CHAPTER

8

Axonal Transport

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OUTLINE

INTRODUCTION

The complex functional architecture of neurons includes many specializations in cytoskeletal and membranous components. Each of these specializations is dynamic, constantly changing and being renewed at a rate determined by the local signaling environment and cellular metabolism. In this context, the processes of axonal transport are key to neuronal dynamics. Recent advances have provided important insights into the molecular mechanisms underlying axonal transport and its regulation, although many questions remain. Continued exploration of these phenomena will improve our understanding of neuronal dynamics in development, regeneration, and neuropathology.

NEURONAL ORGANELLES IN MOTION

The axon comprises a major portion of the total volume and surface area of most mature neurons and may extend for several thousand cell-body diameters. Since the genetic material and nearly all of the protein synthesis machinery are localized to the cell body, a supply line needs to be maintained to provide structural and functional materials to discrete functional compartments along the length of the axon. Insights as to how neurons accomplish this task were obtained by real-time imaging of living axons with video-enhanced light microscopy (Brady et al., 1983, 1985) (Fig. 8-1).

Such video images reveal an array of neuronal organelles moving down the axon toward the nerve terminal (anterograde direction), as well as returning to the neuronal cell body (retrograde direction). The movements create patterns
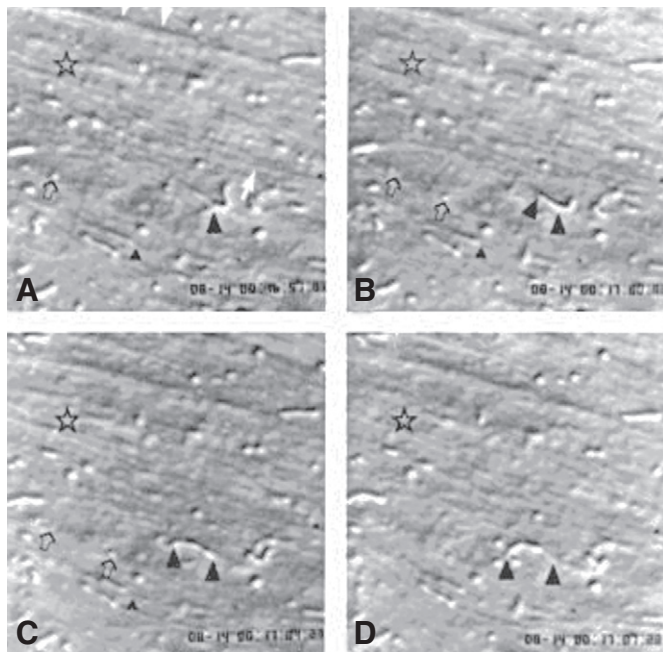

FIGURE 8-1 **Sequential video images of fast axonal transport in isolated axoplasm from the squid giant axon.** In this preparation, anterograde axonal transport proceeds in the direction from upper left to lower right (from 10 o'clock toward 4 o'clock). The field of view in these stills is approximately 20 μm, and the images were recorded in real time on videotape. Representative still images were taken at 3–4 second intervals to illustrate the transport of membrane-bounded organelles (MBOs). The large, sausage-shaped structures (filled triangles) are mitochondria. Medium-sized particles (open arrows) most often move in the retrograde (right-to-left) direction. Most structures of this size are lysosomal or prelysosomal organelles. The majority of moving particles in these images are faint and moving rapidly (2 μm/sec), so they are difficult to catch in still images; however, in the region above the star, a number of these organelles can be visualized in each panel. The entire field contains faint parallel striations (like those indicated by the white arrows in panel a) that correspond to the cytoskeleton of the axoplasm, primarily microtubules. The movement of MBOs occurs along these structures, although organelles can occasionally be seen to switch tracks as they move (note the position of the mitochondrion indicated by large triangles in a–d). (From Brady et al., 1982.)

as engrossing as the ant farms of our childhood and initially appear chaotic. Some organelles glide smoothly, while others move in fits and starts. On closer examination, an underlying order emerges: The organelles moving in the anterograde direction are typically fainter and smaller but more numerous than those moving in the retrograde direction, and all organelles appear to travel along gently curving fibrils. Occasionally, two organelles are seen to travel in opposite directions along the same fibril, appearing destined for a head-on collision but seeming to pass through each other; other organelles hop from one fibril to another. The images imply, and experimental evidence confirms, that these organelles represent membrane-bound packets of materials en route to a variety of intraneuronal destinations. Unseen in these images because their movements and changes occur orders of magnitude more slowly, structural elements of the axonal cytoskeleton are equally dynamic (Baas & Buster, 2004).

The life cycle of these organelles, their kinetics, their molecular cargo, the molecular motors driving their transport, and the substrates along which these movements track constitute interrelated aspects of what is broadly termed axonal transport. A primary aim of this chapter is to provide an understanding of this form of intraneuronal organelle traffic. Achieving this goal requires an appreciation of the dynamics and structure of the relevant neuronal components involved. Studies of how cellular structures and components move from the compartment where they are synthesized to where they are utilized comprise an area of intensive research in cellular and molecular neurobiology. To comprehensively encompass this topic, we examine how our concepts of axonal transport evolved to our present understanding of this complex and dynamic field.

DISCOVERY AND DEVELOPMENT OF THE CONCEPT OF FAST AND SLOW COMPONENTS OF AXONAL TRANSPORT

The size and extent of many neurons presents a special set of challenges

Protein synthesis for the entire neuron largely takes place in the cell body, which may represent only 0.1% of the total cell volume. Therefore, the growth and maintenance of neuronal processes requires timely, efficient delivery of material to axonal and dendritic domains. The idea that materials must be transferred from cell body to axon was first suggested by Ramon y Cajal and other pioneers during the early part of the 20th century. For many years, the existence of such transport processes could only be inferred.

The first experimental evidence for axonal transport resulted from studies on peripheral nerve regeneration, which were stimulated by the desire to improve treatment of limb injuries sustained during World War II. In the classic work of Weiss and Hiscoe (1948), surgical constriction of a sciatic nerve branch led to morphological changes in the nerve that directly implicated the cell body as the source of materials needed for axon regrowth. After several weeks, the

axon appeared swollen proximal to the constriction, but shriveled on the distal side. Following removal of the constriction, a bolus of accumulated axoplasm slowly moved down the nerve at 1–2 mm/day, very nearly the rate observed for outgrowth of a regenerating nerve. Weiss and Hiscoe concluded that the cell body supplies a bulk flow of material to the axon. This view dominated the field for two decades, but the characteristics of this slow "flow" of material did not seem adequate to explain some aspects of nerve growth and function.

Cell biologists subsequently provided convincing arguments for the necessity of this form of intracellular transport. Neuronal protein synthesis was almost completely restricted to the cytoplasm surrounding the nucleus and, to some extent, to dendrites (translational cytoplasm, which includes polysomes, rough endoplasmic reticulum and the Golgi complex, see Chapter 7), and in normal conditions, ribosomes are undetectable in the axon (Brady, 1993). This information implies that all or nearly all materials necessary for axonal function have to be supplied by mechanisms involving transport from the neuronal cell body. Thus, axonal transport must be a normal, ongoing process in neurons.

By the mid-1960s, the use of radioactive tracers had confirmed the existence of a slow "bulk flow" component of transport. Using autoradiography, Droz and Leblond (1962) elegantly showed that systemically injected 3H-amino acids were incorporated into nerve cell proteins and transported along the sciatic nerve as a wavefront of radioactivity. These methods demonstrated that newly synthesized proteins were transported, but some responses of the neuron occurred too rapidly to be readily explained solely by such a slow "flow."

Shortly thereafter, radiolabeling and histochemical studies demonstrated that faster rates of transport occur (Grafstein & Forman, 1980). Unlike slow transport, the faster components move material bidirectionally, both toward and away from the cell body. Both endogenous proteins and exogenously applied labels were detected moving at fast transport rates. These findings expanded the concept of axonal transport: materials move in both anterograde and retrograde directions and transport rates vary by as much as three orders of magnitude (Table 8-1).

At first, emphasis was placed on the characterization of fast and slow axonal transport. The kinetics of axonal transport were analyzed by injection of radiolabeled amino acids into the vitreous of the eye or the dorsal root ganglia (to "label" sensory neurons), or into ventral spinal cord (motor neurons). In the case of fast transport, a wavefront of labeled protein was detected traveling away from the cell body at rates of 250–400 mm/day in mammals. Using this same approach, slow transport rates were shown to approximate 1 mm/day. Rates for fast axonal transport were also determined by measuring the amount of a transported substance, such as acetylcholinesterase or norepinephrine, accumulating at a nerve constriction over a few hours, well before bulk accumulation of axoplasm was detectable. These two approaches for studying axonal transport—locating a radiolabeled wave by analysis of successive nerve segments and monitoring the accumulation of materials at a constriction

over time (Fig. 8-2) generated considerable information on the kinetics, as well as on the metabolic and ionic requirements, of axonal transport (Grafstein & Forman, 1980). Such findings formed the basis for a more detailed characterization of the axonally transported materials and for studies of the underlying molecular mechanisms.

Fast and slow components of axonal transport differ in both their constituents and their rates

Fast transport is bidirectional: many proteins that are distributed by fast anterograde transport are also returned in the retrograde direction. In contrast, proteins transported at slow rates are degraded when they reach their destination and are not detected in the retrograde component. Biochemical fractionation and electron microscopic studies have shown that proteins moved by anterograde and retrograde fast axonal transport are predominantly membrane associated, whereas proteins moved in the slow axonal transport component are recovered in soluble fractions or in cytoskeletal pellets (Tytell et al., 1981).

When labeled polypeptides traveling down the axon are analyzed by SDS polyacrylamide gel electrophoresis, materials traveling in the axon can be grouped into five distinct rate components (Tytell et al., 1981). Each rate component is characterized by a unique set of polypeptides moving coherently down the axon (Table 8-1, Fig. 8-3). As specific polypeptides associated with each rate class were identified, most were seen to move only within a single rate component. Moreover, proteins that have common functions or interact with each other tend to be moved together. These observations led to the proposal of the Structural Hypothesis (Tytell et al., 1981). This model can be stated simply: proteins and other molecules move down the axon as component parts of discrete subcellular structures rather than as individual molecules (Table 8-1).

