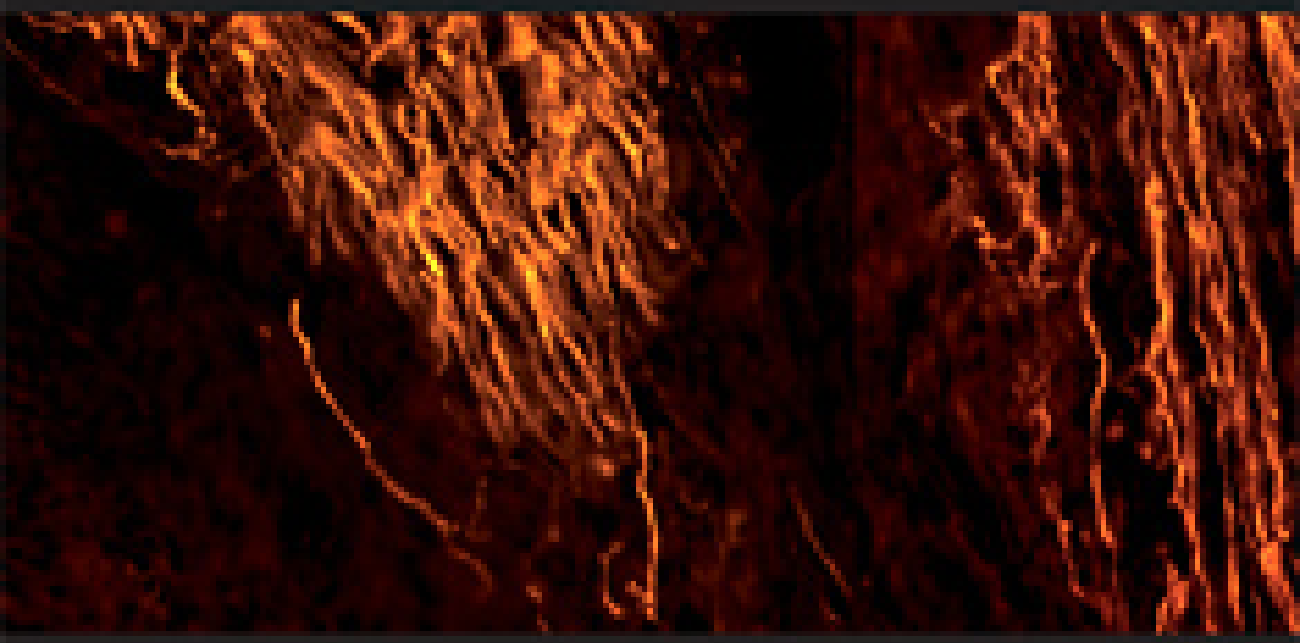

Neurobiology of Peripheral Nerve Regeneration

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.

Our laboratory is grateful for support from the Alberta Heritage Foundation for Medical Research, Canadian Institutes of Health Research, Canadian Diabetes Association and Muscular Dystrophy Association of Canada.

> "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.

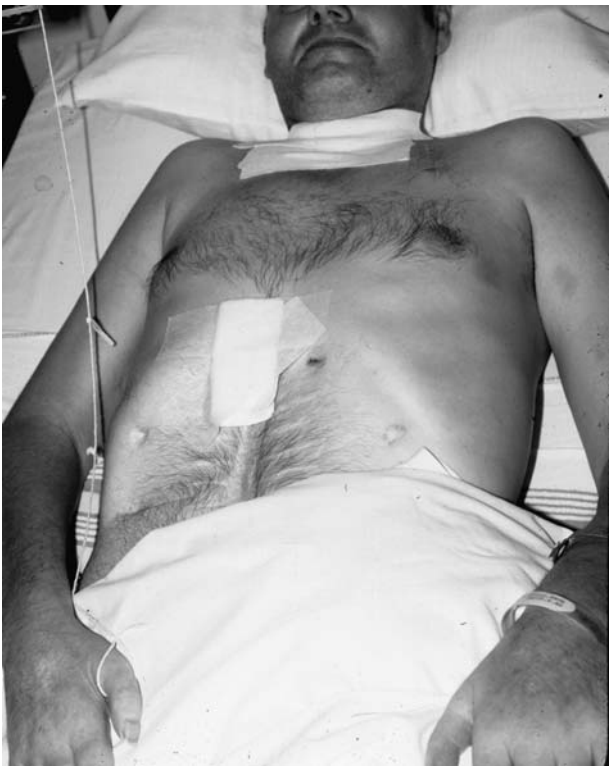

Figure 1.1 A patient with severe Guillain–Barré syndrome with axonal damage and paralysis of all of his limbs. He required intensive care unit support to breathe for a period of 1 year before recovering.

