Mini-Review

The Transport and Assembly of the Axonal Cytoskeleton

Peter J. Hollenbeck

Medical Research Council, Cell Biophysics Unit, London WC2B 5RL, England

Nerve cells display a number of unusual properties which are generally viewed as adaptations to their extreme length and regional specialization. The dense and highly organized neurofilament (NF)[1] and microtubule (MT) arrays which serve to support nerve axons provide one example; another is the phenomenon of axonal transport, by which macromolecules synthesized in the cell body move outward along the axon in several relatively discrete components with widely different velocities (3, 22, 32, 95; Fig. 1). During the 1970s, a crucial connection was made between these two properties of nerve cells by the demonstration that the slowest moving component of axonal transport (called slow component a [SCa]) conveys primarily tubulin and the three proteins comprising NFs (18, 23, 27, 33, 41, 52, 78, 96). This was achieved by a synthesis of the biochemical and ultrastructural data available at that time; since then, intensive study of neuronal cytoskeletal proteins has generated a succession of models of their transport, assembly, and turnover (39, 43, 58, 83, 93, 94). Recently, a number of studies have indicated that the components and interactions of the neuronal cytoskeleton may be more complex than previously thought. This review will attempt to evaluate whether current models are sufficient to accommodate the results of radiolabeling studies of axonal transport, ultrastructural studies, and recent work on the dynamics of the MT and NF systems. The principal questions to be addressed are (*a*) in what state are cytoskeletal proteins transported in axons; (*b*) how are their assembly and interactions regulated; and (*c*) in what region or regions of the axon does assembly of the cytoskeleton occur?

The State of Moving Cytoskeletal Proteins

Studies by Lasek and co-workers (7, 27, 78, 86) concerning the protein composition and transport behavior of SCa provided the first hypothesis about the state in which cytoskeletal proteins are transported in axons. They identified NF protein and tubulin as the principal components of SCa, and determined that their transport velocities were similar (27, 41). This apparent coordinated transport of cytoskeletal proteins, their restriction to relatively coherent waves, and electron microscopic evidence suggesting that axonal MTs and NFs were highly cross-linked (26, 54, 79, 92, 98) led them to propose that cytoskeletal proteins in the SCa wave traveled down the axon as a cross-linked matrix of assembled MT and NF polymers (7, 11, 40, 43, 86). A number of subsequent studies have compelled modification of this "moving matrix" model by demonstrating that the axonal cytoskeleton is neither continuous nor stably cross-linked. The presence of major discontinuities and asymmetries in the axonal cytoskeleton, such as breaks at nodes of Ranvier (85), and proximodistal gradients in the number of MTs and NFs (61, 99), makes it unlikely that the continuous elaboration of an assembled matrix can account for axonal ultrastructure. Furthermore, two types of data indicate that connections between MTs and NFs revealed by electron microscopy are actually weak or transient. First, it is now clear that tubulin and NF protein are transported neither coordinately nor in nonspreading waves in most neurons. Rather, the relative velocities and distributions of these proteins during axonal transport seem to be highly variable between different types of neurons (16, 19, 53, 55, 61, 65, 68, 84); in particular, in the axons of most peripheral nerves, tubulin is transported at two different velocities, only one of which corresponds to SCa. A second line of evidence comes from observations of the rapid axonal transport of membrane-bounded organelles which indicate that they can pass with little hindrance through axoplasm (1, 13, 42), despite its impenetrable appearance when viewed by electron microscopy (26, 79). These considerations have led to the suggestion that cytoskeletal proteins travel in the form of individual, sliding MT and NF polymers, assembled but not stably cross-linked (39, 94). This model is attractive not only because it can account for a large body of radiolabeling studies on SCa and for the relative insolubility of its components (discussed below), but also because filament sliding, albeit more rapid, is a familiar phenomenon from ciliary and muscle motility.

Both of the above models presume that most, if not all, of the cytoskeletal protein in axons is moving. However, recent studies of slow axonal transport in the optic tract suggest that, at least in some neurons, the majority of NF protein may be in an effectively stationary form. Nixon and Logvinenko (61) have followed the procession of radiolabeled NF protein down the optic tract, with particular attention to the distribution and half-life of the labeled protein left behind as the SCa wave exits this region. (This trailing component is apparent in many studies of SCa, but insufficient experimental precision and resolution have prevented its analysis until recently.) They find that one third of the NF protein entering the tract is deposited in a pool which disappears from axons much more slowly than the SCa wave. Because of its longer

Peter J. Hollenbeck's present address is Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA 02115.