The Structural Hypothesis, which was formulated in response to observations that axonal transport components

TABLE 8-1   Major Rate Components of Axonal Transports

| Rate component	Rate (mm/day)	Structures and composition
FAST TRANSPORT		
Anterograde	200–400	Small vesiculotubular structures, neurotransmitters, membrane proteins and lipids
Mitochondria	50–100	Mitochondria
Retrograde	200–300	Lysosomal vesicles and enzymes
SLOW TRANSPORT		
SCb	2–8	Microfilaments, metabolic enzymes, clathrin complex
SCa	0.2–1	Neurofilaments and microtubules
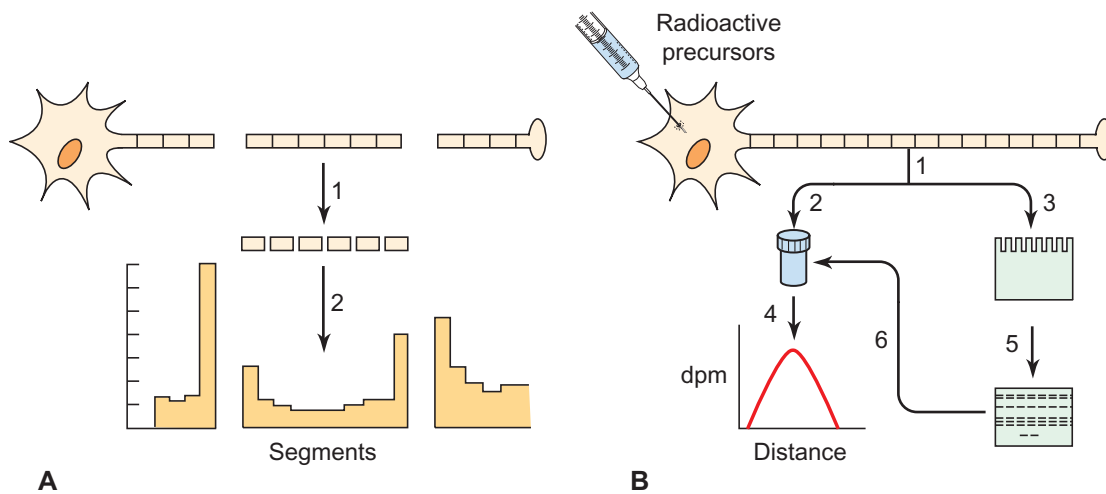
FIGURE 8-2 **Schematic diagram of two common methods for analyzing the component rates of axonal transport. (A)** Accumulation of transported material can be studied at a focal block of axonal transport caused by a cut, a crush, a cold block, or a ligature. The approach described here represents a variation of that employed by Weiss and Hiscoe, and has been used often in studies of fast axonal transport. In this example, two cuts or ligations have been made in order to detect both anterograde and retrograde transport. After some time is allowed for accumulation of transported material close to the cuts or ligatures, nerve segments of uniform length are cut for analysis (step 1). Each individual segment is analyzed either for radioactivity in metabolically labeled nerves or for enzyme activity, and the rate of accumulation at each segment is estimated (step 2). **(B)** With segmental analysis, the nerve must be pulse labeled, usually with radioactive precursors. After an appropriate injection–sacrifice interval to label the rate component of interest, the nerve is also cut into segments (step 1). In some cases, only a single segment is used as a "window" onto the transport process. Each segment is analyzed both by counting the radioactivity in an aliquot (step 2) and by gel electrophoresis (step 3), where each lane corresponds to a different segment. The amount of radioactivity incorporated in different polypeptides can be visualized with fluorography (step 5) and individual bands cut out of the gel (step 6) for quantitative analysis by liquid scintillation counting. The distribution of either total radioactivity or radioactivity associated with a specific polypeptide can then be plotted (step 4) in disintegrations per minute (dpm). (Adapted from Brady, 1986.)
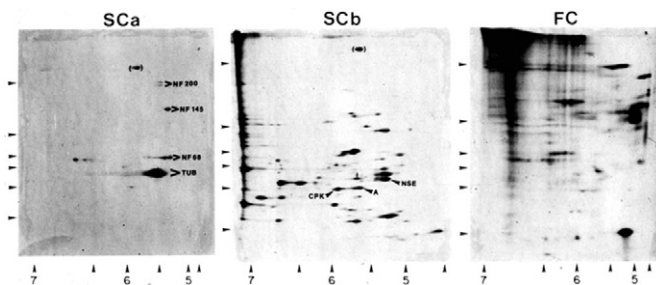

FIGURE 8-3 **Differences in protein composition for specific axonal transport components.** Two-dimensional fluorographs showing the 35S methionine-labeled polypeptides in the three major anterograde rate components of axonal transport: SCa, slow component a; SCb, slow component b; FC, fast component. Note that each rate component not only has a characteristic rate, but also a characteristic polypeptide composition. The discovery that each rate component has a different polypeptide composition led to the Structural Hypothesis. (From Tytell et al., *Science* 214:179–181, 1981; illustration provided by Dr. Michael Tytell.)

move as discrete waves, each with a characteristic rate and a distinctive protein composition, can explain the coherent transport of functionally related proteins and is consistent with the relatively small numbers of motor molecules in mature neurons. The only assumption is that the number of elements that can directly interact with transport motor complexes is limited.

Therefore, appropriate packaging of the transported material after its synthesis is required. Different rate components result from packaging of transported material into different, cytologically identifiable structures. In fact, the faster rates reflect the transport of proteins preassembled as membranous organelles, including vesicles and mitochondria, or of proteins contained in the lumen of these organelles (Fig. 8-4), whereas the slower rate components contain the cytoskeletal proteins. Thus, tubulin and MAPs move as preassembled microtubules and neurofilaments subunits move as neurofilaments, although the moving may be short segments of these cytoskeletal elements (Baas & Buster, 2004). Cytoplasmic proteins that are not integral components of a cytoskeletal element may be linked in some manner to those structures (Fig. 8-5) or may represent molecular complexes of cytoplasmic proteins (Roy et al., 2007). While disputes regarding the size and composition of the transported package for cytoskeletal and cytoplasmic proteins (i.e., polymer, oligomer, macromolecular complex, etc.) continue, the idea that complexes of proteins, rather than individual polypeptides, are moved has gained general currency (Baas & Buster, 2004).

Although five distinct major rate components have been identified, the original categories of fast and slow transport remain useful. All membrane-associated proteins move in one of the fast rate components, while cytoskeletal and cytoplasmic proteins move as part of the slow components. Current studies indicate that the various organelles
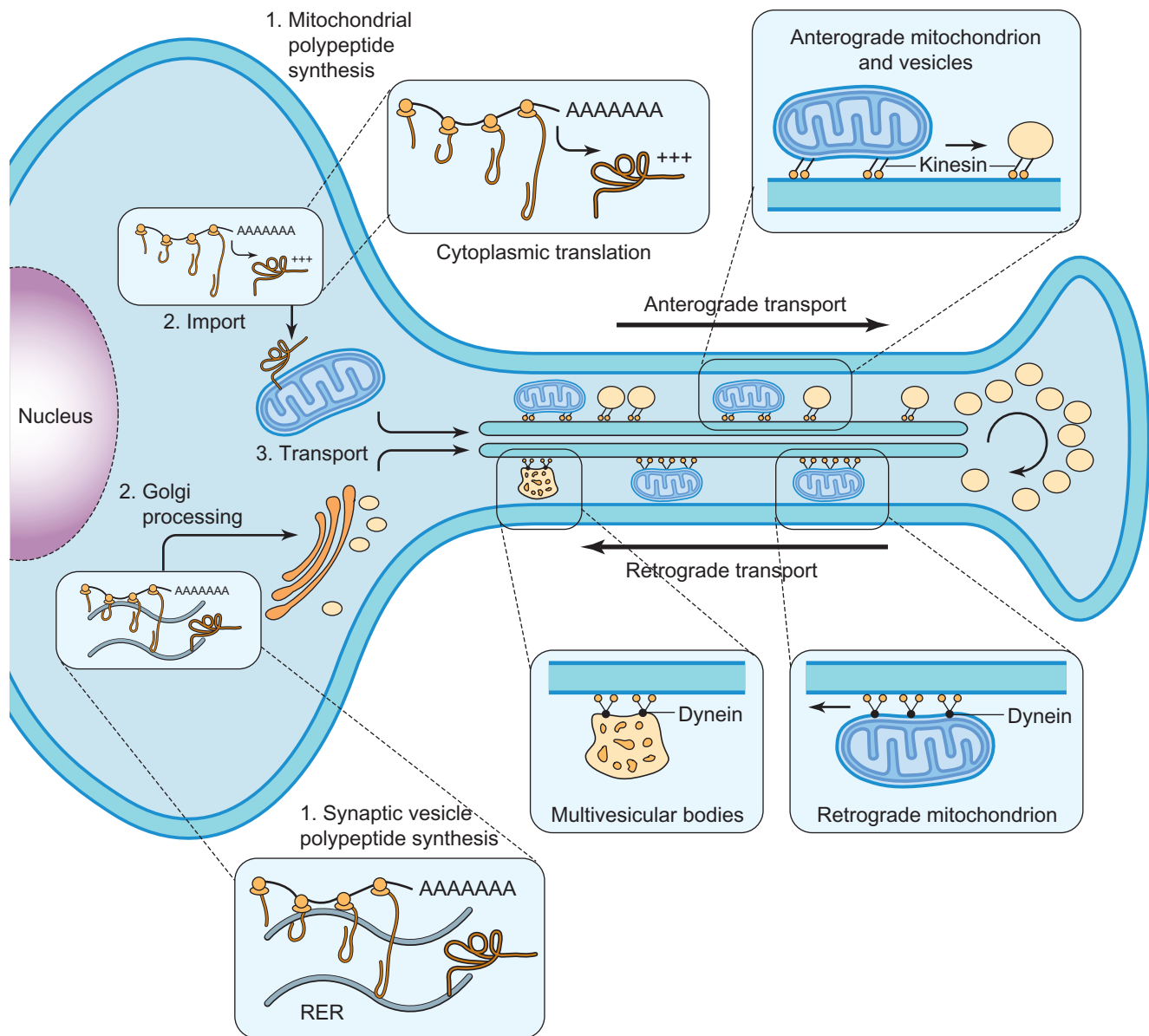
FIGURE 8-4   Schematic illustration of membrane-associated material moving in fast axonal transport. Fast axonal transport represents the movement of membrane-bounded organelles along axonal microtubules in both the anterograde and retrograde directions. Two major classes of membrane-bounded organelles that are synthesized and packaged by different pathways are depicted. Synaptic vesicle polypeptides are translated on endoplasmic reticulum–bound ribosomes, at which time membrane proteins become properly oriented within the lipid bilayer and secretory polypeptides enter into the lumen of the endoplasmic reticulum. These polypeptides are further processed within the Golgi apparatus, where the appropriate post-translational modifications and sorting of polypeptides destined for the axon occur. After these polypeptides are packaged into vesicular organelles and the appropriate motor molecules are attached, the organelles are transported down the axon utilizing axonal microtubules as "tracks" at rates of 200–400 mm/day. Movement in the anterograde direction is believed to be mediated by the molecular motor conventional kinesin, while the force necessary to move retrograde organelles is thought to be generated by cytoplasmic dynein. Unlike vesicular polypeptides, mitochondrial polypeptides that are supplied by the host cell are synthesized on cytoplasmic ribosomes and contain a targeting sequence that directs the polypeptides to the mitochondria. Following assembly and the association of motor molecules, the mitochondria move down the axon at rates of 50–100 mm/day. Mitochondria can also be detected moving back toward the cell body in the retrograde direction. The morphology of retrogradely transported mitochondria is distinctly different from that of mitochondria moving in the anterograde direction and is believed to represent degenerating organelles that are not metabolically active.

