Meissner's corpuscles, and sweat glands. Sweat gland reinnervation paralleled a return of functional sweating. By 6 weeks, however, the density of epidermal axon reinnervation remained incomplete. After a more severe nerve transection injury, the timing of reinnervation was further delayed. Reinnervation first appeared by 35 days after injury. By 6 weeks, the epidermis had only rare short axons innervating it. Sweat gland reinnervation had not recovered fully. The time courses for reinnervation by axons expressing the peptides CGRP, SP, and VIP after crush or transection paralleled that of PGP 9.5 labeled axons.

Tseng and colleagues [695] described reinnervation of the skin in rats that underwent chronic constriction lesions (CCI) of the sciatic nerve. These injuries, discussed in Chapter 3, are models of neuropathic pain and involve the placement of four loose ligatures around the nerve. There is subsequent slow strangulation and degeneration of the nerve. Not all of the axons degenerate in this model, and small unmyelinated axons are relatively preserved. If the sutures were removed by week 4 after the lesion, there was a slow rise in reinnervation of the skin by 6 weeks and 8 weeks but only to less than half of the pre-injury value. Reversal of behavioral abnormalities such as thermal hyperalgesia and mechanical allodynia developed more rapidly and completely. Only rats in the decompressed group recovered. Since CCI is a partial nerve injury model, it does appear that preserved cutaneous axons can serve an important role in guiding regrowing axons back to the skin. As will be described below, however, morphological and behavioral recovery do not necessarily occur simultaneously. This work also illustrates that pain generation is a complex process and does not strictly correlate with the presence or absence of regeneration. Full and complete regeneration, however, usually extinguishes neuropathic pain.

Misdirection of axons

Misdirected axons can fail to reconnect to their target. This outcome is as detrimental to recovery as if no axon regrowth whatsoever had occurred. Surgical approaches can help to prevent axon misdirection. For example, suturing predominantly motor nerve trunks to their own distal stumps, or sensory nerve trunks to sensory distal stumps leads to better outcomes than suturing mixed nerve trunks [660]. This finding illustrates that mixed trunks offer axons

more opportunities to go astray or be misdirected, limiting the success of overall regeneration. Myelinated axons may also inappropriately grow through pathways previously occupied by unmyelinated axons [629]. Which factors misdirect axons away from fully innervating distal stumps is uncertain. Specific cues, some from SCs, can help direct sensory, motor, or autonomic axons to appropriate targets. Guidance also depends on the type of axon being considered and the roadmap to their destination. For example, in one study using retrograde markers, a higher proportion of regrowing axons arrived successfully back into their original tibial nerve trunk after a sciatic nerve injury than arrived later into their original distal skin territory [562]. There may be fewer successful cues for correctly directed regeneration into the distal skin territory compared with more proximal nerve stumps. It is not uncommon to identify sensory deficits in human skin with nerve injuries that have failed to recover even after decades. "Digit splitting" in human hands, with localized loss of sensation to only one half of a ring finger from an ulnar nerve lesion may persist for long periods. Loss persists despite the presence of intact innervation in the nearby other half of the digit, innervated by the median nerve. In this instance, there is a long-term failure of invasion of axons that are meant to collaterally sprout from one half of the finger to the other.

Brushart and colleagues first described how regenerating motor axons preferred to enter nerve branches traveling to muscles, rather than to an equally available branch directed to the skin (cutaneous branch) [71]. This interesting property is referred to as "*preferential motor reinnervation*" (*PMR*) and occurs through active pruning of inappropriately directed motor axons heading down the cutaneous branch [72]. PMR and overall motor axon regeneration are facilitated by exogenous electrical stimulation [7]. Simulation, in turn, is thought to recruit endogenous BDNF that acts through TrkB receptors. Electrical stimulation also may support PMR by upregulating HNK-1 expression. HNK-1 is an acidic glycan carbohydrate moiety that is exclusively expressed by the compact myelin, basal lamina, and cell surface of SCs associated with motor axons in motor fascicles [162]. Thus, HNK-1 promotes preferential outgrowth from motor neurons, but not sensory neurons [441].

While adhesion molecules are discussed in more detail in Chapter 10, I will briefly outline some of their roles in PMR. Franz and colleagues [190] described high levels of neural cell adhesion molecule (NCAM) and its polysialic acid (PSA) moiety in motor axons targeting motor pathways. Mice lacking NCAM failed to exhibit PMR and had less withdrawal of misprojected axons, fewer collateral sprouts, and smaller fields of arborisation. PMR was also abolished in mice with enzymatic removal of the PSA moiety. Alternatively, Saito and colleagues suggested that sensory fascicles did not express HNK-1 but expressed NCAM on

SCs associated with unmyelinated sensory axons [592]. Overall then, while NCAM is expressed both on axons and SCs, there are discrepancies as to whether sensory axons of motor branches preferentially express it. Most studies agree however, that HNK-1 is a motor-associated basement membrane molecule. F-Spondin extracellular matrix adhesive protein is also more selectively enriched in motor branches where it facilitates axon outgrowth [82].

Are new neurons formed in ganglia?

There is no current evidence that endogenous stem cells provide ongoing replacement of lost motor neurons in adults. In sensory ganglia, however, this possibility has been considered. Devor and colleagues [136] suggested that sensory neurogenesis was ongoing in young and adult rats. These authors identified surprising rises in neuron counts within ganglia over time. Their conclusions were disputed by others who criticized the counting approach. The concept has recently resurfaced but has not escaped controversy. Kuo *et al.* [365] described a rise in nestin-labeled lumbar DRG sensory neurons ipsilateral to sciatic nerve injury in adult rats starting at 2 weeks and lasting out to 6 weeks. The numbers increased by adding NT-3. While nestin can indeed be used to identify new neurons *in vitro*, this molecule is also present in endothelial and other cells and may not accurately reflect neural stem cells *in vivo* [495]. Cheng *et al.* [107] examined the uptake of BrdU, a nuclear marker of the cell cycle entry, in DRGs. Populations of proliferating DRG cells were identified that developed after axonal injuries of the sciatic nerve. Most proliferating cells represented perineuronal satellite cells. These cells also exhibited hypertrophy and heightened expression of glial fibrillary acidic protein (GFAP). Some actively proliferating cells might represent stem cells, but this possibility has not been confirmed. Cheng *et al.* [107] also identified rare, mature intact neurons with BrdU uptake, suggesting ongoing replication. The question of whether mature neurons divide remains speculative. It has recently been shown that neurons destined for apoptosis can inappropriately enter the cell cycle (and take up BrdU) prior to death [264]. In turn, apoptosis is recognized to occur in sensory neurons as a retrograde response to axon injury. Overall, it therefore remains controversial as to whether new sensory neurons can arise in ganglia. There have been no investigations as to whether autonomic neurons are capable of replacement.

Support of consolidation

Neurotrophic support is discussed in Chapter 9. In the case of regrowing motor axons, trophic support does not facilitate all aspects of regeneration.

In fact, when targets are recognized, growth factors may delay proper neuromuscular junction development. Peng *et al.* [548] observed that a mixture of BDNF, GDNF, NT-3, and NT-4 and interventions to raise intracellular cAMP levels promoted outgrowth of xenopus motor neurons. All of these stimuli, however, downregulated the processes of agrin deposition and Ach receptor clustering when the motor neurons were cocultured with muscle fibers. Coculturing normally promotes the formation of neuromuscular junctions, and the changes in agrin and Ach receptors are cardinal requirements for muscle innervation. SC-conditioned media restored the requirements and allowed neurons to switch to a synaptogenic state, modifying the actions of the neurotrophin mixture. Thus, the findings confirm that the SCs not only facilitate growth of axons, but also guide axons in their attempts to form stable neuromuscular junctions.

SCs that support regrowing axons elaborate different menus of growth factors, depending on the type of nerve they are associated with. Thus, for example, denervated ventral roots expressed mRNAs for pleiotrophin and GDNF and supported motor axons in preference to cutaneous axons. In contrast, cutaneous nerves upregulated NGF, BDNF, VEGF, HGF, and IGF-1 and preferred to support cutaneous axons. Interestingly, these menus are modified over time if cross-connections among regenerating nerve trunks are made [280].

Myelination

Remyelination of peripheral axons during regeneration is thought to recapitulate events during development. The reader is referred to striking and helpful depictions of its progress illustrated in other sources [23,661]. Overall, myelination is a unique biological process in which SCs initiate the synthesis of large amounts of lipid membrane material upon contact with axons. Moreover, since axons must acquire a significant radial diameter before myelination begins, it is not just axon contact that is relevant. During development, the critical diameter of an axon that induces myelination begins at approximately 2 microns. Both myelination and radial axon growth can proceed concurrently at this point. Myelinated axon segments have a 1:1 relationship with the SCs that myelinate them. Thus, individual SCs line up along a given axon and each is responsible for an internodal segment of myelin. Any given internode is only associated with one SC. This differs from CNS oligodendrocytes where a given cell supplies multiple axons.

The lipid material from the SC membrane is spirally wrapped around the axon, then compacted. Spiraling of myelin occurs from the side of the SC membrane facing the axon, and this process slowly “drags” the rest of the SC around with it [79]. Compaction involves narrowing of spiral membranes and extrusion of most

of the SC cytoplasm. The collapsed cytoplasmic surfaces of the SC membrane adjacent to one another form the major dense line, whereas the apposed extracellular faces form what is known as the intraperiod line. Compaction does not occur at some sites including the periaxonal collar, the outer SC loop, the paranodal loops, and the Schmidt–Lanterman incisures. The portion of the SC cytoplasm next to the axon, or periaxonal collar, is termed the inner mesaxon, whereas that on the outer surface of the SC is called the outer mesaxon. All of the events of myelination have specific triggers. For example, P0, the most common protein found in peripheral myelin, helps to form an adhesive lattice during compaction. Its extracellular domain forms interactions with other P0 molecules on the adjacent membrane, helping to form the intraperiod line. Its intracellular domain also interacts with like domains on the opposite membrane side to form the major dense line of myelin. Thus, components of P0 operate as adhesives on both sides of the membranes.

Myelination is not simply a product of an SC and axon interaction. There are important signals from the basement membrane that are critical to initiate myelination. One signal involves an interaction between a heparin sulphate proteoglycan glypican-1 and the α4(V) motif of Type V collagen [112]. Glypican-1, present on the SC surface, is required for both α4(V) binding, and for incorporation into the normal SC basement membrane. The interaction of these molecules, both apparently synthesized by SCs, is a prerequisite for subsequent myelination, as studied in cocultures of embryonic rat sensory neurons and newborn rat SCs. There are several examples of abnormal developmental myelination in transgenic mice that result from failed interaction between basement membranes, adhesion molecules, and SCs [112]. Mice with mutations in the laminin α2, α4, or laminin γ1 subunits, rendering abnormal laminin 2, 8, or both, respectively, all had defective myelination. Similarly, disruption of β1 integrin or dystroglycan impairs proper myelination.

Several other molecules play critical roles in the development and maintenance of myelin. One player is Krox20 (also known as Egr2), a zinc finger protein transcription factor. Mice lacking Krox20 fail to develop peripheral myelin. Later, conditional knockdown of Krox20 disrupted mature myelin sheaths. NRG, discussed in Chapter 5, is essential for Krox20 expression [486]. Further transcription factors with related actions include Oct6 (SCIP/TstI) and Sox10. Oct6 is translocated to the nuclei (where it plays out its role as a transcription factor) of SCs that proliferate during Wallerian degeneration and early regeneration [330]. Mirsky and Jessen [475] provide a list of additional transcription factors that influence SC behavior.

Growth factors have important impacts on myelination both during development and in adults during regeneration. NGF acting through TrkA promotes

myelination in sensory neuron-SC cocultures [96]. Similarly, BDNF facilitates myelination both during development and in the adult. Mice overexpressing BDNF had thicker myelin with a less proportionate increase in axon caliber [683]. High doses of recombinant human GDNF given to rats altered axon–SC units and promoted myelination of normally unmyelinated axons [279]. These interesting findings occurred in the context of enhanced SC proliferation in response to systemic intraperitoneal injections of GDNF (10 or 100 mg/kg) given to adult rats. Normally, in the rat, unmyelinated axons occur in a ratio of approximately 6 axons per SC Remak bundle. After GDNF, this ratio changed to 1.5–3 axons per SC Remak bundle, and a number of the small unmyelinated axons showed early myelination. Thus adult SCs do alter their behavior in response to growth factors and GDNF may play a role in determining when an axon switches from being unmyelinated to myelinated. TGF-β1 also appears important for the maintenance of mature myelin: mice lacking this growth factor had abnormal "honeycomb" myelin with expanded SC cytoplasmic collars [130].

Long-term indices of regeneration

Relatively few studies have examined how indices of regeneration in models recover during long-term follow-up. For example, after sciatic nerve transection in rats, recovery of function may be substantially delayed even up to 6 months. The long-term outcome after injury is influenced by the degree of injury and the time to repair (if carried out). The types of assays examined may also differ in their analysis of long-term recovery. While it is assumed that more accelerated early regeneration translates into more rapid and long-term recovery, this may not necessarily be the case. If there is an unsupportive nerve or target microenvironment (see Chapter 8), or an opportunity for slower axons to "catch up," the long-term outcome may not improve despite more rapid initial regeneration.

McDonald and Kennedy [332,451] examined mid-sciatic nerve transections and repair with a tube conduit in adult rats and transections in mice, respectively. They found that small CMAPs reappeared from the interosseous foot muscles by approximately 6–8 weeks and grew slowly thereafter. By 10 weeks, CMAP amplitudes were less than 25% of pre-injury values in both models. By 20 weeks, values still remained significantly below the normal range. Motor nerve conduction velocities only recovered to 50% of control values by 20 weeks. The findings indicate that the behavior of initial reconnected motor axons may not predict the course of full recovery. In contrast, crush injuries recovered more rapidly [332]. CMAPs reappeared in mice by 3 weeks and by 8 weeks were 67% of values prior to injury. Conduction velocities improved more rapidly.

Analysis of myelinated axon repopulation of injured nerves identifies surprising structural plasticity. For example, after a mid-sciatic crush injury in mice, Kennedy and colleagues [332] identified rises in the number and density of mouse tibial myelinated axons from 2–8 weeks after injury to values higher than intact nerves. Their axons, however, were smaller in caliber and their myelin sheaths thinner. These findings confirmed that individual parent axons gave rise to more than one daughter axon. By contrast, with transection injuries myelinated axon numbers and density had only recovered by about 75% by 10 weeks after injury and regenerated axons were small in caliber with thinner myelin sheaths. The tibial nerve is mixed but contains a large proportion of motor axons. The sural nerve, almost exclusively sensory, had similar patterns of change. McDonald *et al.* [451] examined myelinated axon repopulation of rat sciatic nerves and their branches after 20 weeks distal to 3–5mm gaps following sciatic transection and temporary placement of silicon regeneration conduits. In the sciatic nerve distal to the injury and in all its three branches, the number of myelinated axons exceeded those of the intact nerve as in the mouse model above. The axons, however, were smaller in caliber indicating immaturity in radial growth characteristic of multiple daughter axon branches.

The important caveats about electrophysiological and structural indices of regeneration also apply to behavioral techniques. For example, measurements of recovery of grip strength or walking may not coincide. Reinnervation of proximal muscles below the knee may allow some function of long digit flexors and extensors that impact grip strength. Thus, after mid-sciatic transection, McDonald identified the first evidence of recovery of grip by 8 weeks, whereas none was found with a walking track until 16 weeks. This correlates with other reports. Walking track analysis of functional foot reinnervation in mice after transection by Yao and colleagues indicated very limited recovery out to 90 days after injury, despite immediate resuture. Crush injuries recovered within approximately 20 days. Dysfunction from contracture of the foot muscles is a complication of prolonged nerve injury [759].

It is difficult to conclude that sensory recovery differs substantially from that of motor function using electrophysiological or behavioral criteria. Again, the types of assays all have important caveats in their interpretation. By 20 weeks after mid-sciatic transection in rats, McDonald found that SNAPs and sensory conduction velocities from fibers reinnervating the digits remained substantially below control values. The morphology of the sural sensory nerve trunk did not necessarily predict electrophysiological properties of their more distal digital axons. Neither mean myelinated axon diameter nor the number of myelinated axons correlated with the amplitude of the digital SNAP. McDonald

also found that withdrawal latencies to thermal stimuli of the hindpaw shortened somewhat by 4 weeks after injury. No further improvement was observed. Mechanical hyperalgesia of the hindpaw appeared within only 1 week of sciatic transection, a finding that most certainly reflects collateral sensory sprouting from adjacent and intact saphenous axons. Hyperalgesia persisted for 20 weeks thereafter. Similarly, skin pinch tests applied to the lateral aspect of the paw with a forceps (graded for flinching with or without vocalization) returned rapidly after transection, also indicating surprisingly rapid collateral reinnervation. Overall, there did not appear to be a correlation between how many myelinated axons repopulated the sural nerve with measurements of thermal or mechanical sensation. Each kind of measure requires separate interpretation.

Recovery from forelimb lesions have had less attention. Galtrey and Fawcett [208] tested regeneration and functional recovery in rats following combined median and ulnar lesions above the elbow. Nerves underwent crush, transection, and primary repair (immediate direct suture of the proximal to distal stump), transection, and crossed repair (median to ulnar and ulnar to median), or transection without repair. At 15 weeks following injury, crush lesions exhibited near complete recovery of most indices of regeneration: the staircase test, pawprints, grip strength, horizontal ladder, von Frey testing, and cold sensitivity. For most of these tests recovery of function was crush > primary repair > crossed repair > no repair. Even with primary repair after transection, some tests, such as cold sensitivity remained very abnormal by the 15 week endpoint (<15% of normal). Behavioral tests that depend on more complex function and proximal limb muscles recovered more fully. Central remodeling and adaptation to distal deficits compensated for failed distal regeneration.

In conclusion, long-term studies of reinnervation demonstrate surprising complexity. It is important to emphasize that apparent structural recovery may not translate into behavioral improvement. Interpretation of recovery requires consideration of the type of axon being tested and the assay for recovery that is chosen. Comprehensive sets of testing are undoubtedly required for predictions of the overall outcome. In motor fibers, early reconnection of proximal muscles that operate the paw may allow earlier recovery of walking and grip. Similarly, with sensory testing, there may be surprisingly rapid recovery because of collateral reinnervation. A drawback of collateral reinnervation, however, is that it may be associated with painful allodynia.

Plasticity of the central nervous system

The central nervous system responds to injury and regeneration of the peripheral nervous system. This may occur in several ways. Motor neurons,

for example, have extensive dendritic arbors that extend throughout the anterior gray matter horn of the spinal cord, allowing them to form synaptic connections with interneurons or afferent neurons. After axotomy, there is remodeling of these arbors and they undergo shrinkage and retraction. Similarly, there is a loss of axon boutons that normally connect with these dendrites. Regrowth of the arbor and reconnection to synaptic boutons occur with peripheral reinnervation [62–65]. Even with long-term reinnervation, however, there are redistributions of the type and localization of the boutons associated with motor neuron dendritic branches [65,770].

The central nervous system offers representative topographical maps of somatic sensation and motor function at several levels. Topographic representation (somatotopic) of sensation is found, for example, in the dorsal horn of the spinal cord, thalamus, and cortex. By using electrophysiological approaches (e.g., magnetoencephalograpy) and high resolution imaging (fMRI), these maps can be identified and examined in response to peripheral nerve lesions. Such studies indicate that maps are highly plastic and change, sometimes rapidly, to alterations in sensory input. Anesthesia of body parts shifts the map toward greater emphasis on the nonanaesthesitized body. Similarly, a peripheral nerve lesion may permanently alter the size and distribution of its sensory map in the cortex. After the peripheral nerve lesions that accompany transection, phantom sensations of the missing body part may be associated with altered cortical or subcortical sensory maps, sometimes generating pain. The sequence of cortical events following a focal lesion of a peripheral nerve in a human, such as the median nerve has been reviewed [421]. In the distribution of the cortex previously representing the nerve territory, there is initial loss of activity, followed by expansion of the representation of adjacent intact territories into it. These newly occupied territories are refined over time. Restitution of the previous cortical map can occur after a crush injury, if reinnervation is complete. In contrast, transection injuries with permanent misdirection of axons are not associated with eventual return of normal cortical maps. Instead, a mosaic of discontinuous cortical representation zones develops progressively over time [714]. Transplanted hands can re-acquire portions of previously shrunken cortical maps [218]. There is less information about cortical reorganization of motor maps from selected peripheral motor lesions. Since most nerves are mixed, containing motor and sensory axons, it may be difficult to distinguish their relative impact on the cortex.

The overall significance of cortical maps is that they may offer an opportunity to offset “permanent” peripheral nerve deficits by central compensation. The neurobiology that underlies cortical remodeling is not fully understood. One might appreciate, however, its capability to offer insights into compensation

for peripheral lesions; patients with irreversible peripheral lesions may benefit from improved function and less pain.

Summary

The consolidation and maturation of regeneration are at least as important as early regenerative events in dictating recovery. At this time, major limitations of understanding persist in how motor axons and sensory axons reinnervate their targets over the long-term. Both collateral reinnervation and central nervous system cortical remodeling may modify long-term benefits, but neither completely compensate for severe injuries such as transection. SCs remain important partners in later aspects of regeneration and specific trophic molecules may help. Repopulation of parent neurons with stem cells at the level of the anterior horn or ganglia may appear attractive but face challenges identical to those of native neurons during reinnervation of distal targets. Local events that influence how distal axons behave will play major roles in functional reconnection, irrespective of whether they arise from old or new neurons.

Suggested reading

Armati, P. J. (2007). *The Biology of Schwann Cells*, Cambridge, UK: Cambridge University Press [23].

Lundborg, G. (2004). *Nerve Injury and Repair. Regeneration, Reconstruction and Cortical Remodeling*. Elsevier [421].

Mirsky, R. & Jessen, K. R. (1999). The neurobiology of Schwann cells. *Brain Pathology*, **9**, 293–311 [475].

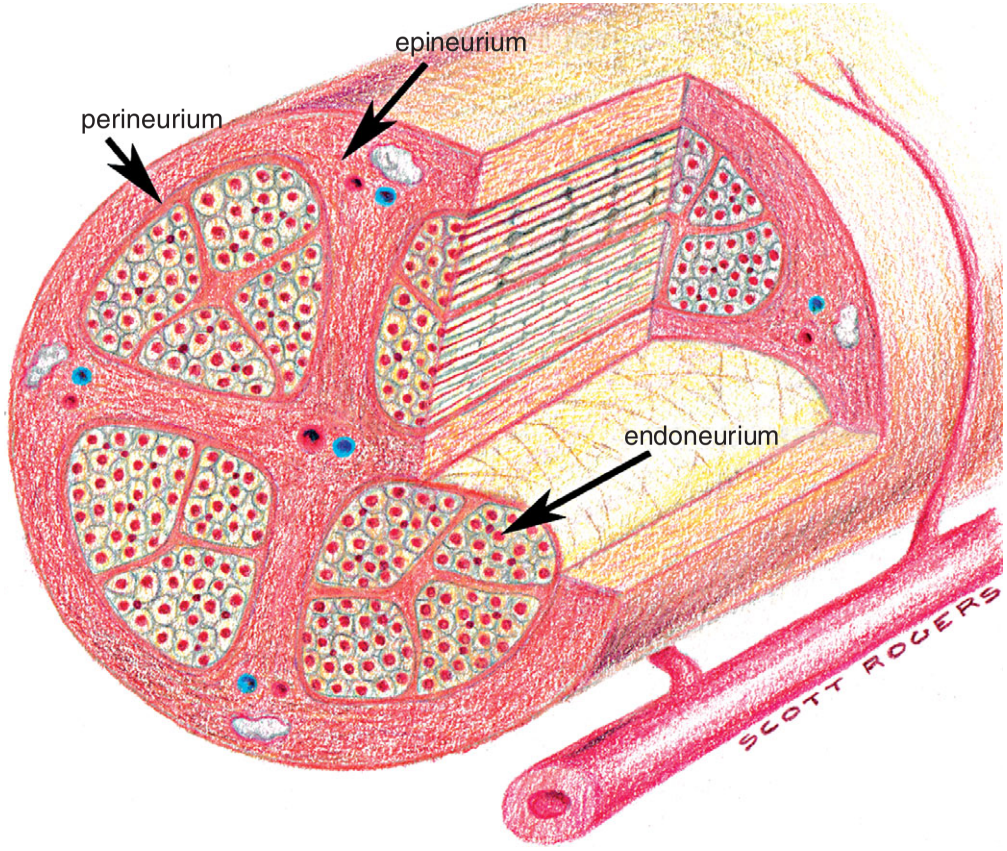

Figure 2.2 An illustration of the overall organization of the peripheral nerve trunk and its compartments. (Illustration by Scott Rogers.)

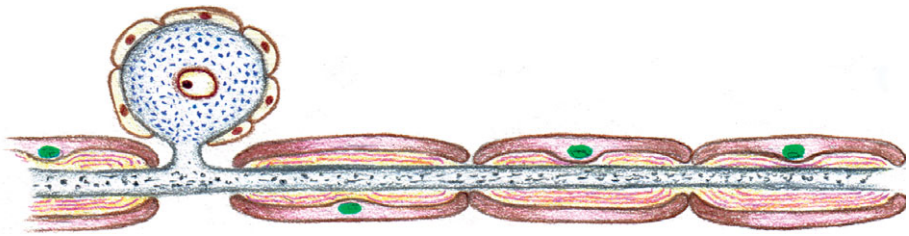

Figure 2.11 An illustration of a primary sensory neuron that resides in a dorsal root ganglia. The neuron is described as "pseudounipolar" with a single branch from the perikaryon (cell body) that then divides into central and peripheral branches. The perikaryon is surrounded by closely apposed perineuronal satellite cells. (Illustration by Scott Rogers.)

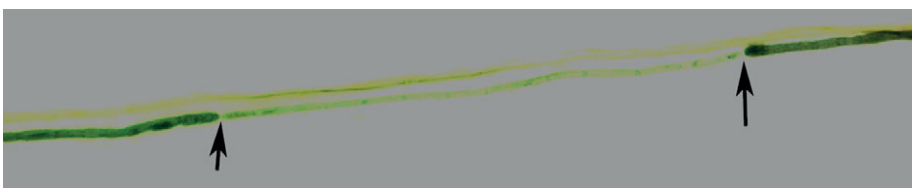

Figure 3.1 An image of a teased myelinated fiber illustrating segmental demyelination between two nodes of Ranvier (arrows). Demyelination accounts for neurapraxic nerve injuries.

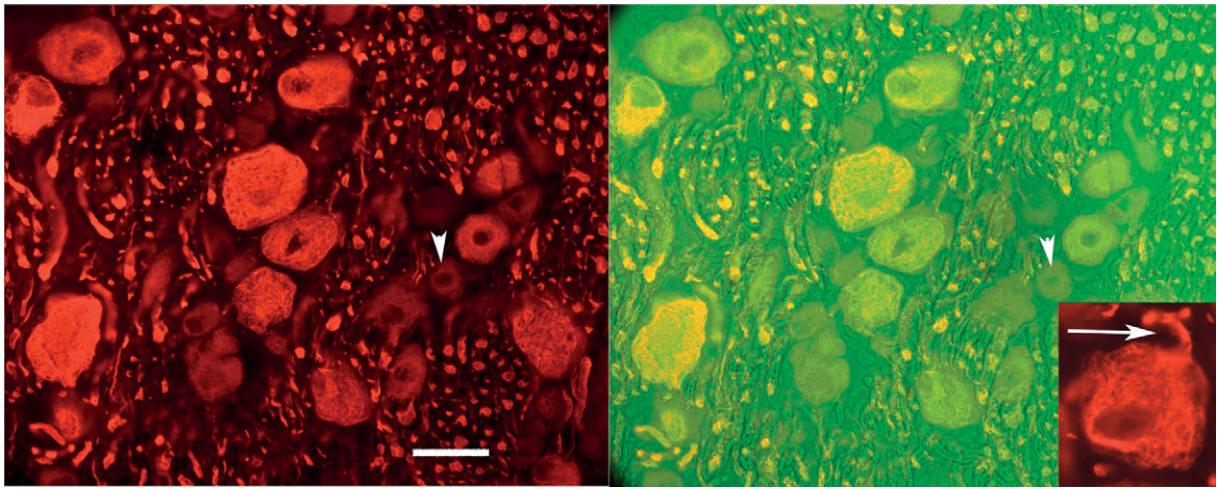

Figure 2.13 An image of DRG sensory neurons labeled by immunohistochemistry with an antibody directed against the heavy subunit of neurofilament (red). The arrowhead points to a smaller neuron with less prominent neurofilament staining (sometimes erroneously called "neurofilament negative"). The right (green) panel is the same section with transmitted light. The inset shows a single large neuron with a "unipolar" process (arrow) emerging from the perikaryon. Note also that the neurofilament label shows a patchy and complex network within the perikaryon that is thought to determine overall neuronal shape. (Bar = 20 microns) (Image taken by Chu Cheng, Zochodne laboratory.)

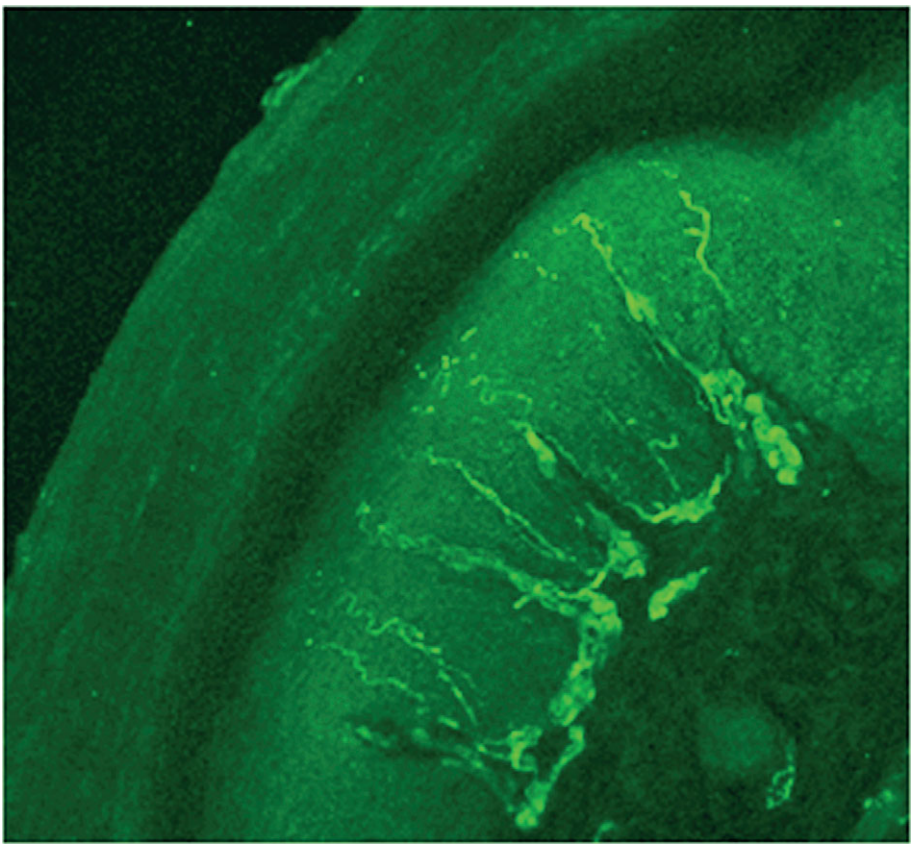

Figure 2.15 An image of the skin of a normal mouse footpad labeled by immunohistochemistry with an antibody directed against the axon marker PGP 9.5 (green). Note the dense ramification of tortuous axons up from the dermal plexus into the epidermis. The epidermis is also covered by thick callus. (Image taken by James Kennedy, Zochodne laboratory and reproduced with permission from [335].)

