Slow axonal transport: the polymer transport model

Peter W. Baas and Anthony Brown

About 20 years ago, Lasek and colleagues proposed that cytoskeletal proteins are transported along axons as assembled polymers, and cytosolic proteins are transported coordinately by association with these polymers. Over the years, this hypothesis has met with repeated challenges from workers who favour a model in which cytoskeletal proteins are actively transported along axons as free subunits or small oligomers that assemble into stationary cytoskeletal polymers. Here, we discuss the major evidence relevant to this controversy and explain why we believe that polymer transport remains the most plausible model for slow axonal transport.

Pioneering studies on slow axonal transport

Studies using radioisotopic pulse-labelling techniques have established that axonal transport is an intrinsic property of neurons that continues throughout their life and that it occurs by an active process rather than diffusion. These studies have also established that hundreds of proteins move down axons in a small number of discrete rate components, each representing the coordinated movement of a distinct subset of axonal proteins[1]. Proteins associated with membranous organelles move at rates of up to several hundred mm per day in the fast components of axonal transport. Tubulin, neurofilament and a few other proteins move at modal rates of ~1 mm day^{-1} in 'Slow Component a' (SCa). Actin and more than 200 other 'cytosolic' proteins move at modal rates of several mm per day in 'Slow Component b' (SCb). Together, SCa and SCb constitute slow axonal transport.

In pulse-labelling experiments, both SCa and SCb form bell-shaped waves that spread as they progress down the axon (Fig. 1). Each wave represents the concerted movement of many distinct proteins whose individual waveforms coincide[1]. Models that envisage individual protein molecules moving independently down the axon require that each protein molecule interact independently with the transport machinery but move coherently with many other proteins. A more plausible explanation is that the motile units of axonal transport are macromolecular complexes[2]. In this model, each rate component represents the movement of a distinct cytological structure composed of a distinct subset of axonally transported proteins. Fast transport is considered to represent the movement of membranous organelles and associated proteins, and slow transport the movement of cytoskeletal polymers and associated proteins[2]. Originally, the cytoskeletal polymers were considered to move *en masse* as a crosslinked network[1]. Later, this model was refined to incorporate the idea of individual polymers moving independently[3]. The spreading of the wave during transit is consistent with this view and suggests that individual polymers move at a range of rates within each rate component[4].

Support for this polymer transport hypothesis came initially from ultrastructural studies. For example, axons regenerating after transection show a transient decrease in the amount of neurofilament protein transported into the axon, resulting in a local decrease in neurofilament number and axon diameter that arises close to the cell body and propagates down the axon at the rate of slow axonal transport[5]. A similar correlation between the amount of axonally transported protein and neurofilament number has also been observed in a variety of neuropathies that alter neurofilament protein transport[6,7]. Also consistent with their transport as polymers, neurofilaments accumulate proximal to axon constrictions and become depleted distally[8,9].

The subunit transport hypothesis

This hypothesis was first articulated in 1986 by Bamburg and colleagues who found that application of drugs that alter microtubule dynamics to the distal tips of axons arrested their growth, but application of similar concentrations to cell bodies had no apparent effect[10]. Assuming that the sole action of these drugs was to inhibit microtubule assembly, they concluded that axon growth is dependent upon net microtubule assembly at the axon tip and proposed that tubulin is transported down axons in an unassembled form. More recent analyses have shown that the drug treatments used in this study actually cause substantial microtubule disassembly and that milder drug treatments, which prevent net microtubule assembly but induce less disassembly, do not prevent axon growth[11]. Axon growth is therefore not dependent upon microtubule assembly at the distal tip. Thus the Bamburg study actually provides no evidence for the subunit transport hypothesis.

Visualization of cytoskeletal polymer movement in axons

The late 1980s saw a new approach for studying slow axonal transport in which fluorescent or caged-fluorescent cytoskeletal proteins are introduced into neurons for incorporation into cytoskeletal polymers. A short segment of the axon is then marked by photobleaching or photoactivating the tagged proteins and monitored for potential movement. Studies on tubulin in cultured chick sensory neurons[12] and neurons in zebrafish and grasshopper embryos[13,14], as well as on tubulin, actin and neurofilament proteins in cultured mouse sensory neurons[15–17], all reported no detectable movement of the marked zone. However, studies on tubulin in the axons of cultured *Xenopus* motor neurons revealed clear anterograde movement of the marked zone, shown to represent the transport of microtubule polymers, at rates consistent with those expected for slow transport[18,19].