transported anterogradely are moved along the axon by one or more motor molecules (see below). The differing rates of fast anterograde transport appear to result from the varying sizes of organelles. Increased drag on larger structures results in a slower net movement, leading to more frequent pauses for larger organelles like mitochondria (Fig. 8-1). Less is known about molecular components mediating slow axonal transport.
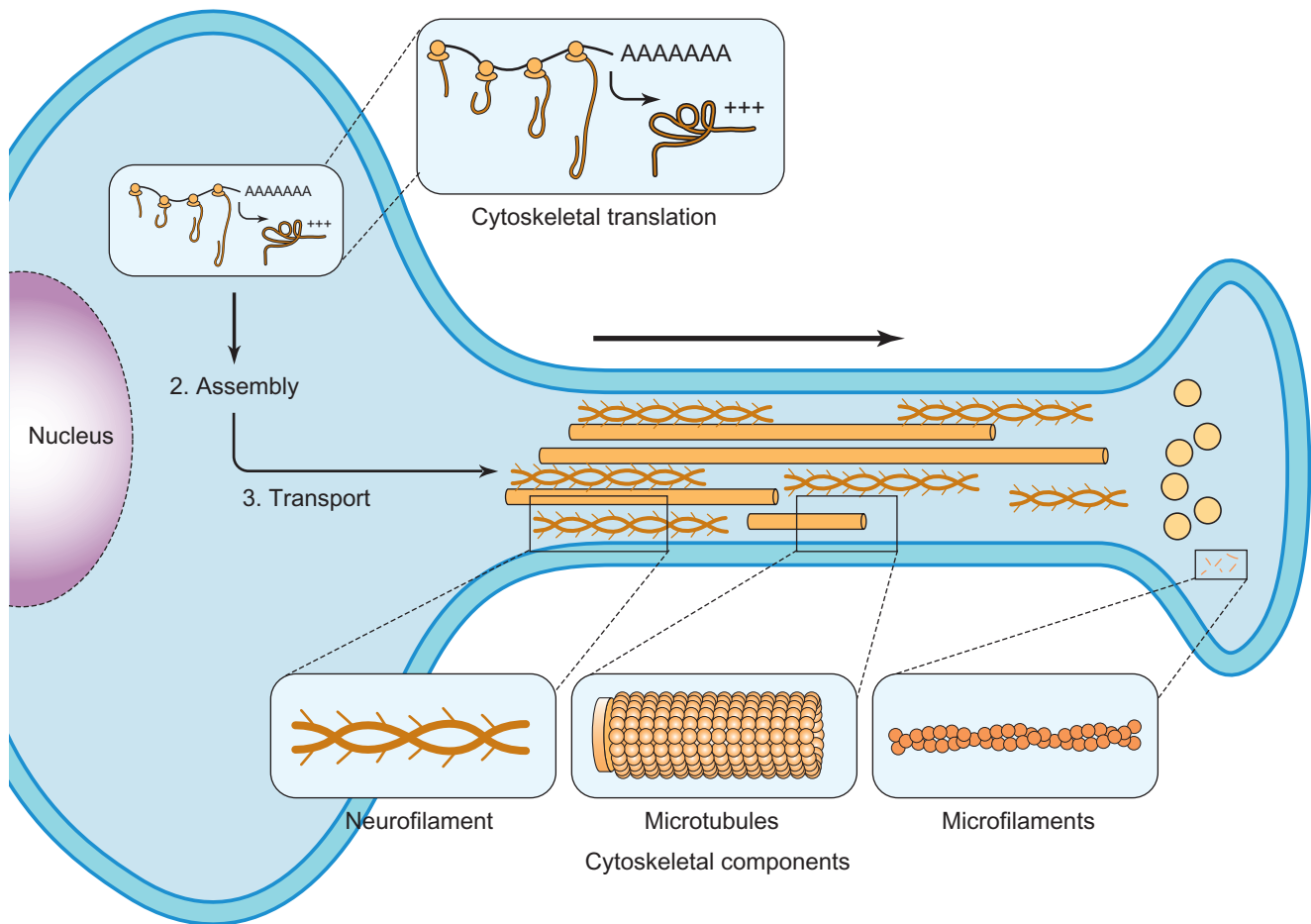
FIGURE 8-5 **Schematic illustration of the movement of cytoskeletal elements in slow axonal transport.** Slow axonal transport represents the movement of cytoplasmic constituents, including cytoskeletal elements and soluble enzymes of intermediary metabolism, at rates of 0.2–2 mm/day, which are at least two orders of magnitude slower than those observed in fast axonal transport. As proposed in the Structural Hypothesis and supported by experimental evidence, cytoskeletal components are believed to be transported down the axon as short polymers, not as individual subunit polypeptides. Cytoskeletal polypeptides are translated on cytoplasmic polysomes in the neuronal cell body and then assembled into polymers prior to transport down the axon in the anterograde direction. In contrast to fast axonal transport, no constituents of slow transport appear to be transported in the retrograde direction. Although the polypeptide composition of slow axonal transport has been extensively characterized, the motor molecule(s) responsible for the movement of these cytoplasmic constituents have not yet been identified.

Features of fast axonal transport demonstrated by biochemical and pharmacological approaches are apparent from video images

Video microscopy of isolated squid axoplasm, as described briefly at the beginning of this chapter, directly confirmed the bidirectionality of fast transport that had been inferred from the accumulation of radiolabeled materials on both sides of a crush, and established that the populations of organelles moving in each direction are different (Tsukita & Ishikawa, 1980). Inhibition of axonal transport by agents that disrupt microtubules (MTs) is consistent with movement of organelles along fibrils identified as MTs by correlated video (Fig. 8-1) and electron microscopy. Video microscopy also reveals that organelle movement can continue in apparently normal fashion in axons isolated from their cell bodies and divested of a plasma membrane. The implication is that transport must be driven by local energy-generating mechanisms, as predicted from observations that application of a cold block or metabolic poison (dinitrophenol or cyanide) to a discrete region of a nerve inhibits transport locally (Grafstein & Forman, 1980; Tsukita & Ishikawa, 1980).

FAST AXONAL TRANSPORT

Newly synthesized membrane and secretory proteins destined for the axon travel by fast anterograde transport

However, not all membrane proteins are destined for the axon. As a result, the first stage of transport must be synthesis, sorting and packaging of organelles (see Chapter 7). Once
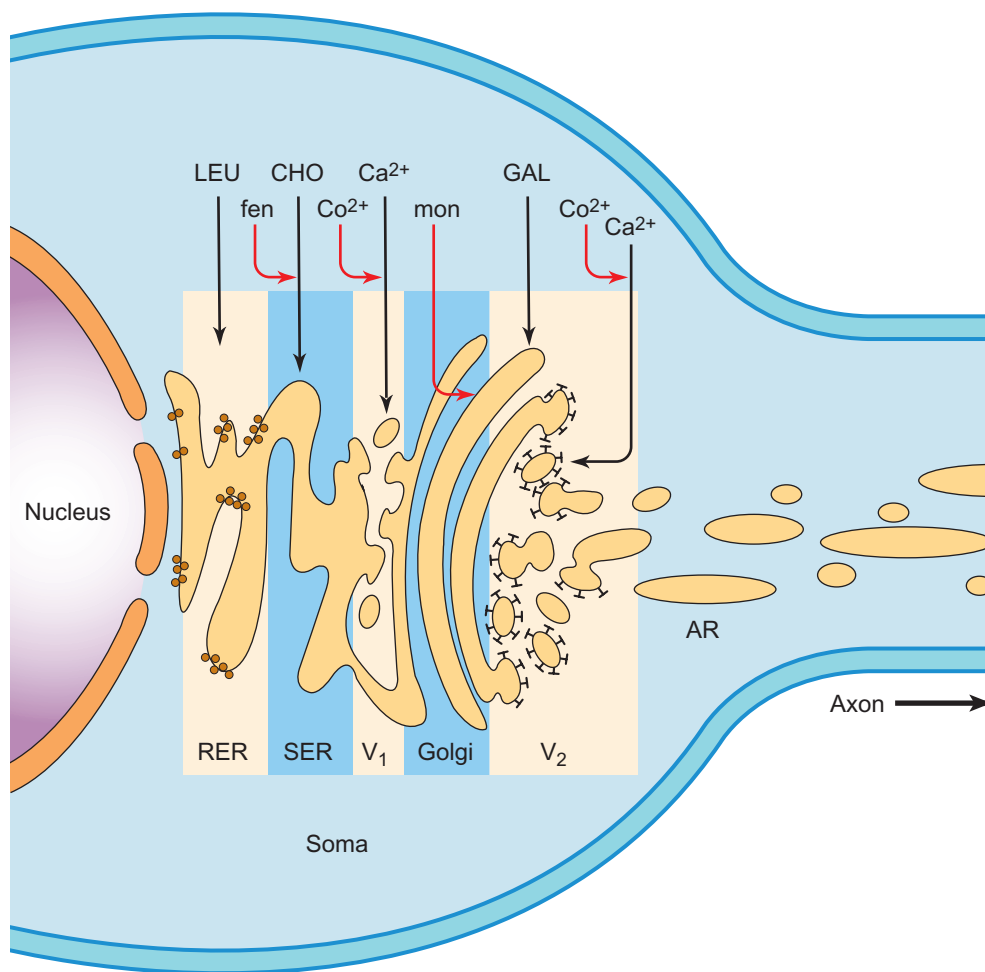
FIGURE 8-6 **Intracellular trafficking in neurons.** Summary of pharmacological evidence indicating that newly synthesized membrane and secretory proteins in neurons reach the axons by a pathway similar to that utilized for intracellular transport in non-neuronal cells. Incorporation sites are indicated for several precursors of materials in fast axonal transport: leucine (LEU) for proteins, choline (CHO) for phospholipids, and galactose (GAL) for glycoproteins and glycolipids. Sites of action for several inhibitors are also indicated, including fenfluramine (fen), monensin (mon), and Co^{2+}. One possible site for Ca^{2+}-mediated vesicle fusion is at the transition from rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) to the Golgi apparatus (GA) via one type of transition vesicle (V1). The subsequent budding of vesicles (V2) off the GA, presumably mediated by clathrin-coated vesicles, is a second site for Ca^{2+} involvement. The microtubule guides for the axonal tubulovesicular structures and the axoplasmic reticulum (AR) moving in fast axonal transport are not shown.

assembled and packaged with the appropriate lipid and protein components, membrane bounded organelles (MBOs), including transport vesicles and tubulomembrane structures, must then be committed to the transport machinery (i.e., through addition of the appropriate molecular motor) and moved down the axon. Finally, membrane proteins must be targeted and delivered to functionally heterogeneous domains in the axon, including presynaptic terminals, axolemma, and nodes of Ranvier, among others. Axonal constituents include integral membrane proteins, secretory products, membrane phospholipids, cholesterol and gangliosides. As predicted by the Structural Hypothesis and as apparent in video microscopy, rapid transport of membrane-associated proteins is achieved after packaging materials into MBOs (Figs. 8-4 and 8-6). Clearly, an understanding of how MBOs are formed in the cell body and routed to the fast-transport system in axons is essential.