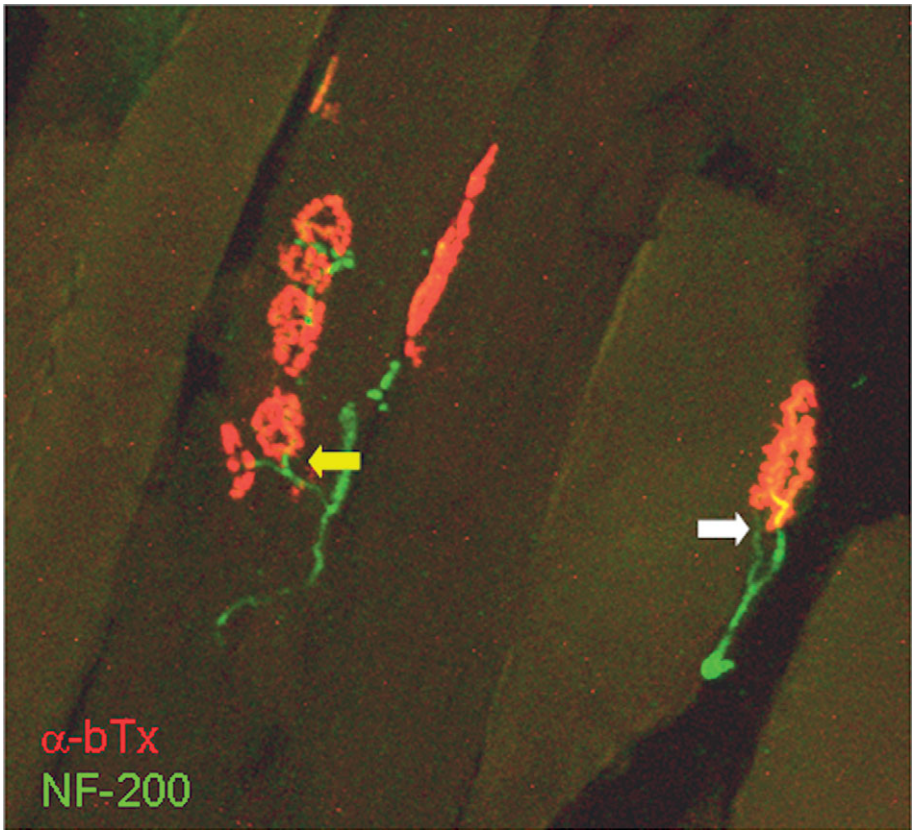

Figure 2.16 Images of neuromuscular junctions from the mouse hindlimb labeled by immunohistochemistry with an antibody directed against neurofilament (NF200, green) identifying terminal motor branches. The junction itself is labeled with α bungarotoxin (α-bTx, red) that binds the presynaptic terminals. (Image taken by Noor Ramji, Zochodne lab and reproduced with permission from [571].)

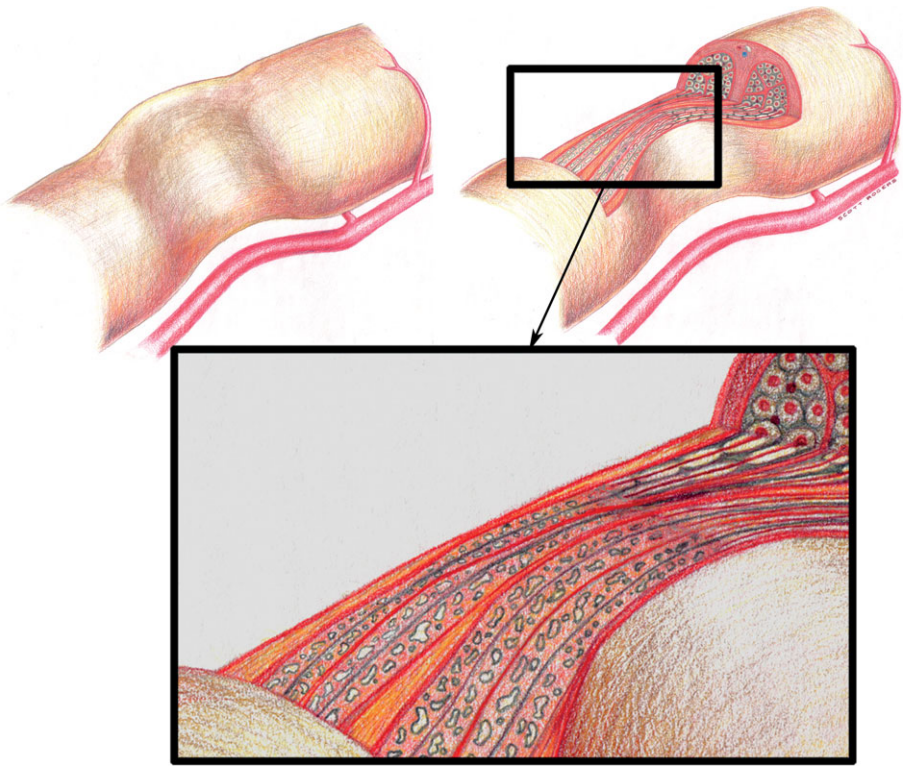

Figure 3.2 An illustration of early events following a peripheral nerve trunk crush (axonotmesis). Distal to the center of the crush zone, Wallerian-like degeneration begins with breakdown of myelin and axons. (Illustration by Scott Rogers.)

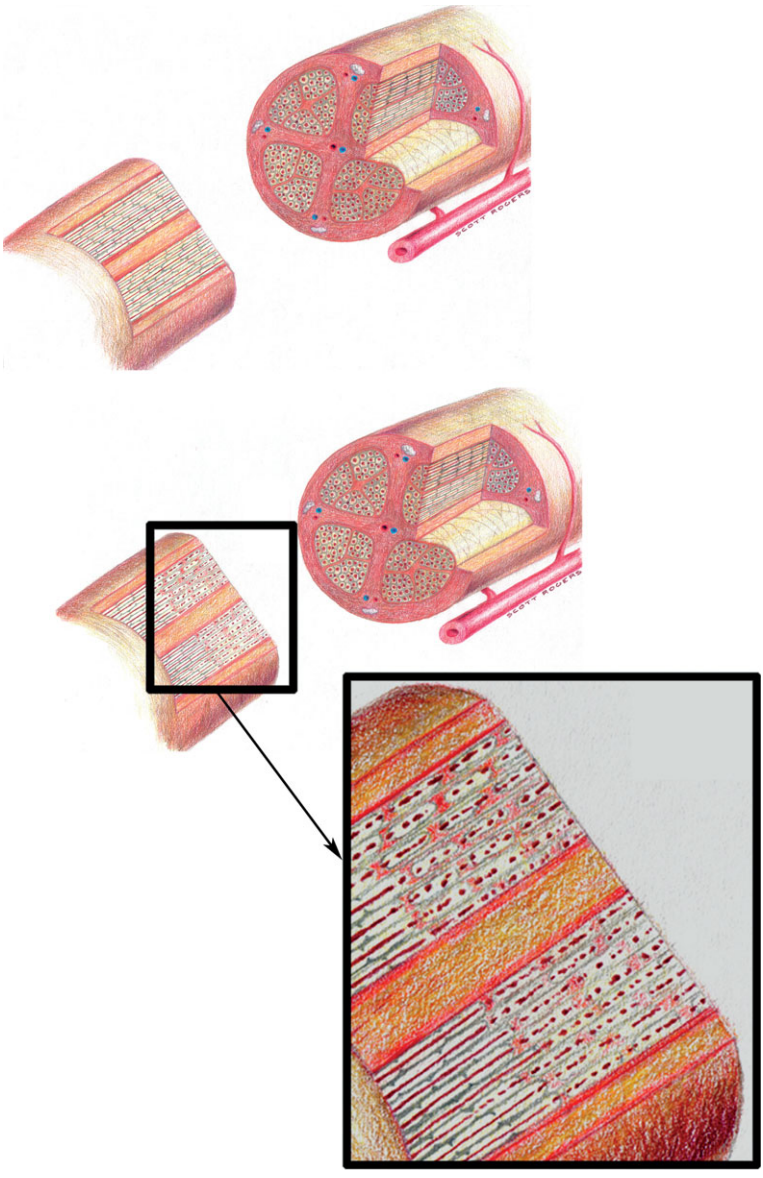

Figure 3.3 An illustration of early events following a peripheral nerve trunk transection (neurotmesis). In the distal stump, Wallerian degeneration begins with breakdown of myelin and axons. (Illustration by Scott Rogers.)

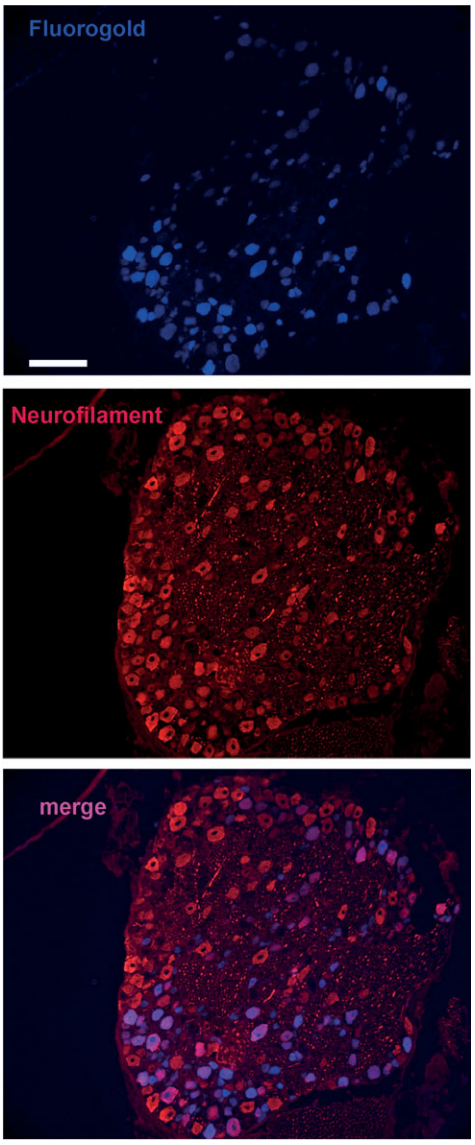

Figure 4.4 Images of DRGs retrogradely labeled with fluorogold (FG, blue) after a sural nerve injury. The sections are colabelled with an antibody against neurofilament (red). (Image taken by X.-Q. Li, Zochodne laboratory.)

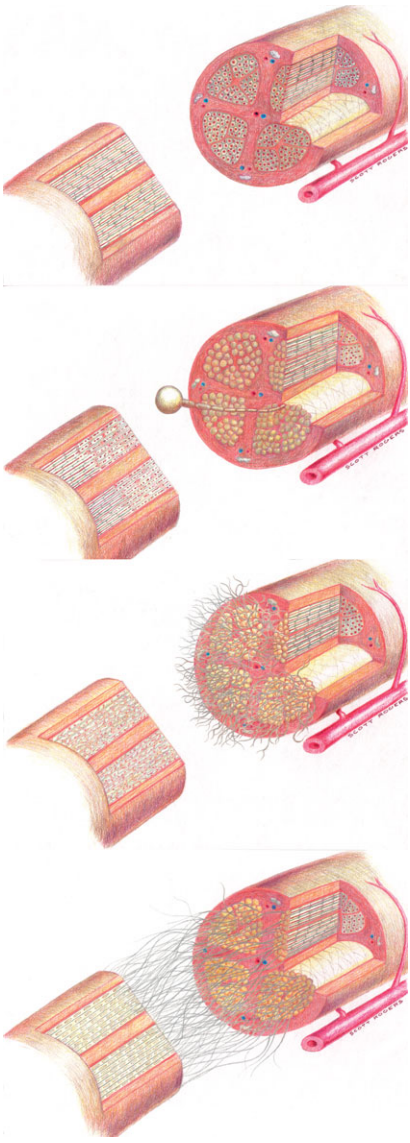

Figure 5.1 Illustration of the sequence of events during early regeneration from a nerve trunk transection injury. The figures from top to bottom show transection, followed by axonal endbulb formation, early regenerative sprouts, and bridging of the transection zone. (Illustration by Scott Rogers.)

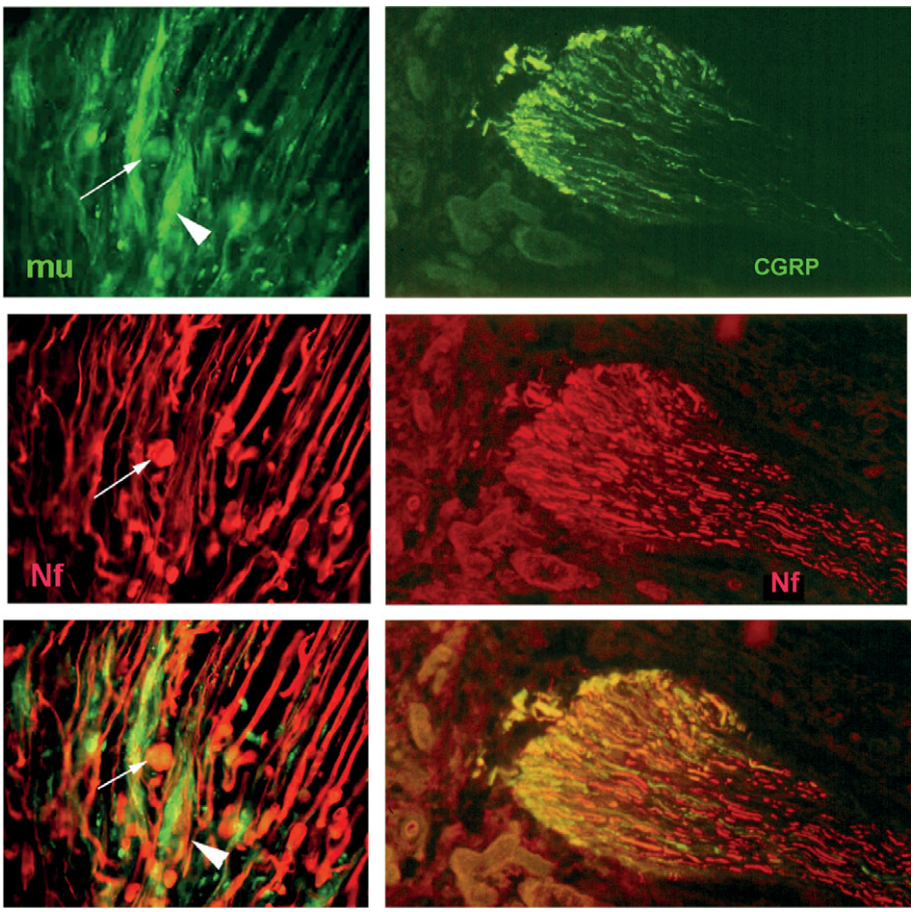

Figure 5.2 Images of axonal endbulbs using immunohistochemistry to label their specific constituents. The panels on the left show discrete (arrow) and irregular (arrowhead) endbulbs proximal to a chronic constriction injury lesion that contain the mu opioid receptor, double labeled with an antibody directed against neurofilament (Nf). The panels on the right show CGRP containing endbulbs proximal to a sciatic nerve transection also double labeled with neurofilament. (Images reproduced with permission from [694] and from [788].)

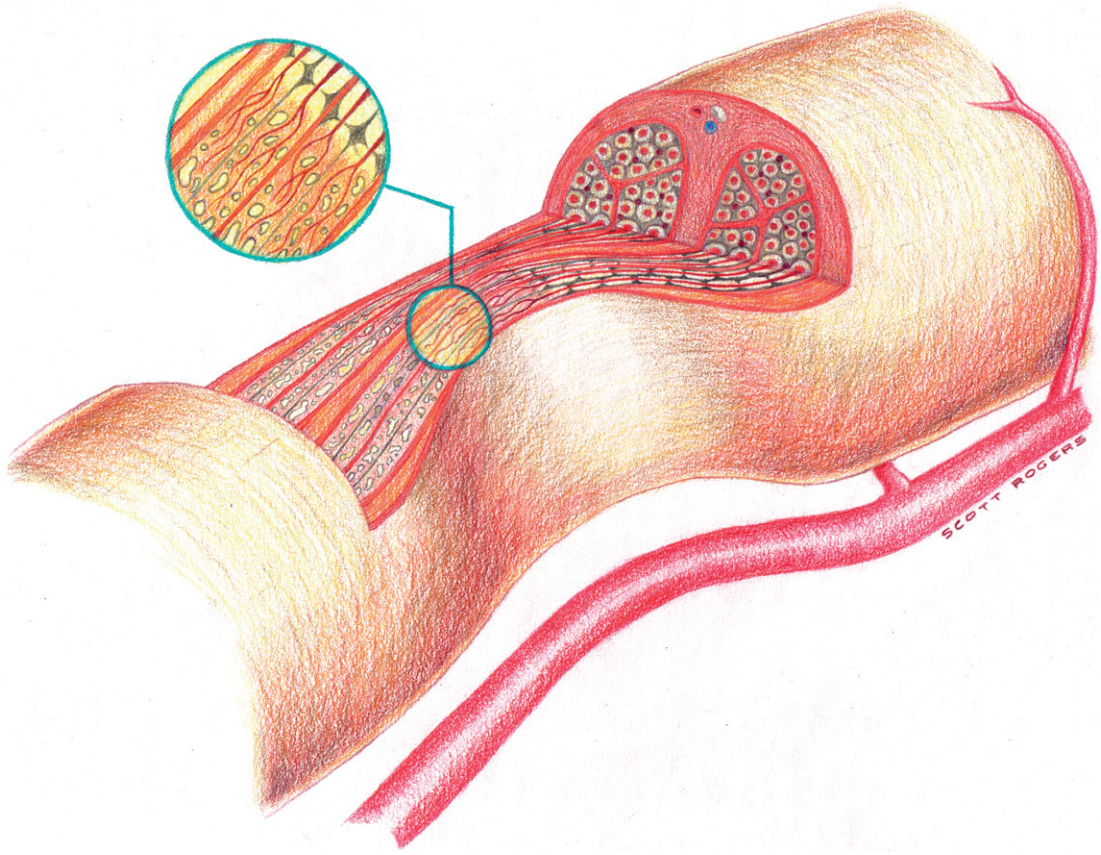

Figure 5.4 Illustration of early axon sprouting following a nerve trunk crush injury. Note that each parent axon can send multiple daughter sprouts. (Illustration by Scott Rogers.)

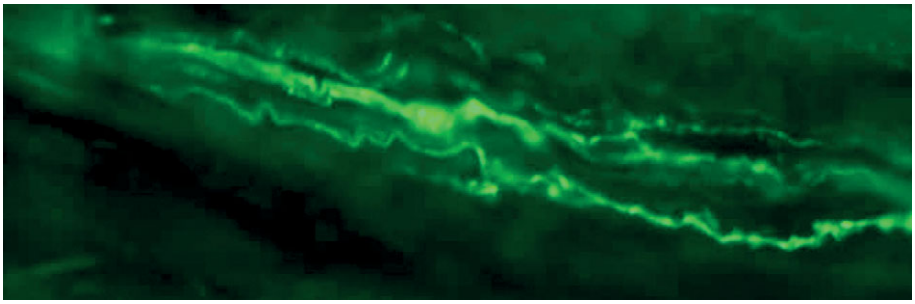

Figure 5.5 Images of axonal sprouts after a crush injury of the rat sural nerve using immunohistochemistry to label CGRP. CGRP is displayed prominently in early regrowing axons. (Image taken by X.-Q. Li, Zochodne laboratory.)

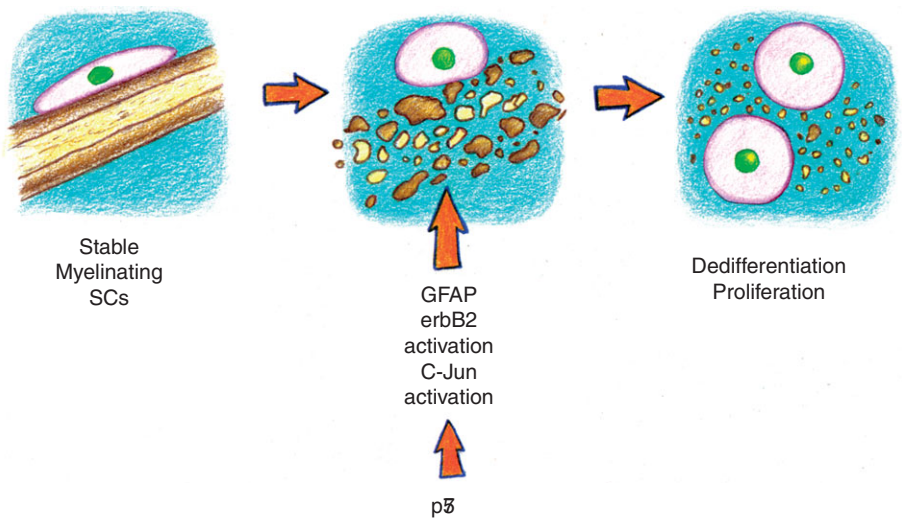

Figure 5.11 Illustration of changes in SC phenotype after nerve injury. (Illustration by Scott Rogers.)

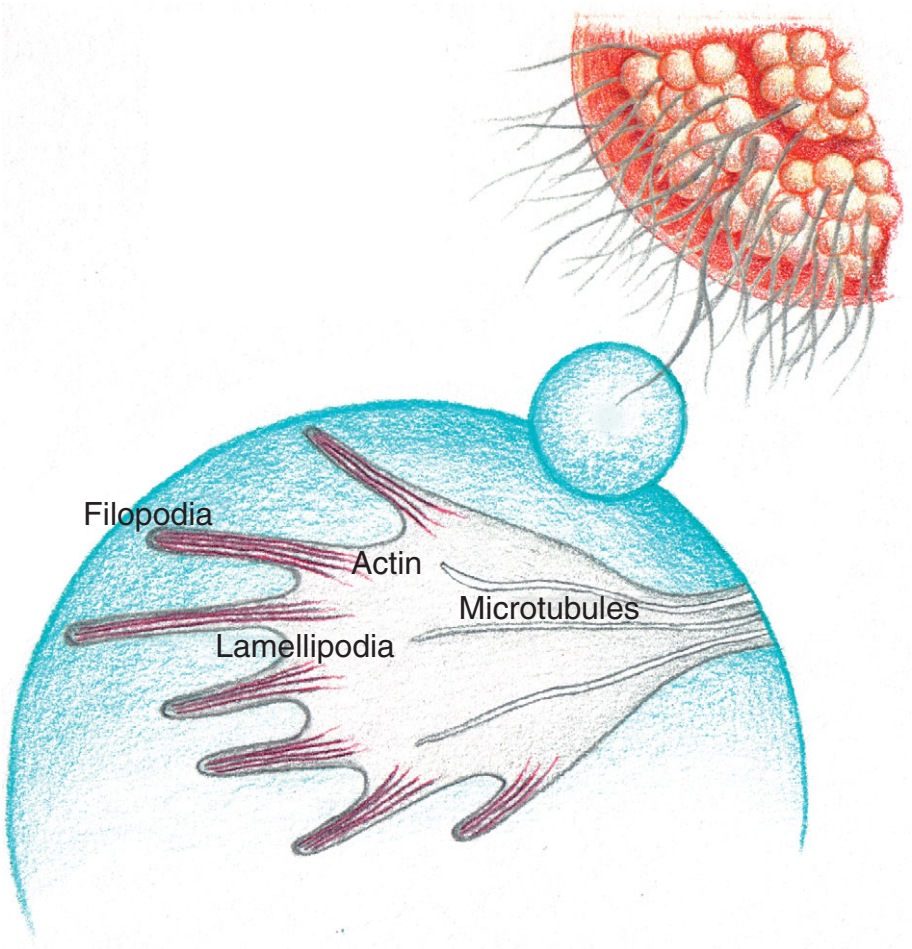

Figure 5.7 Illustration of axon sprouts and a growth cone with its main components labeled. (Illustration by Scott Rogers.)

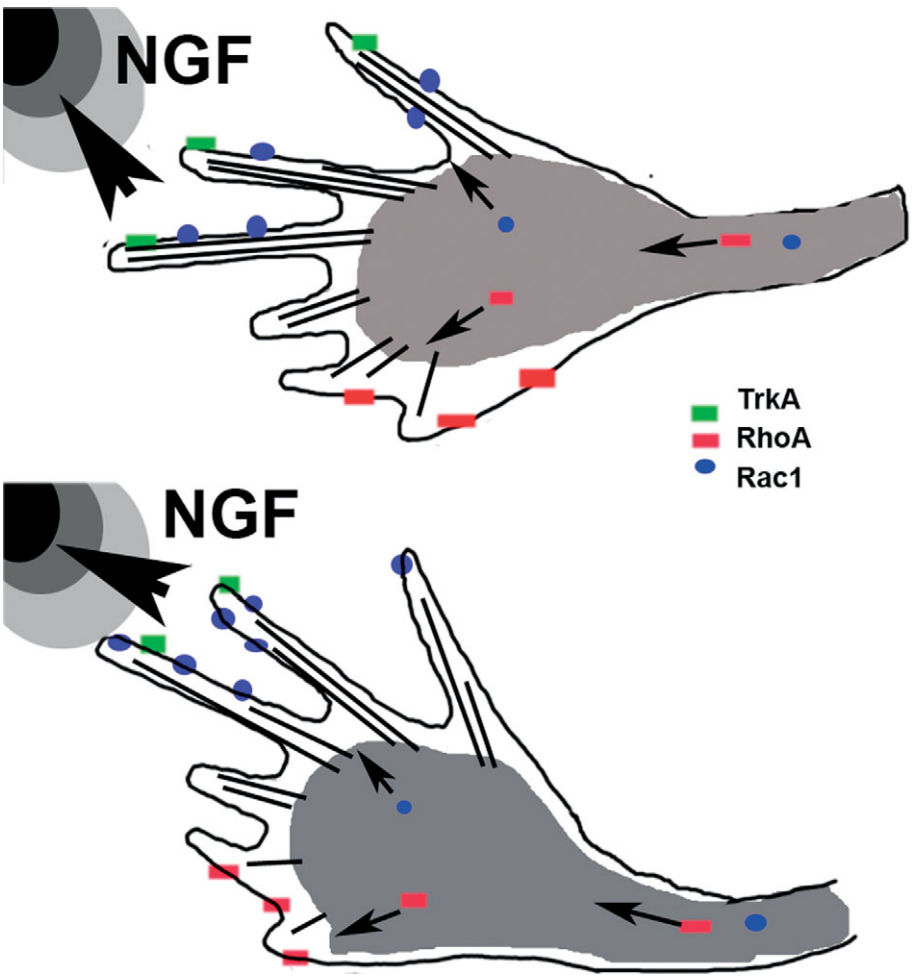

Figure 5.9 Simplified drawing illustrating how growth cones change the direction of their trajectory in response to localized gradients of growth factors such as NGF acting on their TrkA receptors (green). RHOA GTPase (red) is thought to mediate active repulsion and may be localized to growth cone membranes that are collapsing and not advancing whereas RAC1 facilitates outgrowth and may be localized to the membranes of advancing parts of the growth cone.

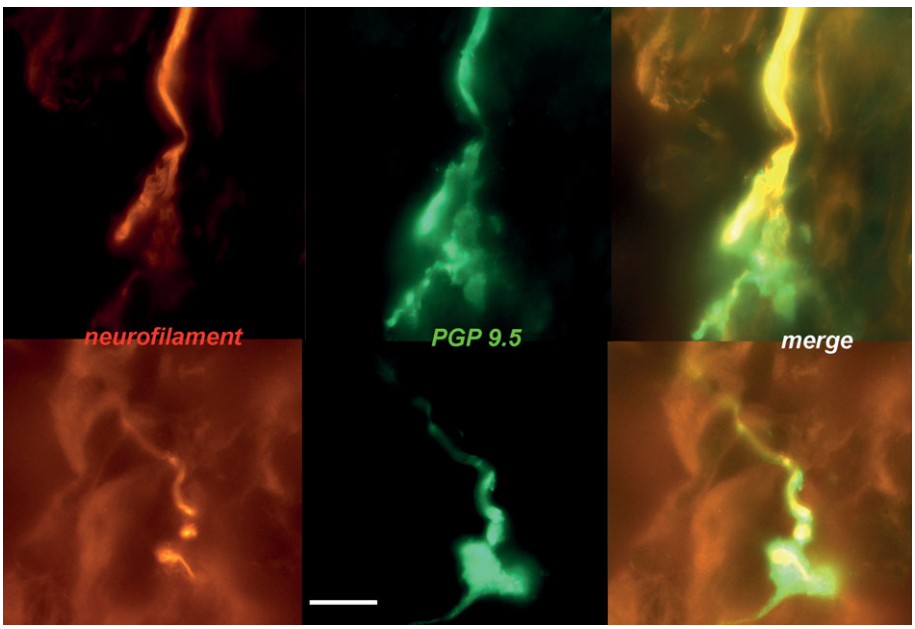

Figure 5.10 Examples of complex *in vivo* growth cones found at the proximal stump of a transected rat sciatic nerve and labeled using immunohistochemistry for neurofilament (red) or PGP 9.5 (green). Note that the appearance is substantially different from growth cones studied *in vitro*. The PGP 9.5 labels the full extent of the structure, whereas neurofilament is only found in portions of the growth cone and in its proximal stem. (Bar = 20 microns) (Reproduced with permission from [450].)

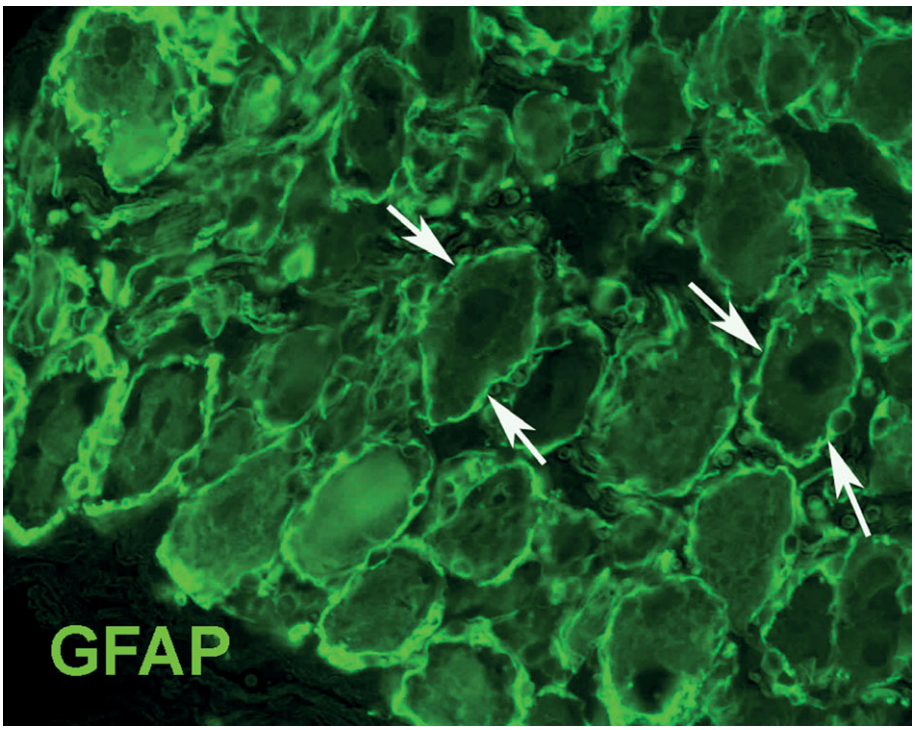

Figure 5.12 Immunohistochemical images of a DRG proximal to a sciatic nerve injury carried out 5 days earlier labeled with GFAP (glial fibrillary acidic protein). GFAP labels glial cells including perineuronal satellite cells. These cells, closely apposed to and surrounding sensory neurons, become hypertrophic and upregulate their content of GFAP after an injury (arrows). (Images taken by Chu Cheng, Zochodne laboratory.)

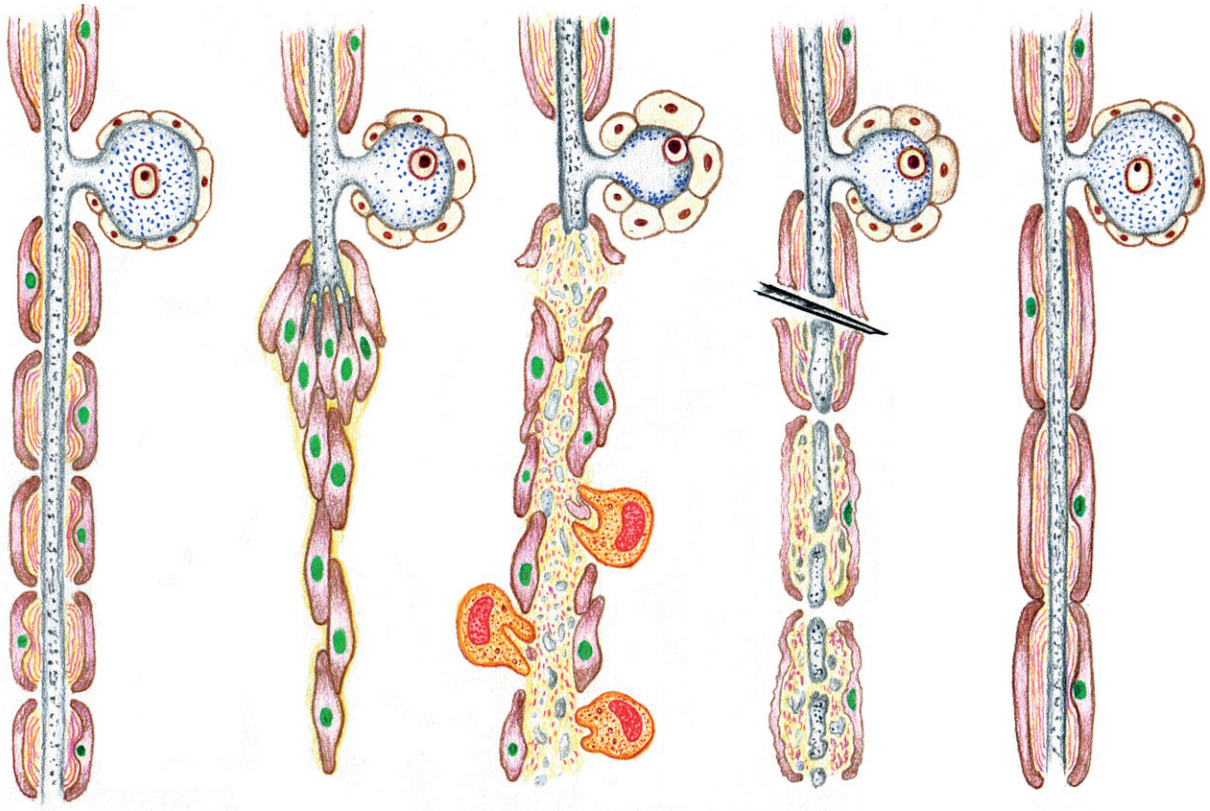

Figure 5.13 Illustration showing the sequence of changes in sensory neurons and SCs after a transection injury. Note the development of hypertrophic perineuronal satellite cells. Neurons have peripheral displacement of their Nissl substance and their nuclei. Invasion of hematogenous macrophages accompanies proliferation and dedifferentiation of SCs preparing the way for new axon outgrowth. (Illustration by Scott Rogers.)