1. *Abbreviations used in this paper*: MT, microtubule; NF, neurofilament; SCa, slow component a.

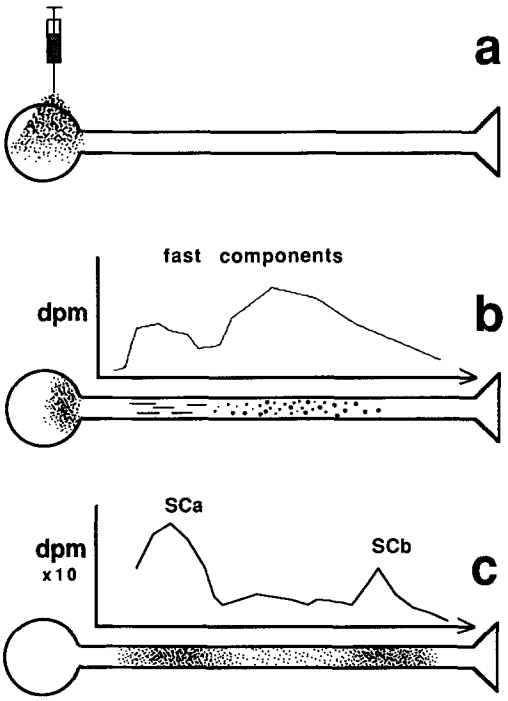

Figure 1. A schematic description of how the different velocity components of axonal transport can be resolved into relatively discrete waves of anterogradely moving radioactivity. After application of labeled amino acids to the region of the neuronal cell bodies (*a*) and a short delay for protein synthesis to occur, the components of fast transport, vesicles (200–400 mm/d) and mitochondria (~50 mm/d), move down the axon (*b*). Days to weeks later, moving at a velocity which is approximately two orders of magnitude slower, two additional components become apparent (*c*): slow component b (*SCb*, 2–8 mm/d), the faster of the two, has a complex protein composition which includes actin and, in some axons, tubulin; SCa (*SCa*, 0.2–1 mm/d), the slower, contains mainly tubulin and NF protein. The polypeptide composition of the different components can be determined by excising the appropriate segment of the nerve at an appropriate interval after labeling, and performing electrophoresis and fluorography. This technique for analyzing axonal transport is described in detail in reference 12.

residence time, this pool should actually represent the majority of NF protein in these axons at steady state. In addition, the stationary pool of NF protein is distributed nonuniformly along axons, its density increasing distally in a pattern that closely parallels the gradient of filament density seen in ultrastructural analysis of optic tract axons. These data have given rise to a model of the NF cytoskeleton in which a nonuniform and essentially stationary network is maintained by NF protein conveyed by SCa (58, 61). Three aspects of this model are significant: first, it accounts for both the structural stability and plasticity of form of the axonal cytoskeleton, a stationary infrastructure providing the former and SCa the latter; second, it suggests a potential regulatory role for the complex posttranslational modifications of NF protein (discussed below); and third, it places no constraints upon the form of the moving component of NF protein; i.e., it need not be polymer, since the polymer visible by electron microscopy can be accounted for by the stationary component.

Recent work by Weisenberg and colleagues suggests that a nonpolymeric, motile form of cytoskeletal protein may exist in neurons (89–91). They have isolated particulate structures from brain MT preparations which, like SCa, are composed primarily of insoluble tubulin and NF protein. These particles, designated SCAPs, have an associated microtubule-stimulated ATPase activity (20) and show ATP-dependent movement along MTs in vitro at velocities comparable to those of SCa in vivo. Isolated SCAPs are irregular globular structures 20 nm in diameter; although there is no direct evidence that SCAPs exist in vivo, similar particles have been seen in association with MTs in ultrastructural studies of squid axoplasm (38). Weisenberg et al. (90, 91) propose that the movement of SCAPs along axonal MTs in vivo could account, fully or in part, for the SCa wave of transport. In their view, SCAP particles could convey cytoskeletal proteins along axons in a closely associated but nonpolymeric form; the proteins could then be released and incorporated into the cytoskeleton in distal regions of the axon, perhaps by covalent modification.