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Some authors view the lack of movement in most of these studies as evidence for the subunit transport hypothesis. However, this approach should detect active transport of cytoskeletal proteins whether they move as subunits or polymers, so a lack of movement does not provide evidence for the form in which they move. Furthermore, we favour studies that demonstrate polymer movement over those that do not because it is difficult to imagine how such movement could be generated artifactually. Hirokawa has suggested that the microtubule movement in *Xenopus* axons is an artifact of the rapid growth and low adhesiveness of these axons in culture and that it reflects passive dragging by forward movement of the growth cone at the distal tip of the axon[16]. We disagree because marked microtubules move in *Xenopus* axons during pauses in growth cone advance, indicating that microtubule translocation proceeds independently of the forward movement of the growth cone[18].

If the movement of microtubules in *Xenopus* axons reflects slow axonal transport, why has cytoskeletal polymer movement not been observed in other neurons using the same approach? The existence of different transport mechanisms in different species is an unsatisfactory explanation because it implies fundamental differences among vertebrate neurons. A more likely explanation was suggested by Sabry and colleagues who noted that, while active transport is normally required to support axon growth, diffusion could theoretically supply sufficient subunits for net assembly of cytoskeletal polymers at short distances from the cell body[13]. They noted that all the photobleaching/photoactivation studies to date have been performed relatively close to the cell body and that the distance at which diffusion becomes limiting is least for *Xenopus* axons because they grow far more rapidly in culture than the other axons studied. It may be that polymer translocation is less apparent in regions where diffusion is not limiting and that polymer movement may have only been observed in *Xenopus* axons because only in these axons were the observations made far enough from the cell body.

Two groups recently took a more direct approach to study potential axonal movement of cytoskeletal polymers. Terasaki and colleagues injected fluorescently labelled taxol-stabilized microtubules or phalloidin-stabilized actin filaments into squid giant axons[20]. Although individual polymers could not be resolved, the fluorescent label moved predominantly in an anterograde direction at rates equivalent to slow axonal transport. Miller and Joshi injected chemically fixed fluorescently labelled microtubule fragments into the cell bodies of cultured neurons[21]. They observed no movement and concluded that microtubules are stationary in neurons. However, this study is inconclusive because the lack of movement could have resulted from technical problems, such as the inability of chemically fixed microtubules to interact appropriately with the transport machinery.

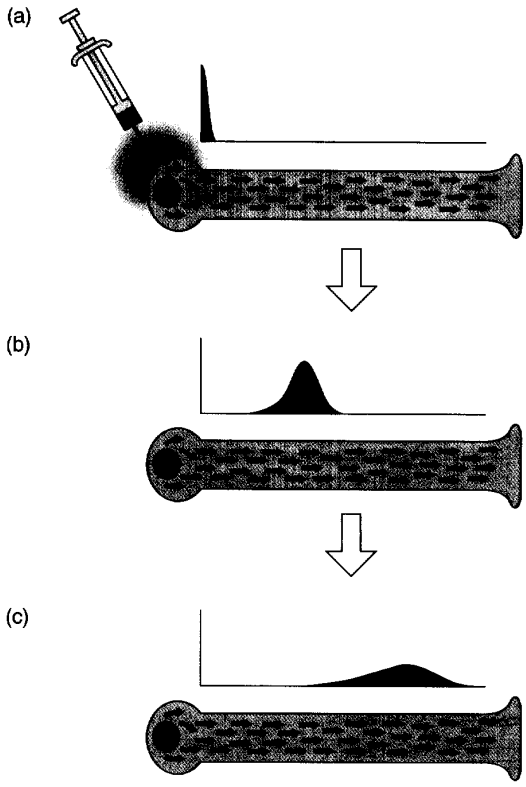

FIGURE 1

The kinetics of slow axonal transport. Radioisotopically labelled amino acids are injected into the vicinity of a neuronal cell body (a), resulting in a brief 'pulse' of newly synthesized radioactive proteins that are conveyed into and along the axon by slow axonal transport. The nerve containing the axons is then sliced into segments, which are analysed biochemically to identify specific transported proteins. This schematic diagram illustrates the movement of a protein or protein complex within a single rate component, which might represent 'Slow Component a' (SCa) or SCb. The labelled proteins (red arrows) spread out and intermingle with the unlabelled proteins (green arrows) as they all move anterogradely down the axon (b). The distribution of the pulse of labelled proteins can be described as a unimodal bell-shaped wave that spreads as it moves distally (a–c). The spreading of the wave indicates that the proteins move at a range of rates; the rate of movement of the peak represents the modal rate for the population[4].