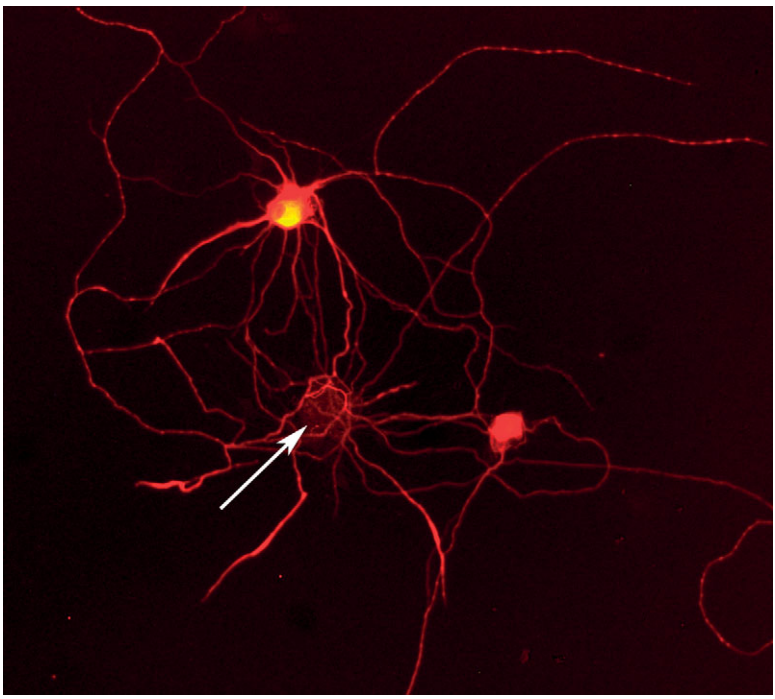

Figure 5.14 Adult rat peripheral sensory neurons *in vitro* showing extensive neurite formation. The neurons underwent a preharvesting preconditioning injury and are examined at day 3. The arrow points to a collection of glial cells in the culture that have attracted neurites to grow towards it. The image illustrates the tropic properties of supporting cells during axon outgrowth. (Culture prepared by Sophie Dong, Zochodne laboratory.)

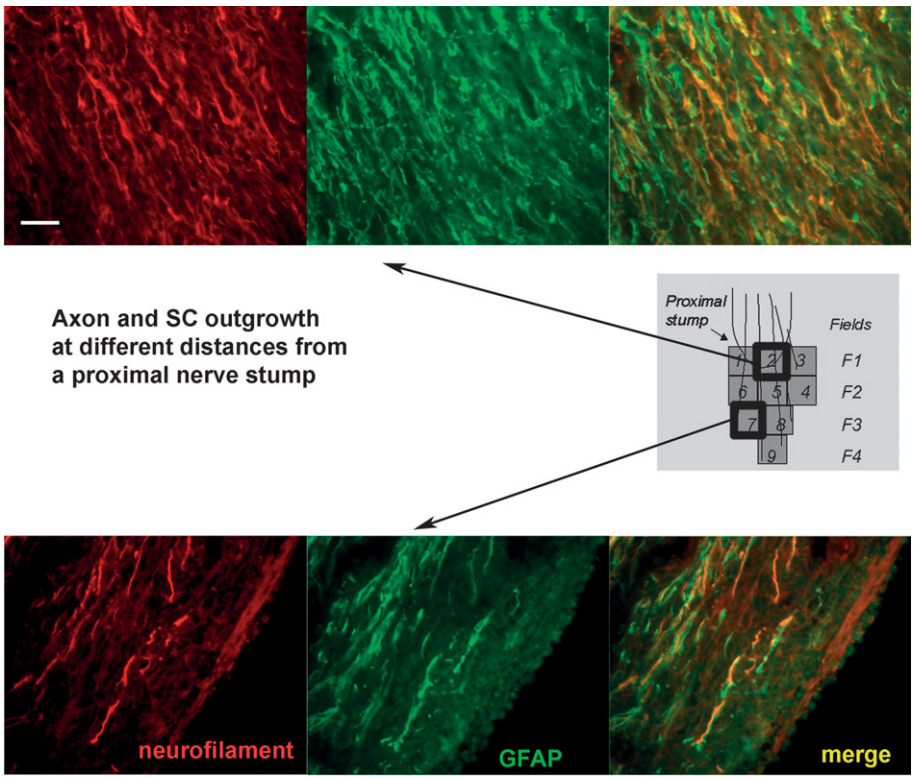

Figure 5.15 Immunohistochemical images of axon (labeled with an antibody directed against neurofilament) and SC (labeled with an antibody to GFAP) outgrowth at day 7 from the proximal stumps of transected rat sciatic nerves. The images in the top panel illustrate extensive outgrowth close to the proximal stump and the images in the bottom panel are further distally. Note the close relationship between axons and SCs. (Images taken by Y. Y. Chen, Zochodne laboratory.)

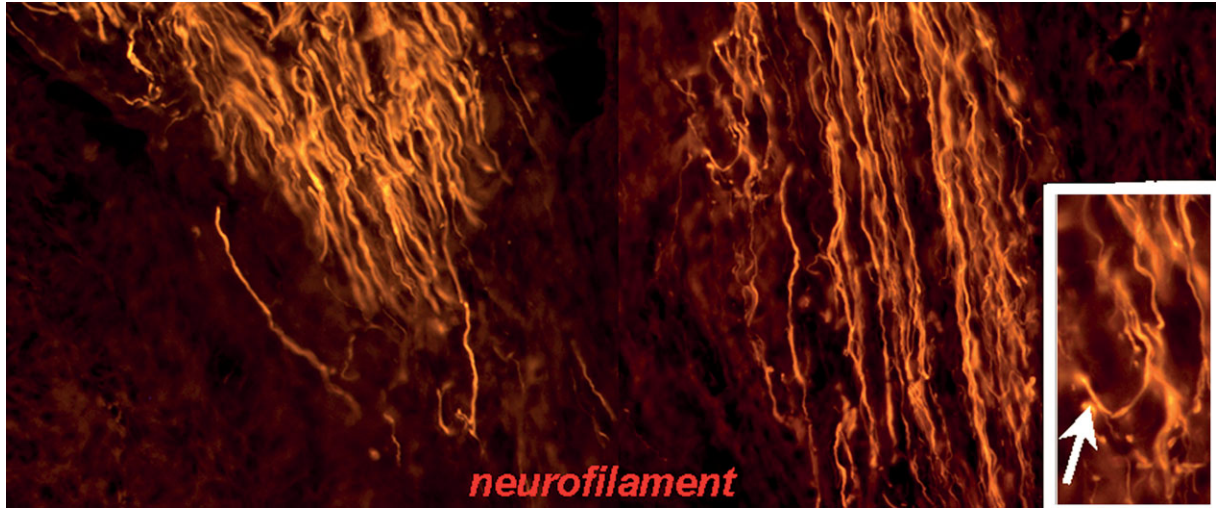

Figure 5.17 Immunohistochemical images of axons emerging from the proximal stump of a transected rat sciatic nerve (connected by a conduit) at day 7 labeled with an antibody to neurofilament. Note the rapid fall off in axon numbers as they penetrate the regenerative bridge. The inset and arrow shows an axon in a misdirected, or "wrong way" trajectory. (Reproduced with permission from [450].)

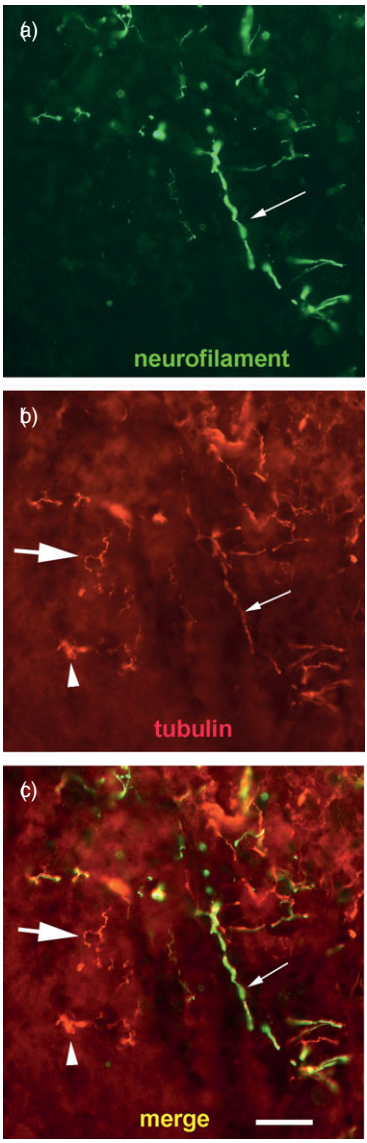

Figure 5.18 Immunohistochemical images of fine axons at the most distal end of regenerative outgrowth by day 7 following transection of a rat sciatic nerve (connected by a conduit). The axons are labeled by antibodies to βIII tubulin and neurofilament. The small arrow shows a double labeled profile. The arrowhead and large arrow show a tubulin, but not neurofilament labeled axon profile that is highly irregular and partly misdirected. The images illustrate that very fine distal axon outgrowth at the regenerative front may not label with a neurofilament antibody. (Bar = 50 microns) (Image taken by Chu Cheng, Zochodne laboratory and reproduced with permission from [450].)

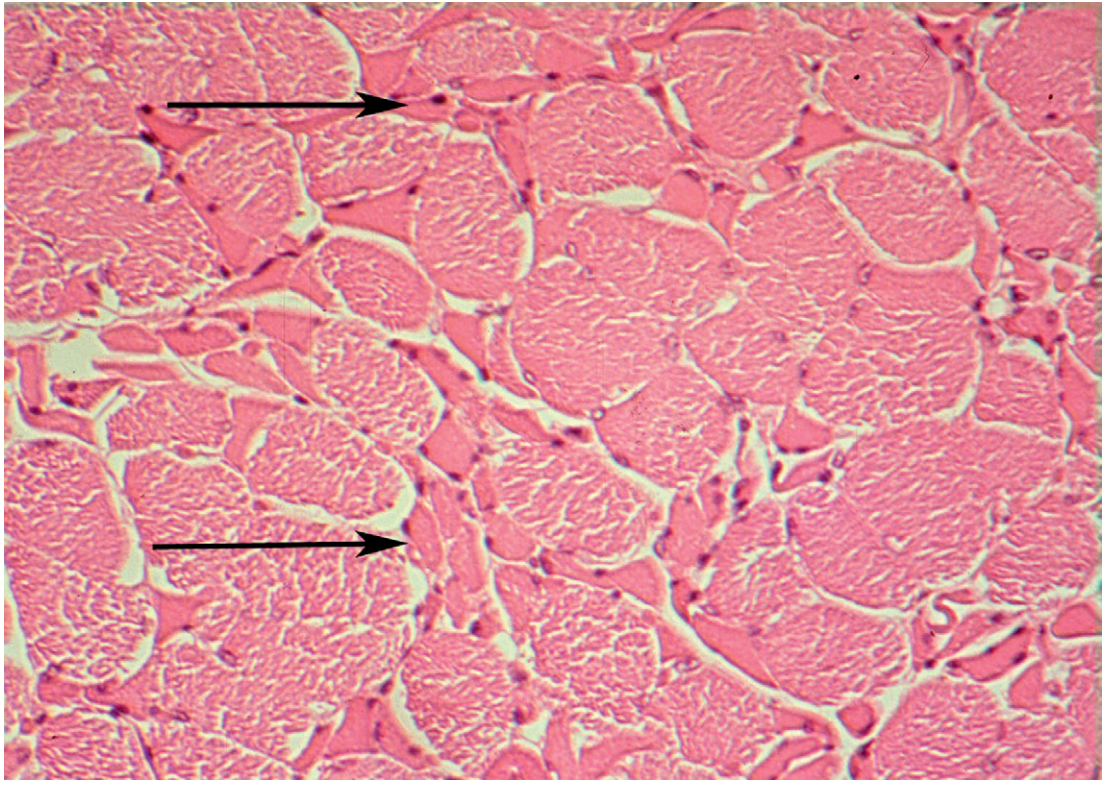

Figure 6.2 A photomicrograph of a sample of ileopsoas muscle from a patient with a severe progressive axonal polyneuropathy showing grouped fiber atrophy (arrows). (Reproduced with permission from [785].)

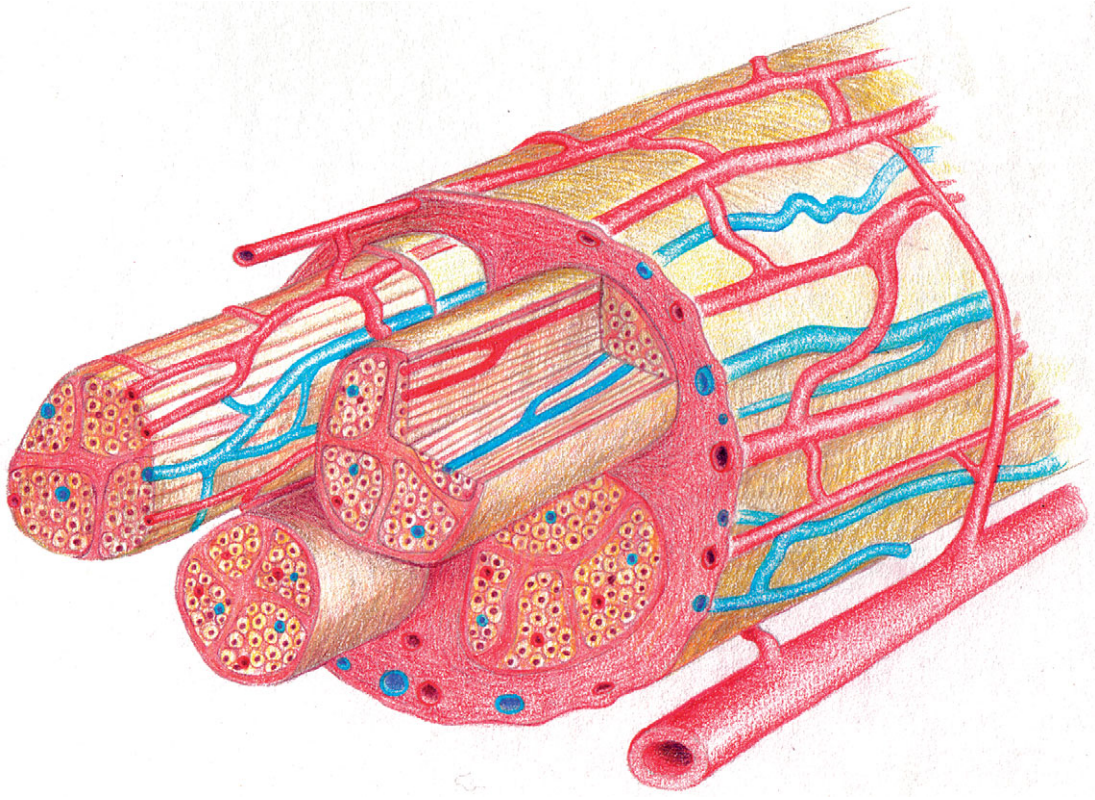

Figure 7.1 Illustration of the microvascular supply of the peripheral nerve trunk. (Illustration by Scott Rogers based on Figure 3.4 by [421].)

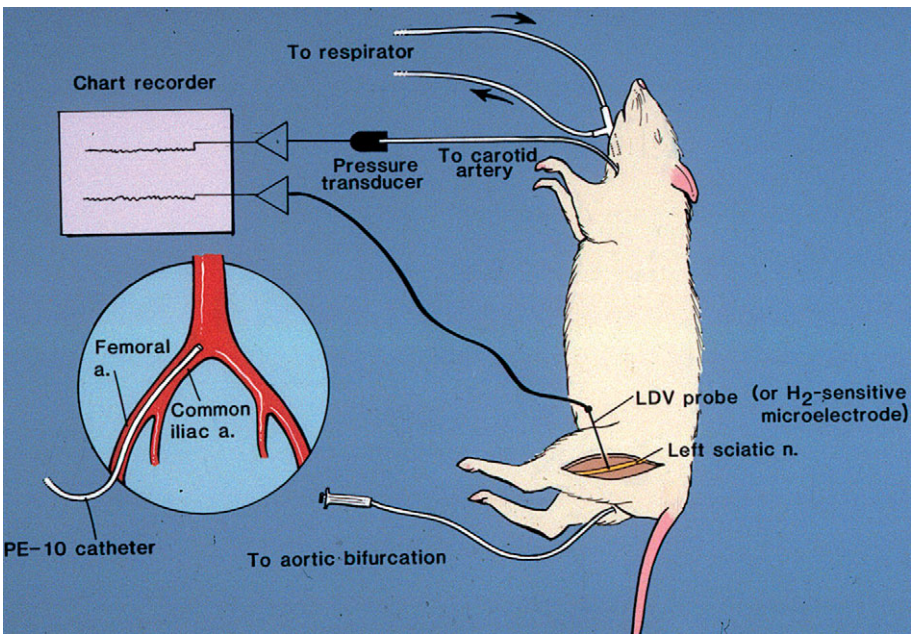

Figure 7.4 An example of the experimental setup used to study nerve blood flow in the rat using either a laser doppler flowmetry probe or a hydrogen sensitive polarographic microelectrode. In this setup, vasoactive agents were tested by infusing them into the arterial supply of the nerve from the contralateral femoral artery. (Reproduced with permission from [801].)

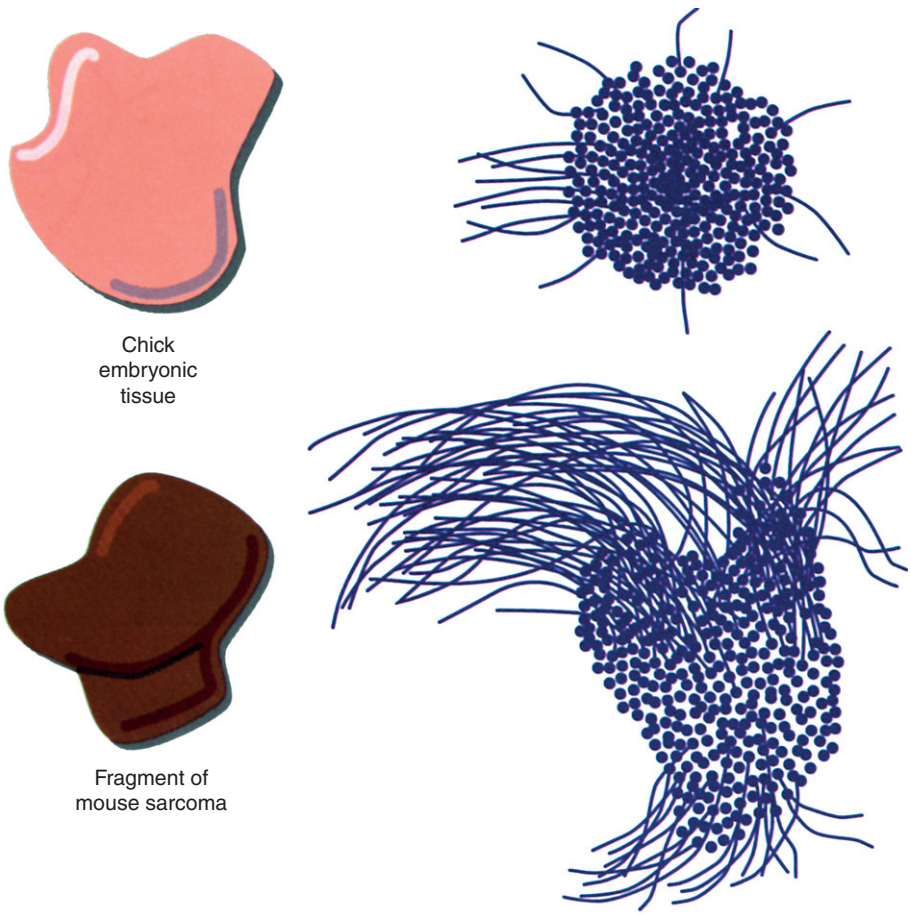

Figure 9.1 Illustrations of the original findings of Levi-Monalcini and colleagues that led to the discovery of NGF. Axons emerging from explanted sympathetic ganglia grow toward mouse sarcoma cells that secrete a gradient of NGF. (Based on the original figures by Levi-Montalcini [389] and reproduced with permission from [782].)

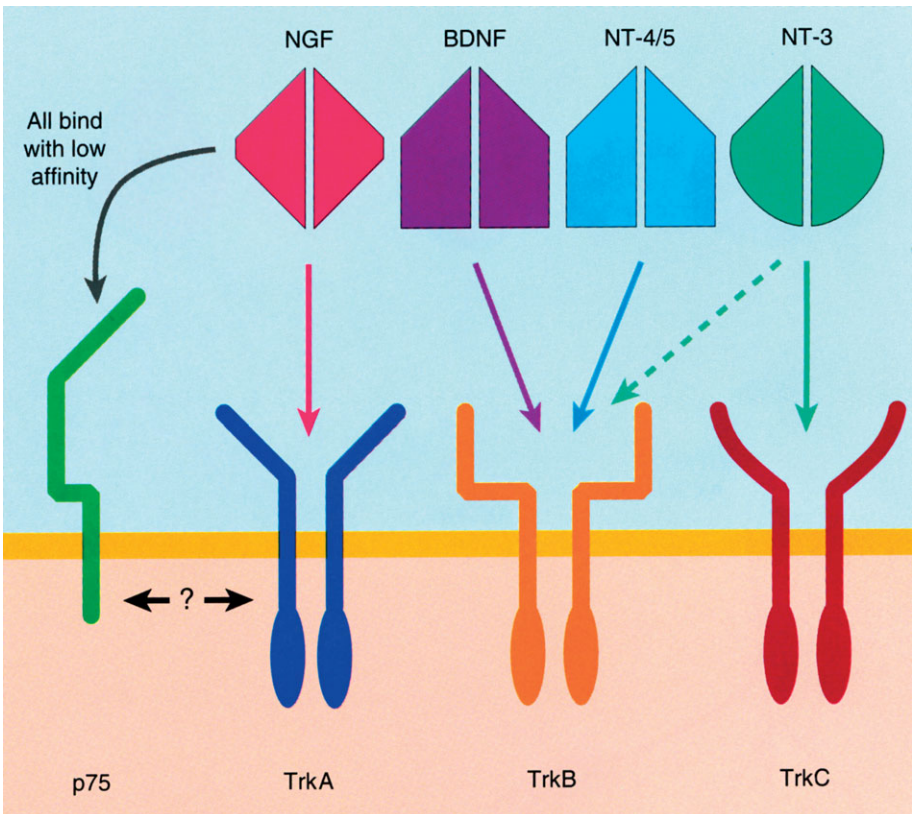

Figure 9.2 Illustration of members of the neurotrophin growth factor family and their receptors. (Reproduced with permission from [782].)

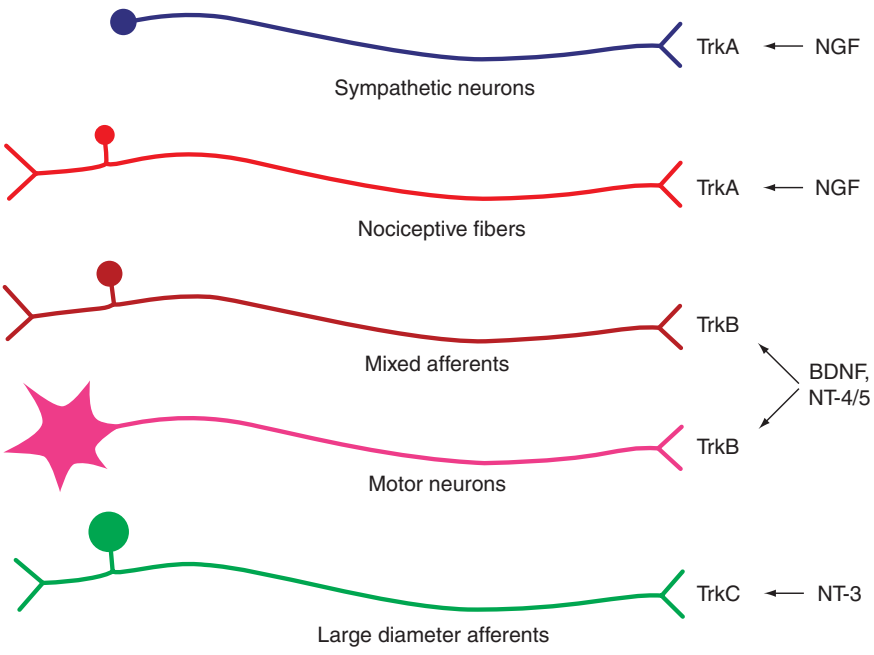

Figure 9.3 Illustration of the peripheral neuron subtypes responsive to members of the neurotrophin growth factor family. (Reproduced with permission from [782].)

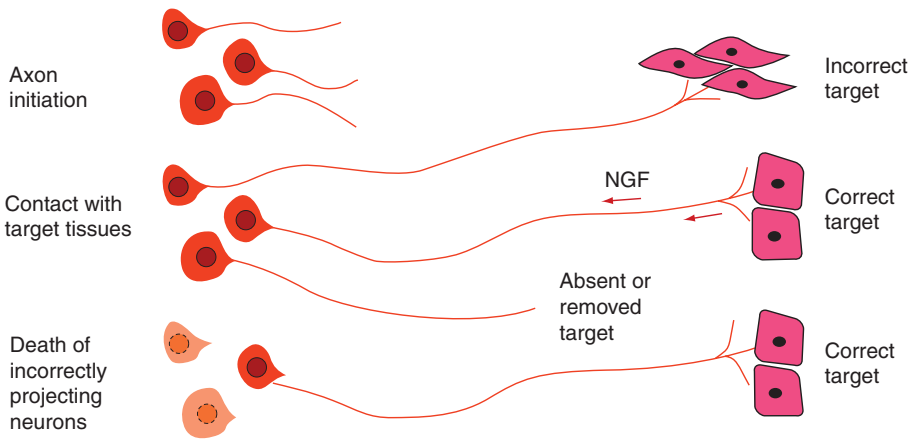

Figure 9.4 Illustration of the neurotrophic hypothesis. Neurons from axons that arrive at a target source of growth factor survive whereas those that fail to arrive or grow to incorrect targets undergo apoptotic cell death. (Reproduced with permission from [782].)

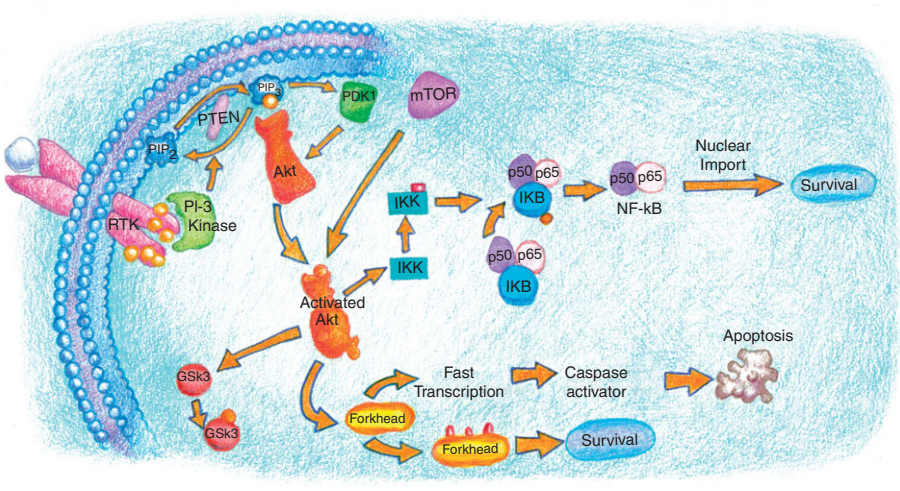

Figure 9.5 Illustration of the PI3K-Akt neuron survival pathway activated by growth factor receptors containing a receptor tyrosine kinase (RTK) intracellular signaling domain. (Illustration by Scott Rogers based on diagrams posted by EMD/Calbiochem (www.emdbiosciences.com).)

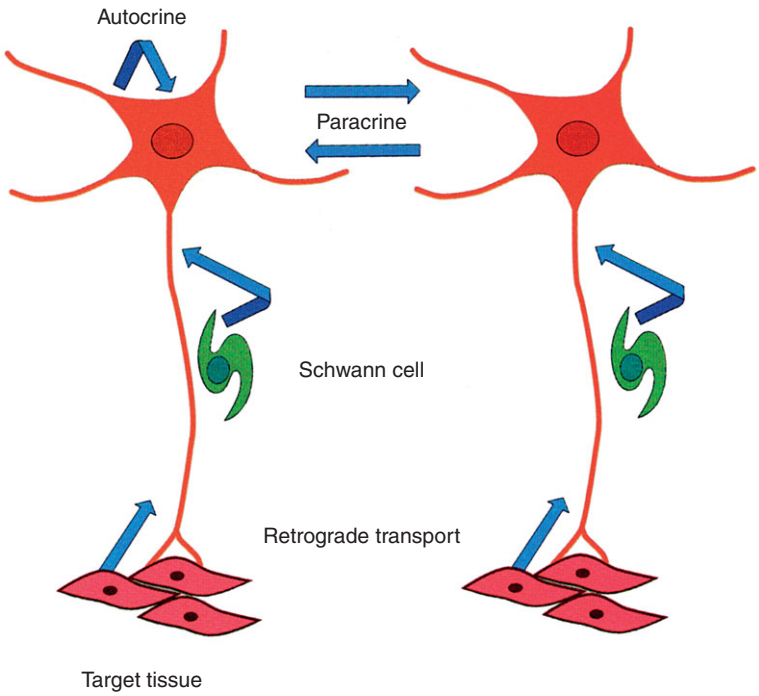

Figure 9.6 Illustration of sources of growth factor support. (Reproduced with permission from [782].)

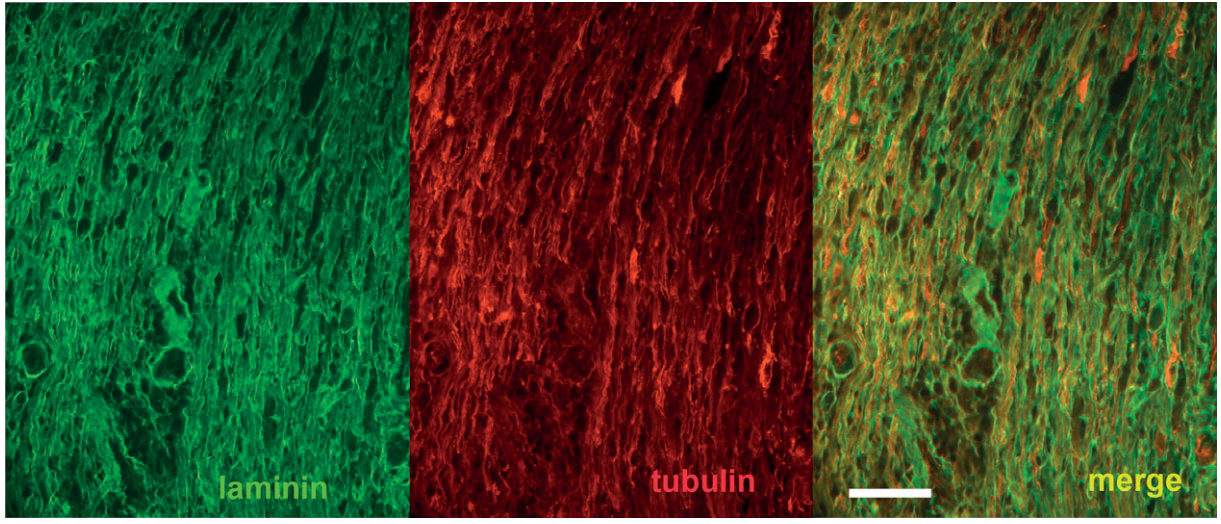

Figure 10.1 Immunohistochemical image of the relationship between laminin (labeled with an anti-laminin antibody, green) and axons (labeled with an anti-neurofilament antibody, red) in a peripheral nerve stump. Laminin is associated with basement membranes of SCs surrounding axons and with blood vessels. (Bar = 50 microns) (Image taken by Chu Cheng, Zochodne laboratory.)