Passage through the golgi apparatus is obligatory for most proteins destined for fast axonal transport

In all cell types, secretory and integral membrane proteins are synthesized on polysomes bound to the endoplasmic reticulum. Secretory proteins enter the lumen of the reticulum, whereas membrane proteins become oriented within the membrane bilayer. In contrast, components of the cytoskeleton and enzymes of intermediary metabolism are synthesized on so-called free polysomes, which are actually associated with the cytoskeleton. As reviewed in Chapter 7, fast-transported proteins leave the endoplasmic reticulum in association with transfer vesicles that bud off and undergo Ca^{2+}-dependent fusion with the Golgi apparatus. Newly formed membrane-associated proteins must be transferred from the endoplasmic reticulum to the Golgi apparatus for
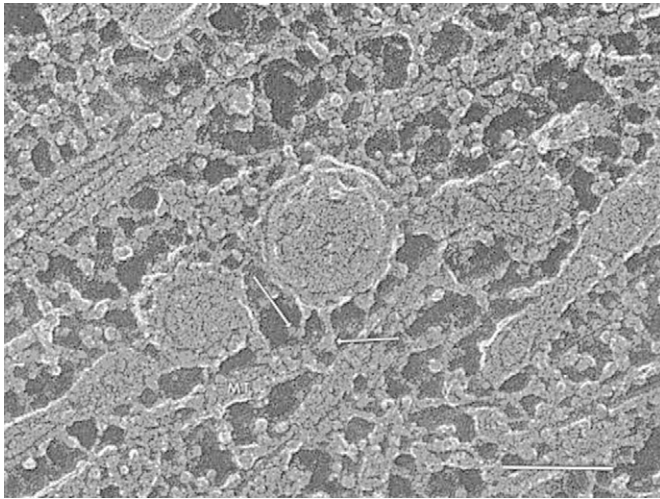
FIGURE 8-7 **Axonally transported vesicles and the axonal cytoskeleton in longitudinal section.** Quick-freeze, deep-etch electron micrograph of a region of rat spinal cord neurite rich in membrane-bounded organelles and microtubules. Arrows point to rod-shaped structures that appear as approximately 17-nm cross-bridges between organelles and microtubules. The bar at the lower right indicates 100nm. (From Hirokawa et al., 1989)

processing and post-translational modification, including glycosylation, sulfation, and proteolytic cleavage, as well as for sorting to selected neuronal compartments (van Vliet et al., 2003).

Pharmacological studies demonstrated a requirement that most proteins destined for fast axonal transport traverse the Golgi stacks, where membrane proteins are post-translationally modified, sorted and packaged (Hammerschlag et al., 1982) (Fig. 8-7). This suggests that proteins moving in fast axonal transport must either pass through the Golgi complex or associate with proteins that do. Clathrin-coated vesicles mediate transfer from the Golgi apparatus to the fast axonal transport system (Maxfield & McGraw, 2004) (Fig. 8-6). Coated vesicles, however, are rarely observed in axons. Accordingly, clathrin, the major coat protein, is primarily a slow transport protein (Garner & Lasek, 1981). Thus, Golgi-derived coated vesicles shed their coats prior to undergoing fast transport, and travel down the axon either as individual uncoated vesicles or other MBO structures (Fig. 8-6). Transport of MBOs along MTs to their various axonal destinations is mediated by the kinesin and dynein superfamilies of motor proteins (see below) (Hirokawa et al., 2009). Membrane and secretory proteins become associated with MBOs either during or immediately following their synthesis, and then maintain this association throughout their lifetime in the cell. For example, inhibiting synthesis of either protein or phospholipid leads to a proportional decrease in the amount of both protein and phospholipid moving in fast axonal transport, whereas application of these inhibitors to axons has no effect on transport. This suggests that fast axonal transport depends on *de novo* synthesis and assembly of membrane components. Recent data suggest the attachment of specific motor proteins to their transported cargoes might involve the activity of small GTPases (Szodorai et al., 2009).

Anterograde fast axonal transport moves synaptic vesicles, axolemmal precursors, and mitochondria down the axon

Fast anterograde axonal transport represents the movement of MBOs along MTs away from the cell body at rates ranging from 200–400mm/day or 2–10μm/second in mammalian neurons (Brady, 1991). Anterograde fast axonal transport provides newly synthesized components essential for neuronal membrane function and maintenance. Ultrastructural studies have demonstrated that the material moving in fast anterograde transport includes many small vesicles and tubulovesicular structures, as well as mitochondria and dense core vesicles (Smith, 1980; Tsukita & Ishikawa, 1980). Material in fast anterograde transport is needed for supply and turnover of intracellular membrane compartments (i.e., mitochondria and endoplasmic reticulum), secretory products, and proteins required for the maintenance of axonal metabolism. The net rate appears to be largely determined by size, with the smallest MBOs in almost constant motion, while mitochondria and larger structures frequently pause, giving a lower average rate (Brady et al., 1985).

A variety of materials move in fast anterograde transport including membrane-associated enzymes, neurotransmitters, neuropeptides and membrane lipids. Most are synthesized in the cell body and transported intact, but some post-translational processing events occur in transit. For example, several neuropeptides are generated by proteolytic degradation of propeptides (see Chapter 20). This biochemical heterogeneity extends to the MBOs themselves. The population of fastest-moving small organelles (200–400mm/day) is particularly varied in function and composition. Some correspond to synaptic vesicle precursors and contain neurotransmitters and associated proteins, while others may contain channel proteins and other materials destined for delivery at the axolemma. Biochemical and morphological studies have provided a description of the materials transported in fast axonal transport, but are not as well suited for identifying the underlying molecular mechanisms involved in their translocation.

Video microscopic experiments in isolated axoplasm allowed the study of molecular mechanisms underlying fast axonal transport through direct observation of MBO movements. Fast axonal transport continues unabated in isolated axoplasm from giant axons of the squid *Loligo pealeii* for hours (Brady et al., 1985). The lack of plasma membrane in isolated squid axoplasm allows for an evaluation of the molecular mechanisms for fast axonal transport through biochemical and pharmacological approaches. Such studies extended earlier observations on properties of fast anterograde transport and facilitated the discovery that motor proteins are responsible for axoplasmic transport of MBOs (see below) (Brady et al., 1985; Lasek & Brady, 1984).

Retrograde transport returns trophic factors, exogenous material, and old membrane constituents to the cell body

MBOs moving in retrograde transport are structurally heterogeneous and, on average, larger (>200nM) than the

structures observed in anterograde transport, which are commonly tubulovesicular (Smith, 1980; Tsukita & Ishikawa, 1980). Multivesicular or multilamellar bodies are common in retrograde transport, and these are thought to represent materials to be delivered to lysosomes in the neuronal cell body. The larger size of most retrogradely moving MBOs reduces their rate of transport by increasing drag due to interactions with cytoplasmic structures (Brady et al., 1985).

Both morphological and biochemical studies revealed different MBOs moving in anterograde and retrograde transport (Smith, 1980; Tsukita & Ishikawa, 1980). Repackaging of membrane components apparently accompanies turnaround or conversion from anterograde to retrograde transport. The mechanisms of repackaging are incompletely understood, but certain protease inhibitors and neurotoxic agents inhibit turnaround without affecting either anterograde or retrograde movement. Experiments involving the use of specific protease inhibitors implicate a thiol protease in this process (Sahenk & Lasek, 1988; Smith & Snyder, 1991). For example, protease treatment of purified synaptic vesicles affects directionality of their movements in axoplasm and presynaptic terminals. Also, exogenously injected fluorescent synaptic vesicles normally move in the anterograde direction, but protease pretreatment of these vesicles results in retrograde transport (Schroer et al., 1985). More recently, activation of retrograde axonal transport was associated with increases in the activity of PKC (Morfini et al., 2007). However, the precise sequence of molecular events underlying activation of retrograde fast axonal transport is currently unknown.

Uptake of exogenous materials by endocytosis in distal regions of the axon results in the return of trophic substances and growth factors to the cell body (Kristensson, 1987) (see Chs. 7 and 29). These factors assure survival of the neuron and modulate neuronal gene expression (Brady & Morfini, 2010). Changes in the return of trophic substances also play critical roles during development and regeneration of neurites. Retrograde transport also provides a pathway for viral agents to enter the CNS (Berth et al., 2009). Once retrogradely transported material reaches the cell body, the cargo may be delivered to the lysosomal system for degradation, to nuclear compartments for regulation of gene expression, or to the Golgi complex for repackaging.

Molecular sorting mechanisms ensure delivery of proteins to discrete membrane compartments

Pathways by which selected membrane-associated proteins are delivered to the correct destination have been established in general terms. However, many intriguing questions regarding the selection process itself remain. How do certain membrane proteins remain in the cell body (for example, glycosyltransferases of the Golgi) while others are packaged for delivery to the axon? Among transported proteins, how do some reach the axolemma (i.e., sodium and potassium channels) while others travel the length of the axon to the nerve terminal (a presynaptic receptor or synaptic vesicle) or enter the synaptic cleft (a secreted neuropeptide)? Finally, how are MBOs such as synaptic vesicles directed toward axons and presynaptic terminals, but not into dendritic arbors? This question becomes particularly compelling for dorsal root ganglion sensory neurons where the central branch of its single axon has presynaptic terminals, while the peripheral branch of that same axon has none.