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,

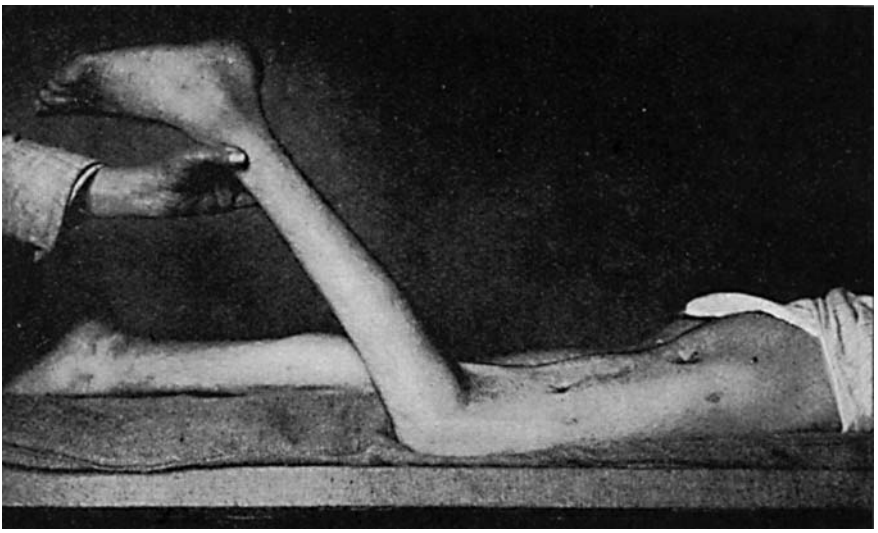

Figure 1.2 A patient with a right buttock bullet wound that damaged his underlying sciatic nerve trunk. The severe axonal disruption caused by the injury resulted in permanent paralysis and atrophy of muscles in the thigh and below the knee with sensory loss in the foot and leg. (From Tinel [681].)

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

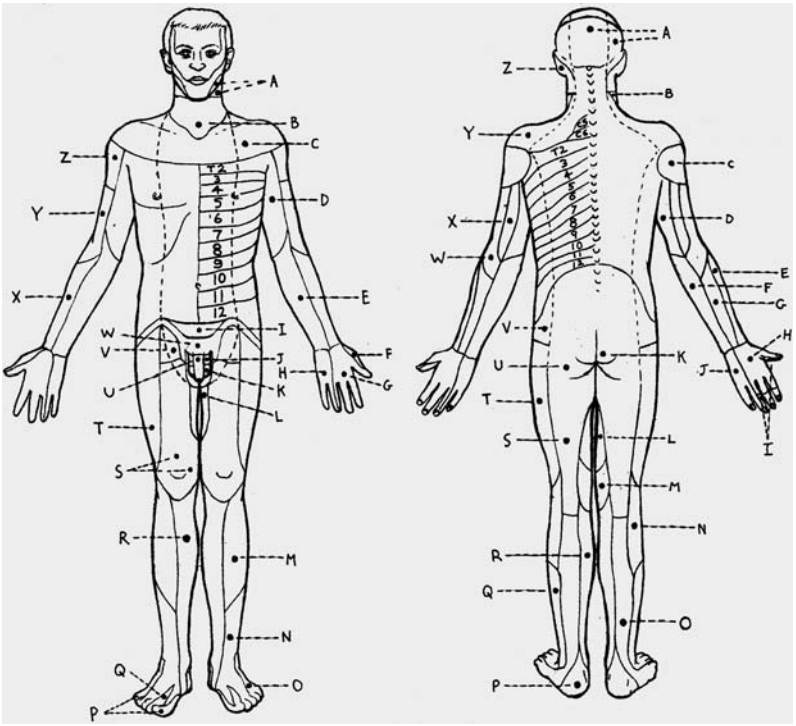

Figure 2.1 An illustration of the peripheral sensory nerve territories of the human body. *Left panel, anterior body:* A – greater auricular nerve; B – anterior cutaneous nerve of neck; C – supraclavicular nerves; D – medial cutaneous nerve or arm and intercostobrachial nerve; E – medial cutaneous nerve 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.