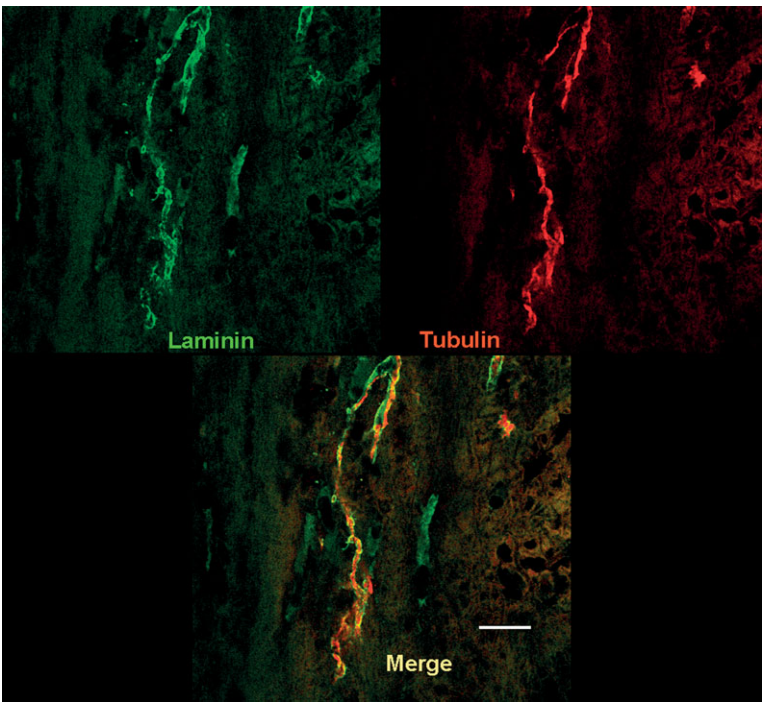

Figure 10.2 Immunohistochemical image of outgrowing axons (labeled with an anti-βIII tubulin antibody, red) at the regenerative front of a transected rat sciatic nerve (connected by a conduit) closely associated with deposits of laminin (labeled with an anti-laminin antibody, green), likely laid down by leading SCs. (Bar = 50 microns) (Image taken by Chu Cheng, Zochodne laboratory and reproduced with permission from [450].)

7

Regeneration and the vasa nervorum

Peripheral nerves are living dynamic tissues that thrive on a nutritive blood supply. The vascular supply of the peripheral nerve, termed "vasa nervorum" participates intimately in regenerative events and influences their success. There are important morphological and physiological differences among microvessels that supply nerve trunk, ganglia, and brain. Each entrains and regulates its vascular supply from differing physiological perspectives, depending on their need for metabolic support. Following injury, vasa nervorum alter their behavior in unique ways that reflect their exposure to molecules released within the microenvironment and that offer insights into the repair process.

Blood flow and microvessels of intact nerve trunks

Nerve trunk blood vessels, or vasa nervorum, are supplied by upstream arterial branches of major limb vessels. Sometimes these arteries and nerves course together as neurovascular bundles. Peripheral nerves also share their abundant blood supply with other structures in limbs such as bone, connective tissue, skin, and muscle. For this reason, major ischemic lesions are likely to target several tissues and cause widespread damage. The redundant and abundant blood suppy of nerve trunks, however, can be advantageous because the interruption of a single artery is unlikely to cause significant ischemia. There are some sites where there is ischemic vulnerability, known as watershed zones. These are found at areas supplied by terminal branches of overlapping arterial trees. For example, a nerve watershed zone has been identified in the proximal tibial nerve of rats [458]. While less readily demonstrable in humans, an analogous area in the proximal sciatic nerves likely is present. The central, or "centrofascicular" portion of some

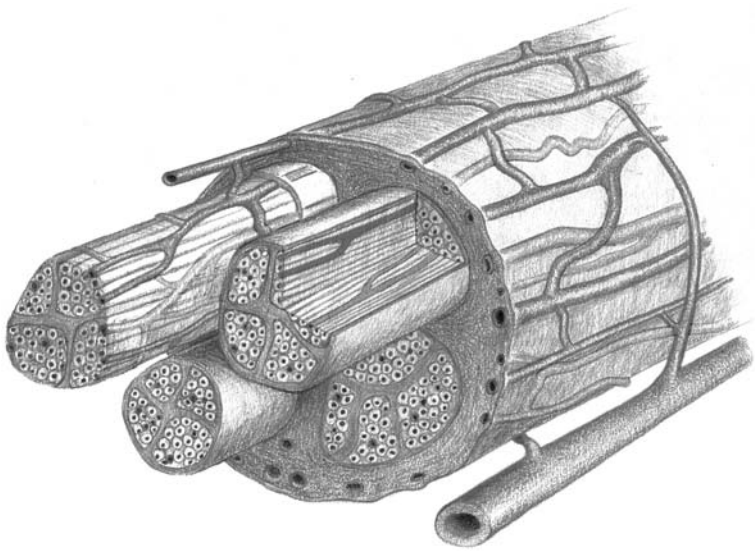

Figure 7.1 Illustration of the microvascular supply of the peripheral nerve trunk. (Illustration by Scott Rogers based on Figure 3.4 by [421].) See color plate section.

nerve trunks may also be a vulnerable type of watershed. If the ischemia is prolonged (>3 hours), axons may be injured and undergo Wallerian-like degeneration. Centrofascicular damage is uncommon in large multifascicular nerve trunks, suggesting that a more complex arrangement of their blood supply determines what parts may be at risk. In these nerves, ischemia instead more commonly renders unpredicatable patterns of multifocal damage that depends on its severity at individual sites within the nerve trunk [155,786].

Peripheral nerve trunks have an overlapping blood supply from microvessels that form multiple connections, or anastamoses (Figure 7.1). The overall pattern, as routinely observed by surgeons exposing nerves, is that of an extensive and complex vascular plexus in the outermost epineurial layer of the peripheral nerve trunk. Connections are found among arterioles or venules or between arterioles and venules, where they are known as arteriovenous (AV) shunts. AV shunts are found primarily in the epineurial plexus but they have also been localized to the endoneurium. By direct examination it may be difficult to distinguish AV shunts, arterioles, and venules in the epineurial plexus. Nonetheless, their existence has been demonstrated in several ways. By injecting microspheres into the arterial vascular supply of the nerve, experiments have shown that AV shunts allow microspheres to bypass capillaries to the venous system and eventually deposit in the pulmonary vascular bed. Mathematical modeling based on experimental measurements of nerve blood flow also predicts their

presence [368,369]. Why such shunts develop, or how they might contribute to nerve trunk integrity is unknown. Redundancy in the vascular supply of the nerve trunk, however, explains why long segments of nerves can be "mobilized" by surgeons with relative impunity.

Vasa nervorum can be assigned into either an epineurial vascular plexus or an intrinsic endoneurial plexus. The structure and physiological properties of blood vessels from these two compartments differ, despite the fact that they are anatomically connected. Epineurial blood flow, supplied by extrinsic arteries, is ultimately responsible for downstream blood flow in the endoneurial compartment. Feeder arterioles supplying the endoneurium arrive directly from adjacent segmental penetrating arterioles. Arterioles within the endoneurium may also be longitudinal in orientation, arising from penetrating vessels at remote sites and traveling parallel to axons.

Different approaches to measure nerve blood flow highlight the differences between epineurial and endoneurial networks. For example, the epineurial plexus entrains higher levels of blood flow, exhibits prominent AV shunting, has innervation of its arterioles, and has a leaky blood nerve barrier. In contrast, the endoneurial vascular supply is constituted largely, though not exclusively, of noninnervated capillaries that respond passively to blood flow changes. The capillaries are associated with pericytes, smooth muscle-like contractile cells, but their influence on local blood flow is uncertain. Endoneurial capillaries are also somewhat larger in luminal caliber than those of other tissue beds [37].

Arterioles in the epineurial plexus are innervated by sympathetic adrenergic unmyelinated axons that mediate local vasoconstriction. These vessels are similarly innervated by axons containing peptides, called peptidergic (Substance P, calcitonin gene-related peptide (CGRP)) that mediate local vasodilatation [20,137]. CGRP, in particular, is a highly potent vasodilator that is capable of relaxing vascular smooth muscle through both nitric oxide dependent and independent pathways [60,565]. Adrenergic and peptidergic innervation provide a form of nerve trunk vascular self-regulation since they arise from their own parent nerve trunk [574]. Moreover, both types of innervation are tonically active, and influence the luminal caliber of arterioles in real time. Since the epineurial plexus feeds the downstream endoneurial compartment, this type of ambient sympathetic and peptidergic control can thereby direct downstream endoneurial capillary blood flow. For example, interrupting sympathetic or adrenergic activity by pharmacological blockade or sympathectomy results in a rise in normal endoneurial nerve blood flow. Alternatively, blockade of peptide receptors (either CGRP or Substance P) is associated with declines in nerve blood flow [795,798,801]. Discrete rather than diffuse zones of vascular regulation mediate these changes because the overall innervation pattern of epineurial arterioles is

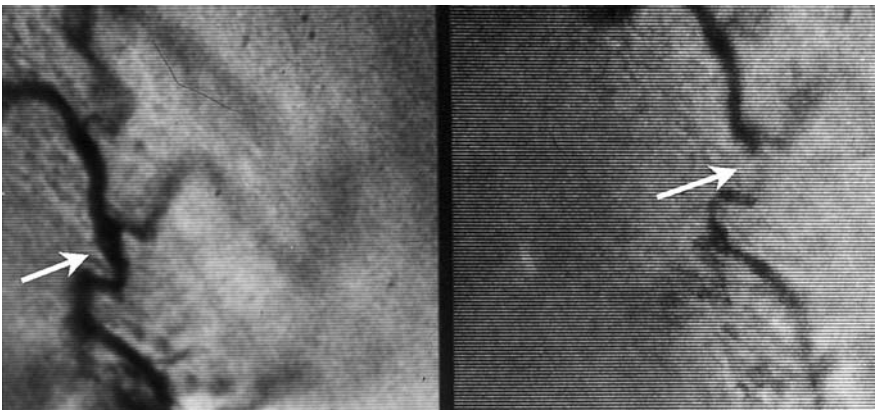

Figure 7.2 Image of an epineurial arteriole supplying the sciatic nerve in a live anesthetized rat showing segmental vasoconstriction to topically applied norepinephrine. The localized response (arrow) despite superfusion of the entire preparation with norepinephrine suggests that there is variation in the density of adrenergic receptors along these arterioles. (Reproduced with permission from [801].)

likely nonuniform and segmental [801] (Figure 7.2). While uneven and segmental adrenergic vascular responsiveness has been demonstrated, the peptidergic innervation pattern has received less attention.

Hypercarbia is associated with a rise in cerebral blood flow in the CNS. In peripheral nerves hypercarbia is associated with mild rises in epineurial plexus blood flow but does not appear to influence endoneurial blood flow [416,576,792]. The role of autoregulation also differs between the CNS and peripheral nerve trunk. Autoregulation refers to the physiological maintenance of blood flow during alterations in mean arterial pressure and it is a feature of CNS blood flow. In peripheral nerve trunks instead there is an almost direct linear relationship (possibly curvilinear) between endoneurial blood flow and mean arterial pressure [416]. Explained differently, rises in mean arterial pressure are accompanied by parallel passive changes in nerve blood flow and constant levels of flow are not maintained. Both the absence of autoregulation and overall lower levels of ambient blood flow in peripheral nerve trunks likely reflect their lower metabolic requirements than brain. Normal endoneurial blood flow ranges from 15–20 ml/100 g per min [416,588], values considerably lower than the 80–120 ml/100 g per min in CNS gray matter. Blood flow in central white matter is somewhat lower than that of gray matter, whereas ganglia entrain higher blood flow than nerve trunks. Their vascular properties are discussed next. Approaches to measure blood flow in small tissues like nerve trunks and ganglia are also considered in detail below.

Blood flow and microvessels of ganglia

Spinal dorsal root ganglia (DRG) are supplied from arteries that emerge from segmental radicular arteries along the spinal column. These branches anastamose with other branches arising from spinal arteries that supply the spinal cord [3]. Unlike the nerve trunk, ganglia do not have an extracapsular plexus comparable to that of the epineurium. Neurons in ganglia are prominently placed in the subcapsular space. From this location, their axons coalesce in the core of the ganglia before emerging into the dorsal nerve root. Few studies involving ischemic damage to ganglia have been carried out and the vulnerability of specific structures is unclear.

DRG have higher levels of blood flow than nerve trunks. Measuring approximately 30–40 ml/100 g per min [792], or 2–3 times that of the peripheral nerve trunk, the higher flow values likely reflect a higher metabolic demand. This difference parallels that of the CNS gray matter containing neuron cell bodies and synapses that entrain higher levels of blood flow compared with white matter. Signficantly, neither nerve trunks nor DRG are as well perfused as spinal cord gray matter, where blood flow ranges approximately 50–60 ml/100 g per min and they are less well perfused than brain cortex discussed above [808]. Oxygen tension values measured in ganglia are shifted to lower values than those of nerve trunks, indicating heightened oxygen extraction. DRG microvessels do not demonstrate significant vasodilation from hypercarbia [796].

In sensory ganglia, unlike nerve trunks, partial autoregulation of ganglion blood flow can be identified. Thus for mean arterial blood pressures between 80 and 120 mmHg ganglion blood flow changes are less than expected and are partly stabilized. One might also expect that, since ganglia have some autoregulation, the influence of adrenergic vasoconstriction would be attenuated. Autoregulation and adrenergic vasoconstriction can have opposite actions on blood vessels. For example, when there are falls in mean arterial pressure from hemorrhage or cardiac failure, rises in adrenergic tone compensate by increasing arteriolar resistance, thereby increasing blood pressure but reducing flow to tissues. In the DRG or CNS, neurons are highly vulnerable to ischemia and blood flow needs to be maintained within a protective range through autoregulation and lessened adrenergic input. Along these lines, DRG blood flow appears to have a negligible input from adrenergic sympathetic fibers [796].

Blood flow within sympathetic ganglia is less studied, but it is likely their flow would be comparable to that of DRG. One study suggested higher local flow than sensory ganglia, but the measures may have been spuriously elevated by flow through the adjacent carotid bifurcation [85].

Measurements of nerve and ganglion blood flow

Accurate measurements of blood flow in small tissue compartments like nerve trunks and ganglia are obviously challenging, particularly when experimental models are used. Relatively few laboratories have used the techniques listed here or have reported rigorously acquired results. Hopefully, newer imaging techniques may soon provide accurate blood flow results within nerve comparable with conventional results obtained over the last 20 years. Over this time, valid classical approaches have included: quantitative microelectrode hydrogen clearance (HC) polarography, laser Doppler flowmetry (LDF), [^{14}C]idioantipyrene distribution or autoradiography, and microsphere embolization. Detailed reviews of the methods and their pitfalls have been published [415,783] and a summary is provided below. Several related approaches that do not provide quantitative blood flow data have yielded important corollary information. These have included direct live videoangiographic imaging of the epineurial plexus and its vessels [801] (Figure 7.2), quantitative morphometric measures of fixed or unfixed peripheral nerves [654,806], and measures of indicator transit times [674].

Since different techniques may provide complementary information, combining them within specific studies has been a powerful way to confirm, supplement, and extend critical findings. For example, the epineurial plexus is particularly suited to sampling by LDF, whereas HC most suitably measures selective endoneurial blood flow measurements. Combining approaches is technically challenging, but one can address interesting problems about how the two compartments interact, for example, with pharmacological challenge or injury.

The technique of *hydrogen clearance polarography* (*HC*) is considered a "gold standard" for quantitative measurements of nerve trunk and ganglion blood flow. HC involves the detection of small currents from platinum microlectrodes that have been polarized (hence polarography) to sense concentrations of H_2. H_2, a highly diffusible gas, is delivered to tissues through an inspiratory gas mixture in paralyzed, ventilated animals. HC microelectrodes must be small in caliber so as not to disrupt the tissue they sample from, and they must also be linearly sensitive to tissue concentrations of H_2. Once the HC microelectrode detects tissue saturation, or a plateau in the concentration of the hydrogen, the H_2 is shut off from the inspiratory gas mixture. The microelectrode then records a washout, or clearance curve from which standardized approaches are used to calculate endoneurial, or composite (weighing in the input of epineurial blood flow and that of AV shunts) local blood flow. The calculations used in this technique are provided in references [128,369,792]. It requires considerable technical expertise, including rigorous physiological support and quantitative characterization. Most laboratories with expertise in its use have used locally

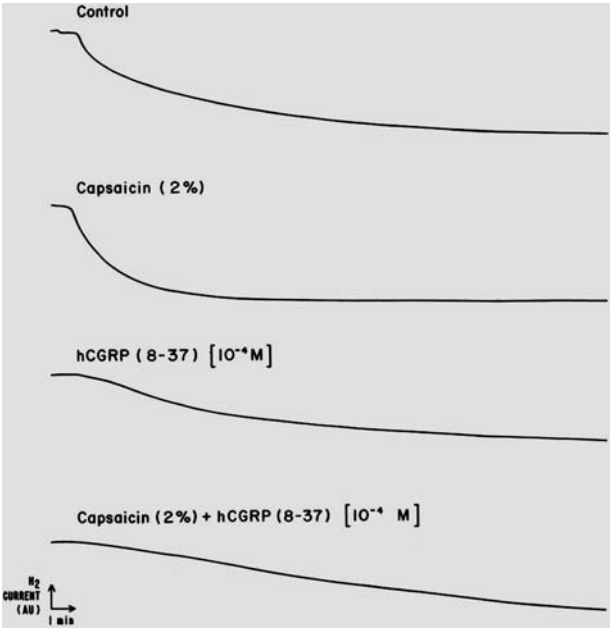

Figure 7.3 Examples of hydrogen clearance curves recorded from the endoneurial vascular compartment of rat sciatic nerves using a polarographic microelectrode. The clearance curves are monoexponential or biexponential. Note the more rapid washout, indicating higher flow, in nerves exposed to capsaicin, an agent that causes acute release of CGRP and SP, vasodilating peptides expressed in terminals innervating epineurial arterioles. In contrast, the lower two curves exposed to hCGRP (8–37), a CGRP antagonist, are flattened indicating slower washout and reduced flow. (Reproduced with permission from [791].)

manufactured linearly sensitive hydrogen microelectrodes with small diameter tips of 3–5 microns. Some laboratories have reported use of smaller commercial microelectrodes that have not undergone rigorous standardization or have used very large microelectrodes that excessively disrupt the architecture of the nerve trunk [288–290,520,642]. HC, when used with these caveats, offers quantitative measures of selective endoneurial blood flow that can be performed serially (Figure 7.3). Each measurement requires 20–30 minutes for the complete washout of hydrogen, whereas rapid real-time variations in blood flow are difficult to detect. Laser Doppler flowmetry, discussed next, allows real-time measurements.

Measurement of blood flow with *laser doppler flowmetry* (*LDF*) involves the placement of afferent and efferent fiberoptic probes (usually combined into one "probe") over the surface of the nerve trunk or ganglion (Figure 7.4). LDF utilizes the doppler principle, in which the efferent fiber emits a laser light

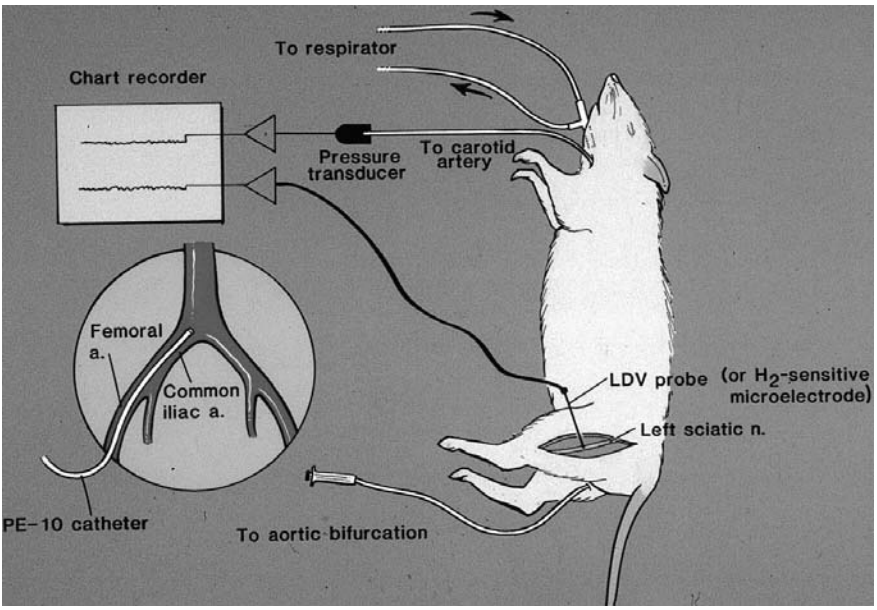

Figure 7.4 An example of the experimental setup used to study nerve blood flow in the rat using either a laser doppler flowmetry probe or a hydrogen sensitive polarographic microelectrode. In this setup, vasoactive agents were tested by infusing them into the arterial supply of the nerve from the contralateral femoral artery. (Reproduced with permission from [801].) See color plate section.

signal to erythrocytes moving in its field of application. The afferent probe measures reflected light, shifted in wavelength by the doppler effect, from the movement of erythrocytes. Thus LDF is used to measure erythrocyte flux, or the product of individual erythrocyte velocities and erythrocyte numbers. LDF is sensitive to real-time changes in erythrocyte flux but vigorous care and controls are required: (i) lighting conditions have a significant bearing on the amplitude of the afferent signal and must be standardized, preferably with room lights and other light sources turned off while the measurement is being made; (ii) multiple sampling along the nerve trunk or ganglion is required, since very slight shifts in the position of the probe can dramatically alter the recorded signal. These kinds of variations are a result of the highly variable anatomy of the epineurial nerve plexus and the lesser likelihood of being able to sample uniform areas. In practice, measurements are best taken from several (a minimum of ten sites) individual adjacent positions [315]; (iii) microtremor from the hand of the experimentalist alters afferent signals, thus mandating the use of a micromanipulator used to position and hold the probe; (iv) strict control of near nerve temperature is required since local nerve temperature during exposure

and experimentation can vary greatly from central body temperature. Since LDF preferentially samples from higher flow blood vessels adjacent to the probe, and since deeper penetration into the nerve is limited, its measurements emphasize flow in the epineurial plexus.

[^{14}C]idioantipyrene distribution or autoradiography is also a gold standard approach for the measurement of nerve and ganglion blood flow [459,588]. Iodoantipyrine is freely diffusible among tissues and, after systemic administration, its distribution can be measured in nerve. Its segregation into the endoneurial or epineurial compartments can also be quantitated in specific regions with autoradiography. Unlike HC, however, single and not serial measurements are available in experimental preparations.

Microsphere embolization involves the administration of radiolabeled microspheres through the bloodstream. Blood flow is calculated by using the distribution of their radiolabel to nerve samples. The approach has been criticized for generating lower blood flow values in peripheral nerve trunks, and this may be a technical limitation of the approach [561,662,680]. The reason for lower blood flow measurements is that AV shunts found in vasa nervorum allow through passage rather than capture of indicator microspheres, underestimating their distribution. Other isotope distribution techniques have used [^{3}H] desmethylimipramine, and [^{14}C] butanol [99,652] and do not suffer from this limitation.

Specific *morphological techniques* to examine vasa nervorum are available. Standard sampling of nerves with fixation, embedding, and transverse sectioning are used to appraise blood vessel structure, numbers, and density. High-quality EM allows measurements of basement membrane thickening, smooth muscle duplication, and other aspects of vessels [437]. While fixed tissues have also been used to measure luminal areas of blood vessels, the approach obviously does not reflect dynamic luminal caliber in physiological situations [603]. One technique that may compensate in part for fixation problems involves perfusion of live anesthetized preparations with a mixture of India ink, gelatin, and mannitol [66]. After euthanasia and completing the infusion (identified by staining of cutaneous vessels), the preparation is cooled at −5°C, allowing the intraluminal mixture to congeal and harden. The nerves are then harvested, fast frozen in molds of OCT (optimal cutting temperature compound), and cryostat transverse sections made. Luminal profiles are outlined in black India ink. Such profiles, obtained from unfixed tissues, can be counted and sized, both in the endoneurium and the epineurium [805,806]. Finally, another approach uses fluorescein imaging of blood vessels in live, anesthetized preparations or humans and can provide information about vascular density and other properties.

Several methods are available for measuring *oxygen tensions*. One approach uses oxygen-sensitive microelectrodes. Some of these microelectrodes operate

through the principle of polarography, analogous to the approach discussed above using hydrogen-sensitive microelectrodes. Since individual point measures of oxygen tension in tissue have questionable relevance, an appraisal of tissue oxygenation requires the construction of oxygen tension histograms sampled from multiple sites and depths [697,794].

Specific acute ischemic nerve injuries

Peripheral nerve trunks are much more difficult to render ischemic than the CNS. Nerve trunks resist acute ischemia given their rich overlapping vascular supply and lesser metabolic demands. Despite this resistance, however, there are well-characterized clinical neuropathies that involve nerve ischemia. Examples include compartment syndromes, discussed earlier in this text. There are autoimmune conditions, known as vasculitis, involving inflammation of blood vessels, including those of peripheral nerves. In these neuropathies, damage can be highly variable, targeting one nerve and not another, in a pattern known as "mononeuritis multiplex."

There are some forms of focal diabetic nerve injury, resembling vasculitis, that probably arise secondary to ischemia. For example, a type of diabetic neuropathy known as lumbosacral plexopathy is thought to result from focal ischemia of the lumboscacral plexus that supplies the lower limb. Leg paralysis is the unfortunate result for patients [157,567]. Altered physiological properties of the vasa nervorum in diabetes that develop even in early disease, contribute to these types of injury. Defective vasorelaxation is a common theme and may arise from impaired endothelial nitric oxide release, availability, or signaling. Given these properties, diabetic nerves exposed to minor or innocuous levels of ischemia are considerably more vulnerable to damage [512,513].

Several forms of experimental neuropathy are used to understand and model the impact of ischemia on nerves. *Multiple arterial ligation* to the hindlimb results in ischemic nerve damage, provided enough of the redundant vascular supply is targeted by the procedure [129,355–359,458,605]. In these models, the mid-portion of the sciatic nerve trunk is the most susceptible area to arterial interruption. The time window required for ischemia to cause permanent axonal injury appears to be at least 3 hours, substantially longer than what is required for CNS damage. After ischemia, reperfusion may account for additional ongoing damage [605], a phenomenon referred to as reperfusion injury.

Ischemic neuropathy has also been studied after *microsphere embolization* of the arterial supply of the nerve trunk, an approach described by Nukada and colleagues [514–517]. In essence, this approach involved seeding the arterial branches to the vasa nervorum with microspheres that occluded multiple feeding vessels and caused ischemia. Within 12 hours following ischemia,

myelinated axons examined by LM became dark with or without light cores, then developed swelling and secondary myelin thinning. Later myelin changes led to overt demyelination and axons became "attenuated," or atrophic. Overall, the ischemic changes were identified 12 to 48 hours after the ischemic insult but diminished by 7 days. They were then followed by Wallerian-like axonal degeneration.

Other models of ischemic neuropathy have used *intra-arterial infusions of arachidonic acid or topical application of endothelin* (*ET*) over a portion of the epineurial vascular plexus [542,797]. Both of these agents cause severe vasoconstriction of vasa nervorum. ET, the most potent vasoconstrictive agent known, generates intense, albeit transient ischemia. A further form of nerve ischemia occurs in the *chronic constriction injury* (*CCI*) model of neuropathic pain previously discussed [41]. CCI is associated with ischemia from the placement of four loose ligatures around a nerve trunk with gradual swelling and strangulation of the blood supply [631].

Nerves rendered completely ischemic or hypoxic, however, do not demonstrate *immediate* conduction failure or block! There is an important property of axons known as *resistance to ischemic conduction failure* (*RICF*). RICF refers to the property of nerve trunks under conditions of complete ischemia to nonetheless retain their excitability for a period of time. In nerve trunks supramaximal stimulation generates a NAP, the amplitude of which correlates with the number of excitable axons that contribute to it. After complete interruption of their blood supply *in vivo*, axons within a nerve trunk develop a gradual loss of their capability to propagate the NAP. Thus, for a 50% decline in the amplitude of a NAP, it takes 20–30 minutes of complete ischemia or hypoxia: this time is referred to as the duration of RICF. Some conditions, such as diabetes, are associated with a prolonged RICF. In contrast, *in vitro* excised nerves without a blood supply, kept moist in physiological saline but exposed to air, retain their excitability for much longer periods. Unlike nerves embedded deeply in a tissue compartment, excised nerves are maintained by atmospheric oxygen.

After nerve trunks lose their excitability during complete ischemia, axonal *ischemic conduction block* can be detected. Conduction block refers to a physiological, rather than structural interruption of action potentials traveling along the nerve trunk. Like the more commonly considered conduction block from demyelination of internodes, ischemic axonal conduction block is identified using electrophysiological techniques. Stimulation distal to the site of the block identifies normal excitability with normal distally evoked NAPs or CMAPs. In contrast, stimulation above or proximal to the level of ischemia is incapable of exciting the distal nerve or muscle because the action potentials are blocked across the involved zone. This phenomenon is occasionally captured in patients with ischemic axonal lesions from vasculitis [314,543]. Short periods of complete ischemia can induce transient conduction block without permanent damage.

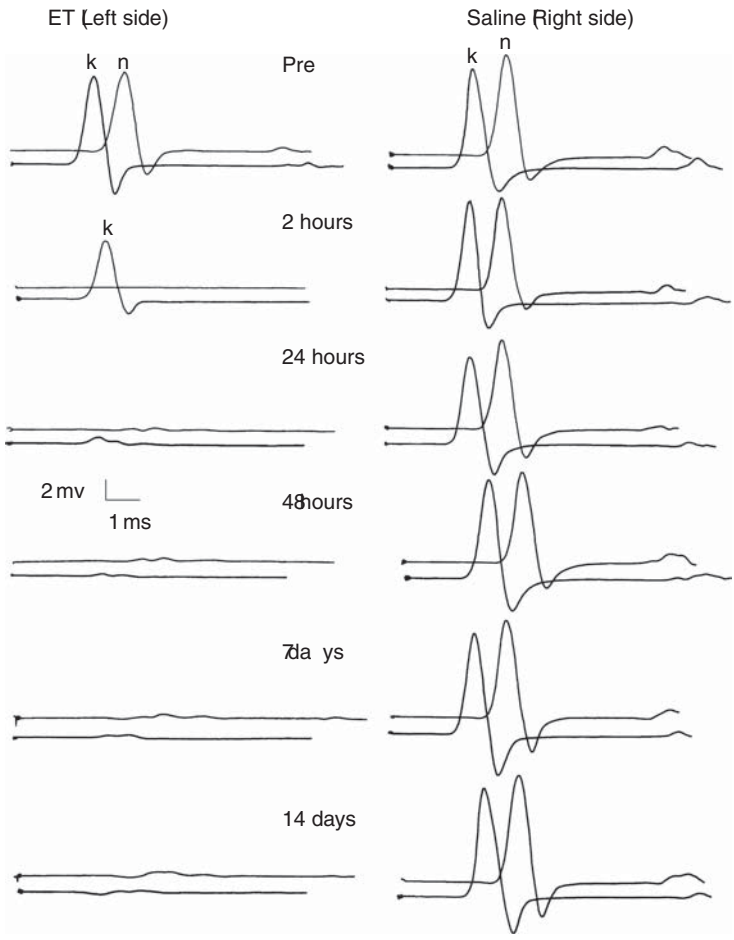

Figure 7.5 An example of acute conduction block from ischemia followed by axonal degeneration. The tracings are CMAPs recorded from the interosseous muscles of the foot of a rat following supramaximal stimulation of the sciatic nerve at the sciatic notch (n), or more distally at the knee (k). Between these stimulation sites, the nerve was exposed to endothelin, a topical and potent vasoconstrictor on the left and carrier alone on the right. Note that there is acute conduction block at 2 hours because the distal nerve (k) remains excitable but stimulation more proximally (n) is blocked. At 24 hours and thereafter, all excitability is lost because Wallerian-like degeneration ensues from severe axonal damage at the ischemic site. This example is from a rat with experimental diabetes. (Reproduced with permission from [789].)