The answers to these questions remain incomplete, but some mechanisms have begun to emerge. Some information comes from studies on polarized epithelial cells, where the identity of destination signals to deliver newly synthesized proteins selectively to basolateral or apical membranes can be assayed (Sampo et al., 2003). These mechanisms are relevant to the neuron, because viral proteins that normally go to epithelial basolateral membranes end up in neuronal dendritic compartments, while those targeted to apical compartments are typically transported into the axon (Bradke & Dotti, 2000). However, these mechanisms appear to be complex. Signals may be "added on" as post-translational modifications including glycosylation, acylation or phosphorylation, or "built in," in the form of discrete amino acid sequences. For example, addition of mannose-6-phosphate to proteins directs them to lysosomes, while specific amino acid sequences have been identified that direct proteins into the nucleus or into mitochondria. In general, the targeting signals are likely to direct proteins to specific organelles, whereas other mechanisms, including attachment of specific motor proteins to selected MBOs, appear to direct organelles to the appropriate neuronal subdomain (Szodorai et al., 2009).

Once an organelle is committed to the dendritic or axonal compartment, additional mechanisms allow for their delivery at their final sites of utilization (Brady, 1993; Morfini et al., 2001). For example, synaptic vesicles needed for neurotransmitter release should go to presynaptic terminals. The problem is compounded because many presynaptic terminals are not at the end of an axon, particularly in CNS neurons. Often, numerous terminals occur sequentially along a single axon making *en passant* contacts with multiple targets. Thus, synaptic vesicles cannot merely move to the end of axonal MTs. Similar complexities arise with membrane proteins destined for the axolemma or a nodal membrane (Brady, 1993; Morfini et al., 2001).

One proposed mechanism for targeting of MBOs to synaptic terminals involves the synapsin family of phosphoproteins, which are concentrated in the presynaptic terminal (Greengard et al., 1994). The dephosphorylated form of synapsin I binds tightly to both synaptic vesicles and actin microfilaments (MFs), while phosphorylated synapsin I does not. Accordingly, dephosphorylated synapsin I inhibits axonal transport of MBOs in isolated axoplasm, while phosphorylated synapsin at similar concentrations has no effect (McGuinness et al., 1989). It is thought that when a synaptic vesicle passes through a region rich in dephosphorylated synapsin, it may be cross-linked to the available MF matrix by synapsin I. Such cross-linked vesicles would be removed from fast axonal transport and effectively targeted to the presynaptic terminals, which is a synapsin I- and MF-rich domain.

More recent studies have begun to map additional pathways for regulation of fast axonal transport based on selective, localized activation of kinase and phosphatase activities within neurons (Morfini et al., 2001; Morfini et al., 2009). Some of these phosphotransferase activities have been shown to target specific molecular motors and to regulate some of

their functional activities (Morfini et al., 2009). For example, activation of the kinases GSK3 and JNK3 in axons leads to phosphorylation of specific subunits of the motor protein kinesin, and these events results in detachment of kinesin from its transported cargo (Morfini et al., 2002) or from MTs (Morfini et al., 2009), respectively. Other phosphotransferases and cytoskeletal proteins indirectly modulate fast axonal transport by regulating the activity of enzymes that in turn modify molecular motors (Lapointe et al., 2009; Morfini et al., 2004). In this way, molecular pathways leading to the local delivery of MBOs in axons have started to be identified. Such mechanisms are consistent with observations of differential phosphorylation of neurofilament proteins and microtubule associated proteins throughout specific axonal subdomains (see Chapter 6).

Finally, this section has focused almost entirely on axonal transport, but dendritic transport also occurs (Horton & Ehlers, 2004). Since dendrites usually include postsynaptic regions while most axons terminate in presynaptic elements, dendritic and axonal transport each receive a number of unique proteins. An added level of complexity for intraneuronal transport phenomena is the intriguing observation that some mRNAs are routed into dendrites (Martin & Zukin, 2006) and local protein synthesis occurs at postsynaptic sites (Steward, 1995), where it participates in the regulation of synaptic plasticity (Glanzer & Eberwine, 2003; Steward & Schuman, 2003). While ribosomal components and mRNA are largely excluded from axons of mature neurons, some experimental evidence suggests that some protein synthesis could take place in axons of developing neurons (Lin & Holt, 2008). The biological significance of this process remains unknown, but has been proposed to play a role in axonal guidance and regeneration. The importance of dendritic mRNA transport and local protein synthesis is underscored by the demonstration that the mutation associated with Fragile X syndrome affects a protein important for transport and localization of mRNA in dendrites (Bassell & Warren, 2008). Similar processes of mRNA transport have been described in glial cells, particularly oligodendrocytes and Schwann cells (Kalwy & Smith, 1994). Remarkably, a recent report demonstrated that protein synthetic machinery from Schwann cell processes could be transferred into axons after injury (Court et al., 2008).

SLOW AXONAL TRANSPORT

Cytoplasmic and cytoskeletal elements move coherently at slow transport rates

Two major rate components have been described for slow axonal transport, representing movement of cytoplasmic constituents including cytoskeletal elements and soluble enzymes of intermediary metabolism (Brady, 1993). Cytoplasmic and cytoskeletal elements in axonal transport move with rates at least two orders of magnitude slower than fast transport. In favorable systems, the coherent movement of neurofilaments and microtubule proteins provides strong evidence for the Structural Hypothesis.

Striking evidence was provided by pulse-labeling experiments in which NF proteins moved over periods of weeks as a bell-shaped wave with little or no trailing of NF protein. Similarly, coordinated transport of tubulin and MAPs makes sense only if MTs are being moved, since MAPs do not interact with unpolymerized tubulin (Baas & Brown, 1997).

Slow Component a (SCa) largely comprises the cytoskeletal proteins that form NFs and MTs. Rates of transport for SCa proteins in mammalian nerve range from 0.2–0.5 mm/day in optic axons to 1 mm/day in motor neurons of the sciatic nerve, and can be even slower in poikilotherms such as goldfish. Although the polypeptide composition of SCa is relatively simple, the relative contribution of SCa to slow transport varies considerably. For large axons (i.e., alpha motor neurons in the sciatic nerve), SCa is a large fraction of the total protein in slow transport, while the amount of material in SCa is relatively reduced for smaller axons (i.e., optic axons) (Oblinger et al., 1987). The amount and phosphorylation state of SCa protein in axons is the major determinant of axonal diameter (de Waegh et al., 1992).

Slow Component b (SCb) represents a complex and heterogeneous rate component, including hundreds of distinct polypeptides ranging from cytoskeletal proteins like actin (and tubulin in some nerves, see Oblinger et al., 1987) to soluble enzymes of intermediary metabolism (such as glycolytic enzymes). The structural correlate of SCb is not as easily identifiable as the MTs and NFs of SCa. Actin is presumed to form MFs, but actin represents only 5–10% of the protein in SCb and a significant fraction of axonally transported actin is deposited in the membrane cytoskeleton of the axon. Most proteins in SCb may be assembled into labile aggregates that can interact transiently with the cytoskeleton. Recent studies with live cell imaging of fluorescent SCb proteins in transport are consistent with this idea (Roy et al., 2007).

Although SCa and SCb can be identified in all nerves examined to date, their rates and protein compositions varies among different nerve populations. For example, SCa and SCb are readily resolved as discrete waves moving down optic axons, but differences in rate are smaller in the motor axons of sciatic nerve, where the two peaks partially overlap. Moreover, virtually all tubulin moves as a single peak in SCa in optic axons, but significant amounts of tubulin move at both SCa and SCb rates in sciatic motor axons (Oblinger et al., 1987). In each nerve, certain polypeptides may be used to define the kinetics for a given slow component of axonal transport. For SCa, those signature polypeptides are the NF triplet proteins, while actin, clathrin and calmodulin serve a similar role for SCb.

Axonal growth and regeneration are limited by rates of slow axonal transport

The rate of axonal growth during development and regeneration of a nerve is roughly equivalent to the rate of SCb in that neuron (S.T. Brady, 1993). This suggests that critical roles are played by slow axonal transport in growth and regeneration. During development, SCb proteins are prominent and relatively little NF protein is detectable. Tubulin can be

detected moving at both SCb and SCa rates. Once an appropriate target is reached and synaptogenesis begins, there is a dramatic upregulation of NF protein synthesis and a gradual slowing of slow transport.

Axonal regeneration involves a complex set of cell body and axonal responses to a lesion. Downregulation of NF triplet proteins and upregulation of specific tubulin isotypes are hallmarks of cell body responses to a lesion. In CNS neurons that fail to regenerate, changes in NF and tubulin expression are reduced or absent. Since changes in protein expression do not alter axonal cytoskeletal composition until after a regenerating growth cone has formed and extended for some distance, such changes in expression do not affect neurite growth, but rather may reflect activation of a cellular program for neurite growth. Axonal MTs must be disassembled in order to reorganize the cytoskeleton for growth, and then reassembled for neurite extension. During axonal growth or regeneration, the expression of specific tubulin genes is upregulated. In addition, there are characteristic changes in the axonal transport of tubulin, with an increase in the fraction of tubulin moving at SCb rates. During development and in regeneration, MAPs also are differentially expressed.

Properties of slow axonal transport suggest molecular mechanisms

Information about molecular mechanisms underlying slow axonal transport is relatively limited. They are energy dependent and require intact MTs. Accordingly, transport of NFs can be pharmacologically uncoupled from MT transport without eliminating slow transport (Griffin et al., 1995). Recent imaging studies in cultured neurons confirmed these findings, further showing that transport of NFs (Francis et al., 2005) and various proteins in SCb does not depend on actin (Roy et al., 2008). In contrast, pharmacological agents that disrupt MTs appear to block slow transport of all components. While this does not rule out a role for the MF cytoskeleton in slow transport movements, MTs appear to be required for the transport of other elements of the cytoskeleton.

The macroscopic transport velocity rates measured by radiolabeling experiments should not be taken to reflect maximum rates of the motors involved. As with mitochondrial transport, the net rate velocity of slow component proteins reflects both the rate of actual movement and the fraction of a time interval that a structure is moving (Brady, 2000). The large size and elongated shape of cytoskeletal structures and their potential for many interactions means that net displacements are discontinuous. If a structure is moving at a speed of 2 μm/sec, but on average only moves at that rate for 1 out of every 100 seconds, then the average rate for the structure will translate to an net rate of only 0.02 μm/sec (Baas & Buster, 2004).

Recently, methods for direct visualization of fluorescently tagged MTs and NFs in cultured neurons have been developed (Brown, 2003). In such studies, relatively short MT or NF segments can be seen to move as rapidly as MBOs moving in fast axonal transport, although they move much less frequently. Integration of these infrequent rapid movements of MTs and NFs over time gives a net rate of approximately 1–2 mm/day instead of the 200–400 mm/day seen for MBOs in mammalian nerve. Other studies permitted visualization of microtubules nucleated at the microtubule organizing center and being translocated toward the cell periphery. The combination of studies of axonal transport using radiolabels and direct observations of individual MTs or NFs with video microscopy provides strong experimental evidence that MTs and NFs can and do move in the axon as intact cytoskeletal structures. As discussed below, there are still questions about the specific motors and mechanisms underlying these movements.