Evidence for a centrosomal origin for axonal microtubules

Baas and colleagues have shown that γ-tubulin, a protein required for normal microtubule nucleation, is absent from axons and is concentrated at the centrosome in neuronal cell bodies[22]. They proposed that microtubules are nucleated at the centrosome and released for transport into the axon. Consistent with this, experimental inhibition of microtubule nucleation at the centrosome compromises microtubule formation and axon outgrowth[23]. Additional support derives from a recent pharmacological study that investigated the fate of microtubules nucleated from the centrosome[24]. Neurons were treated with the microtubule-depolymerizing drug nocodazole to disassemble the existing polymer, permitted to assemble a burst of microtubules at the centrosome by removing the drug, then exposed to low concentrations of vinblastine to suppress further microtubule assembly. Microtubules were first observed at the

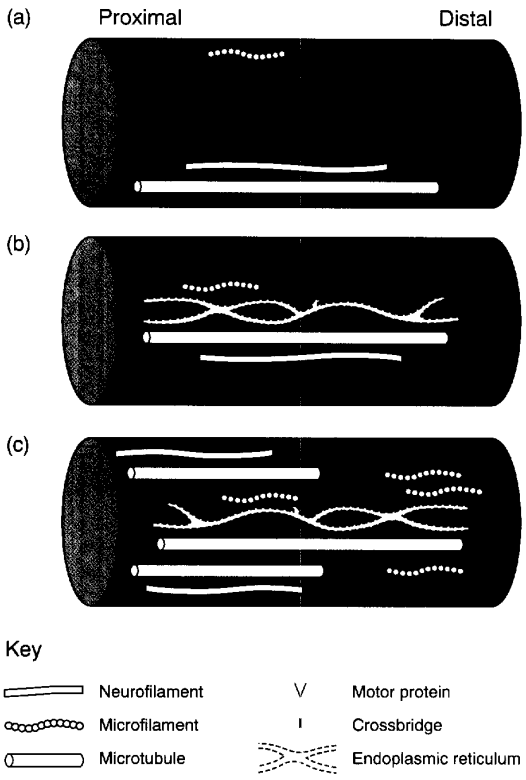

FIGURE 2

Potential substrates for the motor-driven sliding translocation of cytoskeletal polymers in axons include any structure that has a greater resistance to movement than the cytoskeletal polymer that is to be moved. Microtubules and actin filaments probably interact directly with motor proteins, and neurofilaments may move 'piggy-back' style by attachment to other moving polymers[40]. Microtubules could translocate along the plasma membrane or endoplasmic reticulum (ER), which forms a pervasive three-dimensional tubular network in axons[41], or relative to the actin cytoskeleton[42]. The many cytosolic proteins in 'Slow component b' (SCb, not shown) could move by association with the moving polymers. (a) Polymers moving relative to the plasma membrane or its membrane cytoskeleton. (b) Polymers moving relative to the ER. (c) Polymers forming transient crosslinks to relatively immobile axoplasmic structures (such as the plasma membrane or ER), allowing other polymers to slide relative to them.

centrosome, then dispersed in the cytoplasm, and finally at the periphery of the cell body and in developing axons, suggesting that they had been actively transported from the centrosome into the axon.

This interpretation was questioned by Miller and Joshi, who argued that the apparent movement of microtubules from the centrosome could somehow be explained by the assembly of new polymer[21]. This was based on the observation that fluorescent tubulin can incorporate into microtubules in cells exposed to vinblastine. We agree that vinblastine does not entirely inhibit microtubules from exchanging subunits with the soluble tubulin pool (e.g. Ref. 25). However, Baas and colleagues used a significantly higher concentration of vinblastine than Miller and Joshi, and this was shown to prevent the total microtubule mass in the cell from increasing as well as individual microtubules from elongating. Thus any incorporation of fluorescent tubulin into microtubules must have reflected subunit turnover rather than net addition of new polymer. Moreover, microtubule release and transport from the centrosome have now been visualized directly in living epithelial cells, giving strong precedent for Baas' interpretation[26].