Longer periods of ischemia, however, render permanent damage to the axon. After 3 hours of complete ischemia, Wallerian-like degeneration typically develops and there is loss of excitability of the distal segment of the nerve with disappearance of the CMAP evoked from distal stimulation (Figure 7.5).

To summarize then, axons within nerves *in vivo* take 20–30 minutes to lose their excitability after complete ischemia because of RICF. After this period of time, ischemic conduction block may be detected. If the ischemia is prolonged beyond 3 hours, however (and the nerve is not excised and exposed to air), permanent ischemic damage with later Wallerian-like degeneration will ensue. In diabetes, RICF is prolonged out to 30–40 minutes, but beyond this time, Wallerian-like degeneration develops more rapidly. The overall result is that, despite prolonged RICF, diabetic nerves have greater susceptibility to ischemic axonal injury [512,786,789].

It is likely that sensory ganglia in humans also undergo ischemic injuries but they are not commonly characterized. Some acute forms of sensory "radicular" neuropathy, as occur in diabetes, might arise from ganglion ischemia. It is also possible that common degenerative spinal bony changes known as spondylosis, with narrowing and compression of intervertebral foramina might cause nearby ganglia ischemia. Ganglia are more vulnerable than nerve trunks to ischemia because of their difference in metabolic demands.

Experimental models of *sensory ganglion ischemia* are available and like nerve, diabetic ganglia are also more sensitive. For example, ET vasoconstriction after topical application to ganglia caused ischemic necrosis of diabetic sensory neurons, intraganglionic axon damage, and downstream degeneration of distal axon branches [753]. Neurons disappeared and were replaced by nests of Nageotte or had nuclear disruption identifying apoptosis. Alternatively, some neurons had peripheral displacement of their nuclei and loss of neurofilaments characteristic of a retrograde cell body response to damage, as occurs after axotomy. Thus, ganglion ischemia generated three separate pathological reactions: ischemic necrosis of neurons, apoptosis of sensory neurons, and a retrograde cell body response to axonal damage. The time window of ganglion vulnerability to ischemia is unknown.

Local blood flow and the role of ischemia in acute nerve injury

Ischemia may not contribute to routine mechanical nerve trunk injuries. Lesions like crush or entrapment could disrupt local microvessels through direct shearing, compression from edema of the endoneurium, or venous engorgement in the case of entrapment. Alternatively, the rich, overlapping anastamotic blood supply of nerve trunks described above and the prolonged periods of intense ischemia required to cause permanent axonal damage makes nerves resistant to ischemia. There has been no clear demonstration that ischemia occurs or plays a significant role in chronic entrapment or acute traumatic nerve injuries. In these circumstances, it seems unlikely that a single compressive lesion would

disrupt the nerve vascular supply continuously for 3 hours or longer. Instead, most types of chronic entrapment or compression likely damage nerves through repeated mechanical injury. Unlike ischemia, local demyelination is prominent in these lesions with later progression to axonal degeneration.

Direct investigations of blood flow at sites of nerve crush or transection have not identified ischemia, even immediately after injury, or later as edema develops [790,793]. Instead, blood flow is well maintained and rises to produce hyperemia (high local blood flow). At crush sites, endoneurial nerve blood flow begins to rise within 3 hours of injury, and further increases by 24 hours to peak at 48 hours following injury. Partial nerve ligation, or complete nerve transection renders similar changes. Following transection, hyperemia becomes particularly intense within the proximal nerve stump but also occurs in the distal nerve stump [281]. Hyperemia develops within both the endoneurium, demonstrated by HC and in the epineurial vascular plexus observed using LDF studies. Finally, early hyperemia demonstrated using physiological techniques also correlates with morphological evidence, indicating vasodilatation of epineurial and endoneurial microvessels. Given all of these findings, it is not suprising that, in a set of experimental studies, application of hyperbaric oxygen to treat presumed hypoxia failed to ameliorate damage after nerve injury [246].

Early after injury, CGRP and nitric oxide (NO) associated with axon endbulbs contribute toward nerve hyperemia. Other mechanisms maintain elevated nerve blood flow at later time points. By 7–14 days after injury, for example, blood flow measured in both the epineurial and endoneurial compartment persist above normal levels. By this time, large rises in the numbers of vessels, particularly in the epineurium, can be identified and are found both in the proximal and distal nerve stumps (Figure 7.6). Moreover, accompanying this robust angiogenesis are local rises in VEGF mRNA expression [281]. These features of peripheral nerve repair parallel the changes of general wound healing.

It is also surprising that even long zones of nerve crush (e.g., 20 mm in length) do not develop ischemia in experimental studies (Xu, Zochodne *et al*., submitted data). In this type of injury, it might be expected that vascular disruption and edema would be particularly intense. These injuries not only retained normal or increased levels of local blood flow but also supported robust regenerative activity.

Despite these comments, there are instances of chronic compression or acute compression where ischemia of the nerve trunk is likely responsible for axon damage. These include large hematomas or mass lesions (or compartment syndromes discussed above) that destroy significant portions of the vascular plexus. None of the lesions or injuries discussed above may accurately reflect the complex microenvironment of actual clinical lesions. Local edema and

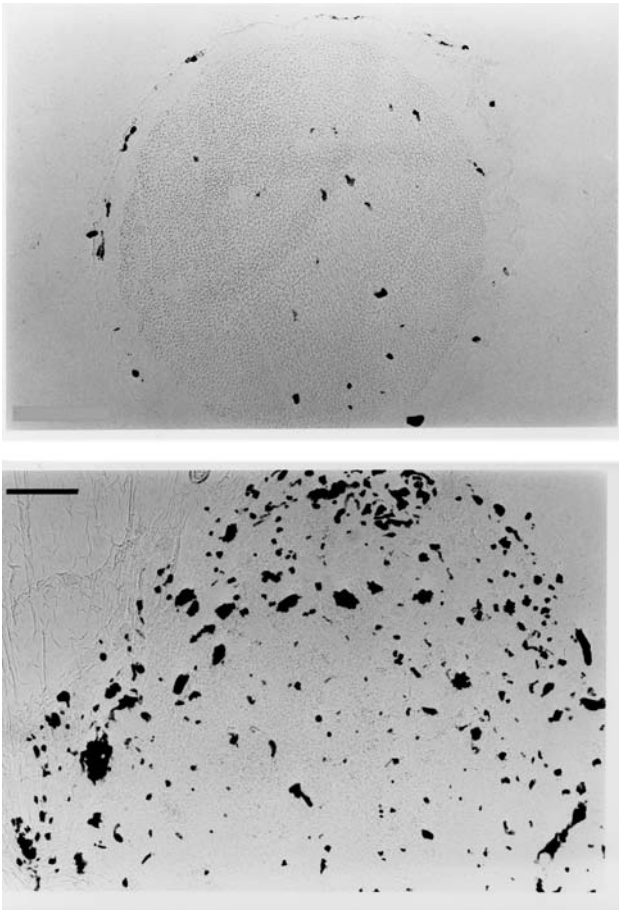

Figure 7.6 Photomicrographs of peripheral nerves perfused with India ink to outline their blood vessels as black profiles. The top panel shows a normal sciatic nerve of a rat and the bottom image is from a proximal neuroma stump of a transected nerve at 14 days. Note the very extensive investment of new blood vessels indicating angiogenesis after injury, especially in the epineurial space. The images are unfixed transverse sections of the sciatic nerve harvested after perfusion under anesthesia. (Bar = 200 microns) (Reproduced with permission from [805].)

vascular interruption could have an impact on multiple sources of vascular supply to the nerve trunk. Extravasation of blood (and hemoglobin) along long segments of the nerve trunk can quench NO mediated vasodilation. After stretch, also a common clinical lesion, greater disruption of vessels may occur. While quantitative studies of nerve blood flow have not been examined after stretch, preliminary work using luminal dye studies suggests it can disrupt the vasa nervorum [420].

Blood flow following long-standing nerve injuries

There are changes in blood flow of peripheral nerves that have sustained chronic injuries. Distal nerve stumps that have not been reinnervated over several months become less receptive to later reinnervation by axons, a problem that is discussed further in Chapter 8. One of the factors that contribute to this unreceptive microenvironment may be chronic declines in blood flow.

Changes in blood flow after chronic nerve injuries have been examined in several models. Local blood flow was examined in 6-month neuromas formed at the proximal stump of transected rat sciatic peripheral nerves [755]. Histologically, these were complex lesions, with some zones retaining prominent vascular investment, but nearby zones devoid of both blood vessels and axons. Similarly, quantitative measures of flow within neuromas were highly variable with hyperemia in some areas, but relative ischemia in others. Minifascicles appeared in well-vascularized portions. It is possible that axons prefer to grow in well-perfused areas of neuromas, or alternatively entrain new blood vessels to accompany them.

Long-term experimental neuromas exhibit features observed in human neuroma specimens. These kinds of chronic lesions were illustrated in a classical pathological monograph by Lyons and Woodhall [431] depicting nerve segments removed from soldiers injured in warfare. Resected several months after injury during attempts at nerve repair, the specimens highlight zones of fibrosis, atrophy of whole fascicles, and disrupted nerve trunk architecture. Where specifically examined, there were also instances of prominent thrombosis involving nerve blood vessels, suggesting chronic ischemia.

Chronically denervated distal nerve stumps also develop ischemia. As in neuromas, declines in their local blood flow may present an unsuitable microenvironment for regeneration. In experimental studies of distal nerve stumps that were not reinnervated, there were substantial declines in local endoneurial and epineurial blood flow 3–6 months after injury [281]. The number and caliber of epineurial microvessels declined at these later time points and levels of mRNA for VEGF reverted to baseline values. In contrast, nerve trunks that had undergone immediate resuture had preserved endoneurial nutritive blood flow. Resutured nerves however, did exhibit persistent abnormalities of blood flow in the epineurial plexus. Progressive fibrosis and scarring in the epineurium after injury may permanently remodel its vascular plexus: it is uncertain whether this change renders repaired nerve trunks more vulnerable to later ischemic or mechanical injury.

Vascular changes in long-term damaged nerve trunks may also be secondary to chronic loss of axons rather than the cause of poor regeneration. It is possible

that long-term declines in blood flow simply reflect a microenvironment with lower metabolic demands. Alternatively, molecular connections among axons, SCs, and endothelial cells of the vasa nervorum could also influence how severely disrupted nerves are eventually supplied with microvessels. These may include shared trophic factors, such as VEGF, or basement membrane components.

Summary

Peripheral nerve trunks and ganglia are supplied by blood vessels termed vasa nervorum that possess unique physiological and structural qualities. These qualities have an important impact on the response of the nerve trunk to injury. Fortunately, the vascular supply of peripheral nerves is rich, redundant, and difficult to render ischemic. Most focal injuries of nerve likely do not involve ischemia Nonetheless, ischemic injuries to nerve from complete interruption of the blood supply of a peripheral nerve can develop in some situations and render acute axonal conduction block across the injury zone. If prolonged, distal axonal degeneration ensues. Chronic nerve trunk lesions are associated with substantial declines in local blood flow that may contribute to an unfavorable microenvironment for regeneration.

Suggested reading

Zochodne, D. W. (2002). Nerve and ganglion blood flow in diabetes: an appraisal. In *Neurobiology of Diabetic Neuropathy*, ed. D. R. Tomlinson. pp. 161–202. San Diego: Academic Press [783].

Zochodne, D. W. & Ho, L. T. (1991). Unique microvascular characteristics of the dorsal root ganglion in the rat. *Brain Research,* **559**, 89–93 [792].

8

Delayed reinnervation

What kind of reception do regenerating axons or SCs encounter when they enter nerve trunks that have not housed axons for substantial periods of time? Are target organs receptive to reactivation after months or years of denervation? Unfortunately, the structural and molecular consequences of prolonged denervation substantially diminish the likelihood for reinnervation. This chapter deals with delayed reinnervation, a common and all too frequently unavoidable problem in patients.

Clinical scenarios and long-term denervation

There are several reasons why axons may encounter denervated distal stumps or target organs months or years after an injury. The first is obvious. The most optimistic rates of axon recovery range between 1 and 3 mm/day or an inch per month. Many severe human nerve trunk injuries occur in large proximal nerves, such as the sciatic nerve in the thigh or buttock [557] (see Figure 1.2). These lesions rarely allow successful recovery of sensation or motor connections to muscle endplates in the distal leg or foot. In the case of a lesion of the sciatic nerve at the level of the thigh, it would require over a year for axons to regenerate an approximate distance of 800 mm to the foot unimpeded. Moreover, distal portions of the nerve trunk would not receive new axons for many months. This analysis, however, oversimplifies the true scenario that may exist. Estimates of regeneration are based on the time the *first* axons meet their target, whereas a substantial population of axons must connect to their targets for functional reinnervation. While this proportion might not have to match the original complement of axons, sufficient number must be available and must be capable of sending collateral sprouts to target organs in the muscle or skin. We now

know, however, that axons do not regenerate in a single wave or front. Rather, early pioneer axons are followed by axons that advance at staggered rates. In a crush injury, it is usually assumed that the "pathways" or Bands of Bungner suitable to support regenerating axons are intact and allow rapid re-entry of axons into a distal stump. This injury is associated with better regeneration, but nonetheless only occurs along very protracted timelines. Simple crush injuries, however, are only a small proportion of clinical injuries. Higher grade nerve lesions with superimposed ischemia or added stretch impose new and added constraints to axon regeneration.

Many injuries are not strictly localized to one site but may have separate insults added in tandem. One of the most vivid examples of this seen by the author was a victim of a bear attack and mauling while cycling in the Canadian Rockies. The animal bit through and resected tissue that spanned much of the victim's arm from shoulder to elbow. Some of the nerve trunks that normally course through this territory (ulnar and radial nerve) were completely destroyed rendering denervated nerves and targets in the forearm and hand. The median nerve, however, was found to be grossly connected and preserved in the bed of severely damaged, swollen and resected muscle, skin, and bone. The combination of lesions involving stretch, ischemia, or crush along several centimeters of its length can only be estimated. Surgical action to graft nerves and bridge such lesions is challenging. In an acute situation with prominent tissue swelling, a high risk of infection, and other problems, the probability of a successful result is greatly diminished.

The second major reason that axons encounter long-term denervated territories is dictated by clinical circumstances. In the case of blunt nerve trunk injuries, it is not recommended that the injury site be immediately opened, exposed, and the integrity of the underlying nerves examined. Such an approach would add to further traumatic damage already present in and around the nerve trunk. Yet this type of early exposure, with electrophysiological recordings around the injury zone might be essential to determine if the lesion is neurapraxic, and likely to recover well without intervention, or has axon interruption, i.e., axonotmesis. In the case of a neurapraxic lesion, the finding of conduction block with retained excitability and function of the distal nerve trunk indicates that full recovery can ensue. If the lesion is accessible by non-invasive electrophysiological studies (e.g., nerves beyond the mid-upper arm and in the forearm or leg below the knee), then conduction block can be identified early after the injury. Thus, the identification of a predominantly neurapraxic lesion with conduction block is important in directing surgeons not to intervene, since spontaneous recovery is expected. In the case of aoxonotmesis, however, the choice is less obvious. Some of these lesions, like the simple crush above, may

recover spontaneously with time, particularly if the distance to the target is short. Unfortunately, simpler approaches, including electrophysiological ones, to determine which axonotmetic lesions are likely to regenerate enough for a functional benefit are unavailable. Both the passage of time and careful follow-up are required to determine if an axonotmetic lesion is beginning to reinnervate distal muscles. In the author's experience, some distal targets may be *reinnervated by a single motor unit*, without the benefit of additional reconnected motor units several years in the future. Therefore, these lesions may require re-exploration, their associated neuromas resected and a decision made to either transect and resuture "refreshed" ends or graft the lesion. The decision to resect and repair is therefore difficult when a lesion might recover better without intervention. If further transection and resuture is required, the timetable for regeneration begins anew.

A third major reason that axons encounter long-term denervated territories is because they are common after transection injuries or neurotmesis. These injuries impose additional barriers to regeneration and reinnervation of distal stumps. After nerve trunk transection and immediate resuture, it is estimated that only 10% of axons reach their target, despite high-quality reconnection [744]. Moreover, regeneration rates following transection are slower than the rates discussed above. In a swollen and bleeding acutely injured limb, it may be very difficult to identify sundered ends of peripheral nerves and to reconnect them microscopically. In the past, attempted high-quality reconnections of nerves have been postponed for 3 months or more to provide optimal conditions for surgical success.

In conclusion, all of these scenarios are common and approaches to them do differ. The reader is referred to several excellent texts published on this topic [349,421,660].

Proximal nerves and perikarya following axotomy

The proximal nerve stump and the pool of neurons supplying peripheral nerves undergo several changes if a nerve trunk injury is chronic and does not regenerate. With time, there may be further retrograde loss of parent neurons. For example, while it is debated, axotomized motor neuron numbers may decline by 60% over long periods [179]. This loss, in turn, reduces parent axons available to send sprouts into the distal stump. RAG expression also gradually declines in this situation; a proximal axon will not exhibit as robust an outgrowth capability as it might shortly after an injury. Fortunately, collateral sprouting can compensate for some degree of irreversible neuron loss.

Lago and Navarro's report [370] that proximal nerve stump axons regenerating into an isolated nerve stump maintain their population of axons for long

periods is more encouraging. Transected sciatic nerve axons were allowed to regenerate into sections of nerves not connected to target tissues and subsequently formed neuromas. The regenerating axons remained intact, did not drop out, and maintained their expression of immunohistochemical markers (ChAT, CGRP, GAP43/B50) for up to 9 months later. These findings indicate that long-standing transected nerves, as might occur after amputation, retain the capability of reconnecting to a distal target for many months. The authors suggested that these intact proximal axons might potentially be interfaced with prosthetic devices.

Although axons may persist in proximal stumps that are not reconnected, Furey *et al.* [204] show that motor neurons are less likely to extend new axons following prolonged axotomy. The experimental design differed from that of Lago and Navarro above. Femoral nerves were transected and either implanted into a blind tube to prevent regeneration or into a cut saphenous nerve without a target. The capability of motor neurons to regrow into a fresh distal stump was then tested after a second surgical procedure. Chronically axotomized motor neurons were compared with motor neurons allowed to regrow toward their targets (primary resuture of the transected femoral nerves). Their ability to regrow was measured with retrograde tracers. It was found that motor neurons deprived of contact with their targets were less likely to regrow at a later time. The authors also emphasize that counts of regrowing axons may not accurately reflect the population of regenerating motor neurons because up to ten axons may grow from a single parent motor neuron.

Prolonged disconnection from target tissues is a common clinical scenario that impairs later regeneration. Parent neurons may disappear from retrograde loss or may simply fail to respond to a regenerative stimulus. While these neurons may send out multiple daughter sprouts, their fidelity and success of functional reconnection cannot compensate for loss of parents.

Long-term denervated nerve trunks

For the first 2 or more weeks after a nerve trunk injury, active distal Wallerian-like degeneration ensues. SCs proliferate, migrate, change their phenotype, and elaborate growth factors. There is an influx of macrophages, a local rise in blood flow, and angiogenesis. These events are reviewed in previous chapters. Overall, the behavior of cells and the synthesis of cytokines indicate an active inflammatory milieu.

When reinnervation is prevented, these features dissipate over time. The microenvironment of denervated distal trunks eventually becomes unsupportive of regeneration [656]. Even after 2 months of delayed repair, 20% fewer motor

neurons regenerated axons in the femoral nerve [658]. SC proliferation tapers, and they elaborate lower levels of growth factors and cytokines. With further time, the number of SCs decline, they lose their reactive phenotype, and become atrophic [179]. SC molecular markers associated with an activated status decrease: erbB2, erbB4, and p75 [397,768]. While SCs can later be partly reactivated, they remain less supportive for axons seeking to regrow into the distal nerve stump. Associated basement membranes and Bands of Bungner eventually disappear in denervated distal stumps [215]. Interestingly, while smaller numbers of atrophic SCs cannot adequately support new axons, they are capable of remyelinating those axons that do eventually reach them [656]. SCs are also conditioned by the types of axons that regrow within them and thereafter choose suitable axons. For example, Sulaiman and colleagues [658] found that motor axons will enter and reinnervate a graft that originated from a sensory nerve trunk. If, however, sensory axons are simultaneously allowed to reinnervate it (i.e., into the other end of the graft), motor axon ingrowth is substantially reduced. The sensory axons appear to sensitize the residual SCs to support the original axons they once associated with. In this case, sensory axons rapidly instructed the nerve graft to recapitulate the allegiance to sensory, not motor axons. These studies show that both chronic denervation of the distal stump and also "sensitizing" the stump with sensory axons substantially impairs motor regrowth. It seems likely that regrowing motor and sensory axons from mixed nerves compete for SC support.

Declines in a number of cell types have been described in chronically denervated nerve stumps. For example, the numbers of overall proliferating cells fell between 3 and 6 months in chronically denervated distal nerve stumps [626]. Macrophage numbers also declined. In studies of chronically denervated rat sciatic nerves lasting 3–6 months, blood flow within both the endoneurial and epineurial compartments fell below levels of intact nerves [281]. When chronically denervated nerves are examined grossly, they appear atrophic, fragile, and translucent. There is atrophy of endoneurial fascicles and collagen replaces considerable portions of the nerve architecture (Figures 8.1, 8.2). Long-term denervated nerve stumps become "fibrotic" because of collagen deposition; the epineurium and perineurium abundantly express Types I and III collagen mRNA in denervated stumps. Axon re-entry into chronically denervated distal stumps is associated with a concurrent increase in mRNA of Type I collagen [626].

Chronic denervation is associated with reductions in the the mRNAs of growth factors synthesized by SCs, mast cells, and macrophages. For example, GDNF levels fall progressively (after a large rise soon after injury) in the SCs of distal nerve stumps up to 6 months if they are not reinnervated [278]. Hall showed that chronically denervated SCs had lower proliferation rates,

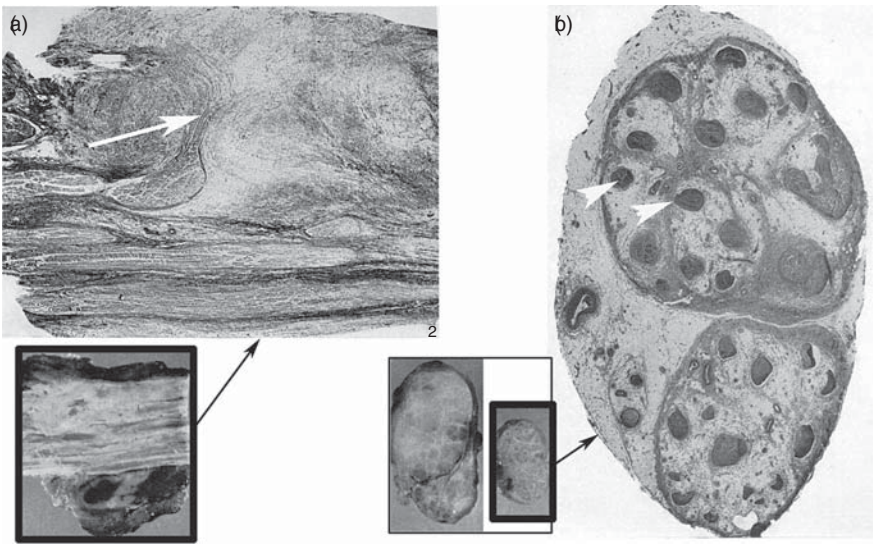

Figure 8.1 Images of peripheral nerve specimens from WWII soldiers wounded in battle by missile injuries that were resected during attempts at surgical repair several months later. The example in A is a longitudinal section of a sciatic nerve illustrating severe disruption of the nerve architecture and a neuroma in continuity (white arrow). From a similarly injured sciatic nerve, the image in B is from the nerve distal to an injury (the insets are proximal, left, and distal, right, boxed, to the injury) where no significant axonal regrowth has developed. Note the fascicles are small and atrophic and it is likely that they consist mainly of fibrotic collagen. These distal stumps do not provide a favorable microenvironment for regrowth. (Reproduced with permission from [431].)

less migratory potential, and attenuated expression of cell adhesion molecules (N-CAM and N-cadherin). Some of these features could be restored with neuregulin (GGF) [248]. VEGF mRNA, a transcript that rises to high levels with angiogenesis early after injury, declines to low level baseline values during prolonged denervation [281]. Finally, chronically denervated nerve stumps may impose additional barriers to regeneration through the expression of chrondroitin sulphate proteoglycans (CSPGs). CSPGs collapse growth cones and inhibit nerve regeneration. Neural stem stells that expressed MMP-2 (matrix metalloproteinase-2) facilitated regrowth into chronically denervated nerve stumps by degrading CSPGs [258].

Denervated muscle

Muscles deprived of their innervation undergo substantial alterations. These include structural changes in muscle fibers, as well as alterations in electrophysiological and biochemical properties. Most are reversible during

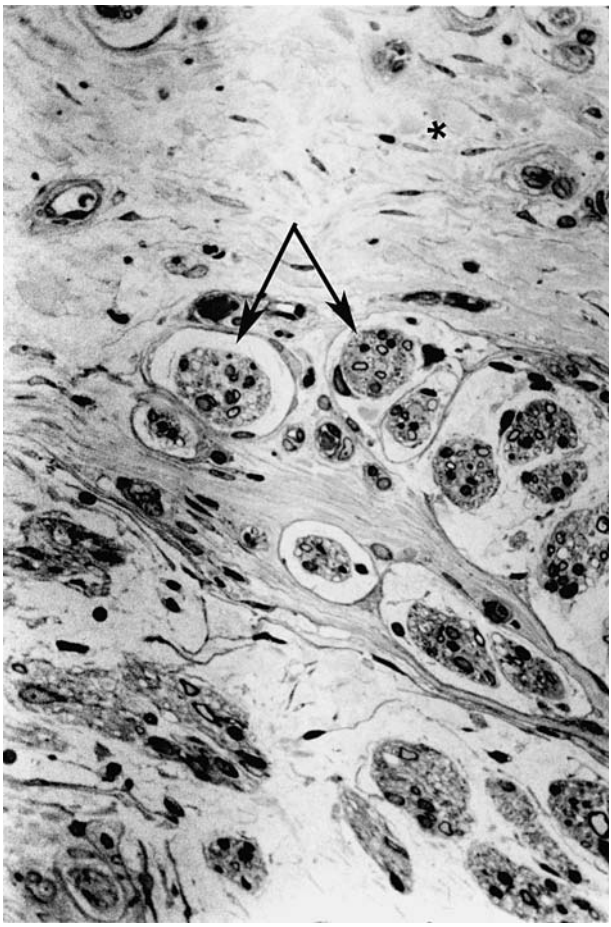

Figure 8.2 Examples of isolated minifascicles (arrows) from a chronic (6 month) experimental neuroma from a rat sciatic nerve. Much of the field is not invested by axons or blood vessels (asterisk). The image is from a semithin section, embedded in epon and stained with toluidine blue.

reinnervation. Their onset is more rapid when the nerve injury is closer to the muscle. There is debate over whether denervation changes occur as a result of loss of activity, loss of input by motor nerves, or from loss of axon trophic molecules (see review [471]). Experimentally, rigorously applied forms of disuse can recapitulate several features of denervated muscle. Many changes of denervation can be reversed by direct stimulation of muscles, a traditional clinical approach for "conditioning" muscles during recovery from a nerve injury. In the case of the neuromuscular junction, however, there is evidence that specific molecules are involved in the remodeling during reinnervation. These include

IGF-II, agrin, and neuregulin. CNTF may act as a "myotrophin" in denervated muscles, capable of attenuating atrophy and reducing loss of twitch and tetanic tensions associated with denervation [261].

The weight and force of muscles decline to 30% of their original value after 6 months of chronic denervation [199]. In the author's experience, dramatic atrophy of human muscles can occur within a similar time frame during denervation. The changes in muscle, however, do not indicate resistance to reinnervation. Failure of motor axons to return to chronically denervated endplates is the predominant reason for poor motor reinnervation. Intrinsic changes in the target muscles such as fibrosis and calcification occur much later. Recovering and reconnecting motor axons can also enlarge their motor units by five times or more [199].

Denervated muscles thus develop atrophy of individual muscle fibers and gross loss of muscle weight. Moreover, these changes may be accelerated in slow twitch muscles. By light microscopy, atrophic denervated muscle fibers form "nuclear knots" consisting of chains of nuclei occupying very little cytoplasm. Ultrastructural changes in muscle may occur within days of denervation. There is disorganization of sarcomeres and disruption of myofibrils. Sarcoplasmic reticulum changes include hypertrophy of terminal cisternae, proliferation of transverse tubules, and rises in triad numbers [471]. Eventually, there may be frank loss of muscle fibers. The gross weight of muscles is not, however, an accurate index of denervation or reinnervation. Its use in experimental work should be discouraged; replacement of muscle fibers by connective tissue and other technical factors may independently alter weight measures. Morphological features of reinnervated muscles, such as fiber caliber, provide more accurate estimates of denervation and reinnervation. Other late morphological changes of denervated muscle are vacuolar degeneration, hyaline degeneration, and progressive disintegration of fibers [471]. Electromyography of long-term denervated muscles in humans (10–20 years or more) identifies electrical silence and a "woody"or fibrotic gross consistency. This state is severe and likely at this time to be an irreversible state of affairs. Reinnervation is then essentially impossible.

The behavior of *terminal SCs*, discussed in detail in Chapter 6, is important in promoting appropriate reinnervation. Since terminal SCs guide axons back to denervated endplates, their loss may result in failed axon pathfinding. This has been identified (see review [319]) in situations where denervation is prolonged. The plasticity of terminal SCs, so apparent during early reinnervation or partial denervation with concurrent reinnervation, may gradually dissipate. Endplates may therefore be abandoned. Regenerating motor axons, strictly guided by terminal SCs are unable to identify these abandoned sites, and they are not reinnervated [319].

Electrophysiological recordings, described in Chapter 4, can also provide information about delayed or frustrated reinnervation. CMAPs decline in amplitude in direct proportion to the loss or degree of dysfunction of the motor axons innervating the endplates being recorded from. Recovery of CMAPs similarly reflects the extent of axon reconnection, but collateral sprouting to other denervated muscle endplates also occurs at this time. Rises in CMAPs during reinnervation therefore reflect both reconnection of regenerated axons as well as collateral sprouting (see Chapter 6). Serial studies with persistent small amplitude CMAPs or NAPs that do not recover or change with time indicate that further regeneration has halted.

Needle electromyography may provide further information. With long-term denervation, some have argued that the amplitude of fibrillation potentials gradually declines, reflecting individual muscle fiber atrophy. Muscles with very prolonged denervation fail to generate an injury discharge with movement of the needle electromyography electrode, a property of normal muscle.

Contractile changes develop within denervated muscles. These include the acquisition of fast contractile properties by slow twitch muscles whereas fast twitch muscles are not altered. Overall, contraction in both fiber types is slowed, and tension development rate and velocity are reduced [471]. Denervated muscles have decreased uptake of glucose, impaired binding of insulin, depletion of glycogen, altered activity of glycolytic enzymes, and decreased glucose oxidation [81,147,151,275,312,494]. Relatively few studies of denervated muscles in humans are available since even rapid freezing of biopsy specimens can alter their biochemistry. A noninvasive approach is to analyze muscle bioenergetics using NMR (nuclear magnetic resonance) spectroscopy, a technique that measures levels of phosphocreatine (PCr), inorganic phosphate (Pi), ATP, and ADP. Denervated forearm muscles in patients with nerve injuries or motor neuron disease were studied by P31 NMR spectroscopy. Subjects placed their arms into a small bore (26cm bore, 1.89T) magnet [810]. Patients were compared with normal controls and to patients with forearm disuse resulting from wearing orthopedic casts for bony injuries. Denervated muscles had reduced ratios of PCr/Pi, indicating a decline in the bioenergetic reserve of the muscle. Denervated muscles also had a rise in pH. The caveat is that these changes are not specific and other primary muscle disorders are capable of generating them. The time course of denervation related changes was examined experimentally by Dort *et al.* [148]. Declines in PCr began within 1 week of denervation of the rat facial muscle and continued out to 8 weeks following injury.

