Slow axonal transport: fast motors in the slow lane
Jagesh V Shah and Don W Cleveland*

The bulk of neuronally synthesized proteins destined for the axon is transported in a phase of transport ~100 times slower (1 mm/day) than the vesicular traffic of fast axonal transport (100 mm/day). Of late, a number of studies have shed considerable light on the controversies and mechanisms surrounding this slow phase of axonal transport.

A long-standing controversy has centered on the form of the transported proteins. One major transport cargo, neurofilament protein, has now been seen in a number of contexts to be transported primarily in a polymeric form, whereas a second cargo tubulin is transported as a small oligomer. The development of techniques to visualize the slow transport process in live cells has demonstrated that instantaneous motions of transported neurofilaments, and presumably other slow transport cargoes, are fast, bidirectional and interspersed with long pauses. This and additional biochemical efforts indicate that traditional fast motors, such as conventional kinesin and dynein, are responsible for these fast motions.

Introduction

Most of the proteins required for neuronal homeostasis are synthesized within the cell body. Many of these proteins are required in the axon or at the nerve terminal far from the point of synthesis. Most synapse-bound cargoes are transported directly to their destination(s) through the fast transport phase including glycolytic enzymes, clathrin, dynein and the Cu-Zn superoxide dismutase to name a few. Most proteins have generally been reported to travel in either of the two transport components SCa or SCb. For all such slowly transported proteins studied, the transported material is well approximated by a bell-shaped concentration profile. For SCa proteins this is ~0.1–1 mm/day and for SCb proteins it is ~5–10 mm/day, speeds that themselves slow markedly during aging and disease (see, for example, [3,4]). It should be borne in mind that these classes are defined by the speed of movement of the cytoskeletal proteins and as we will see below some individual cargoes actually have characteristic transit speeds that do not neatly fit into SCa or SCb.

Given the apparent bulk movement of slowly transported proteins, an initial attractive hypothesis was that the entire axoplasm moved as a coherent block, with some diffusion of the profile over time. To test this hypothesis, many investigators used photobleaching [5] and photoactivation [6] methodologies to locally mark axoplasm and visualize its movement; however, in each case the marked region failed to move, making the coherent transport model an unlikely possibility. More recent work has exploited a newly emerging method, microtubule speckle microscopy, to label axonal microtubules with many fiduciary marks [7]. By introducing low concentrations of fluorescently labeled tubulin into living cells, in this case a developing frog (*Xenopus laevis*) embryo, the stochastic nature of tubulin polymerization produces microtubules with a speckled appearance, with fluorescent regions alternating with nonfluorescent ones. Using this method, the entire axonal...
The microscopic array is now marked (in contrast to local marks from photobleaching or photoactivation) and bulk motion of axonal cytoskeleton can be assessed. Such microscopy technique again failed to demonstrate anterograde motion of the axonal microtubules [7], hammering the final nail in the coffin of the coherent axoplasm transport model.

If the axoplasm in its totality does not move, then the transport process must take place through the independent motion of individual units. But what are these units? For soluble proteins, this cargo form has been presumed to be small: possibly small particles or even individual polypeptides. For cytoskeletal elements, however, their form could be either polymeric or monomeric. The idea of polymer transport of cytoskeletal elements arose alongside the initial coherent transport model, whereas the monomer transport hypothesis seemed more aligned with the characteristics of fast transport, albeit at a much slower rate. Thus began a longstanding controversy that has continued for 20 years. As we review here, the answer is that both monomeric and polymeric cytoskeletal elements can move — a rather diplomatic resolution to what has been an impassioned debate.

Beyond the microtubule speckle microscopy method, a very useful alternative to the radiolabeling technique has proven to be the squid giant axon. Previous work has demonstrated that material, such as cytoskeletal proteins and polystyrene beads, can be directly injected into the axoplasm of the squid giant axon and is transported anterogradely at slow rates [8]. A recent study observed the motions of fluorescently labeled mammalian neurofilament polymers and tubulin in the squid giant axon to assess the form of the transported cargo [9**]. By measuring the spread of the fluorescent material during its anterograde transport, a rough diffusion constant for the transported material was calculated. From this it was inferred that injected neurofilament protein was transported as a polymer. Tubulin, on the other hand, appeared to be transported as small oligomers or monomers. In parallel work, Terada and colleagues [10**] used a sophisticated measurement technique termed fluorescence correlation spectroscopy (FCS) to accurately determine the diffusion constant of transported fluorescent tubulin that had been injected into the squid giant axon. In its simplest form, the FCS technique measures the motions of many fluorescent proteins within a small volume and computes their diffusion constant(s) based on correlations between these small motions over many time intervals. The sophisticated technique confirmed the somewhat cruder measurement — that tubulin was in fact being transported in a small oligomeric form.