Like membrane proteins, cytoplasmic and cytoskeletal proteins are differentially distributed in neurons and glia. Progress has been made toward identification of targeting mechanisms and some general principles have begun to emerge. Since cytoplasmic constituents move only in the anterograde direction, a key mechanism for targeting of cytoplasmic and cytoskeletal proteins appears to be differential metabolism (Brady, 1993). Concentration of actin and other proteins in presynaptic terminals can be explained by slower turnover in the presynaptic terminal relative to NF proteins and tubulin. Proteins with slow degradative rates in the terminal would accumulate and reach a higher steady-state concentration. Thus, alteration of degradation rates for a protein can change the rate of accumulation for that protein. For example, some protease inhibitors cause the appearance of neurofilament rings in affected presynaptic terminals (Roots, 1983).

Although slow axonal transport of cytoskeletal proteins has received the most attention, all other cytoplasmic proteins must be delivered to specific neuronal compartments of the neuron as well. Many of these have been defined as part of the "cytosol" or soluble fraction that results from biochemical fractionation. These include the enzymes of glycolysis and regulatory proteins like calmodulin and HSC70 (Brady, 1993). However, in pulse-chase radiolabel studies, soluble proteins move down the axon as regularly and systematically as cytoskeletal proteins. Again, this coherent transport of hundreds of different polypeptides appears consistent with the Structural Hypothesis and indicates a higher level of organization of cytoplasmic proteins than has been traditionally assumed (Lasek & Brady, 1982). Such organization is likely necessary to facilitate interactions with motor proteins and targeting mechanisms and to assure a reliable delivery of all required proteins to the axon at appropriate stoichiometries.

MOLECULAR MOTORS: KINESIN, DYNEIN AND MYOSIN

Prior to 1985, the only molecular motors characterized in vertebrate cells were muscle myosins and flagellar dyneins. Myosins had been purified from nervous tissue, but no clear functions were established. The pharmacology and biochemistry of fast axonal transport created a picture of organelle transport distinct from muscle contraction or flagellar beating. Moreover, the biochemical properties of fast transport were inconsistent with both myosin and dynein (Brady et al., 1985; Brady, 1991).

The characteristic biochemical properties of different molecular motors aided in their identification

One striking difference between fast axonal transport and myosin- or dynein-based motility emerged from studies with ATP analogues. Adenylyl-imidodiphosphate (AMP-PNP), a nonhydrolyzable analogue of ATP, is a weak competitive inhibitor of both myosin and dynein. However, when AMP-PNP is perfused into axoplasm, bidirectional transport stops within minutes (Brady et al., 1985; Lasek & Brady, 1984). Both anterograde and retrograde moving organelles freeze in place on microtubules, and "pearls on a string" structures became apparent. Inhibition of fast axonal transport by AMP-PNP in isolated squid axoplasm indicated that fast axonal transport involved a novel class of motors and further suggested that this new motor should have a high affinity for microtubules in the presence of AMP-PNP. The polypeptide composition of this new motor molecule was soon defined and it was christened kinesin (Brady et al., 1985; Vale et al., 1985). This discovery raised the possibility of other novel motor molecules and soon molecular biology techniques allowed the discovery of additional classes of molecular motors (Aizawa et al., 1992). The proliferation of motor types has transformed our understanding of cellular motility.

With all mouse and human genes identified, it is currently known that each class of molecular motor proteins corresponds to large protein families with diverse cellular functions (Miki et al., 2003). Both the kinesin family (Hirokawa et al., 2009) and myosin family (Brown & Bridgman, 2004; Kalhammer & Bahler, 2000) have been defined and their proteins grouped into subfamilies. Finally, the elusive cytoplasmic version of dynein was identified and a multigene family of flagellar and cytoplasmic dyneins defined (Asai & Wilkes, 2004). Members of a given motor protein family share significant homology in their motor domains with the defining member (kinesin, cytoplasmic dynein or myosin), but they also contain unique protein domains that are specialized for interaction with different cargoes or differential regulation (Hirokawa et al., 2009) . This large number of motor proteins may reflect the number of cellular functions that require force generation or movement, ranging from mitosis to morphogenesis to transport of vesicles. In this chapter, we focus on major motor proteins known to be important for axonal transport or neuronal function, starting with conventional kinesin.

Kinesins mediate anterograde fast axonal transport in a variety of cell types

Since their discovery, much has been learned about the biochemical, pharmacological and molecular properties of kinesins (Brady & Sperry, 1995; Hirokawa et al., 2009). Conventional kinesin is the most abundant member of the kinesin superfamily in vertebrates and is widely distributed in neuronal and non-neuronal cells. The holoenzyme is a heterotetramer comprising two heavy chains of 115–130 kDa and two light chains of 62–70 kDa. Structural studies have shown that kinesin is a rod-shaped protein approximately 80 nm long, with two globular heads connected to a fanlike tail by a long stalk. High-resolution electron microscopic immunolocalization of kinesin subunits and molecular genetic studies both indicate that kinesin heavy chains are arranged in parallel with their amino terminals forming the heads and much of the stalk (Hirokawa et al., 1989). The kinesin heavy chain heads (also known as kinesin-1 or KIFs) comprise the motor domains, containing both ATP and MT binding motifs. This motor domain is the most highly conserved region within the kinesin family. Binding of kinesin to MTs is stabilized by AMP-PNP, and this property remains a major hallmark of kinesins. Kinesin light chains (KLCs) localize to the fan-like tail and may also contribute to part of the stalk. The α-helical coil–coiled domains that are present in both heavy and light chains form the stalk itself. KLCs appear to be unique to conventional kinesin, but are highly conserved across species. These subunits are thought to be involved in organelle binding and may also play a role in targeting to different types of MBO (Stenoien & Brady, 1997).

A large body of evidence implicates conventional kinesin as a motor molecule for fast axonal transport. Kinesin-1 is an MT-activated ATPase with minimal basal activity. MTs will glide across kinesin-1–coated glass surfaces with motor movement toward the MT-plus end. Since axonal MTs have a uniform polarity with their plus ends oriented towards the cell periphery, the directionality of kinesin is consistent with an anterograde transport motor. Immunofluorescence and electron microscopy studies showed that conventional kinesin is associated with MBOs that are, in turn, associated with MTs (Hirokawa et al., 1989; Pfister et al., 1989) (Fig. 8-7). While these properties of conventional kinesin are consistent with a role in axonal transport of MBOs, they are insufficient to prove the hypothesis that this motor is responsible for fast axonal transport. Such proof came from inhibition of conventional kinesin function in isolated axoplasm by antibodies against KLC subunits (Stenoien & Brady, 1997). Since KLCs are only associated with conventional kinesin, the ability of anti-KLC antibodies to inhibit transport is compelling evidence that conventional kinesin is involved in fast axonal transport. Finally, reduction of kinesin heavy chain levels using antisense oligonucleotides and gene deletion studies also implicate conventional kinesin in axonal transport processes (Amaratunga et al., 1993).

In neurons and non-neuronal cells, conventional kinesin is associated with a variety of biochemically heterogeneous MBOs, ranging from synaptic vesicles to mitochondria to lysosomes (Hirokawa et al., 1989; Pfister et al., 1989). In addition to its role in fast axonal transport and related phenomena in non-neuronal cells, conventional kinesin appears to be involved in constitutive recycling of membranes from the Golgi to the endoplasmic reticulum. However, kinesin is not associated with all cellular membranes or MBOs. For example, the nuclear membrane, membranes of the Golgi complex and the plasma membrane all appear to lack conventional kinesin (Hirokawa et al., 1989; Pfister et al., 1989). Current evidence suggests the interactions of conventional kinesin with membranes are thought to involve KLCs and/or the carboxyl termini of heavy chains. However, neither this selectivity nor the molecular basis for binding of kinesin and other motors to membranes are well understood.

Cloning and immunochemical studies of conventional kinesin subunits have demonstrated that multiple isoforms of kinesin heavy and light chains occur in brain. Three kinesin heavy chain genes (also known as kinesin-1 or KIF5) are expressed in mammals, including a ubiquitously expressed one (kinesin-1b) and two kinesin heavy chain genes (kinesin1a and c) mainly enriched in neuronal tissues (Miki et al., 2003). Radiolabeled kinesin-1s move down the axon at different net rates that correlate with different MBO types, such as synaptic vesicles and mitochondria (Elluru et al., 1995). In addition, biochemical fractionation studies showed differential association of kinesin-1s with specific organelles (Deboer et al., 2008). At least two different genes exist for KLCs (KLC1 and KLC2) in nerve cells and differential splicing for one of these genes generates various isoforms differentially expressed in tissues (Cyr et al., 1991). Heterogeneity in kinesin heavy and light chain subunits may allow for transport of different MBO types and ensure that organelles are delivered at their correct destinations in the axon. Current evidence suggests that the different combination of subunits may produce functionally diverse forms of conventional kinesin and allow transport of different types of organelles in mature neurons (Deboer et al., 2008).

Mechanisms underlying attachment of motors to transported MBOs remain elusive

Metabolic labeling and sciatic nerve ligation experiments indicate that all axonally transported kinesin associates with MBOs *in situ*. Intriguingly, mitochondria, synaptic vesicle precursors, coated vesicles and post-Golgi carriers all move anterogradely and all have conventional kinesin associated with their surface, even though these MBOs have few or no integral membrane proteins in common (Leopold et al., 1992). Similarly, cytoplasmic dynein associates with retrogradely moving MBOs of unique biochemical composition, including multivesicular bodies, late endosomes and lysosomes. These observations suggest that specific targeting of molecular motors to selected types of MBOs. Supporting this notion, immunoelectron microscopic experiments showed a discrete localization of conventional kinesin on the surface of MBOs (Leopold et al., 1992). However, molecular mechanisms underlying the interaction of molecular motors with biochemically heterogeneous MBOs remains uncertain (Akhmanova & Hammer, 2010). One possibility that has emerged recently for targeting kinesins to organelles is the class of small GTPases, particularly members of the Rab family (see Chs. 7 and 21) (Szodorai et al., 2009).