Long-term denervation also results in contractures of muscle tendons that reduce joint mobility and may fix the limb in postures that interfere with function. In the human foot, a shortened Achilles tendon leads to persistent plantar flexion

of the foot. In rodent models, persistent flexion–contraction of the paw digits may develop. Functional tests of motor recovery are complicated by these changes [759].

Long-term denervated skin and other organs

There are long-term changes in other endorgans with prolonged denervation. In patients with chronic polyneuropathies, skin atrophy and loss of hair may closely parallel the extent of sensory loss. Denervated skin is also prone to breakdown and ulceration. In part, this occurs because desensitized skin may undergo unrecognized injuries. Patients may not sense the presence of a nail or a stone in their shoe. Loss of sudomotor innervation also contributes to ulcerations in denervated skin. Normal sweating likely moisturizes and protects the skin. Skin deprived of sweating can become thickened, cracked, and susceptible to injury and infection.

Sensory nerves also supply the skin and other organs with neuropeptides that influence its microcirculation and healing. Two of the major neuropeptides considered are SP (Substance P) and calcitonin gene-related peptide (CGRP). Neurogenic inflammation refers to the participation of sensory nerve terminals in the inflammatory response of the tissues they innervate. All of the principal components of inflammation, described as the "triple response of Lewis," are involved: tumor (swelling from edema, plasma extravasation), rubror (redness from rises in local blood flow), dolor (pain), and calor (heat from rises in blood flow) [395]. The triple response refers to the timing of these changes, following a firm stroking of the skin. Lewis' classical description is that of an initial dull red line followed by a bright red halo called the flare response, then third, swelling (edema) with blanching of the line. When tissues are denervated, they are deprived of neurogenic inflammation and the overall inflammatory response is attenuated. Since all of the features of inflammation are important in proper wound healing, denervated skin and other tissues have impaired healing.

A number of properties of healing wounds are impaired during denervation [32]. These include delayed contraction of their edges to close the wound, reduced microvascular responses, delayed and prolonged inflammatory phases, hypertrophic scarring, and delayed epithelial cell migration. Rises in blood flow supply the demands of proliferating and activated cells and help with clearance of debris and micro-organisms. Plasma extravasation delivers blood-borne trophic and other molecules as well as macrophages to the injury site. These properties, in turn, depend on neuropeptides released by neurogenic inflammation. SP contributes to vasodilation and plasma extravasation. CGRP is a very potent vasodilator [60]. Both peptides may signal mast cells to release

histamine to promote further vasodilation and plasma extravasation. Mast cells also release proteases and other growth factors within the injury milieu. In the absence of "peptidergic" sensory axons that normally innervate the skin, all of these properties of neuropeptides that are required for healing are absent.

Overall then, denervated skin that fails to mount an appropriate inflammatory response to an injury experiences prolonged, impaired healing. Chronic ulceration with superimposed infections result. Bone infection or osteomyelitis, in turn, may be associated with loss of distal limb viability, requiring drastic measures such as amputation. In diabetic patients with chronic polyneuropathy, amputation due to nonhealing infected ulcers and osteomyelitis is a serious and frequent complication. Fluctuating discoloration, particularly with a dependent limb is a common development. Altered vasomotor control due to denervation probably accounts for these changes. For example, loss of adrenergic vasoconstriction of skin blood vessels may result in its dark blue discoloration (e.g., of the toes and feet in sensory neuropathy). Loss of the muscle supply to a limb may also cause edema because of impaired venous return, a function of the normal contracting muscles of a limb.

Terminal sensory axon SCs, described in Chapter 6, can survive and support regrowth to correct targets after denervation [152]. With prolonged denervation, however, these specialized SCs become atrophic and disappear. Munger described persistent changes in several skin sensory organs including Pacinian corpuscles, Meissner's corpuscles, Merkel touch domes, and the piloneural complex associated with guard hairs after prolonged denervation [483].

Collateral sprouting, described in previous chapters, is an important mechanism for reinnervating skin or muscle when their nerve supply is permanently interrupted [152]. While nociceptive axons undergo brisk collateral sprouting, large caliber axons supplying touch/pressure may not. The hindpaw is thought to exhibit less collateral sprouting of cutaneous axons than the dorsal trunk skin. The presence of fewer available intact neurons in adjacent territories also diminishes the formation of collateral sprouts.

Summary

Both distal portions of the peripheral nerve trunk and target organs may experience prolonged loss of their axon investment. This is a major clinical problem following peripheral nerve injuries. First, repair is not always accomplished immediately and second, regrowing axons require time to extend long distances. Long-term denervated muscles have altered structural, electrophysiological, and biochemical properties. Prolonged denervated skin has impaired

healing in part related to loss of neurogenic inflammation. In both muscle and skin, specialized terminal SCs are important sources of guidance during regrowth but may disappear with prolonged denervation.

Suggested reading

Fu, S. Y. & Gordon, T. (1997). The cellular and molecular basis of peripheral nerve regeneration. *Molecular Neurobiology*, **14** (1–2), 67–116 [200].

Gordon, T. & Fu, S. Y. (1997). Long-term response to nerve injury. *Advances in Neurology*, **72**, 185–199 [227].

9

Trophic factors and peripheral nerves

The concept that molecules can signal neurons and axons and convince them to behave in new, even innovative ways is exciting. Trophic factors are molecules, usually proteins, that act on specific cell receptors to induce changes in protein synthesis, outgrowth, or survival. In the nervous system, nerve growth factor (NGF) leads the classic family of growth factors known as "neurotrophins." A working definition of a neurotrophin is "an endogenous soluble protein regulating survival, growth, morphologic plasticity or synthesis of proteins for differentiated function of neurons" [256]. NGF was discovered in 1951 by Rita Levi-Montalcini and subsequently characterized by Stanley Cohen, culminating in the award of the Nobel Prize in Physiology or Medicine in 1986 to both investigators [388,389]. Using sarcoma tumor in the mouse model, Levi-Montalcini and Viktor Hamburger established that NGF was a soluble factor from mouse sarcoma tumor that was capable of inducing hyperplasia in sympathetic ganglia [389,390] (Figure 9.1). The original articles describing these discoveries are recommended and make for fascinating reading [390]:

> The growth promoting effect of the tumor is mediated by a diffusible agent. This mode of action was suggested by the observation that sympathetic ganglia which are located rostrally to the tumor and not connected with the tumor by nerve fibers, are also conspicuously enlarged. The overgrowth of the ganglia is the combined result of an increase in cell number and in cell size. The enlarged ganglia send large sympathetic fiber bundles into the adjacent viscera. Branchial and abdominal viscera which normally receive only a sparse innervation or none at all are inundated with fiber masses during the second and third weeks of invasion.

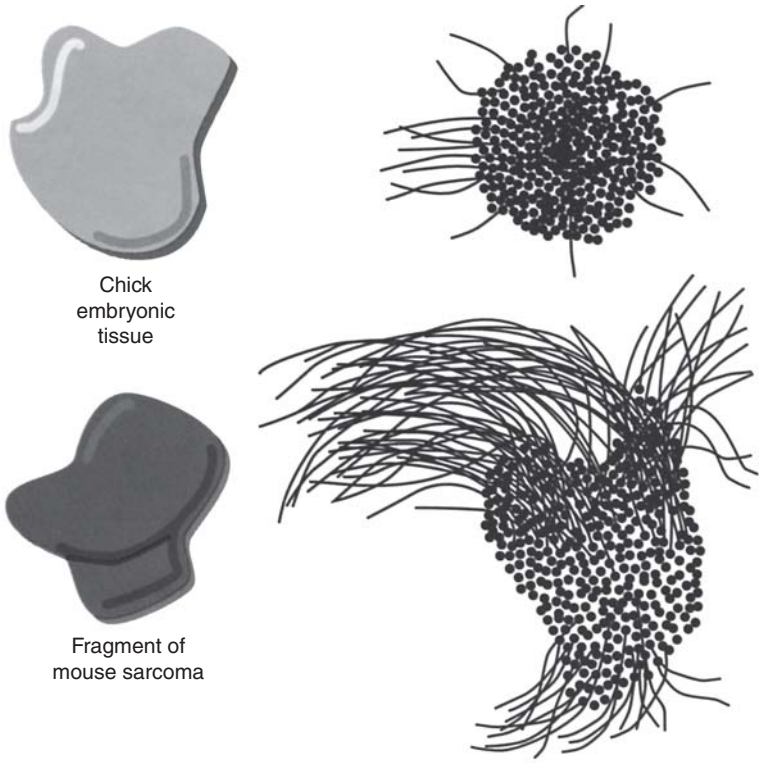

Figure 9.1 Illustrations of the original findings of Levi-Monalcini and colleagues that led to the discovery of NGF. Axons emerging from explanted sympathetic ganglia grow toward mouse sarcoma cells that secrete a gradient of NGF. (Based on the original figures by Levi-Montalcini [389] and reproduced with permission from [782].) See color plate section.

Growth or trophic factors support neurons through several mechanisms that include retrograde transport from target tissues, paracrine support from other cell types adjacent to neurons, or autocrine support from themselves or neighbor neurons. Growth factors can be elaborated by a variety of cell types including neurons, SCs, perineuronal satellite cells, and other cells of target organs.

The topic of growth factors and neurons is large and this discussion is limited to its impact on the adult peripheral nervous system. The reader is also referred to Boyd and Gordon's [58] comprehensive review of this topic. Multiple and overlapping roles exist for growth factors during development both in the peripheral and central nervous systems, and in synaptic plasticity. These important roles for growth factors are not addressed here.

The neurotrophin family

Since the publication of the original NGF papers, the neurotrophin family has expanded to include several new members all sharing an interaction with the p75 neurotrophin receptor (previously known as the "low affinity" NGF receptor) but operating on more specific Trk ("tropomyosin-related kinase"; tyrosine kinase) receptors. They are brain-derived neurotrophic factor (BDNF) [31,386], neurotrophin-3 (NT-3) [276,436], and neurotrophin 4/5 (NT-4/5) [42]. Neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) are regarded as additional members but NT-6 is nonsoluble and both are only found in fish [230,371]. There is high sequence homology (approximately 50%) among the neurotrophin members [58].

In the classical scheme, NGF binds to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC (Figure 9.2). Work by Verge and others, however, has established the classical schema is incomplete. These interactions are actually more complex and expression of Trk receptors can change following injury [231,324]. Some neurotrophins can interact with other Trks, e.g., NT-3 with TrkB and TrkA. Overall, Trk receptors mediate differentiation, survival, and loss of proliferative capacity through their intracellular cascades. TrkA receptors have been typically

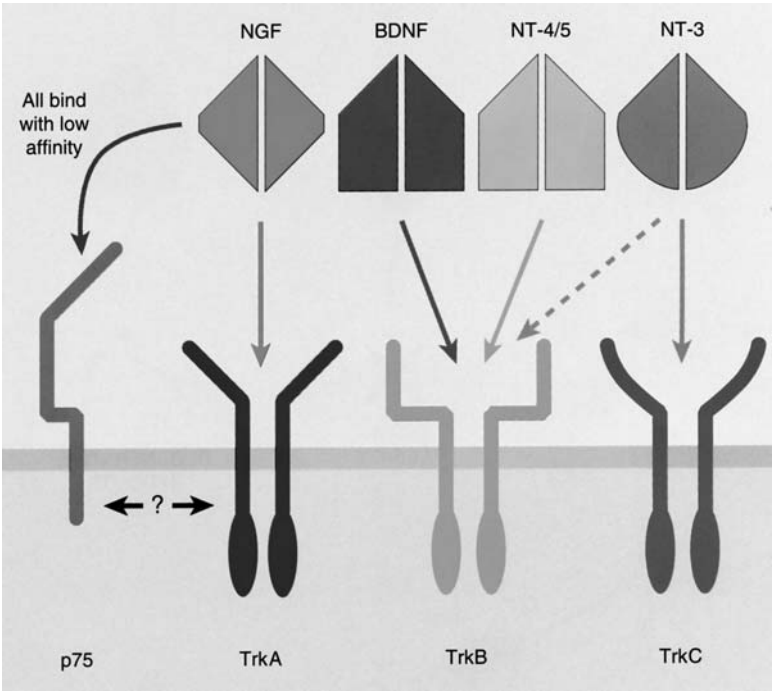

Figure 9.2 Illustration of members of the neurotrophin growth factor family and their receptors. (Reproduced with permission from [782].) See color plate section.

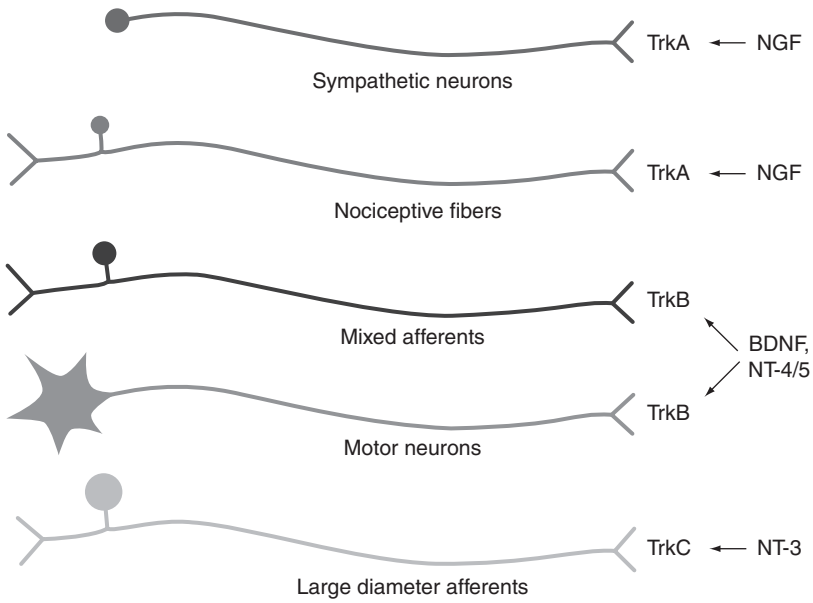

Figure 9.3 Illustration of the peripheral neuron subtypes responsive to members of the neurotrophin growth factor family. (Reproduced with permission from [782].) See color plate section.

localized to small sensory neurons with unmyelinated axons that mediate nociception and temperature sensation and contain Substance P (Figure 9.3). TrkA are also found on NGF responsive postganglionic sympathetic neurons, as originally described by Levi-Montalcini. TrkB receptors are located on medium-sized sensory neurons and motor neurons, while TrkC receptors are located on large caliber sensory neurons and motor neurons with large myelinated axons [325,457]. As might be expected, Trk receptors are also found in the central nervous system, and the distribution of the receptors (and the response of the neurons bearing them!) vary dramatically during development. At different stages of development, the neurotrophins and their receptors have specific responsibilities. For example, NT-3 and TrkC are required for the development of muscle spindle afferents and larger caliber sensory neurons. Many investigators have argued that their importance is much greater during development than in adulthood. For example, axotomy interrupts the retrograde transport of NGF and other growth factors from target tissues and massive retrograde cell death in neonatal sensory neurons occurs. In contrast, adult nerves subjected to the same injury suffer relatively little retrograde apoptosis (see Chapter 3), indicating that they have less reliance on ambient growth factor transport. There is also extensive literature on neurotrophin family members and on their modulation of synaptic transmission.

Neurotrophin growth factor receptors [325] are differentially distributed among neuron populations. TrkA, high affinity NGF receptors, are found on 41% of lumbar DRG neurons. TrkB, the cognate receptor for the BDNF on 33%, and TrkC, the receptor for NT-3 on 43%. The low affinity neurotrophin receptor, p75, is capable of binding all neurotrophin family members (Figure 9.2) and is found in 79% of DRG neurons. There are two TrkA isoforms that have overlapping expression. This clearly establishes that extensive colocalization of Trk expression exists. Of all sensory neurons, 10% coexpressed TrkA and TrkB, 19% coexpressed TrkA and TrkC, and 18% coexpressed Trk B and Trk C. Trilocalization of Trks was rare, but Trk and p75 colocalization was tightly coupled. Neurons expressed only one Trk 34% of the time and more than one in 40% of lumbar DRG neurons. Interestingly, approximately 26% of DRG neurons that were small and medium in size did not express Trks and represented the IB4, or GDNF responsive subpopulation. A small number of neurons and satellite cells also expressed truncated isoforms of Trks (especially TrkB,C) that do not have an intracellular signaling domain [579]. These splice variants may allow the receptors to have more promiscuous binding to other, nonpreferred neurotrophins, but their function is unknown. They may modulate interactions of the ligand with full-length receptors (dominant negative). They may also alter rates of degradation of ligand–receptor complexes or limit the overall concentrations of the neurotrophins available to act on normal Trk receptors [325].

Unlike other neurotrophins, BDNF is synthesized in intact DRG sensory neurons and its levels rise within 1 day following injury [686]. Motor neurons do not express BDNF. It is likely that all neurotropins, however, are internalized by axons and are retrogradely transported from target tissues. BDNF undergoes both anterograde and retrograde axoplasmic transport, whereas NGF only exhibits retrograde transport [142,262,606,607]. The transport of NT-3 is controversial, but it does undergo rises in local expression by target tissues (such as muscle) after injury, possibly from local synthesis [686]. The classical tenet of the "neurotrophic factor hypothesis" is that such target-derived support, delivered by retrograde transport, is essential to the maintenance of neurons. During development or regeneration, neurons compete for these sources of support, and axons that fail to secure them undergo apoptosis (Figure 9.4).

Neurotrophin receptors and signaling cascades

Neurotrophins are capable of signaling neurons locally, for example, at growth cones, or centrally at either the cell body membrane or within its cytoplasm after transport [679]. The initial synthesis of NGF is in the form of a proNGF dimer molecule. The proNGF chains are then cleaved to form β subunits, and two γ and α subunits are added to form a storage form of the molecule

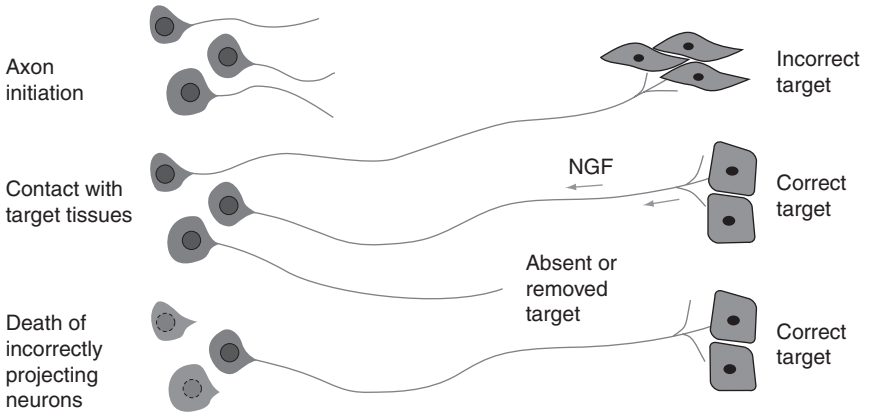

Figure 9.4 Illustration of the neurotrophic hypothesis. Neurons from axons that arrive at a target source of growth factor survive whereas those that fail to arrive or grow to incorrect targets undergo apoptotic cell death. (Reproduced with permission from [782].) See color plate section.

[15,16,453,622]. Finally, each β subunit joins an identical subunit to form a noncovalently linked dimer [782] that is freed from its subunits and released as the active form of NGF. Each β subunit is a necklace of 118 amino acids connected by three covalent sulphur bonds on cysteine residues. The three-dimensional structure of each NGF β subunit includes three antiparallel strands that form a surface where they can therefore associate into a dimer, facilitated by a cysteine knot motif. The antiparallel strands within each subunit are joined by four loops that are unique among neurotrophin family members.

The mature NGF dimer is distinguished as having the attributes of a classic signaling molecule. More recent evidence, however, has also indicated that its precursor proNGF can act as a signaling molecule with a repertoire of actions different from its cleaved substrate [178]. Its overall levels may therefore be determined by the efficiency of proteolytic conversion to mature forms. ProNGF binds p75 receptors with higher affinity and may, as a consequence, have more widespread actions than classical NGF [293,383]. Several forms of proNGF have also been described [178].

Mature or classical NGF binding to TrkA results in receptor dimerization and autophosphorylation of its intracellular domains. Trks are concentrated in caveoli-like structures that facilitate their interaction with proteins on lipid rafts [291]. Ligand binding stimulates their internalization through clathrin-coated pits and macropinocytosis [579].

Other members of the neurotrophin family also operate as noncovalently linked homodimers. Like NGF, their ligation of Trk receptors causes receptor dimerization, then autophosphorylation of an intracellular "activation loop" of the receptor. Specific phosphorylation sites (called Y490 in the case of TrkA)

interact with proteins containing SH2 (src-homology-2), that in turn act as scaffolds to activate one of four pathways: PI3-kinase, PLC-γ, and Ras. Another, but less well-studied pathway involves SNT [suc1-associated neurotrophic factor-induced tyrosine phosphorylated target] [58]). Some of the protein intermediaries include shc, Gab-1, Grb2, and Sos (for review see [58]).

All of the mature neurotrophins (as well as proNGF) thus interact with p75, a member of the tumor necrosis receptor superfamily (Figure 9.2). P75 is a multifunctional molecule, facilitating neurotrophin signaling but also capable of promoting cell death. It interacts with its neurotrophin ligand and with the Trk receptors in complex patterns (see review [579]). Thus, p75 may inhibit activation of Trks by nonpreferred neurotrophins, thereby enhancing the specificity of neurotrophin–Trk interactions. It may also potentiate activation of Trks when there are low levels of ligand, and it may promote retrograde transport of neurotrophins. Overall, while the interaction of Trk and p75 therefore facilitates neurotrophin signaling, whether this requires a direct physical interaction to do so is also debated [735].

In the absence of its Trk interaction, p75 also promotes apoptosis and cell death through an intracellular "death" domain and it facilitates RHO GTPase-mediated growth cone collapse (see Chapter 5). Proneurotrophins, such as proNGF, bind with high affinity to p75, but they do not activate Trk receptors. Other proteins interact with p75 and Trks. For example, Necdin is a multifunctional signaling protein that facilitates the interaction between TrkA and p75 for sensory neuron survival signaling [366].

The PI3K (phosphatidylinositol 3-kinase) pathway, introduced in Chapter 5, is one of the main downstream targets of neurotrophin signaling (Figure 9.5). PI3K interacts with protein complexes that form in association with the cytoplasmic side of the cell surface. PI3K converts PI(2)P to PI(3)P (phosphatidylinositol trisphosphate) that, in turn, phosphorylates and activates the serine/theonine kinase Akt (also known as PKB). Akt then acts through a variety of pathways to influence cell survival and outgrowth including the inhibition of GSK3β (that, in turn, inhibits growth cone advance). Akt also activates IκB promoting its degradation and dissociation from *NF-κB*, another important player in sensory neuron survival [182,249]. A novel pathway involves an NGF-p75 interaction, independent of TrkA, that phosphorylates and inhibits phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [22]. PTEN, also introduced in Chapter 5, is a phosphatase that inhibits PI(3)P formation and pAkt to thereby facilitate GSKβB signaling. By "knocking down" PTEN, elevating pAkt, and thereby inhibiting GSKβ signaling, NGF-p75 can facilitate outgrowth independent of TrkA. The end result of these many steps is an elevated level of pAkt. Thus either TrkA signaling, or the p75 pathway, may increase pAkt.

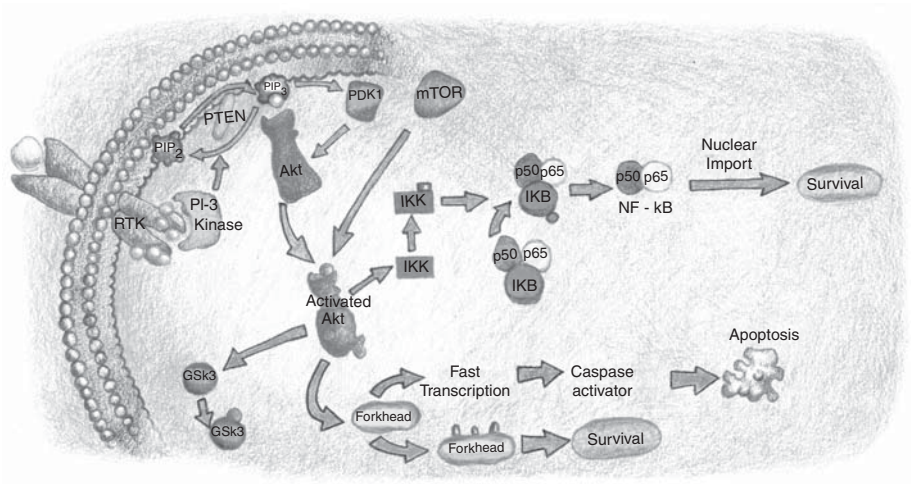

Figure 9.5 Illustration of the PI3K-Akt neuron survival pathway activated by growth factor receptors containing a receptor tyrosine kinase (RTK) intracellular signaling domain. (Illustration by Scott Rogers based on diagrams posted by EMD/Calbiochem (www.emdbiosciences.com).) See color plate section.

A related growth factor signaling pathway involving *PLC-γ1* operates through inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) to mobilize Ca^{2+} and PKC. While its impact is uncertain, its actions on PKC may also be important in a number of additional pathways including localization of GAP43/B50 to the membrane of the growth cone.

The third major pathway of neurotrophin signaling involves the *extracellular signal-regulated protein kinases (ERKs), a subfamily of the mitogen-activated protein kinase (MAPK) cascade*. Growth factor receptors, as discussed above, interact with Ras. Ras then activates the protein kinase Raf, that in turn phophorylates MEK1 or MEK2 (MEK = MAPK and ERK kinases) to phosphorylate and activate ERK1 and ERK2. This pathway eventually activates the intermediate early genes fos and jun. ERK5 is an additional target of neurotropin signaling and there are likely other related intermediaries with varying evidence for their involvement [321]. ERKs influence transcription within nuclei through several additional molecules, in particular, cAMP response element binding protein (CREB) [579]. Neurotrophins elevate cAMP levels, in turn, activating a series of pathways that support neurite outgrowth and involve PKA and CREB. All of these findings therefore illustrate that CREB is a major mediator of neurotrophin actions [670].

There are a number of other proteins and signaling cascades that are either influenced by neurotrophins or work together with them. Poly ADP-ribose polymerase (PARP) acts on histone H1 of the chromatin structure to allow

neurotrophin signals access to DNA. This access can thereby influence DNA transcription and repair [711]. Neurotrophins activate RAC and CDC42 GTPases, growth cone molecules that facilitate neurite outgrowth through PI3K. Moreover, NGF is capable of bypassing inhibition by CSPGs, an inhibitor pathway that acts through RHOA GTPase. Trk receptors can be phosphorylated without neurotropin ligation. For example, adenosine or PACAP signals through G-protein-coupled receptors to activate Trk [570]. NGF signals converge with those mediated by preconditioning and laminin–integrin signaling on GSK3β–APC [780]. Further pathways that may operate independently of neurotrophins upstream but eventually converge on the same transcriptional pathways, such as CREB, include JAK-STAT, Bcl-2, and PKC (see review [670]).

Other growth factors and the peripheral nervous system

The list of trophic molecules that influence peripheral neurons continues to expand. While many are proteins, others like purines, nucleosides, and nucleotides [283,502] are not. The term "pleiotrophic" has been applied to this broad group, highlighting their widespread actions beyond the nervous system. This is not to be confused with a specific growth factor pleiotrophin (PTN) described below. Alternatively, many growth factors originally linked to other tissues, such as epidermal growth factor (EGF) or erythropoietin (EPO), were later discovered to also act on the nervous system. A current list of several of these growth factors is given in Table 9.1.

One class of growth factors, also called neurotrophic or neuropoeitic cytokines, share a common receptor subunit known as gp130. The association of this subunit with other specific receptor subunits confers ligand specificity. This family also shares a single, four-α-helix bundle protein structure that, unlike neurotrophins, does not form dimers [259,260]. The ligand–receptor complexes signal through the cytoplasmic tyrosine kinase Janus-kinase (JAK) and signal transducer and activator of transcription (STAT) pathways. The STAT3 transcription factor promotes neurite outgrowth. The suppressor of cytokine signaling (SOCS3), a member of another large protein family, dampens STAT3 actions through feedback inhibition [469].

Members of the neuropoeitic cytokines include *ciliary neurotrophic family* (*CNTF*), a growth factor that supports the survival of parasympathetic, motor, sensory, and sympathethic neurons [299]. CNTF can rescue embryonic and neonatal motor neuron cell loss after axotomy but the actions in adults are less robust [232,398,537,593]. Its receptor complex consists of gp130, LIFRβ, and CNTFRα. Other members of this family include *leukemia inhibitor factor* (*LIF*; receptors gp130 and LIFRβ), *interleukin-6* (*IL-6*; receptors gp130 (2 subunits) and IL6Rα), and *cardiotrophin-1* (*CT1*; receptors gp130 and LIFRβ). CT1 is a survival factor for

Table 9.1 *Neurotrophin and non-neurotrophin growth factors*

Name	Receptor	Peripheral neuronal target
Neurotrophin growth factors		
NGF	TrkA, p75	Small sensory, sympathetic neurons
BDNF	TrkB, p75	Sensory neurons, motor neurons
NT-3	TrkC, p75	Large sensory neurons
NT-4/5	TrkB, p75	
Non-neurotrophin growth factors		
1. Acting through gp 130 receptor complex		
CNTF	LIFRβ, CNTFRα	Motor neurons, probably others
LIF	LIFRβ	?Several types
CT-1	LIFRβ	?Several types
IL-6	IL6Rα	?Several types
2. Growth factors discovered in other tissues		
FGFs	FGFR1–4	?Several types
EGF	EGFR	?Several types
TGFβ	TGFβR-I-III	?Several types
BMPs	BMPRII, ALK2,3,6	?Several types
PDGF	PDGFRα and β	?Several types
HGF	Met TK	?Several types
MSP	c-Ron	?Several types
VEGF	Flk-1	?Several types
EPO	EpoR	Sensory neurons, ?others
3. Insulin-related growth factors		
Insulin	IR	Motor, sensory, ?sympathetic
IGF-1	IGF-IR	Motor, sensory, ?sympathetic
IGF-II	IGFII-R	?similar to IGF-1
4. Other cytokines		
IL-1	IL-1R1, 1R2, 1RAcP	?Several types
?IL-11		?Several types
5. Glial-derived neurotrophic factors		
GDNF	GFRα-1, c-RET	Motor neurons
Neurturin	GFRα-2, c-RET	
Artemin	GFRα-3, c-RET	Sympathetic, sensory neurons
Persephin	GFRα-4, c-RET	
6. Other growth factors		
PTN	LRP-1, others	?Several types

developing motor neurons, but not adult motor neurons [538,593]. Oncostatin-M (OSM) and neurotrophin-1/B-cell stimulating factor-3 are additional members [259,614]. The roles of IL-6 and LIF in SC support, inflammatory signaling, and regeneration are discussed in Chapters 3 and 5.

Glial-derived neurotrophic factors (*GDNFs*) include GDNF, neurturin, artemin, and persephin, members that share approximately 40% homology [30]. GDNF was first described as a potent surval factor for midbrain dopamine cells [406,407]. Like neurotrophins, GDNFs have a cysteine knot motif that facilitates their formation into homodimers. GDNFs, however, bind to a unique receptor complex consisting of a series of GFRα receptors (GFRα-1 for GDNF, GFRα-2 for neurturin, GFRα-3 for artemin, and GFRα-4 for persephin) and c-RET, a tyrosine kinase [597]. When the two receptors and ligand interact, downstream signaling ensues through recruitment of RET into lipid rafts and subsequent activation of its kinase domain. Activation involves association of tyrosine phosphorylated residues on RET with PLC-γ1, Shc, Grb2, and others through to Ras-ERK, PI3K. The reader will recognize that the pathways involved resemble those activated by neurotrophins. Heparin sulphate proteoglycans concentrate extracellular gradients of GDNF to facilitate its interaction with GFRα and RET [597]. Signaling without RET activation may also occur [58]. There are also interesting and important interactions of the neural cell adhesion molecule (NCAM) with GDNFs and its GFRα receptors [597]. Similarly, GDNF can act synergistically with laminin to promote outgrowth of IB-4 NGF unresponsive, TrkA negative adult sensory neurons [698]. Like NGF, GDNF also activates RAC GTPase in growth cones to facilitate lamellipodia formation [202].