The apparent difference in neurofilament versus tubulin has also been recapitulated in vertebrate axons. Radiolabeled axonal segments, obtained using traditional slow transport methodology in chicken neurons, were biochemically fractionated into soluble and particulate fractions to assess the assembled state of the transported cytoskeletal elements. Previously, this fractionation technique was used to convincingly demonstrate that actin can be transported in an unassembled form [11]. The same approach has now been used to show that neurofilaments are transported in a polymeric or particulate form [12*], whereas tubulin is in the soluble fractions, consistent with transport as small oligomers.

Perhaps the most compelling evidence for filamentous neurofilament transport comes from the study of cultured mammalian neurons. Using green fluorescent protein (GFP) tagged neurofilament subunits and live-cell microscopy, two groups have been able to visualize the transport of filamentous neurofilament protein in real time [13**,14**]. The introduction of the GFP-tagged subunits produced neurofilaments that were fluorescently labeled. In axonal regions depleted of neurofilaments or large regions that were photobleached, GFP-tagged proteins were visualized as long cylindrical objects that moved bidirectionally at varying speeds (up to 2 microns per second) and with long intervals of no motion. These long objects were, in fact, filamentous neurofilament protein being transported down the axon. Taken together, in squid, chicken and mammalian systems, these experiments all point to neurofilament polymers as a major transport unit of neurofilament protein. This is in contrast to tubulin, in which the same experimental approaches identified small oligomers as the major transport units.

The transport of neurofilament polymers, however, stands in direct contrast to earlier studies that demonstrated just as compellingly that neurofilament subunit movement does not have to be in the form of an assembled polymer. These earlier studies demonstrated that virally expressed NF-M, the medium-sized protein subunit of neurofilaments, was transported at slow transport rates in the axons of a transgenic mouse with a substantially reduced number of axonal neurofilaments [15]. The transgenic mouse model (expressing NF-H linked to beta-galactosidase) provided many axons that lacked neurofilaments due to trapping of most neurofilaments in the cell bodies. The ectopic expression of NF-M revealed its nonfilamentous transport at a slow rate. Moreover, the few axons in which neurofilaments were not accumulated in the cell bodies provided examples in the same nerves for the normal rate of neurofilament transport. In the absence of polymeric neurofilaments, the NF-M subunit was transported at the same slow speed as in adjacent axons that contained assembled neurofilaments. It seems most likely that neurofilament subunits can be transported as monomers in the slow phase in the complete absence of axonal polymers, but in the normal physiological context the major form of transported neurofilaments may be the polymeric form.

The sum of evidence seems to us to lay to rest the debate of whether the major structural proteins are moved as subunits or polymers. Both are correct. Tubulin and actin...
move as subunits or oligomers; neurofilaments can move as subunits or polymers.

**Motors: fast speeds in the slow lane?**
The slow net velocity of transport as measured by radiolabeling methods represents an average over long time intervals (days to weeks). Insight into the instantaneous rates of transport has emerged from exploiting sympathetic neurons in culture into which plasmids encoding GFP-tagged NF-M were microinjected \[13**,16\]. Live cell details of neurofilament transport come from observations of fluorescent neurofilaments within naturally occurring gaps that act as ‘windows’ or artificial windows created by photobleaching. In each case, fluorescent filamentous neurofilaments could be seen traversing the gap predominantly in an anterograde direction, with short periods of reversal. Movements in either direction were interrupted by long periods of no motion at all. The most surprising result was that when motile, the filaments moved at fast transport speeds (up to 2 microns per second), implicating an as yet unidentified fast motor! Using the same culture system, Roy and colleagues \[14**\] introduced a GFP–NF-H fusion (NF-H, the heavy protein subunit of neurofilaments) via an adenoviral vector. The GFP–NF-H labeled filaments moved with nearly identical characteristics to those of the GFP–NF-M filaments. One important difference between this pair of reports is in the interpretations regarding the apparent retrograde motions that were observed. In the earlier work with the NF-M fusion, the observed retrograde motions were short in duration and spatial extent and were considered to be a result of elastic recoil \[13**\]— filamentous neurofilaments being stretched anterogradely and then suddenly snapping back. In the later work with the NF-H fusion, however, the observed retrograde motions of transported neurofilaments were much longer in duration and spatial extent \[14**\], making the elastic recoil hypothesis unlikely, allowing the authors to propose that a small part of neurofilament transport may be made up of retrograde motions.