Recent evidence for microtubule transport in cultured neurons

Yu *et al.*[27] and Slaughter *et al.*[28] used a novel strategy to study microtubule transport that avoided the use of drugs. They injected biotinylated tubulin into cultured neurons either prior to or shortly after axon formation and allowed it to diffuse throughout the neuron. After a period of axon growth, the distribution of labelled tubulin in microtubules was analysed. The presence of labelled tubulin in microtubules indicated assembly or turnover since the injection, so any unlabelled microtubules in the newly grown region of the axon must have assembled prior to the injection and then moved into this region. This approach was expected to underestimate the number of transported microtubules because some transported microtubules could have turned over subunits. Nevertheless, varying amounts of unlabelled polymer were found in the newly grown region of the axon. The unlabelled microtubules were intermingled with labelled microtubules, confirming that the labelled tubulin had reached the most distal regions of the axon. These studies provide strong evidence that microtubule polymers are actively transported from the cell body into the axon and down its length.

Recent challenges to the polymer transport hypothesis

Miller and Joshi injected fluorescent tubulin into cultured neurons in the presence of very low vinblastine concentrations[21]. A portion of the fluorescent microtubules in the cell body appeared to progress down the axon over a period of ~30 minutes, leaving a region of unlabelled microtubules between them and the cell body. The authors argued that these observations support a model whereby a wave of tubulin subunits is actively transported down the axon, exchanging completely with stationary microtubules during transit. No supporting evidence was presented because all of their analyses were performed after extraction of free tubulin subunits. In our view, a more plausible explanation is that a pulse of microtubules from the cell body entered the axon and moved down its length. In fact, labelled tubulin is known to diffuse rapidly down axons, resulting in a diminishing concentration with distance from the cell body, and with no indication of the kind of wave proposed by these authors (e.g. Ref. 29).

Funakoshi and colleagues used photoactivation to mark tubulin molecules within a discrete region of the axons of cultured mouse sensory neurons and then immunoelectron microscopy to analyse the distribution of photoactivated tubulin subunits[30]. No labelled tubulin was observed outside of the photoactivated region in neurons that were extracted to

remove tubulin subunits one hour after photoactivation. By contrast, labelled tubulin was found outside of the photoactivated region in unextracted neurons one minute after photoactivation, with more label on the distal side than the proximal side. This was interpreted as evidence that microtubules in these axons do not move and that tubulin subunits are actively transported in an anterograde direction. We disagree because we do not regard an asymmetrical distribution of tubulin subunits at one time point as sufficient evidence for active transport. Furthermore, the lack of labelled polymer outside of the photoactivated zone after one hour is surprising because axonal microtubules are dynamic and we would expect some incorporation of photoactivated tubulin subunits into these polymers even if they were stationary. This suggests that the immunodetection technique used may not have been sufficiently sensitive to rule out microtubule translocation.

Campenot and colleagues recently adapted the radioisotopic pulse-labelling approach to cultured rat sympathetic neurons using a compartmentalized culture dish assembly[31]. As expected, a wave-shaped pulse of labelled tubulin progressed down the axon. The leading edge of the wave moved at a rate of at least 4 mm day^{-1}, but we calculate that the mean rate was ~0.9 mm day^{-1}, consistent with the kinetics of tubulin transport in other radioisotopic studies. The authors asserted that this movement was too rapid to be accounted for by polymer transport. We do not understand this reasoning because the mean rate for tubulin transport in their studies is actually very similar to that of microtubule movement in *Xenopus* axons[18] and is far slower than the rates of microtubule translocation that have been measured in non-neuronal cells (e.g. Ref. 26). Thus we would argue that translocation of microtubules is fully capable of accounting for the transport observed.

Finally, Terada *et al.* transfected a modified neurofilament protein into neurons of transgenic mice lacking neurofilament polymers in their axons[32]. With time, the transfected protein was detected at substantial distances down the axon. Since this neurofilament subunit could not assemble into polymers, the authors reasoned that it must have been transported down the axon as free subunits or small oligomers. However, this argument does not distinguish between active transport and diffusion. Soluble proteins diffuse over long distances down axons, as evidenced by studies in which fluorescent molecules diffuse over hundreds of microns within hours of their introduction into the cell body[33]. Thus the appearance of detectable levels of a protein at distant sites along an axon is not evidence for its active transport or for the form in which it is transported.