Posttranslational Modification of Cytoskeletal Proteins

Both tubulin and NF protein undergo posttranslational modifications which undoubtedly play a role in regulating the dynamics of the axonal cytoskeleton. The major modification of NF proteins is phosphorylation (17, 28, 29, 71, 80), involving several protein kinases (31, 46, 70, 76, 77, 81) and occurring on the tail regions of NF subunits which extend from the wall of the filament (30). Immunocytochemical and biochemical studies have shown that NF phosphorylation has a pronounced regional heterogeneity: unphosphorylated NF proteins are mainly confined to the region of the cell body, while phosphorylated forms are largely restricted to axons (5, 72, 82). Although substantial phosphorylation of NF proteins occurs in the vicinity of the cell body soon after synthesis (10, 63), it is clear that there is additional phosphate added during subsequent axonal transport (5, 21, 59, 60, 62, 64). In concert with dephosphorylation in the axon, this results in a net gain of phosphate by the high and middle molecular weight NF subunits, and extensive turnover of phosphate groups on the middle and low molecular weight subunits during their transport (49, 59). A possible role for axonal phosphorylation has been suggested by recent studies of the transport of the high molecular weight NF subunit in optic tract axons (49). Here four differentially phosphorylated forms of this subunit can be resolved, and they show different transport behavior; the least phosphorylated form predominates in the moving SCa wave, while the highly phosphorylated forms are left behind in the trailing, stationary NF component. This suggests that phosphorylation may regulate the transition of NF protein from a moving to a stationary phase, and that the turnover of NF-bound phosphate in axons may reflect an equilibrium between these two components.

Tubulin is subject to two forms of posttranslational modification of the α-subunit which are thought to be important for MT dynamics: acetylation (45, 50, 51, 73) and removal of the carboxy-terminal tyrosine (2, 37, 75). Although neither modification is coupled to MT assembly, much evidence suggests that MTs containing tubulin which is acetylated or detyrosinated (or both) are more stable, and that modified tubulin is likely to be part of a stable or long-lived polymer (15, 25, 36, 44, 50, 74, 87, 88). In neurons, acetylation occurs throughout the cell bodies and neurites and specific antibodies reveal a uniform distribution of acetylated tubulin along neurites (6), despite some evidence that modification

occurs progressively during transport (14). However, immunocytochemical studies of actively elongating neurons in culture have shown that the growth cone region contains predominantly unmodified α-tubulin; i.e., neither acetylated nor detyrosinated (76). It is unclear whether this tubulin has been transported to the distal region of neurites in unmodified (and presumably nonpolymeric) form, or whether it has arrived there in modified form and been locally deacetylated and retyrosinated. The resolution of this question, and of the role which posttranslational modification plays in axonal transport of tubulin, requires determination of the sites of assembly of the cytoskeleton in neurons.

Sites of Assembly

Our picture of the neuronal cytoskeleton depends critically upon where assembly of subunits into polymer occurs: assembly in the vicinity of the cell body would be consistent with transport of polymers, while assembly elsewhere in the axon implies that subunits could be conveyed to distal sites in nonpolymeric form. A body of indirect evidence favors assembly of both MTs and NFs near the cell body. Most of the tubulin (~75%) and NF protein (>90%) in axons is insoluble in buffers which stabilize polymer (9, 10, 56, 57), and kinetic studies using pulse-labeled neurons in culture have shown that the majority of tubulin and NF proteins enter an insoluble form soon after synthesis (10), probably before they have traveled a significant distance along the axon (8). However, studies of developing neurons have suggested that substantial MT assembly occurs distally, far from the cell body (4, 35, 47), and involves a component of tubulin which may never have been polymerized (76). Both kinds of data could be explained by a combination of MT transport and MT lability, in which free tubulin would arise in distal regions of the axon by local depolymerization of MTs, perhaps accompanied or regulated by retyrosination and deacetylation. An alternative explanation would be the presence of an insoluble but nonpolymeric form of motile cytoskeletal protein; as discussed above, such a form exists (90, 91).