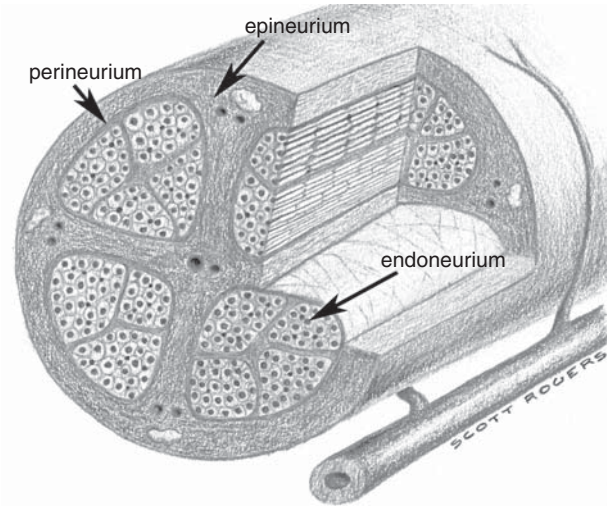

Figure 2.2 An illustration of the overall organization of the peripheral nerve trunk and its compartments. (Illustration by Scott Rogers.) See color plate section.

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

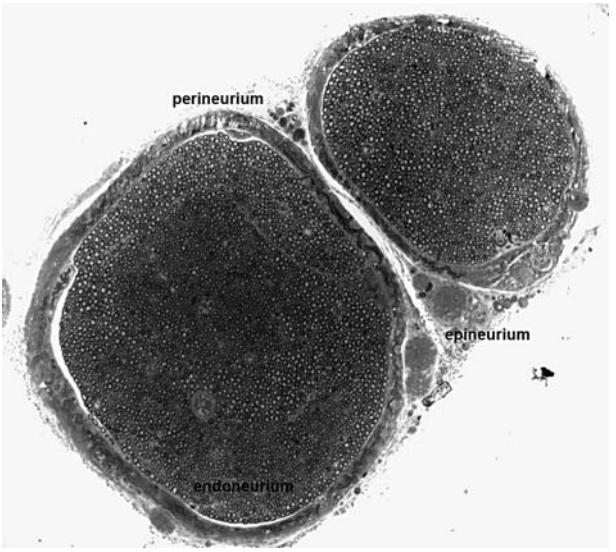

Figure 2.3 A photomicrograph of a sciatic peripheral nerve trunk. Note the presence of two major fascicles with myelinated axons within the endoneurium. The section is a low-power view of a semithin section that was harvested from an adult rat, fixed, embedded in epon and stained with toluidine blue.

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

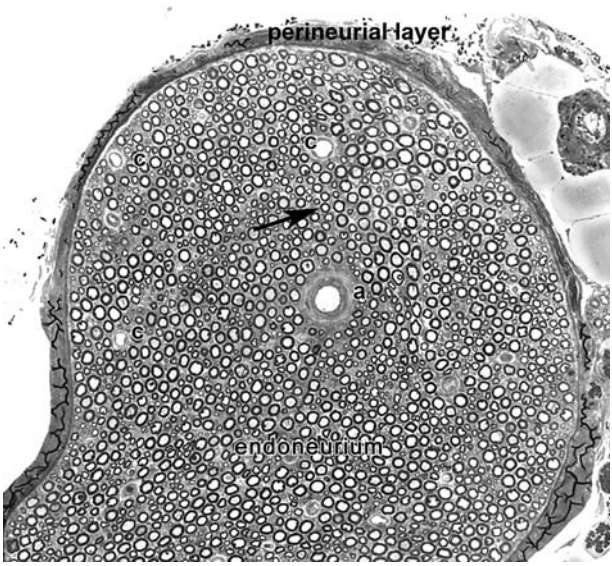

Figure 2.4 A photomicrograph showing the structures within the endoneurium of a nerve trunk fascicle. The arrow points to a myelinated axon. Note that there are populations of larger and smaller myelinated fibers. The section also shows endoneurial capillaries (c) and an arteriole (a). The section is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