In recent years, a myriad of proteins have been proposed to directly or indirectly bind to KLCs and serve as cargo receptors for conventional kinesin (Hirokawa et al., 2009) (Akhmanova & Hammer, 2010). In most cases, the initial identification of proposed polypeptides was accomplished by approaches aimed to detect isolated, high-affinity protein–protein interactions, including two-hybrid system assays and immunoaffinity purification procedures. Intriguingly, a large proportion of candidate receptors identified to date were shown to bind directly to the tandem repeat (TR) region of KLCs *in vitro*. Although a role of the TR domain in the tight binding of conventional kinesin to membranes is well established (Stenoien & Brady, 1997; Tsai et al., 2000), this domain can bind to many polypeptides in a nonspecific manner *in vitro*, including the heterologous, highly soluble protein GFP (Lazarov et al., 2005). Also, TRs are present in all KLC splice variants (Cyr et al., 1991), and thus the association of specific KLCs with selected MBOs appears inconsistent with TRs being the only link between conventional kinesin and MBOs. Regardless, it is unclear how conventional kinesin would be targeted to different classes of MBOs with no common polypeptides on the sole basis of a TR-mediated interaction. These issues raise concerns about the physiological significance of many candidate receptor proteins identified to date.

Most candidate receptor proteins proposed to date fail to fulfill the criteria expected for a conventional kinesin receptor, including kinectin (Brady & Sperry, 1995) and APP (Lazarov et al., 2005). Ultrastructural observations indicate that a significant stretch of the kinesin's heavy chain stalk domain interacts with their membranous cargoes, with metabolic labeling experiments and biochemical fractionation experiments supporting this idea. The available experimental evidence suggests that both the variable tail domain of kinesin heavy chains and the carboxy terminus of KLCs might help targeting biochemically heterogeneous forms of conventional kinesin to selected MBOs. The TR domain of KLCs, on the other hand, likely contributes to the tight binding of conventional kinesins to MBOs (Stenoien & Brady, 1997). Additional work is needed to establish the precise functional role of each conventional kinesin subunit in this process.

Multiple members of the kinesin superfamily are expressed in the nervous system

Kinesin has been purified and cloned from many species, including *Drosophila*, squid, sea urchin, chicken, rat, and human. Both heavy and light chain subunits of conventional kinesin are highly conserved throughout. However, once the sequence of the kinesin motor domain was available, related proteins with homology only in the motor domain began to be identified. Kinesin-related proteins (KRPs) were first identified in yeast and fungal mutants with defective cell division, but many others are now known (Hirokawa et al., 2009) with more than 40 different genes expressed in mouse and human. A careful analysis of kinesin superfamily sequences from many species led to the definition of a standardized kinesin nomenclature for 15 defined families of kinesins (Lawrence et al., 2004), where conventional kinesin is the founding member.

Motor proteins of the kinesin superfamily all have well-conserved motor domains, but KRPs are highly variable in sequence and structure. Even the position of the motor domain in the overall sequence varies. Kinesin-1 and many other family members have amino terminal motor domains, but other KRPs have motor domains at their carboxyl termini and some have centrally located motor domains. This variation in structure has functional significance. Most motor proteins tested featuring amino and central motor domains have been found to move toward the MT plus end, while those with their motor domain located at the carboxy terminus move toward the MT minus end. Many KRPs are known only

from their sequences and expression profile, but only a few have been examined for function. Many KRPs are involved in various steps of cell division, but precise cellular functions are still being defined for many motors.

Systematic cloning strategies based on the conserved motor domain sequences have identified a remarkable number of KRPs expressed in brain (Miki et al., 2003). Members of several KRP families expressed in brain have been implicated in forms of MBO transport. Kinesin-2 family members have been implicated in assembly and maintenance of cilia and flagella and mutations in these motors can lead to sensory defects and polycystic kidney disease (Scholey, 2003). Kinesin-2 motors are heterotrimers with two related heavy chain subunits and a larger accessory subunit. Kinesin-3 family members were proposed as a synaptic vesicle motor because kinesin-3 mutants in the nematode *C. elegans* had defects in synaptic vesicle localization. Various other kinesin family members expressed in nervous tissue have been implicated in the transport of other MBO classes (Hirokawa et al., 2009). The extent to which these kinesins reflect unique transport mechanisms rather than functional redundancy within the kinesin family is not known.

Curiously, functions proposed for some brain KRPs are very different from functions proposed for similar or identical KRPs in non-neuronal cells. For example, members of the kinesin-13 family have been implicated in both mitotic spindle function and in axonal membrane transport. Similarly, a mouse kinesin-4 was reported to associate with unidentified MBOs in neurites, but its chicken homolog binds to chromosomal DNA and mediates chromosome movements in the mitotic spindle. Finally, a kinesin-6 was originally found to have a role in mitotic spindle function, but members of the kinesin-6 family were also implicated in the transport of MTs into dendrites (Baas, 2002). Although kinesins were the last family of motor proteins to be discovered, the kinesin family has proven to be remarkably diverse. Fifteen distinct subfamilies in the kinesin family have been identified, all with homology in their motor domain (Lawrence et al., 2004). Within a subfamily, however, the more extensive sequence similarities are presumed to reflect related functions. At present, many questions remain about the function of these various motors in the nervous system.

Cytoplasmic dyneins have multiple roles in the neuron

The original identification of conventional kinesin as a plus end–directed microtubule motor suggested that it is involved in anterograde transport, but the identity of the retrograde motor remained an open question. Since flagellar dynein was known to be a minus end–directed motor, interest in cytoplasmic dyneins was renewed. Identification of the cytoplasmic form of dynein in nervous tissue came as an indirect result of the discovery of kinesin.

Although dynein binding to MTs is not stabilized by AMP-PNP, both cytoplasmic dynein and kinesin associate with microtubules in nucleotide-depleted extracts and both are released by addition of ATP. Early studies with ATP-free MT-enriched extracts showed that they are substantially enriched in a minor high molecular weight microtubule associated protein that was originally called MAP1c. Biochemical analysis showed that MAP1c was not related to the well-defined microtubule-associated proteins MAP1a and1b, but instead was closely related to flagellar dynein heavy chains. This discovery led to purification and characterization of brain cytoplasmic dynein (Paschal et al., 1987). Like flagellar dyneins, the cytoplasmic dynein holoenzyme is a high–molecular weight protein complex comprising two heavy chains, two dynein intermediate chains, four light intermediate chains and various light chains that form a complex of more than 1,200 kDa (Brill & Pfister, 2000).

As with the kinesins, dynein heavy chains are a multigene family with multiple flagellar and cytoplasmic dynein genes (Asai & Wilkes, 2004). The 530 kDa dynein heavy chain contains the ATPase activity and MT binding domains of dynein. There may be 10–15 dynein heavy chain genes in an organism, but the large size of the dynein heavy chain primary sequence slowed genetic analyses. At present, dynein genes are grouped as members of either flagellar or cytoplasmic dynein subfamilies. The three intermediate (74 kDa), four light intermediate (55 kDa) and a variable number of light chains present in dyneins may also have flagellar and cytoplasmic forms.

The two or more cytoplasmic dynein heavy chain genes could be involved in different cellular functions, but much dynein functional diversity may be due to its many associated polypeptides (Susalka & Pfister, 2000). The intermediate and light chains of cytoplasmic dynein are thought to be important both for regulation and for interactions with specific cellular structures (Brill & Pfister, 2000). In addition, a second protein complex known as dynactin copurifies with cytoplasmic dynein under some conditions (Schroer, 2004). The dynactin complex is similar in size to dynein and contains multiple subunits that include p150Glued, dynamitin, an actin-related protein, and two actin capping polypeptides, among others. The p150Glued polypeptide interacts with both dynein intermediate chains and the actin related subunits. Dynamitin may play a role in the binding of cytoplasmic dynein to different types of cargo. Finally, the actin related protein (Arp1) forms a short filament that may include actin as well as actin-capping proteins. This short filament may interact with both p150Glued and components of the membrane cytoskeleton like spectrin. Dynactin may mediate cytoplasmic dynein binding to selected cargoes, including the Golgi complex and the membrane cytoskeleton. The wide range of functions associated with cytoplasmic dynein is matched by its complexity and its ability to interact with accessory factors (Susalka & Pfister, 2000). Additional proposed functions include a role in mitosis and in anchoring and localizing the Golgi complex.

A number of studies have implicated cytoplasmic dynein as playing a role in retrograde axonal transport (Brady, 1991; Hirokawa, 1998). *In vitro* motility studies demonstrate that cytoplasmic dynein generates force towards the minus ends of MTs, consistent with a retrograde transport motor. Cytoplasmic dynein also accumulates on the distal side of a nerve ligation, coincident with retrogradely transported MBOs. Finally, retrograde transport has been reported to be more sensitive than anterograde transport to UV-vanadate treatment. Since exposure of dynein to UV irradiation in the

presence of vanadate and ADP cleaves the dynein heavy chain, this has been a signature of dyneins, although other ATP binding proteins may be affected as well.

In the nervous system, the most frequent role proposed for dynein is a motor for retrograde axonal transport, but its properties are also consistent with a motor for slow axonal transport (Ahmad et al., 1998). Consistent with this possibility, studies on the axonal transport of radiolabeled cytoplasmic dynein indicated that most cytoplasmic dynein and dynactin moved with SCb (Dillman et al., 1996). The ability of dynactin to interact with both cytoplasmic dynein and the membrane cytoskeleton suggests a model in which dynactin links dynein to the membrane cytoskeleton, providing an anchor for dynein-mediated movement of axonal microtubules (Ahmad et al., 1998; Susalka & Pfister, 2000). Some anchoring role for the membrane-associated cytoskeleton in the mechanisms of slow axonal transport is likely, since neurons require interaction with a solid substrate for neurite growth. Taken together, a variety of studies suggest that cytoplasmic dynein has a wide variety of functions in the nervous system from anchoring the Golgi to retrograde transport of MBOs to transport of MTs into axons. Thus, cytoplasmic dynein appears to fulfill many cellular functions that require minus end–directed MT movements. As observed for conventional kinesin, phosphorylation-based regulatory mechanisms for cytoplasmic dynein have been documented in neurons (Morfini et al., 2007).

Different classes of myosin are important for neuronal function

Myosins are remarkably diverse in structure and function. To date, 15 subfamilies of myosin have been defined by sequence homologies (Kalhammer & Bahler, 2000). The brain is an abundant source of non-muscle myosins and one of the earliest studied. Despite their abundance and variety, the roles of myosins in neural tissues have only recently begun to be defined (Bridgman, 2009; Brown & Bridgman, 2004). Myosin II is in the same subfamily as the myosins found in muscle thick filaments and it forms large, two-headed myosins with two light chains per heavy chain. Although myosin II is abundantly expressed in brain, little is known about its function in the nervous system. In other non-muscle cells, myosin II has been implicated in many types of cellular contractility and may serve a similar function in developing neurons. Intriguingly, myosin II remains abundant in the mature nervous system, where examples of cell contractility are less common.