The fast velocities observed for neurofilament transport have also been recapitulated in squid axoplasm. In extruded squid giant axon axoplasm, the motility of large refractile protein complexes along axonal microtubules was observed \[17*\]. The motion was anterograde, at speeds of up to 1 micron per second, consistent with fast transport velocity. Most importantly, immunofluorescence staining of these motile objects revealed that they contained neurofilament subunits, bolstering the evidence for the action of a fast motor in slow neurofilament transport.

Taken together, these experiments in cell culture and squid axoplasm demonstrate that the ‘slow’ transport process actually proceeds by ‘fast’ motions sometimes interspersed with long pauses. The result would be the slow net velocity measured by radiolabeling techniques and would obviate the need for a special slow velocity motor. Seen this way, slow transport may thus simply utilize many of the motors already identified for fast transport, but with duty cycles, or on-off rates, resulting in periods of nonmotility.

As to the identities of the motors powering neurofilament transit, in the squid system the microtubule molecular motor kinesin was found to co-localize with the motile neurofilaments from the extruded axoplasm \[17*\]. This finding is consistent with FCS measurements of tubulin transport in the squid giant axon in which the injection of a function-blocking anti-kinesin antibody or induced disassembly of microtubules blocked anterograde movement of tubulin \[15\]. Disruption of the actin microfilament system or the poisoning of myosin, the actin filament based motor, however, did not affect tubulin transport, indicating that microtubules provide the main transport substrate for slow transport.

The identification of slow transport motors in vertebrate systems is somewhat more problematic than in the squid system, due in part to technical difficulties and to the large number of genes encoding microtubule-based molecular motors \[18\]. The injection of a function-blocking anti-conventional kinesin antibody into a murine neuroblastoma cell line disrupted the transport of a NF-M–GFP fusion protein into extending neurites \[19*\], albeit this could arise as a result of secondary effects. Further biochemical analysis using immunoprecipitations from the murine neuroblastoma cell line revealed a possible interaction between the murine conventional kinesin isoform and neurofilaments, and that this interaction may be mediated by phosphorylation \[20*\]. The specificity of such an interaction, however, is not established, a continuing problem especially when dealing with interactions with cytoskeletal elements.

The identification of motors participating in slow transport came from the biochemical purification of native neurofilaments \[21**\]. Immunoelectron microscopy of these neurofilaments, isolated from mammalian spinal cord, revealed a distinct subpopulation that were densely labeled along their contours with antidynein antibodies, whereas other filaments in the same preparation had no detectable cytoplasmic dynein. Presumably, this bipartite labeling of dynein is indicative of a specific interaction with a particular subpopulation of neurofilaments, probably those that are being actively transported in the axon. Furthermore, some filaments in these preparations can translocate bidirectionally along microtubules *in vitro* (presumably the ones coated with cytoplasmic dynein). This motility is disrupted by traditional motor poisons (such as EDTA or AMP-PNP), requires ATP and can be inhibited by function-blocking microtubule motor antibodies \[21**\]. The *in vitro* motility characteristics of the purified neurofilaments are very similar to those of GFP–neurofilament subunit fusions visualized in cultured neurons \[13**,14**\]. The bidirectional movement requires at least one anterograde motor, almost certainly one of the kinesin family, but the
specific one(s) have not yet been identified. Success in this quest will represent solution of one key mystery in the mechanism of slow transport.

Fast motors — slow transport: how does it work?

Even with the advent of live cell and in vitro systems, the pulse radiolabeling technique still represents the true in vivo context and the gold standard for identifying slow transport cargoes. This method, however, is biased towards prevalent proteins — such as those of the cytoskeleton — making an extensive catalogue of slowly transported cargoes difficult. Some groups have performed additional purification steps from the radiolabeled axonal segments to enrich for a particular protein to measure its transport rate [11,22,23]. The results of these studies are surprising. Whereas many proteins are categorized into SCa or SCb, there are proteins that do not fall easily into either component; for example, the differentially phosphorylated versions of a single protein, MAP1B, are transported at different rates [24••]. The unphosphorylated form is transported at a rate consistent with SCa, whereas the phosphorylated form moves at a speed greater than SCb but still much slower than fast transport.