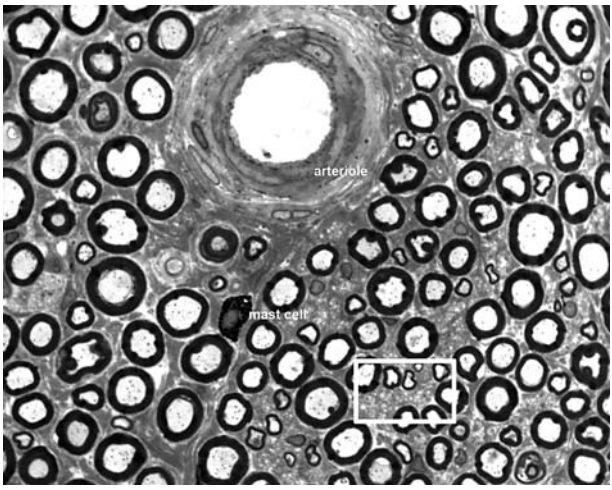

Figure 2.5 A high-power, oil immersion photomicrograph of endoneurial constituents including a mast cell, arteriole, and myelinated axons. Note that some axonal ultrastructural components can be resolved in the large myelinated axons (most likely microtubules and mitochondria) and in the white box, groups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

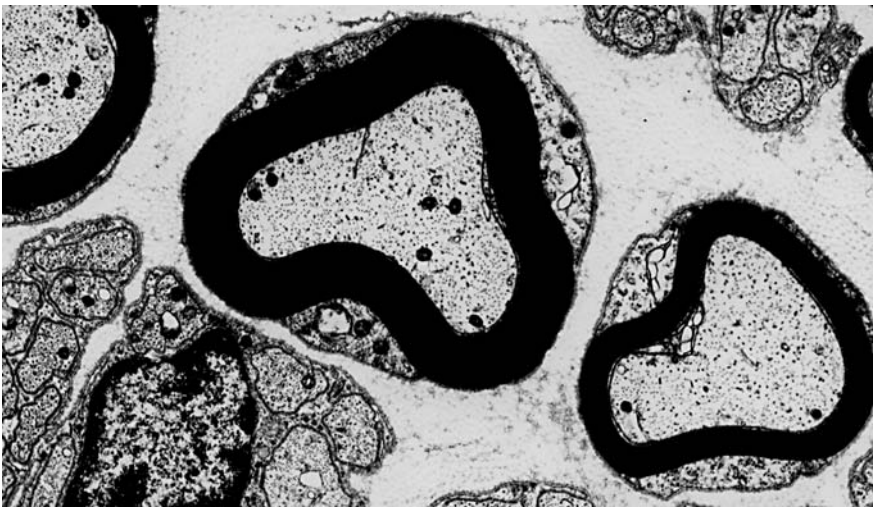

Figure 2.6 An electron micrograph showing two medium caliber myelinated axons with some SC cytoplasm around each. In the lower left part of the image, two clusters of unmyelinated axons (Remak bundle) is seen.