The second myosin type identified in nervous tissue was the myosin I family. It was first described in protists and subsequently purified from brain. Myosin I is a single-headed myosin with a short tail that uses calmodulin as a light chain (Kalhammer & Bahler, 2000). In many cell types it has been implicated in both endocytosis and exocytosis, so it may play an important role in delivery and recycling of receptors. Myosin I is enriched in microvilli and may also be involved in some aspects of growth cone motility, along with myosins from other subfamilies. In both cases, it may link MF bundles to the plasma membrane through a membrane-binding domain. The myosin I family has also been implicated in mechanotransduction by the stereocilia of hair cells in the inner ear and vestibular apparatus. A myosin I isoform, myosin Iβ, has been localized to the tips of stereocilia, where it appears to mediate sensory adaptation by opening and closing the stretch-activated calcium channel (see Chapter 53).

Two other myosin types have been implicated in hearing and vestibular function (Libby & Steel, 2000). The defect in the Snell's waltzer mouse was found to be a mutation in a myosin VI gene that produces degeneration of the cochlea and vestibular apparatus. Myosin VI is localized to the cuticular plate of the hair cell under stereocilia. Similarly, mutations in a myosin VII gene are responsible for the shaker-1 mouse and several human genetic deafness disorders. This myosin, myosin VIIa, is found in a band near the base of the stereocilia distinct from distributions of myosin Iβ and myosin VI.

Another myosin type that plays a role in nervous tissue is myosin V (Kalhammer & Bahler, 2000). Of the myosins identified in brain, myosin I and V are the strongest candidates to act as organelle motors, and myosin V has been reported in association with vesicles purified from squid axoplasm. Myosin V is the product of the mouse dilute locus. Mice carrying the mutant dilute allele show defects in the movement of pigment granules, and this results in dilution of their coat color. These mice also exhibit complex neurological defects that may be due to altered endoplasmic reticulum localization in dendrites. In addition, an interaction between kinesin and myosin V has also been reported. Curiously, a form of myosin V found in yeast complements a mutation in a KRP gene, suggesting an interaction between these two motor molecules. Finally, there is evidence that myosin V plays a role in growth cone motility, where it is enriched in filopodia. Unlike MT-based motors, myosins appear to transport MBOs for short distances within discrete, actin-rich subcellular compartments, including presynaptic terminals (Brown et al., 2004).

Matching motors to physiological functions may be difficult

The three classes of motors are similar in their biochemical and pharmacological sensitivities in many respects (Brady, 1991). However, some hallmark features can be used to identify a motor. In the case of kinesin, the most distinctive characteristic is stabilization of binding to MTs by AMP-PNP. The affinities of myosin for MFs and dynein for MTs are weakened by treatment with either ATP or AMP-PNP. As a result, if a process is frozen in place by AMP-PNP, then kinesins are likely to be involved. If kinesins are not involved in a process that requires MTs, then dyneins are likely to be involved. Similarly, processes requiring MFs suggest that myosins are required. In the case of fast axonal transport, we know that MTs are required and that this process is completely inhibited by AMP-PNP, thus implicating the kinesin family. The development of new pharmacological and immunochemical probes specific for different motors will facilitate future studies.

Although many motor proteins are found in nervous tissue, there are few instances in which we fully understand their cellular functions. The proliferation of different motor molecules and the existence of numerous isoforms raise the

possibility that some physiological activities require multiple motors. There may be cases in which motors serve a redundant role to ensure that the physiological activity is maintained in the event of a loss or deregulation of a given motor protein. Finally, the existence of so many different types of motor molecules suggests that novel physiological activities requiring molecular motors may be as yet unrecognized.

AXONAL TRANSPORT AND NEUROPATHOLOGY

A growing literature has emerged that links defects in fast axonal transport to synaptic dysfunction and neurodegeneration in a variety of diseases (Morfini et al., 2009; Roy et al., 2005). Inhibition of fast axonal transport results in loss of synaptic function and "dying-back" degeneration of axons. The earliest suggestions that inhibition of fast axonal transport could result in neurodegeneration came from studies on exposure to neurotoxins that inhibit transport. For example, the neurotoxicant acrylamide has been found to inhibit kinesin function directly. Similarly, neuropathies are a common side effect of the cancer therapeutic agent vincristine, which depolymerizes axonal microtubules.

More recently, genetic evidence for a role of axonal transport in neurodegeneration has been obtained. A loss-of-function mutation in the kinesin heavy chain isoform kinesin-1a (also known as KIF5A) results in a hereditary form of spastic paraplegia, a disease characterized by progressive dysfunction and degeneration of upper motor neurons (see Box 8-1). Similarly, mutations in dynein have been implicated in "dying back" degeneration as well. For example, certain mutations in dynein heavy chain lead to sensory neuropathies in mammals (Dupuis et al., 2009). Curiously, mutations in the dynactin subunit p150^{Glued} can produce symptoms of motor neuron disease (Laird et al., 2008). In many cases, mutant motor proteins are expressed in many neuronal and non-neuronal cells, (i.e., the ubiquitously expressed cytoplasmic dynein heavy chain), but the disease phenotype preferentially affects specific neuronal populations. Moreover,

different mutations in the same molecular motor protein subunit can cause different pathologies. For example, while some mutations in p150^{Glued} primarily cause sensory neuron disease, a different set of mutations results in Perry's syndrome, which instead is characterized by degeneration of cortical and extrapyramidal neurons (Farrer et al., 2009). A molecular basis for the increased vulnerability of selected neuronal populations to mutations in specific motor subunits is currently unknown, but may result from unique functional specializations of these neuronal cell types. Regardless, a strong body of genetic evidence supports the notion that deficits in fast axonal transport suffice to cause "dying-back" degeneration of neurons (Morfini et al., 2009; Roy et al., 2005).

Significantly, defects in synaptic function and "dying-back" degeneration of axons represent early pathogenic events characteristic of several major adult-onset neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and polyglutamine expansion diseases, among others. Accordingly, alterations in fast axonal transport have been documented in all these diseases (Morfini et al., 2009; Roy et al., 2005). These diseases are not associated with mutations in molecular motors, so other mechanisms are thought to underlie the abnormalities in fast axonal transport observed in each disease. A physiological change common to all of these diseases is alterations in the activity of specific protein kinases (Wagey & Krieger, 1998). Interestingly, many of the kinases altered in neurodegeneration have been implicated in regulation of motor protein function, providing a potential pathogenic mechanism (Morfini et al., 2009). The emerging complexity and variety of regulatory pathways for fast axonal transport appears consistent with a common pathogenic mechanism underlying different familial forms of adult onset neurodegenerative diseases like Alzheimer's and Huntington's diseases. Such diseases represent a novel class of neurological disease that can be collectively characterized as "dysferopathies" from the Greek word for *transport* or *carry* (Morfini et al., 2007; Pigino et al., 2009). Given the unique dependence of neurons on axonal transport for development and maintenance of neuronal function, these processes provide an explanation for the selectivity of these pathologies for neurons.

AXONAL TRANSPORT DEFICITS AND PATHOGENIC MECHANISMS IN HEREDITARY SPASTIC PARAPLEGIAS

Matthew Burns, Gerardo Morfini

An increasing number of neurodegenerative diseases have been shown to follow a "dying-back" pattern, in which neurons begin to degenerate at the synapse and axon, and then slowly die back towards the cell body. Dying-back neuropathies are characterized by a loss of synaptic and axonal connectivity, development of axonal swellings and abnormal accumulation of transported organelles (MBOs). These early pathological events precede neuronal cell death, but correlate well with the onset of early symptoms of disease (Morfini et al., 2009). This pattern of cell degeneration suggests that early pathological events in the

synaptic and/or axonal compartments may be central to the pathogenesis of disease.

One instructive example of dying-back neuropathy is seen in the hereditary spastic paraplegias (HSPs). HSPs are a genetically diverse group of disorders where mutations in one of over 40 different genes result in degeneration of the corticospinal tracts and dorsal column fibers (Fink, 2006). Patients exhibit adult-onset progressive muscle weakness and spastic paralysis of the lower limbs and often require a walker or wheelchair. There is currently no medical treatment, and pathogenic mechanisms are poorly

AXONAL TRANSPORT DEFICITS AND PATHOGENIC MECHANISMS IN HEREDITARY SPASTIC PARAPLEGIAS （*cont'd*）

understood. Recent insights into the role of axonal transport deficits, however, may be central to understanding pathogenesis in HSPs and other dying-back neuropathies.

Autosomal dominant mutations in the SPG10 gene encoding kinesin-1a (also known as KIF5A), one of three isoforms of kinesin-1, results in one such form of HSP (Reid et al., 2002). The significance of this mutation is manifold. First, patients are heterozygotic for the mutation, so only one allele of kinesin-1a is affected, leaving intact the expression of the other SPG10 allele as well as the other two isoforms of kinesin-1. The kinesin 1a-null mouse dies at birth (Xia et al., 2003). This expression pattern suggests that a partial reduction in fast axonal transport is sufficient to cause neurodegeneration. Second, SPG10-HSP is an adult-onset disease. This suggests that a partial reduction in axonal transport may not produce clinical symptoms in affected neurons for years or even decades. Third, although kinesin-1a is expressed throughout the brain, only upper motor neurons degenerate. This selective degeneration suggests that reductions in specific components of axonal transport can produce degeneration of specific neuronal populations while leaving other neuronal populations relatively unaffected. More recent data have given further insight into the role of axonal transport in neurodegeneration. The SPG10 mutation affects the ability of kinesin1-a to bind to microtubules, suggesting that alterations in kinesin function may play a central role in SPG10 pathogenesis (Ebbing et al., 2008).

Axonal transport deficits have been implicated in other forms of HSP as well. Mutations in the microtubule-severing protein spastin are the most common cause of HSP, accounting for over 40% of diagnoses. Recent data show an inhibitory effect of pathogenic spastin mutations on both anterograde and retrograde fast axonal transport (Solowska et al., 2008), raising the possibility that additional forms of HSP may involve changes in axonal transport.

Deficits in axonal transport have been implicated in other forms of dying back neuropathy (Morfini et al., 2009). Recent data has implicated axonal transport deficits in the pathogenesis of the motor neuron diseases amyotrophic lateral sclerosis (ALS) and Huntington's disease. Similarly, both Alzheimer's and Parkinson's diseases display a dying-back pattern as well as evidence of alterations in axonal transport. More work is needed to more clearly elucidate the role of axonal transport in the pathogenesis of these and other dying-back neuropathies, but the common features shared among these various neurodegenerative diseases may provide an avenue for therapeutic intervention based on an understanding of the prominent role played by deficits of axonal transport in neurodegeneration.

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