How can we reconcile the many molecules and many speeds? One possibility is that slow transport cargo—motor interactions are promiscuous, that is, cargoes can bind to many different motors with varying affinities. The difference in biochemical affinities would give rise to differing duty cycles — or time spent moving versus being motionless. The high affinities of fast transport cargoes for their fast transport motors would be the logical extension of such a model and explain why these cargoes are always moving and rarely detached from their motor(s). The speed of slowly transported proteins would be a function of the affinity of the molecules for the motor or some intermediate on which they are ‘piggy-backing’. This would account for the wide disparity in transport velocities in the literature since the affinities would vary based on the local properties of axons. The absence of the typical biological specificity in such a model may be the reason that this fundamental process has remained so poorly understood for over 40 years.

Conclusions

It is anticipated that tests of the involvement of specific kinesins and the cargoes they affect will be forthcoming. They are expected to result from transport experiments in mice genetically deleted for an individual or combinations of molecular motors, in vitro efforts following the addition of motor-specific toxins, or by the direct isolation of motor–cargo complexes. The identification of these slow transport motors should, in turn, open up a host of avenues of investigation to understand how and why slow transport is so slow.

Acknowledgements

We are grateful to the members of the Cleveland and Goldstein laboratories for stimulating discussions.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest

•• of outstanding interest


By injecting fluorescent cytoskeletal proteins into the squid giant axon, both transport and spreading were directly measured, allowing the estimation of a crude correlate of diffusion constant (cruder than FCS, see [10]). Injection of mammalian neurofilament protein and tubulin indicated that neurofilaments are transported in polymeric form and tubulin is transported in an oligomeric form. Along with [10••,12,13••,14•], this largely settles the debate regarding the form of axonal cytoskeletal transport — neurofilaments are moved predominantly as polymers whereas tubulin is moved as small oligomers.


Using a sophisticated technique, FCS, diffusion constants of injected fluorescent tubulin could be ascertained during transport. The measured diffusion constants indicate that the transported form of tubulin in the squid giant axon is in small oligomers, bolstering earlier results from [9••] and [12•]. Furthermore, the injection of function-blocking kinesin antibodies or microtubule disassembly are found to disrupt tubulin transport, whereas actin filament disruption actin filament motor poisoning do not. Together, these findings indicate that axonal microtubules and their associated motors represent the primary transport substrates for tubulin.


Using biochemical fractionation in concert with radiolabeling in chicken neurons, transported neurofilament proteins are found to be part of a particulate fraction, consistent with transport as polymers. Tubulin, however, is in the soluble fraction, consistent with transport in a small oligomeric form. Similar conclusions were found in [9••] from experiments in squid giant axon.


This report represents the first live visualization of slow transport in a cultured cell system. The introduction of a GFP–NF-M fusion by DNA microinjection produced labeled transported filaments moving anterogradely at fast transport speeds while spending the majority of their time not moving. This is in stark contrast to previous reports (for example, see [15]). Moreover, the authors propose that slow transport results from fast motions, presumably due to fast motors, interspersed with periods of no motion. Retrograde motions were also seen and proposed to be the result of elastic recoil of the filaments.

This paper reports nearly identical results to those in [13**] with the same culture system, but using a GFP–NF-H fusion introduced by adenovirus. Far more retrograde motions are seen than in [13**] and these motions appear to be mediated by a retrograde motor.


After extruding axoplasm from a squid giant axon, refractile protein complexes were visualized to move along microtubules. Immunofluorescence analysis of the motile objects revealed that they contained neurofilament protein and kinesin. As in [19*], this offers evidence for conventional kinesin isoforms being implicated in neurofilament transport.


Live cell observations show NF-M–GFP being transported into extending neurites. Transport is blocked by the microinjection of anti-kinesin antibodies. Immunoprecipitation using an anti-kinesin antibody indicates a possible link between neurofilaments and kinesin.


The purification of native neurofilaments from mammalian spinal cord results in two important findings. Firstly, there exist two populations of purified neurofilaments, those with dense labeling of a dynein epitope along their contour and those with no labeling, as assayed by immunoelectron microscopy. Secondly, the purified neurofilaments are filamentous and can translocate along microtubules in vitro. Motility is bidirectional and occurs with speeds consistent with fast motors (~0.5 microns per second). The characteristics of the motility are similar to those seen in the axons of cultured neurons [13**,14**]. This paper and two others [17*,19*] represent the first reports of neurofilaments biochemically interacting with microtubule motors.


Using traditional radiolabeling techniques in concert with immunoprecipitation, the phosphorylated form of MAP1B is shown to be transported faster than the dephosphorylated forms and, in fact, faster than all other slow transport cargoes. This finding highlights the complex nature of the slow transport process. Ultimately, any molecular model of the slow transport process must account for such observations.