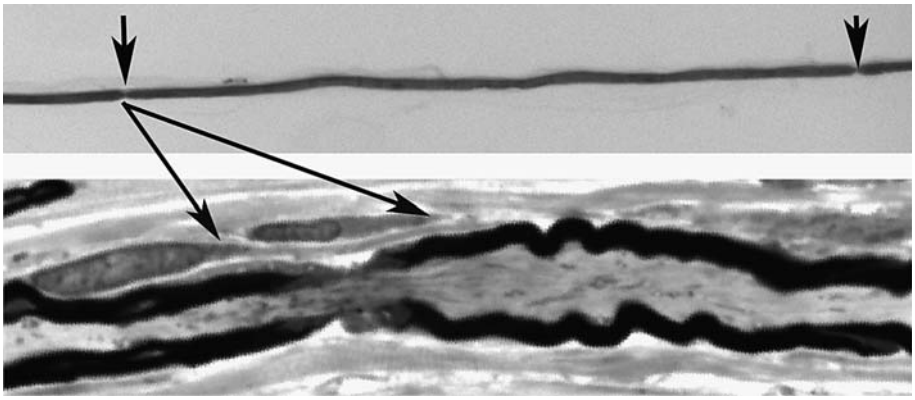

Figure 2.7 A single teased myelinated axon is shown above with the small arrows pointing to nodes of Ranvier (top image). The segment between the arrows is called an internode. Below is a higher power view showing a node. Note the darkened axon in the node region from more closely packed microtubules 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

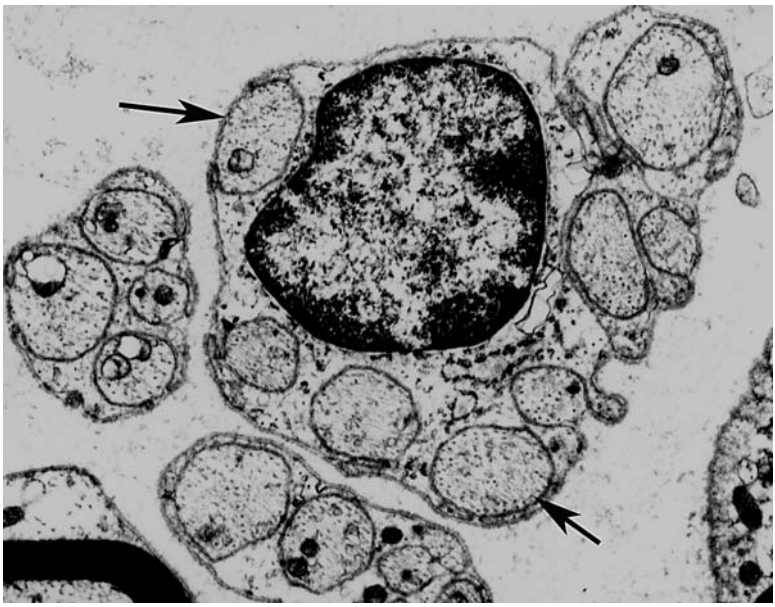

Figure 2.8 An electron micrograph of a Remak bundle of several unmyelinated axons (arrows) associated with a single SC.

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.

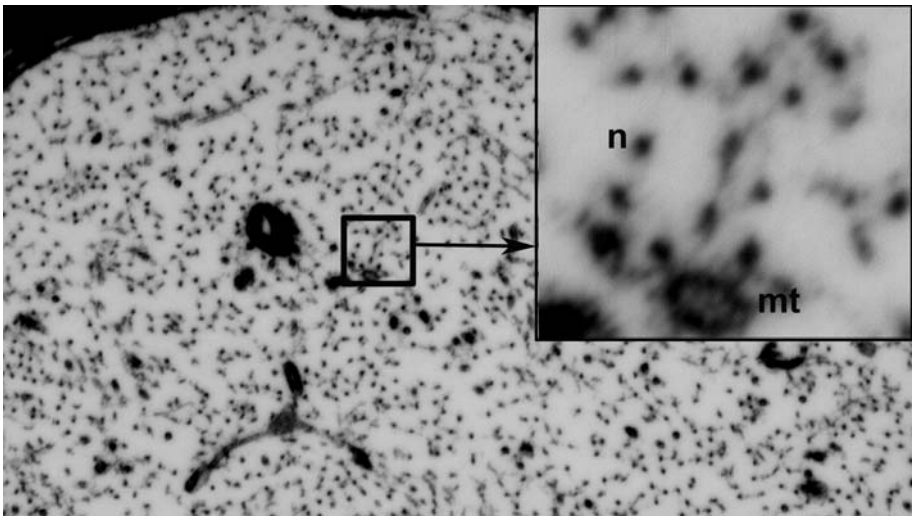

Figure 2.9 An electron micrograph of a myelinated axon illustrating its ultrastructure. The enlarged box identifies neurofilament (n) profiles and a microtubule (mt). Note the faintly resolved sidearms extending from the neurofilaments. The larger structure to the left of the box is a mitochondrion.

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.