GDNF is expressed at only low levels within intact peripheral nerve or ganglia but it is upregulated in Schwann cells after injury. Both GFRα-1 and GDNF levels rise at sites distal to a nerve trunk injury, illustrating another form of axon–SC interchange to support regeneration. GDNF, elaborated by SCs, signals axon outgrowth through the upregulated expression of its receptor on local axons. In contrast, mRNAs for neurturin, artemin, and persephin can be identified in peripheral nerve trunks and ganglia but do not seem to be altered by injury. Similarly, the receptors GFRα2 and RET mRNAs are also present in ganglia and peripheral nerve but are not altered by injury for up to 2 weeks. There was a late rise in GFRα2 in chronically denervated nerve stumps at 3 and 6 months after transection. GFRα3 was upregulated in proximal stumps but not in distal stumps or ganglia after injury [277,278].

When expressed, GDNF acts as a trophic factor for motor neurons, highlighted by a recent review by Bohn [50] entitled "Motor neurons *crave* glial cell line-derived neurotrophic factor"! A number of studies have identified rescue of the retrograde loss of motor neurons after axotomy or rescue of motor neurons in experimental ALS by GDNF. For example, in neonatal mice that overexpress GDNF in astrocytes, facial motor neurons were protected from retrograde loss [777]. Other neurons also "crave" GDNF to a greater or lesser degree. For example, GDNF, neurturin, and artemin support survival and differentiation of sympathetic,

parasympathetic, sensory, and enteric neurons [597]. One interesting feature of artemin is its expression along blood vessels where a chemotactic gradient of the molecule attracts innervation by sympathetic perivascular axons [372]. This may allow formation of sympathetic neuroeffector junctions on blood vessels. Artemin can also increase neurite outgrowth in sensory neurons. Its receptors (GFRα3) are expressed in about a third of adult rat DRG neurons, preferentially in small peptidergic and IB-4 TrkA negative neurons. Artemin also protects sensory neurons *in vivo*. Administered intrathecally after axotomy, artemin prevented changes in peptide expression, upregulation of injury markers (ATF-3), and declines in unmyelinated axon conduction velocity proximal to the injury site [40].

Fibroblast growth factors (*FGFs*) were originally identified through their ability to stimulate fibroblast proliferation. Two main subtypes, FGF-1 (acidic FGF) and FGF-2 (basic FGF) had been recognized but recently the number of FGFs has increased dramatically to 22 [174]. In the extracellular matrix FGFs may be associated with extracellular heparin or heparan sulphate proteoglycan (HSPG). As ligands, FGFs operate on four main tyrosine kinase receptors known as FGFR1–4. FGFR-1 is expressed on neurons and FGFR-2 and 3 are expressed by glial cells. Two downstream docking proteins FRS2α and FRS2β stimulate the Ras/MAPK and PI3K-Akt signaling pathways indicating a convergence with actions of other growth factors discussed above. Eight members of the FGF family, FGF 2, 5, 7, 8, 9, 10, 13, and 14, have been identified in rat lumbar 4 and 5 dorsal root ganglia (DRGs) [396]. Specifically, FGF 2 and 8 are expressed in sensory neurons [309,396,545,558,595,666] and FGFRs are upregulated in DRG neurons following injury [327]. FGF-1 is a known survival factor for injured rat spinal neurons [384] whereas FGF-2 mRNA is increased following embryonic, but not adult spinal cord injuries [563]. FGF-2 and FGFR1–3 mRNAs were all increased in crushed sciatic nerves [238].

Transforming growth factors β (*TGFβ*) are critical trophic molecules that comprise over 50 members. TGFβ1 and 2 are expressed in the peripheral nervous system and in SCs. While TGFs interact with other growth factors, their actions differ since TGFβs inhibit proliferation. TGFβ2 and its receptors (Type I and II) are anterogradely and retrogradely transported in motor neurons [310]. A combination of TGFβ1 and forskolin, a molecule that raises cAMP levels, increased the growth of motor neurons regenerating into chronically denervated nerve stumps [657]. TGFβ1 is upregulated in the distal stump of injured peripheral nerves by SCs [587]. *Bone morphogenetic proteins* (*BMPs*) are a subfamily of TGFβs that support bone growth but are also expressed in peripheral neurons and SCs. BMP-2 is specifically expressed in motor neurons. BMPs engage serine/threonine kinase receptors (BMPRII, ALK2,3 and 6) and activate Smad proteins. BMP-4 and BMP-6 potentiate

NT-3 and neurturin-mediated neurite outgrowth and survival in chicken embryonic sympathetic and nodose ganglia [11]. Local administration of BMP-2 improved facial nerve axon regeneration 4 weeks following crush in rabbits [724].

Epidermal growth factor (*EGF*) plays critical roles in early behavior of neural precursor cells but may have a lesser role in adult nerve regeneration [372]. Heregulin polypeptides, also known as ***neuregulins*** were previously introduced in Chapter 5, and share an EGF domain. The NRG gene splice products have included previously identified proteins called neu differentiation factor (NDF), heregulin, acetylcholine receptor inducing activity (ARIA), and glial growth factor (GGF) [272]. Neuregulins are largely growth factors for SCs. *Platelet-derived growth factor* (*PDGF*) and its receptors are synthesized by neurons and SCs [372].

Erythropoietin (*EPO*) is released by peripheral nerve SCs in response to nitric oxide and signals EPO-R receptors on peripheral axons. The receptors are also expressed on sensory neurons in DRGs [87]. EPO protects axons from degeneration, prevents sensory neuron apoptosis, and promotes axonal regeneration [87,88,338].

Insulin, and closely related *insulin-like growth factors* (*IGFI and II*), support the metabolism, growth, differentiation, and survival of neurons and other cells. Insulin receptors (IRs), consisting of an α and a β subunit, are widely expressed on peripheral sensory and motor neurons. Ligand binding of the α subunit leads to tyrosine autophosphorylation of the β subunit, activation of the catalytic domain, and phosphorylation of substrates, including the insulin receptor substrate (IRS; IRS-1 and IRS-2) proteins and Shc [546,738]. Phosphorylation at multiple IRS serine/threonine and tyrosine sites generates a signaling scaffold or docking pathway [737]. As might be expected, IRS-1 is frequently colocalized with insulin and IGF-1 receptors, and therefore activates a variety of familiar messengers including: PI3K-Akt, Shc,Grb2,S6 kinase, PKCε kinase, MAP2 kinase, Raf1 kinase, and cfos [177,186,257]. PI3K-Akt signaling, activated by insulin and other growth factor receptor tyrosine kinases, blocks apoptosis through interactions with BAD, caspase-9, NFkB, and the forkhead transcription factor FKHRL1 [70,163,496] (for reviews see [189,251]). At low nanomolar physiological concentrations, insulin stimulates neurite outgrowth using insulin receptors, while at supraphysiological doses, insulin also binds to Type 1 IGF-Rs.

IGF-1 receptors (IGF-R) are present on neurons and help to retrogradely transport IGF [125]. Expression of IGF-1 and IGF-R occurs in small (<25 μm) diameter DRG neurons [125]. IGF-1Rs are also expressed on Schwann cells [109] and promote myelination [110]. Like insulin, IGFs bind to α subunits of IGF-Rs extracellularly to activate the intracellular β subunit, which induces a sustained tyrosine phosphorylation of Shc associated with Grb2 while simultaneously activating ERK [341]. IGF-1 also induces a transient tyrosine phosphorylation of

IRS-2 and an association of IRS-2 with Grb2. IRS-2, and Grb2, but not Shc are concentrated at the tip of the growth cone where the IRS-2–Grb2–PI3K complex regulates extension and membrane ruffling [341]. Like insulin, IGF-1 also protects neurons against oxidant stress and apoptosis [589]. Thus, IGF-1 also upregulates uncoupling protein 3 (UCP3), a member of the mitochondrial transporter superfamily found within the inner membrane of mitochondria [245]. UCP3, in turn, regulates the production of reactive oxygen species (ROS) in mitochondria to protect neurons from oxidant damage.

Pleiotrophin (*PTN*) is a member of the mid-kine (MK) family of heparin binding growth factors [313]. PTN is also known as heparin binding growth-associated molecule or heparin binding neurotrophic factor. Both PTN and mid-kine, the other family member, are cysteine- and basic amino acid-rich proteins with 50% amino acid homology. They operate on several receptors including low density lipoprotein receptor-related protein (LRP-1, see Chapter 5), anaplastic lymphoma kinase (ALK), protein tyrosine phosphatase (PTP), and N-syndecan. Both PTN and MK promote neurite outgrowth [318,402]. In the peripheral nervous system, PTN can be identified in nerve stumps distal to crush in mice and is localized to SCs, macrophages, and endothelial cells [49]. PTN is upregulated in acutely but not chronically denervated distal sciatic nerve stumps [467].

Hepatocyte growth factor (*HGF*), also known as "scatter factor" because it can induce epithelial proliferation, is a pleiotropic molecule that acts through the Met tyrosine kinase receptor (see review [435]). It has been most extensively examined during development. HGFs offer their support to spinal motor neurons through self-synthesis and an autocrine loop [372,435]. HGF is also expressed in adult denervated Schwann cell populations [280], where it acts as a mitogen [361]. HGF may have synergistic interactions with NGF by acting through shared downstream pathways such as PI3K, PLC-γ, and Ras/MAPK pathways, but it may not enhance the actions of other neurotrophins [435]. Macrophage stimulating protein (MSP) is a growth factor closely related to HGF [372].

Vascular endothelial growth factor (VEGF) [93,649] is widely known for its role in vascular angiogenesis, but it also has direct actions on peripheral neurons. VEGF is expressed in a subset of small ganglia neurons and its receptor fetal liver kinase receptor (flk-1) is found in sensory ganglia neurons, growth cones as well as SCs. VEGF stimulates axon outgrowth from adult explant cultures of sensory ganglia through the MAPK pathway, and also increases the survival of neurons and satellite cells [634]. VEGF also promotes SC proliferation; two studies have examined *in vivo* outgrowth of axons from nerve transection sites using local exogenous VEGF treatment. In one, SC but not axon outgrowth increased [635], whereas in the second study, both were enhanced [274]. While speculative, it is

an attractive idea that VEGF could operate to coax concurrent outgrowth of axons and blood vessels (since it signals both) during some nerve injuries.

How neurotrophins and other growth factors impact regeneration

Neurotrophins ligate receptors both in the periphery and growth cones, and at the level of the cell body [679]. Within ganglia, they are released into the extracellular milieu by neighboring neurons or satellite cells and interact with perikaryal receptors. In contrast, retrogradely transported neurotrophins from the periphery in endocytotic vesicles may offer direct signals to the nucleus of the neuron [262,727]. Some peripheral signaling, however, is also completely local, without the necessity for transport to the nucleus (Figure 9.6).

Ignoring local signaling for the moment, neurotrophins and other growth factors influence the repertoire of nuclear genes expressed in response to axotomy, also known as RAGs (regeneration associated genes; see Chapter 5). These are complex interactions, however, since some trophic factors seem to shut off RAGs (e.g., NGF applied to axotomized sensory axons) [707,708] suggesting that they compensate for factors no longer available from axoplasmic transport. In contrast, other neurotrophins applied, for example, in the CNS, contrarily

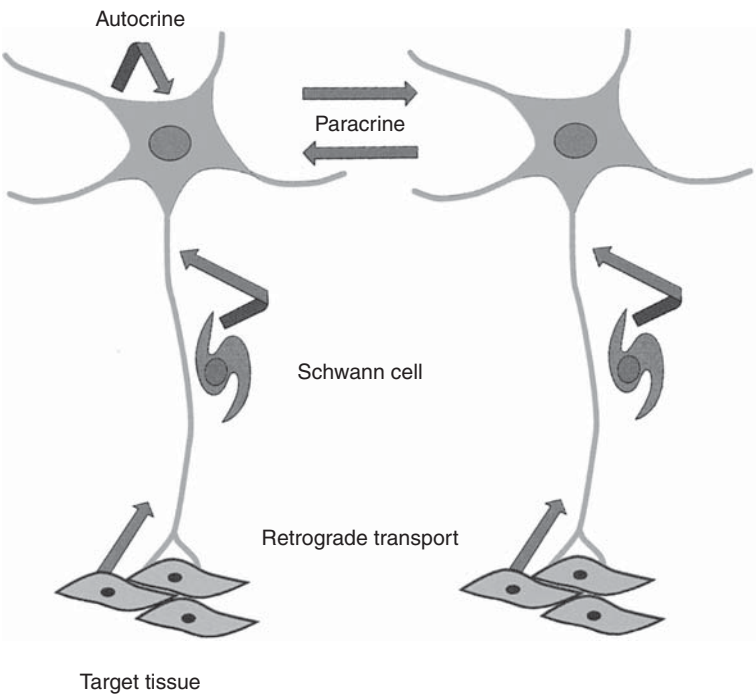

Figure 9.6 Illustration of sources of growth factor support. (Reproduced with permission from [782].) See color plate section.

increase RAG expression (e.g., BDNF applied to sectioned CNS rubrospinal neurons) [216,350]. This paradox may simply speak to the differences between central and peripheral nerve regeneration. In the PNS, RAGs show no hesitancy in being upregulated after an axonal injury. In contrast, CNS axotomized neurons appear to experience dramatic delays recognizing that their axons are damaged and require a response! The delayed RAG response by CNS neurons is one of several differences between central and peripheral regeneration (see [179]).

In peripheral sensory neurons NGF and GDNF (see below) delivered intrathecally to access perikarya, each prevented rises in the RAG known as ATF3, a stress-activated transcription factor [28]. As one might expect, the particular neurons impacted by these growth factors were selective. Thus, NGF prevented ATF3 upregulation in small- to medium-sized TrkA neurons, whereas GDNF impacted ATF3 in neurons known to be GDNF responsive and that express the receptor molecule P2X3 (also known as IB4 GDNF responsive neurons). Whether down-regulating the ATF3 RAG is beneficial for regenerating neurons is unclear. ATF3 is induced by JNK and forms heterodimers with leucine zipper transcription factors. Its actions have both protective and detrimental properties. For example, overexpressing ATF3 in adult sensory neurons increased neurite outgrowth in the presence of both NGF and GDNF [612]. The case of ATF3 is significant in that it illustrates how the RAG program can be substantially altered by growth molecules.

At the growth cone, with minutes of its application, NGF can signal changes in actin dynamics, specifically its localization to leading tips and its polymerization [226]. Through this mechanism, only small amounts of NGF are required to initiate changes in lamellipodia behavior [669]. Moreover, the rapid response to NGF can occur prior to the neurotrophin even reaching the central zone of the growth cone let alone the nucleus! Studies using Cy3-labeled NGF have illustrated that NGF signals begin on ligation of its receptor before internalization.

Other factors and second messengers influence how neurotrophins signal locally at the growth cone or at the nucleus after retrograde transport. NGF may activate Erk1, Erk2, and Erk5 in the cell body when it is applied to distal axons, whereas only Erk5 is activated if NGF is applied to the cell body [726]. Endosomal recycling of TrkA is important for both retrograde transport of endocytotic vesicles containing neurotrophins and for local NGF signaling. Several molecules may influence Trk endosomal recycling. One example is *Rab7*, a member of the RabGTPase family that controls both endosomal trafficking but also regenerative signaling of TrkA. Mutations of Rab7 are associated with inherited polyneuropathy [599]. Another molecule that appears important for retrograde degradation-resistant TrkA and TrkB transport by endosomes is

known as *Pincher,* the pinocytotic chaperone. Pincher mediates internalization of activated Trks within endosomes from plasma membrane ruffles. These are later converted to multivesicular bodies that can signal continuously during retrograde transport retrogradely to the nucleus, where they then activate Erk5 to promote neuron survival [701].

Neurons cultured in Campenot chambers (see Chapter 4) allow segregated testing of signaling in perikarya or axons that are bathed in separate media [90]. Concentrations of NGF placed into axon side chambers but not in the perikaryal media, directed axons to grow toward the NGF source [91]. NGF had synergistic actions with NT-3 but not with BDNF in promoting axon outgrowth into Campenot side chambers.

Local protein synthesis plays a critical role in influencing axon behavior during regeneration (see Chapter 5). While not fully explored, neurotrophins and other growth factors likely have a substantial influence on axon protein synthesis. For example, NGF and BDNF increase β-actin, peripherin, and vimentin mRNA transport into axons [742]. Beyond their actions on transport, however, it is likely that growth factors can also promote translation. The subsequent localization of newly synthesized proteins to particular parts of the growth cone may also be influenced by growth factors. This localization, such as the asymmetric distributions of microtubular or actin-associated proteins helps to determine the direction of growth. Thus neurotrophins also have a significant bearing on guidance, or tropism and chemotaxis by influencing growth cone dynamics [547,699]. NGF, NT-3, NT-4/5, and BDNF can generate chemoattractive growth cone turning, and in some instances, chemorepulsion.

Overlapping expression of growth factors and their receptors after injury

Neurotrophins do not influence axons in isolation. Their behavior is best understood by examining the cells and timetables that generate them. Some neurotrophins are expressed in neurons, particularly BDNF, where they act as autocrine trophic factors [579]. After peripheral nerve injury, however, Schwann cells and macrophages are thought to be the primary sources of growth factors, though other cells also contribute [266]. For example, mast cells express NGF and BDNF in injured nerves [787]. The likelihood that neighboring cells will release NGF or other neurotrophins, in turn, depends on other stimuli such as IL-1, other cytokines, TGF-β, Wnt, and some specific hormones [76,408,579].

Neurotrophin release and receptor expression after injury are also complex (see review [58]). In axotomized motor neurons, BDNF and IL-6 mRNAs rise, peak, and fall within the first 7–10 days after injury. After the peak of expression, BDNF returns to baseline at a slower rate than IL-6. There are also differences in IL-6 expression between facial and femoral motor neurons. In contrast, NT-3 and

NT 4/5 mRNAs decrease in motor neurons after injury. These variations in expression are not easily understood.

It is essential that growth factor receptors are available to accommodate higher loads of ligand after injuries. TrkB, RET, GFR-α1, and LIFR have sustained rises within motor neurons beginning with days of axotomy. Rises in both ligand and receptor are also essential to support autocrine self-signaling.

Axons must regrow into distal nerve stumps to reach target organs and effect functional reinnervation. The elaboration of growth factors by non-neuronal cells in the denervated distal stump can maintain axon ingrowth before their contact with target organs. After the first week following denervation, slow rises were detected for mRNAs of BDNF, NGF, and NT-4/5. The largest rise was in BDNF with low level sustained rises in p75, and truncated TrkC, TrkB [58,203]. SCs also expressed truncated TrkB receptors that were downregulated in the distal stump after denervation. Both NGF and BDNF were detected in SCs 1–12 days following axotomy in the distal stump following a facial nerve trunk injury [651]. FGF-2 and IGF-1 were detected in distal stumps at days 1–8 in two separate peaks at days 2 and 6. *In situ* hybridization studies confirmed that mRNAs of NGF, BDNF, and FGF-2 were present in SCs and probably also macrophages [238,651]. GDNF and GFRα-1 rose within distal stumps in the first week, then had slow declines. In separate work, mRNAs GFRα-1 continued to rise later, peaking 3 months after injury. Similar, but less robust late rises in GFRα-2 were also identified. There were no rises in neurturin, artemin, or persephin or in the receptors GFRα-3 or RET in distal nerve stumps after transection [278].

In the first week after nerve trunk injury, differing timetables in the responses of other neuropoeitic growth factors in non-neuronal cells of the distal stump were also evident. There were rapid short lived rises over the first few days of IL-6 compared to a sustained rise in LIF and rises in IL-6R. Alternatively, CNTF declined and disappeared, then increased after 10 days. A small rise in the first few days was noted for the neuropoeitic growth factor receptor subunit gp130.

Taken together, these features illustrate a complex and overlapping set of changes operating through several mechanisms. They also indicate substantial redundancy in the support of regenerating axons. There are major roles for early neuronal and perhaps SC elaboration of growth factors and their receptors, and somewhat later release by SCs, macrophages, and mast cells in the distal stump. Not all SCs behave alike in a distal denervated nerve stump. Ventral roots, for example, upregulated pleiotrophin and GDNF but not NGF, BDNF, VEGF, HGF, or IGF-1. These latter factors, in contrast, were upregulated in denervated cutaneous nerves [280]. Target organs also generate growth factors that guide axons to them. For example, denervated muscle supplies NGF, NT-3, and NT-4/5[233]. Finally, following transection nerve injuries, axons grow out from the proximal

nerve stump before they can reach distal stumps containing growth factors. While very early axon sprouts likely do not require trophic support, sustained outgrowth does. Their support may come from proximal stumps where macrophage influx, SC plasticity, and mast cell proliferation occur. Each may contribute growth factors to support outgrowing axons [106,807]. Both NGF and BDNF were detected in SCs of proximal stumps of transected nerves at 3–6 days following axotomy. bFGF and IGF-1 were detected in the proximal stumps at days 1–8 in two separate peaks at days 2 and 6. GDNF was detectable in the proximal nerve stump between days 1 and 6.

Clearance of myelin-related and other regeneration inhibitors from the distal nerve stump is thought to be rapid. Despite these efficiencies, however, it is possible that some inhibitors persist in this milieu. These may include chondroitin sulphate proteoglycans (CSPGs), semaphorins, myelin associated glycoprotein (MAG), and others. Growth factors can overcome such inhibitors. Priming sensory neurons with BDNF or GDNF *in vitro* blocked inhibition of axon regeneration by MAG. Their actions involved increases in cAMP and PKA [84]. In xenopus motor neurons, BDNF, GDNF, NT-3, NT-4, and interventions that increased cAMP in neurons [548] enhanced axon outgrowth. Paradoxically these beneficial interventions also inhibited the formation of neuromuscular junctions. Thus while growth factors support initial outgrowth of motor axons they may not necessarily facilitate proper maturation of new connections to muscles.

Some specific studies of growth factors and their impact on nerve regeneration

Investigators have not discounted the possibility that growth factors might offer exciting avenues for the treatment of nerve injuries. Growth factors, combinations of growth factors, or growth factor mimics have been delivered in a variety of ways to peripheral neurons. Some studies have emphasized exogenous delivery, while others have explored endogenous expression. This section will briefly examine some of those studies. A review of how specific regeneration conduits deliver growth factors to promote regeneration has been published [555]. Briefly, these include: (i) providing soluble growth factors in the lumen of conduits connecting transected nerves; (ii) growth factor delivery from a matrix inside the lumen of a conduit; (iii) growth factor delivery from osmotic minipumps or other approaches; (iv) delivery from microspheres; (v) delivery from the conduit wall. A fibronectin "mat" that is wrapped around the two stumps of the nerve has also been used. When impregnated with NGF, it enhanced regeneration in primates [4]. Gradients of growth factors may be important during early regeneration. These may be set up through the design of embedded conduits or by the delivery approach [331]. As already discussed, SCs or stem cells have been implanted between stumps of transected nerves. Pfister *et al.* [555] discuss the

pros and cons of various approaches. For example, conduits filled with material may exert a physical barrier to regrowth. Gap lengths of greater than 10 mm offer particular challenges to regrowth. Finally optimal conduit design should include biodegradability to limit later constriction and an ongoing foreign body inflammatory reaction [35,36,326].

Some of the most rigorous and intriguing investigations addressing endogenous NGF were carried out over 15 years ago by the Diamond laboratory [139,141]. The investigations addressed how cutaneous nerves, isolated on the dorsal trunk of the rat, undergo collateral sprouting of nociceptive axons when adjacent territories are denervated (see Chapter 4). The approach focused on how axons that might be NGF responsive (cutaneous nociceptive axons) might collaterally sprout and enlarge their territories. Subcutaneous injections of antiserum to NGF prevented the expansion of their cutaneous fields. By contrast, Aα axons subserving light touch did not sprout, consistent with their insensitivity to NGF. When collateral sprouting was already in progress, later application of anti-NGF halted further growth, whereas stopping anti-NGF injections allowed sprouting to resume. Normally, there was an estimated latency of the growth of these receptive fields of 8–10 days, thought to be due to a delayed rise in NGF content in the denervated skin target tissue. In additional work, intradermal NGF injections evoked collateral sprouting in normal fully innervated skin, increased the rate of sprouting into denervated skin, and restored sprouting arrested by anti-NGF. A final critical finding was that collaterally sprouting axons in the skin followed residual perineurial tubes left over from the denervated nerve trunks.

Diamond and colleagues also noted that regenerative axon sprouting, in contrast, completely ignored anti-NGF injections. The experiments used the same isolated dorsal cutaneous nerves, but the remaining T13 branch was crushed and subsequent reinnervation was assessed. Recovery (the rate and area of reinnervation) was independent of anti-NGF. Other types of injuries, such as transection were similarly insensitive to NGF. In some rats given anti-NGF after two separate injuries, collateral sprouting could be halted in one territory, whereas regenerative sprouting was unchanged in another. Neither Aα, Aδ, nor C fibers had regeneration blocked by anti-NGF nor accelerated by exogenous NGF. In combination, this series of experiments indicated that endogenous NGF, synthesized by the skin, supported collateral reinnervation of nociceptive axons but had no bearing on regenerative sprouting.

The impact of exogenous NGF on regeneration does remain controversial. Several investigations have noted somewhat surprising actions of NGF alone, or together with other growth factors, on the regeneration of broad categories of peripheral nerve axons. For example, NGF cDNA delivered intrathecally with a polyethylenimine complex, improved regeneration (density and caliber of

myelinated axons) of sciatic nerves across conduits bridging transections [722]. NGF immobilized by heparin in a fibrin matrix enhanced the regrowth of axons through a conduit connecting a 13mm sciatic nerve transection gap [382]. NGF and GDNF enhanced the proportion of process bearing adult sensory neurons and total neurite length *in vitro* [212]. BDNF and NT-3 had inhibitory effects. It seems difficult to understand how NGF might support neurons that do not express TrkA. As discussed above, NGF-p75 signaling may substitute, or it may be that in some experiments the NGF preparation contained the proneurotrophin precursor. Another possibility is that NGF directly signals SCs to migrate out from injury sites first, which in turn seduce axons to follow them [19].

The idea that BDNF influences regeneration is less controversial. BDNF rescues adult motor neurons following injuries [347,509,510]. Similarly BDNF is capable of reversing axotomy-related declines in the motor neuron molecules as ChAT and AchE (see Chapter 5) [347]. The actions of BDNF during nerve regeneration, however, are variable. Exogenous BDNF did not improve some indices of functional recovery after transection and repair [476,621]. BDNF did promote axon regeneration into a chronically denervated distal stump but higher doses had an opposite action and reduced reinnervation [56]. Higher doses of BDNF might antagonize BDNF–TrkB signaling through stimulation of p75. Antibodies to BDNF reduce the number and caliber of regenerating myelinated axons and the distance of their regrowth (pinch test) in crushed sciatic nerves [772]. Similarly, mice heterozygous for TrkB (+/−) (homozygous deletions in mice are neonatal lethal) have normal numbers of intact but attenuated numbers of regenerating motor neurons [55].

Endogenous BDNF may be released by electrical stimulation of nerve trunks (see Chapter 10), an intervention discovered to enhance axon regeneration and preferential motor reinnervation [7]. Thus, TrkB +/− heterozygous knockout mice did not benefit from stimulation. The impact of electrical stimulation through BDNF/TrkB signaling also includes a change in SC phenotype. Stimulation upregulates the HNK-1 carbohydrate epitope of premyelinating SCs that associate with motor axons and may support preferential motor reinnervation [162].

CNTF and BDNF supplied together and linked to collagen tubes had only minor benefits on axon maturation or functional recovery after transection and repair [271]. A chimeric pan-neurotrophin made by combining the active domains of NGF, BDNF, and NT-3 and linked to the BDNF promoter increased axonal sprouting and regeneration of myelinated axons and promoted motor axon regeneration and sensory reinnervation of the skin [294]. Focal application of NGF, BDNF, and IGF-1 reduced inappropriate collateral sprouting of transected motor neurons into incorrect branches [651].

NT-3 applied by fibronectin mat at a site of nerve repair increased axon outgrowth across a 10 mm gap and reduced denervation muscle fiber atrophy [628,640,641]. Several studies have identified an impact of NT-4/5 on regeneration of rat sciatic nerves [170,627,765]. NT-4/5, applied by fibrin glue to a repaired nerve transection site improved walking tracks, myelinated axon diameter, myelin thickness, and numbers of axons up to 60 days 10 mm beyond the injury site [765]. The outgrowth of YFP fluorescently labeled peroneal axons entering grafts from NT4/5 −/− or NT4/5 −/+ mice was of interest [170]. Both transgenic grafts were less supportive of outgrowth than wild-type grafts. BDNF +/− grafts, however, had no impact on regrowth. In grafts treated with fibrin glue containing BDNF or NT4/5, the regenerative deficit in the NT4/5 −/− grafts was reversed and regeneration exceeded that of wild-type grafts. Finally, in p75 (−/−) homozygous mice, with a reduced number of intact sensory neurons during development, there were greater numbers of regenerating motor axons [55] but no overall impact on other facets of peripheral nerve trunk regeneration (Karchewski, Verge, Zochodne, unpublished data).

GDNF promoted reinnervation of chronically denervated nerve trunks but had little impact on early regrowth after transection [57]. Mice lacking IL-6 had delayed regeneration of sensory axons following crush [298,778]. Similar but more persistent delays were identified in mice lacking CNTF [760]. Exogenous CNTF potentiated peripheral nerve regeneration by increasing the distance of axon outgrowth and maturation of myelinated axons during regeneration [591].

The roles of FGFs during peripheral nerve regeneration are diverse (see review [237]) and their impact depends on the receptor expressed. As suggested above, FGF-2 and FGFR1–3 were increased proximal and distal to crush sites within peripheral nerves and FGF-2 and FGFR3 were increased in DRGs following axotomy. Thus, the upregulation of FGF-2 growth factor after injury is well positioned to support regeneration. It promotes neuronal survival and neurite outgrowth when given exogenously or *in vitro*. Alternatively, FGF-2 binding to FGFR3 is also associated with neuron apoptosis and FGF-2 and FGFR3 knockout mice are protected from axotomy-induced apoptosis. FGF-2 acting on FGFR1/2 may inhibit myelination and SC proliferation. The FGF field therefore promises to identify potent and direct but overlapping actions, sometimes conflicting, on neurons and their supporting cells.

Insulin and IGF-1 stimulate survival, neurite outgrowth, and other responses in adult sensory neurons [577]. Unlike other growth factors, insulin and IGFs circulate in the bloodstream and may exert direct actions without the prerequisites of target expression or transport. Insulins have potent actions *in vivo* [75,323,653,754]. Insulin accelerates nerve regeneration, especially when applied intrathecally to access perikarya [689,754] and reverses retrograde atrophy and

axon loss after nerve injury. Insulin directed to sensory neurons intrathecally also promotes reinnervation of the skin in experimental diabetes [690]. IGF-II expressed in muscle stimulates sprouting of motor axons during partial denervation or botulinum paralysis [219,220].

PTN increased motor axon outgrowth from spinal cord explants and in doing so formed "miniventral rootlets." It also protected neonatal motor neurons from axotomy-induced dropout [467]. PTN similarly increased axon regeneration across a transection injury and accelerated the reconnection of motor axons to endplates in rats when delivered locally by HEK cells. All of these properties are believed to be mediated by ligation of the ALK receptor.

The control of remyelination by regenerating axons is also directly influenced by specific neurotrophins. While not extensively studied during adult nerve regeneration *in vivo*, such actions have been documented during development or *in vitro*. Transgenic mice with BDNF overexpression have persistent (into adulthood) increases in myelin content and thickness [683]. NGF promotes myelination by SCs [96]. TGFβ1 may regulate the stability of mature myelin [130]. Mice with a null mutation of the TGFβ1 gene have impaired myelin development rendering structurally abnormal myelin sheaths [130]. A combination of PDGF and IGF-1 enhances myelination but does not improve regeneration across an injured spinal cord [539].

A number of other endogenous or exogenous trophic-like agents have mimicked actions of growth factors. For example, "neotrofin" is a synthesized purine derivative that mimics some of the actions of NGF. Neotrofin induced cutaneous collateral nerve sprouting [283]. Other examples include nucleosides, nucleotides, arachidonic acid, and others [328,502]. Through a screening process, small nonpeptide molecules that are p75 ligands have been discovered that support neuron survival without an interaction with Trks [444].

Summary

A number of protein families influence the regeneration of peripheral neurons. The most widely studied are the neurotrophins that operate through specific tyrosine kinase receptors. Others include the neuropoeitic cytokines, GDNFs, insulin-like molecules, and a series of growth factors better known for their actions on other cell types. Many growth factors that impact peripheral neurons activate overlapping signaling cascades that eventually involve PI3K-Akt, Ras/ERK, and PLC-γ1. Through knockout studies illustrating endogenous actions or investigations using exogenous application, many have potent actions during peripheral nerve regeneration. Their supportive actions may not be confined to neurons but may also operate through the support of SCs and other supporting cells.