The recent development of techniques for directly observing the movement of cytoskeletal proteins in axons may resolve this question. By microinjecting tubulin monomer conjugated with fluorescent dye into cultured neurons, it has proven possible to observe the axonal transport of tubulin at velocities very similar to those predicted by radiolabeling studies of mature nerve (34). In a high resolution variant of this technique, Okabe and Hirokawa (69) have introduced labeled tubulin and then located it by light and electron microscopy at various stages in its transport. They resolve two waves of moving tubulin, the faster of which is soluble in polymer-stabilizing buffer. Labeled tubulin belonging to the slower moving, insoluble component is seen at the ultrastructural level only at the distal, "plus" ends of MTs in the region containing the wave. This would not be expected if assembly of the exogenous tubulin occurred near the cell body; in that case, MTs would contain labeled tubulin proximally or over their entire length. Thus, these results argue that tubulin has moved down the axon in a nonpolymeric form, and has been added to the distal ends of existing, moving MTs. Additional studies using direct visualization techniques of this type should help to determine the exact relationship between the assembly and transport of tubulin, and possibly NF protein as well.

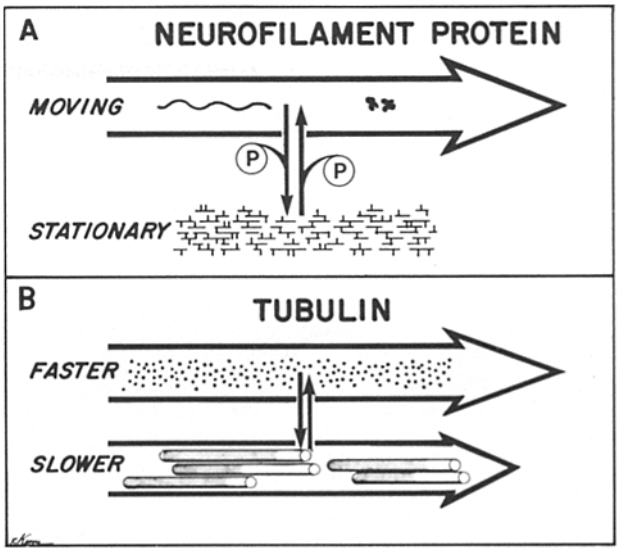

Figure 2. The evidence reviewed in the text gives rise to the following interpretation of the transport and dynamics of cytoskeletal proteins in axons. (*A*) Soon after synthesis, NF protein attains an insoluble polymeric or oligomeric form and moves down the axon in equilibrium with a much slower moving or stationary pool of polymer. The transition between the moving and stationary pool may be governed by the phosphorylation state of NF protein; phosphate turnover on NF protein suggests that the process may be reversible. (*B*) Tubulin travels in two velocity components: a faster moving, soluble, dimeric or oligomeric component; and a slower moving, polymeric component. The equilibrium between these components involves the addition of subunits to the distal, "plus" ends of moving MTs. Soon after polymerization, many axonal MTs become hyperstable (denoted by shading) due to postttranslational modification of subunits and/or the acquisition of MT-associated proteins.

Perspective

After years of analysis of the axonal transport of cytoskeletal proteins by many workers, there can be little doubt of the strength of the hypothesis that proteins travel in coherent waves because they comprise specific subcellular structures (7, 27, 40, 43, 48). But which, if any, of the structures that are seen in the electron microscope represents the moving form of cytoskeletal protein? Although static images reveal a cross-linked MT–NF lattice, and a moving lattice once seemed a likely model, we now know that this is almost certainly not the form in which cytoskeletal proteins travel along the axon. It is now attractive, and consistent with much indirect evidence, to conclude that individual polymers are the moving forms of tubulin and NF protein. The recent studies discussed above suggest that this is probably correct but incomplete: not all that's polymer need move, and not all that moves need be polymer (see Fig. 2). It seems increasingly likely that the transport of the neuronal cytoskeleton involves more than one motility phenomenon, and several forms of regulation. In addition, the synthesis, transport, and arrangement of tubulin and NF protein differ widely between different neurons (53, 65, 68); between different processes of the same neuron (24, 66, 67); and between the same processes in development, regeneration, or maturity (24, 67, 94, 97). In view of this, it is no surprise that workers studying different systems observe and emphasize different phenomena. In neurons as elsewhere, ultrastructure is generated

by a combination of intracellular motility and self-assembly; it is probable that diverse structures result in part from differently organized and regulated forms of transport.

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