The molecular mechanism of slow axonal transport

Recent studies on insect Sf9 cells have shown that motor proteins can transport microtubules into developing cytoplasmic processes[34,35], establishing the feasibility of the polymer transport model. Future progress will depend on the identification of specific motor proteins or complexes that can attach to cytoskeletal polymers within the axon and translocate these polymers relative to an axoplasmic substrate (Fig. 2).

Our confidence in the polymer transport model is greatly strengthened by its ability to explain essential features of slow axonal transport using fundamental principles that have precedent in other cell types. By contrast, the subunit transport model offers no such explanations and invokes principles that have no known precedent. The scientific literature abounds with examples of motor-driven polymer transport (e.g. Refs 26, 36 and 37), and there are also clear precedents for the association of a wide range of cytosolic proteins, such as those transported in SCb, with the cytoskeleton (e.g. Ref. 38). Polymer transport can also account for fundamental features of the axonal cytoskeleton, such as the thirteen-protofilament lattice structure and uniform polarity of axonal microtubules[39]. It is hard to imagine that neurons would abandon this powerful and fundamental mechanism for establishing cytoskeletal arrays and evolve an entirely unprecedented mechanism based on the active transport of cytoskeletal proteins as free subunits or oligomers.

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Slow axonal transport: the subunit transport model

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A central problem concerning slow transport of cytoskeletal proteins along nerve axons is where they are assembled and the form in which they are transported. The polymer and subunit transport models are the two major hypotheses. Recent developments using molecular and cellular biophysics, molecular cell biology and gene technology have enabled visualization of moving forms of cytoskeletal proteins during their transport. Here, we argue that these studies support the subunit transport theory.

Development of the polymer and subunit models

Weiss and Hiscoe[1] discovered axonal transport using a sciatic nerve ligature preparation and suggested that it was related to axonal outgrowth or regeneration. Metabolic labelling studies on rat sciatic nerves *in vivo* showed that one facet of it, slow axonal transport, is dependent on active mechanisms[2–5]. The profile of the transported proteins retains its shape and sharp boundary at the leading edge during the chase period, and the proteins move over a long distance in the axon. The synchronous distribution of numerous labelled proteins suggested that there is a general active transport mechanism. Lasek and coworkers proposed that the axonal cytoskeleton polymerizes in the cell body immediately after synthesis and is subsequently transported as a coherent column by a sliding mechanism[2,4,5]. Numerous features of neurofilament (NF) protein and tubulin transport supported this idea, including the constant nature of the distribution profile of labelled NF proteins and tubulin, and the difficulty in detecting free NF monomers within the axon. However, it is important to note that Lasek *et al.* did not discuss or notice the gradual spreading of the labelled protein waves. Recent polymer-sliding hypothesists often neglect this point (e.g. see Ref. 6).

The results of other investigations have challenged the traditional interpretation. Labelling studies using inorganic phosphate revealed readily detectable quantities of unpolymerized NF proteins in axons[7]. Furthermore, the waves of labelled proteins broaden markedly as transport progresses and may break into several ill-defined waves, with some labelled proteins moving ahead of the principal peak and a significant fraction trailing behind[8,9]. Topical application of microtubule (MT)-destabilizing drugs to different regions of isolated neurons growing in tissue culture showed that the growth cone is most sensitive to drug treatment and that MT polymerization in growth cones is indispensable for axonal growth[10]. The agent used, colcemid, inhibits new MT polymerization. If axonal growth depended on MT polymer sliding, the axons would continue to elongate regardless of the presence of the agent. These data imply that there are at least two forms of NF proteins and tubulin in the axons, polymers and monomers or small oligomers, and that they undergo dynamic exchange. Okabe and Hirokawa[11] obtained evidence that molecules are inserted at the distal ends of microtubules by microinjecting biotinylated tubulin into cultured PC12 cells and also found that small oligomers exchange between stationary MTs and free tubulin pools. They proposed that cytoskeletal proteins are transported predominantly along axons as monomers or oligomers. Thus, the important questions concerning slow axonal transport became: what form do the cytoskeletal proteins take during transport and where do they assemble?[12].

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