Suggested reading

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10

The nerve microenvironment

Nerve regrowth has a context, a microenvironment replete with molecular, physical, and other determinants of success. Some are roadblocks, others are partners. The growth factors considered in Chapter 9 are only part of the story of how nerves fare during regeneration. In this chapter, we consider a number of other salient features of the regenerative milieu that influence regeneration. These range from adhesion and basement membrane molecules, to novel signaling systems to interactions within altered environments as occur during aging, diabetes mellitus, or entry into the spinal cord.

Adhesion molecules

Cell adhesion molecules (CAMs) represent the interface between axons, SCs, and the basement membrane. They provide the essential extracellular "clutch" domains that allow differential movement of membranes and molecules along one another. During nerve regeneration, adhesion molecules permit axon growth with basement membranes, along guiding SC processes, or with other fasciculating axons guided by pioneer axons. In turn, the linkages of CAMs to the intracellular cytoskeleton influence cell mobility. To accomplish their task, CAMs can interact with each other (homophilic) or other molecules (heterophilic). These interactions vary in adhesive strength depending on the location and intensity of their expression, distribution on a given cell, and electrical charge.

Three major families of CAMs are recognized. The *immunoglobulin (IgCAMs) family* contains extracellular β sheets that resemble the variable or constant domains of immunoglobulins (Igs). This family is further subdivided into three types, depending on the type of Ig domains and the presence of fibronectin-like

or other extracellular domains. Some have intracellular signaling kinase moieties or glycosylphosphatidylinositol (GPI) anchors. They can bind in a homophilic or heterophilic fashion. Overall examples include the myelin proteins P0 and myelin associated glycoprotein (MAG), Thy-1, neural cell adhesion molecule (NCAM), and L1. *Integrins*, considered below, are divalent cation-dependent (Ca^{2+} or Mg^{2+}) adhesion molecules that bind in heterophilic fashion. *Cadherins* are calcium-dependent adhesion molecules that usually bind in a homophilic fashion, and have an amino terminal extracellular domain, a single transmembrane domain, and a carboxyl-terminal intracellular domain. Several different types exist, named for the first tissue in which they were identified (though now known to be more widely expressed): N-cadherin or neural cadherin, E-cadherin or epithelial cadherin, and P-cadherin or placental cadherin. Cadherins generate adhesion through extracellular lattice "plates" that form an adhesive dimer across facing cell membranes (see review [121]).

Overall, there are several adhesion molecules expressed by neurons, SCs, and the adjacent basement membrane (BM) that influence axon outgrowth [440]. NCAM is upregulated by SCs distal to an injury site [439]. NCAM is also, however, expressed on axons. Axonal plasticity, in turn, can be enhanced by the addition of a polysialic acid (PSA) post-translational modification. For example, regenerating motor axons destined to motor pathways express high levels of NCAM–PSA [190]. NCAM may facilitate neurite outgrowth through an interaction with the FGF receptor [439,718].

The L1 adhesion molecule is expressed by neurons and SCs and is upregulated by NGF [144,316,613]. Like NCAM, L1 is also upregulated in SCs of the distal stump after nerve injury. HNK-1 (L2) is expressed in SCs and BM and selectively facilitates motor axon outgrowth [441]. To accomplish this task, HNK-1 is only present in SCs associated with motor fascicles. In contrast, sensory fascicles express NCAM in SCs associated with unmyelinated sensory axons [592].

Overall, it is apparent that several redundant types of adhesion molecules foster interactions between axons and SCs. Adhesion molecules are likewise important for later maturation events of peripheral nerves including fasciculation and myelination. For example, P0 and MAG have particular roles in myelination.

Basement membranes

Extracellular basement membranes (BMs) provide the pavement or handholds for axons to move forward. BMs, in turn, require appropriately expressed axon molecules, such as integrins to accurately support and guide axons and growth cones. Hence a two-way cooperation between "fixed" ligands

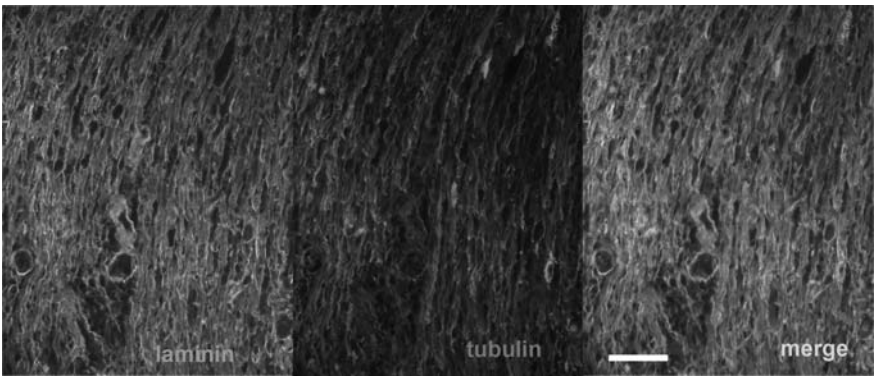

Figure 10.1 Immunohistochemical image of the relationship between laminin (labeled with an anti-laminin antibody, green) and axons (labeled with an anti-neurofilament antibody, red) in a peripheral nerve stump. Laminin is associated with basement membranes of SCs surrounding axons and with blood vessels. [Bar = 50 microns] (Image taken by Chu Cheng, Zochodne laboratory.) See color plate section.

and their receptors is a fundamental requisite. The menu of BM constituents that new SCs and axons are exposed to varies with the type of injury, its duration, and the extent of regrowth. SCs elaborate most BM molecules, but their own migration and guidance may depend on pre-existing molecules. Below we discuss the major extracellular BM constituents identified within peripheral nerves.

Laminins are heterotrimeric glycoproteins with one α, one β, and one γ chain. Up to 14 isoforms from five α, three β, and three γ subunit types have been described. They are cruciform, or cross-like in structure. Endoneurial basal laminae of peripheral nerves contain laminin-2 (merosin; α2β1γ1) and laminin-8 (α4β1γ1) (Figure 10.1). Both appear critical to aspects of nerve regeneration, but they interact with different receptors. Laminin is a preferred axon outgrowth substrate. This was demonstrated by Gundersen during development [243]. In an elegant series of experiments, neonatal chick sensory neurons were given the opportunity to sample and grow along several choices of BM laid down in strips. Axon outgrowth clearly favored laminin strips rather than fibronectin or collagen IV.

Since laminin is essential for peripheral nerve development, severe abnormalities in nerve structure occur when there is abnormal expression of laminin. For example, mice lacking α4 of laminin-8 have abnormal myelination with higher G ratios indicating myelin thinning. They also have abnormal radial sorting of axons within SC basement membranes [716]. Despite their abnormal development, however, mice lacking α4 of laminin-8 had normal axon regeneration after crush.

Although SCs have longer processes when grown on laminin-8, embryonic motor neurons extend on laminin-2, but only poorly on laminin-8. In the case of laminin-2, mice with a disrupted gene encoding the laminin-2 subunit γ1 had severe peripheral nerve motor deficits and tremor [104]. Disrupting this single subunit prevented normal elaboration of laminin-2 and a continuous basement membrane around SCs did not form. SCs in mice without γ1 failed to differentiate and synthesize myelin proteins and did not subsequently sort and myelinate axons. Large bundles of naked, unsorted axons were identified and regeneration was impaired.

Integrins are receptors for extracellular BM molecules, particularly fibronectin and laminin. On SCs, the major laminin receptors are α-dystroglycan and α6β4 integrin. The nomenclature is confusing since both the laminin ligands and the integrin receptors have subunits designated by Greek symbols. Other *integrins* expressed by SCs include α1β1, α2β1, α5β1, α6β1, αvβ3, αvβ1, and β8 [475]. Neurons express α3β1, α4β1, and α5β1 in proprioceptive afferents and α7β1 in cutaneous afferents [240]. The integrin α1β1 is upregulated in SCs distal to an axonal crush or transection, including those that did not previously express it [644]. CD9 is an SC membrane protein associated with integrins and involved in BM adhesion, proliferation, and migration [18,475].

Integrins are involved in more than adhesive binding alone. They interact with a large cascade of molecules to effect intracellular signaling (see review [61]) including RHO GTPases, GSKβ, PI3K, and a series of "platform" intracellular molecules that bind the actin cytoskeleton (ILK, α actinin, talin, filamin, vinculin, Arp 2/3, α parvin, and β parvin). When a ligand binds to an integrin, the receptors become clustered and they recruit actin and its associated proteins to its cytoplasmic side [61,292].

There are several examples of how interactions between integrin receptors on neurons, and their extracellular matrix is critical to regeneration. For example, α7 integrin expressed by injured medium–large diameter sensory neurons is required to facilitate the actions of NGF and NT-3 in supporting axon outgrowth [210]. α7 and β1 subunits of integrin were not simply expressed in neurons, but preferentially localized to axons and growth cones of regenerating facial nerves where they were capable of influencing local behavior. Transgenic mice lacking α7 had a reduction in axon elongation [736]. Uninjured DRG sensory neurons have a low level expression of integrin subunit α6 but more widespread expression of subunits α7 and β1. mRNAs of all three of these integrin subunits, however, were upregulated after a peripheral axotomy and all three were expressed in outgrowing axons [717]. This action was specific for peripheral processes of sensory neurons, since a dorsal root axotomy did not alter their expression. Sensory neurons thus have the capability of distributing

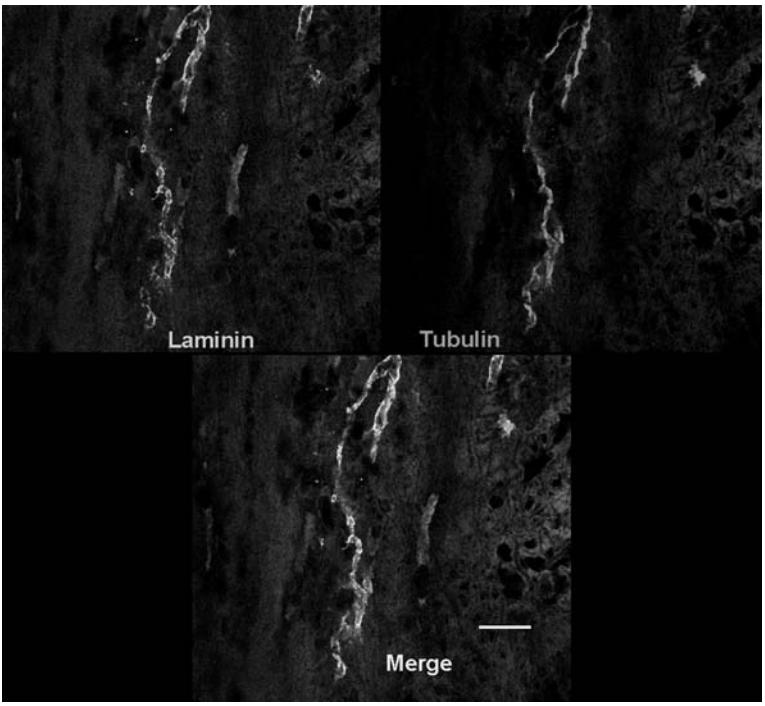

Figure 10.2 Immunohistochemical image of outgrowing axons (labeled with an anti-βIII tubulin antibody, red) at the regenerative front of a transected rat sciatic nerve (connected by a conduit) closely associated with deposits of laminin (labeled with an anti-laminin antibody, green), likely laid down by leading SCs. (Bar = 50 microns) (Image taken by Chu Cheng, Zochodne laboratory and reproduced with permission from [450].) See color plate section.

their integrin receptors to different parts of their structure and it is possible that selective distribution alters how processes respond to injury. In addition, blocking the β1 integrin receptor in the above work interrupted SC adhesion. These findings support the probability that the combination of BM and integrin are decisive in guiding the specificity of axon regeneration. During development, proprioceptive neurons *in vitro* that expressed α3β1, α4β1, and α5β1 extended more robustly on laminin and fibronectin, whereas cutaneous neurons expressed higher levels of α7β1 and preferred outgrowth along laminin [240]. While not yet demonstrated, it may be that regeneration can recapitulate these specific interactions that occur during development.

Laminin or other BM–integrin interactions thus influence neurite outgrowth in several ways (Figure 10.2). Point contacts, analogous to focal adhesions of fibroblasts, are critical to growth cone advance in neurons. Their adhesion to the basement membrane allows the force generation for growth cone movement.

Downstream of integrin activation by laminin or fibronectin, there are roles for RHO family GTPases, discussed in Chapter 5. RAC1, a member of this family, promotes initial point contact after integrin activation. Subsequent stabilization of these contacts requires RHOA/ROK participation. In keeping with RHOA/ROK's inhibitory actions, stabilization implies temporary growth arrest. Semaphorins that collapse growth cones appear capable of disrupting point contact adhesion by inhibiting RAC1 [747].

Laminin is therefore a common facilitator for nerve regeneration, its support being synergistic with the specific growth factors required for outgrowth. Thus, extracellular BM molecules can substantially influence how neurons respond to growth factors. For example, embryonic Bax-/- mice grown on laminin accelerated their growth in response to exogenous NGF. On another substrate L1, there was recruitment of endogenous RHOA within growth cones that prevented enhanced outgrowth by NGF. Inhibition of RHOA restored the responsiveness and permitted NGF to accelerate outgrowth on L1 [411]. Large sensory neurons and CGRP expressing sensory neurons underwent considerable neurite outgrowth on laminin alone and upregulated their expression of the integrin receptor subunit β1 [698]. Both types of neurons had yet further outgrowth when NGF was also applied. Outgrowth also depended on activation of the PI3K-Akt and MEK/MAPK, essential growth factor signaling pathways discussed in previous chapters. In contrast, IB4 NGF-insensitive TrkA negative neurons were more fastidious in their response to growth conditions. These neurons only extended neurites when laminin was combined with added GDNF.

Adding laminin and collagen Type 1 to collagen gel preparations stimulates axon outgrowth from explants [685]. The delivery of exogenous laminin to regeneration conduits spanning rat sciatic nerve transections enhanced regrowth *in vivo* [103]. Laminin was incorporated into the regenerative bridge material. This valuable support, however, does not appear to be offered by vascular laminin despite its location near regrowing axons [450].

F-Spondin is an extracellular basement membrane adhesive protein, synthesized by SCs, that is massively upregulated in nerve trunks distal to injury sites [82]. It is composed of reelin, spondin, and thrombospondin-type 1 repeats (TSR) domains, each of which may have varying influence on axon regrowth. Interestingly, F-Spondin is less upregulated in sensory than in motor branches.

Other constituents of basmement membranes associated with SCs include fibronectin, tenascins, Type IV and VI collagen, heparan sulphate, chondroitin sulphate proteoglycans (see below), and entactin/nidogen [78]. Fibronectin promotes neurite growth but may have less impact on direction finding and outgrowth than laminin [243]. Tenascins (TNs) are extracellular basement membrane glycoprotein "recognition molecules" [244]. Several family members

are described including tenascin-C (TNC), restrictin/J1-160/180 (tenascin-R,TNR), tenascin-X (TNX), tenascin-Y (TNY), and tenascin-W (TNW). TNC was originally linked to inhibition of fibronectin adhesion and decreased neurite outgrowth [114,445]. Subsequent studies have suggested TNC promotes *in vivo* axon outgrowth, since TNC-deficient mice have delayed reinnervation of their vibrissae. In contrast, TNR null mice experience better recovery [244]. TNC is normally found around nodes of Ranvier, is associated with SCs, and is found in the perineurium [439]. Overall, TN (probably mainly TNC) is upregulated in SC basement membranes after injury and found in proximal nerve stumps, regenerating bridges, and distal stumps after a nerve transection injury. SCs elaborate TN, and TNC helps support regeneration by fostering axonal regrowth [439]. In contrast, TNR may decline in injured peripheral nerves [244]. TNR, however, is also expressed in perineuronal nets around motor neurons and it protects motor neurons from microglial-induced cell death [17,244]. TNY is found in rootlets and TNX is particularly widespread on perineurium and endoneurium [445].

Chrondroitin sulphate proteoglycans (*CSPGs*) inhibit axon growth in peripheral neurons. They are concentrated in a narrow band surrounding axon–SC units and within nodes of Ranvier in normal nerves. Importantly, however, CSPGs are substantially upregulated (sevenfold in one investigation) in the distal stumps of peripheral nerves after injury and create an inhibitory barrier for regrowth [812]. CSPG expression in SC BMs becomes thicker and more diffuse throughout the endoneurium. While CSPGs are capable of activating RHO–ROK in adult sensory neurons, they also interfere with integrin signaling as occurs during growth on laminin [780]. Thus CSPGs can disrupt the facilitation of axon regrowth normally associated with laminin substrates [811]. Their inhibition, in turn, can be overcome in preconditioned neurons with NGF that acts through PI3K to inhibit GSK3β. Other myelin inhibitors, such as Nogo, can be overcome by integrin signaling from laminin or RHO–ROK inhibition. Overall then, it is apparent that integrins, RHO–ROK, growth factors, and CSPGs are linked in complex hierarchies that influence outgrowth. To prevent inhibition of axon outgrowth by CSPGs, however, there is also another innovative approach that involves matrix metalloproteinases, discussed next.

Matrix metalloproteinases (*MMPs*) can degrade inhibitory CSPGs and facilitate regeneration. For example, MMPs promote adult sensory neurite outgrowth along the basal laminae of peripheral nerves or the regrowth of peripheral nerves into acellular nerve grafts [180,362]. These roles are exemplified by MMP-2 and MMP-9, members of the MMP family. MMP-2 is expressed in sensory neurons and is transported to growth cones [811]. Like the integrins discussed above, MMPs can be directed intracellularly within neurons to facilitate where needed. Muir and colleagues labeled this unique feature of growth cones as

"focalized" proteolysis. In a similar approach, transplanted stem cells expressing MMP-2 increased axon regrowth into chronically denervated nerves by degrading CSPGs [258]. After crush injury, MMP-2 and MMP-9 levels also rise in distal nerve stumps despite the absence of axons, a property providing a more permissive microenvironment within partly degenerated distal nerve stumps [180]. The findings also indicate that MMPs are not solely expressed in axons, but are also upregulated in distal nerve stumps by other cell types.

A serpin protease known as nexin-1 may also facilitate regeneration. In the report by Lino *et al*. [409], SC expression of nexin-1 had diverse effects on peripheral nerve including control of fibrin deposition, tissue plasminogen activator (tPA) activity, production of mature BDNF, and breakdown of p75. Nexin-1 is thought to inhibit serine proteases including thrombin, trypsin, plasmin, tPA, and uPA. While it is thought to promote SC proliferation and survival, its overall impact may be complex. For example, tPA, one of the substrates it inhibits, is also required to promote nerve regeneration [624,625].

Further molecules and pathways that influence regeneration

The number of additional pathways and molecules that impact nerve regeneration is extensive. The evidence for the importance of any particular molecule varies. Several hormones including estrogen, testosterone, thyroxine, growth hormone, and ACTH are reported to enhance nerve regeneration [116,610,650,712]. Forskolin is thought to increase regeneration by activating adenyl cyclase and increasing cAMP levels [339,340,348].

Cell surface gangliosides are sialic acid-containing glycosphingolipids expressed on distal portions of the axon and other sites within peripheral nerves. They include GM1–GM3, GD1–GD3, and GTs based on the number of sialic acid residues they possess (GM = one; GD = two; GT = 3). There is evidence that GD1a and GT1b facilitate regeneration. When a specific antibody to GD1a ganglioside was passively administered to mice, there was a severe inhibition of regeneration [385]. Abortive "dystrophic" axon swellings (similar to axon endbulbs described in Chapter 3) developed. These findings are relevant to human neuropathies. Antibodies to gangliosides have been detected in patients with immune mediated neuropathies such as Guillain–Barré syndrome. Some antibodies are primarily directed toward axons and others toward myelin or SCs.

Immunophilins (*IPs*) are a family of receptor proteins interacting with ligands that are specific immunosuppressive compounds. IPs were discovered to increase axon regeneration [223–225,500,637,700,721]. The first IP ligands studied in the nervous system were cyclosporin A acting on cyclophilin receptors and FK506 (tacrolimus) acting on FK506-binding proteins (FKBPs). Both cyclosporin A and

tacrolimus are potent immunosuppressive agents used in oncology and organ transplantation. Rapamycin is an additional ligand that binds to a FKBP. IPs ligate FKBP-12, an interaction that mediates their immunosuppressive actions. They also ligate FKBP-52, a receptor thought directly responsible for accelerating nerve regeneration. Non-FKBP-12 binding compounds, including several novel ligands discovered by Gold and colleagues influence nerve regeneration but do not exert immunsuppressive actions [222,560]. Ligand–FKBP-12 interactions suppress transplant rejection largely because of their ability to inhibit calcineurin phophatase in T-lymphocytes. In neurons, their action may not involve calcineurin but instead involves the regulation of calcium flux through ryanodine and inositol 1,4,5-triphosphate (IP3) receptors. Additional downstream pathways include GAP43/B50, Ras/Raf/MAPK, PLC, and PI3K [560,637]. IP ligands increase the number and maturity of regenerating axons, increase collateral sprouting, collaborate with NGF, and may accelerate Wallerian degeneration. They also offer neuroprotection. FK-506 doubled the number of axons and increased the myelin thickness of peripheral axons extending past an injury zone [659]. Some of the machinery for IP signaling, specifically FKBP-12, is upregulated in regenerating neurons [590]. Since both animal models and human patients have benefited from the actions of IPs, they may be valuable agents in situations requiring combined immunosuppression and nerve grafting (e.g., foreign nerve grafts to bridge nerve transections) [637]. Novel IP ligands without immunosuppressive actions may have direct therapeutic roles in nerve injury.

Phospholipase A_2 (*PLA_2*) is a member of a family of enzymes that hydrolyze phospholipids to form lysophospholipids and arachidonic acid [685]. PLA_2 and arachidonic acid may open calcium channels in conjunction with FGFs and CAMs to stimulate neurite outgrowth [718]. In a frog sciatic nerve explant assay, PLA_2 inhibitors attenuated nerve regeneration, whereas stimulating this pathway increased regeneration [685].

Semaphorins and their receptors, the neuropilins (NPs), belong to a family of molecules best known for providing inhibitory guidance cues during development. Levels of Sema3A and Sema3F, along with their receptors NP-1 and NP-2, increase in peripheral nerves distal to crush or transection injuries [602]. NP levels decline between days 8 and 12 after crush but increase on days 24 and 58, coinciding with increased axon ingrowth into the distal stump. SCs express NP-2 mRNA, whereas Sema3F is found in epineurial fibroblasts and in the perineurium. The function and specific cellular localization of all of the semphorin subtypes and their receptors during peripheral nerve regeneration are not yet known. It is likely, however, that their expression may be important in fasciculation and direction finding through Bands of Bungner. Inhibitory cues guide axons in specific trajectories during development.

Another guidance molecule recently identified within peripheral neurons is "repulsive guidance molecule" (RGM), a glycosylphosphatidylinositol-anchored protein involved in the collapse of inappropriately projecting embryonic retinotectal axons [446]. RGM increases in ventral horn neurons after distal axotomy, but may have a potential role as an inhibitor of nerve regeneration, an avenue for exploration [150].

Peptides. Transected peripheral nerve axons accumulate neuropeptides in their endbulbs that may influence regenerative events (see Chapter 3) [784,788]. For example, vasoactive intestinal polypeptide (VIP), expressed prominently in axonal endbulbs, applied between the proximal and distal stump of a mouse sciatic nerve transection gap increased the number and maturity of bridging axons [773]. CGRP is upregulated in newly regenerating outgrowing peripheral nerve axons. Moreover, rises in local axonal CGRP expression contrast with its simultaneous downregulation in perikarya [401]. As endbulbs break down, CGRP egresses from endbulbs into the extracellular space where it may have several actions, including vasodilation of local microvessels as discussed in previous chapters. Little evidence to date, however, has emerged that early CGRP accumulation, persistent expression, or extracellular delivery might have a meaningful impact on subsequent axonal regeneration. Toth and colleagues [691], however, noted that siRNAs to both CGRP and a component of its receptor complex RAMP-1 administered in a regeneration conduit spanning rat sciatic nerve transections completely interrupted nerve regeneration. Moreover, CGRP mRNA was identified within regenerating axons indicating local synthesis of the peptide. These findings have suggested that CGRP elaborated by regenerating axons may offer essential signals to their SC regenerating partners to facilitate regrowth.

Age

Age alters the capacity of the peripheral nervous system to regenerate. Both regenerative and collateral axon sprouting are attenuated by advanced age. The topic has been reviewed in depth (see [337,706]). Almost all facets of peripheral nerve biology change with age. Intact peripheral nerves undergo reductions in conduction velocity and myelin thickness. There are declines in the number of unmyelinated and myelinated axons. The number of sensory receptors as well as sweat gland innervation diminish. Muscle power and autonomic responses diminish. There is axonal atrophy, lower levels of myelin proteins, segmental demyelination, slowing of axoplasmic transport, and decreases in nerve blood flow. There are rises in the thresholds to sensation.

Increased age slows the progress of Wallerian degeneration through impaired macrophage recruitment and decreased SC proliferation. The rate of subsequent

axon regeneration including the numbers of regenerating axons is also attenuated by aging. Decreased levels of trophic factors made by SCs and decreased expression of trophic factor receptors contribute to the deficits. Remodeling of the neuromuscular junction after muscle fiber reinnervation proceeds more slowly, and reinnervating axons have a delay in their myelination. Retrograde neuronal changes are also more pronounced with aging. Finally, cutaneous collateral reinnervation and collateral reinnervation of sweat glands are impaired [138,499].

Diabetes mellitus and regeneration

Diabetes mellitus, the most common cause of acquired disease of peripheral nerves, poses major barriers to successful regeneration. There is a "double hit" in diabetes because neurons and axons spontaneously degenerate during the disease, yet attempts at regeneration are severely compromised. Recent reviews on the topic are available [336, 762]. Several steps during the regenerative process are abnormal in diabetes: axon sprouting [46], elongation [166], interaction with basement membrane adhesion molecules, and either upregulation of RAGs (regeneration associated genes) or downregulation of other genes [46,160,166,300,317,353,413,414,434,448,617,671,672,692,751,752,756,763]. Macrophage entry is slowed in crushed and transected diabetic sciatic nerves but once influx occurs, their clearance from the nerve is delayed [317,332,671]. Slowed Wallerian-like degeneration with a failure to provide the appropriate microenvironment for regrowth is associated with impaired regeneration.

Microangiopathy, disease of vasa nervorum from diabetes may contribute toward an unfavorable microenvironment for regrowth. Rises in blood flow and growth of new blood vessels after a nerve trunk injury are attenuated in diabetes [333]. Diabetes is associated with a series of metabolic abnormalities, each of which may also impact regeneration: oxidative and nitrergic stress, excessive polyol (sugar alcohol) flux through the conversion of glucose into sorbitol, changes in PKC subunits, and defective availability or uptake of growth factors including IGF-1 and insulin. Retrograde loss of parent perikarya may be excessive, reducing the pool of available parent axons for regeneration. Schwann cells that normally provide support for axons may be limited in their ability to proliferate and to synthesize growth factors for axons. Finally, specific alterations of the adhesive properties of basement membrane proteins may influence regrowth in diabetes.

Overall, diabetes mellitus targets almost every step of the regenerative process. Thus, while interventions may be discovered to arrest degenerative diabetic neuropathy, return to function through regenerative activity may be limited.

Unfortunately, diabetic subjects are also prone to focal neuropathies such as carpal tunnel syndrome.

Growth of axons from peripheral neurons in the CNS

Peripheral neurons must navigate portions of the CNS. This occurs when motor axons travel from the ventral horn of the spinal cord into the ventral root, or when the "central" branches of primary sensory neurons enter the dorsal root entry zone (DREZ) of the spinal cord. Large myelinated sensory afferents enter into the posterior columns of gracilis and cuneatus to project to more rostral nuclei. Other sensory afferents enter dorsal horn gray matter to synapse on laminae containing interneurons, projection neurons, or motor neurons. For peripheral neurons, navigation within the CNS poses daunting challenges. The CNS microenvironment is more hostile to their regrowth than the periphery. This important topic is reviewed elsewhere [297].

Steinmetz and colleagues [639] facilitated the entry of several afferent branches into the spinal cord by combining preconditioning with local injections near the DREZ of chondroitinase ABC to digest inhibitory proteoglycans. Simultaneous zymosan was injected into the DRG to generate a "proregenerative" inflammatory state. Neumann *et al*. [504] described enhanced regeneration of sensory neuron central branches into spinal cord white matter when two peripheral branch conditioning (priming) lesions were carried out. The first priming lesion was produced by transection of the sciatic nerve at the same time as an injury to the dorsal column of the spinal cord. The second sciatic priming lesion was given 1 week later. The authors hypothesized that the first lesion enhanced the intrinsic growth capacity of the regenerating central branch sensory axons, and that the second lesion helped to sustain regrowth. Hoang *et al*. [273] demonstrated functional reinnervation of the rat lower urinary tract by spinal cord motor neurons after reimplantation of avulsed L6 and S1 ventral roots into the conus medullaris. Wong *et al*. [746] facilitated the regrowth of central branch sensory axons past a root crush zone and into the dorsal root entry zone by using a viral transfection approach. Local injections of an equine infectious anemia virus (EIAV)-based lentiviral vector expressing the retinoic acid receptor, RARβ2, were made. The injections were associated with functional improvement and with rises in sensory neuron levels of cAMP. Qui *et al*. [564] demonstrated that rises in cAMP, initially mimicking a conditioning lesion operate through PKA to overcome inhibition by MAG/myelin in the CNS. DRG injection of db-cAMP, a stable analog of cAMP, facilitated entry of central branches of sensory neurons into the dorsal columns. Upregulation of arginase I, an enzyme involved in polyamine synthesis was a further result of cAMP facilitation [83].

Polyamines, in turn are linked to the generation of new rounds of transcription that may support new outgrowth. Thus this pathway may have facilitated regeneration of peripheral axons into the spinal cord by reversing myelin/MAG inhibition or by directly altering growth cone cytoskeletal dynamics.

A number of interventions, including conditioning, cAMP, digestion of PSGPs, or other approaches, thus have prompted some ingrowth of axons into the CNS. These successes, however, have had a modest impact at best. Clinical situations requiring regrowth of axons into dorsal columns develop less frequently than peripheral injuries.

Conditioning lesions

A conditioning lesion is an intervention thought to "prime" axons for faster regrowth. It involves a deliberate preliminary injury to an axon followed by a "*test*" injury [187,460,461,518,610]. Classically, conditioning lesions are placed further distally along the nerve beyond the site of the test injury. Conditioning accelerates the outgrowth of neurites *in vitro* or axon outgrowth *in vivo*. It is successful if carried out between 3 days and 2 weeks prior to the "test" injury, and its onset is more rapid if positioned closer to the cell body.

Conditioning is an interesting and robust phenomenon; several mechanisms have been considered. These include rises in cAMP levels, enhanced signaling by integrins, interleukin-6 upregulation, and others [92,167]. Perhaps the most important mechanism in generating a conditioning response is the early induction of RAGs in perikarya, considered in Chapter 5. This shift in gene expression prepares the neuron for regeneration by upregulating growth associated proteins such as GAP43/B50 and HSP27. While conditioning paradigms are of interest in understanding strategies to accelerate regeneration, they are obviously not helpful in treating human nerve damage.

The role of activity

Rehabilitative exercise and activity are assumed to facilitate recovery from peripheral nerve injury. Rehabilitation is crucial in rebuilding muscle mass and preventing complications of recumbency. Experimental work however, suggests that activity may, in fact, be detrimental toward nerve regeneration. Tam and Gordon (see review by Tam and Gordon [664]), demonstrated that activity impaired muscle reinnervation after partial denervation in rats. The growth and spread of the perisynaptic terminal SCs, normally offering guidance to regrowing and sprouting motor axons were diminished by exercise [663]. The combined impacts of excessive flux of calcium and local release of acetylcholine

on perisynaptic SC behavior may account for their impaired behavior after activity [663]. In contrast to activity, brief epochs of exogenous electrical stimulation improved motor and sensory axon regeneration and increased preferential motor reinnervation, discussed in Chapter 6 [73,74]. This form of activation clearly differs from the more continuous recruitment that occurs during exercise.

Summary

This chapter hints at a further orchestrated series of interactions that either support or impair peripheral nerve regeneration. Many novel pathways are likely to be added to this compilation. Broadly speaking, however, adhesive interactions between axons, SCs, and BM play a major role during repair and regrowth. These pathways can be synergistic with those of growth factors. Approaches to degrade CSPGs, to apply immunophilins, and to delineate peptide actions during regeneration are promising avenues toward understanding and treating peripheral nerve damage. The goal is to reiterate a more complete interactive panoply of molecules influencing axon and SC growth during optimal regenerative conditions. New fundamental work will underlie the development of therapeutic approaches for severe peripheral nerve injuries, injuries involving the DREZ or challenging conditions such as diabetes or increased age.

Suggested reading

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