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A Critical Reevaluation of the Stationary Axonal Cytoskeleton Hypothesis

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Neurofilaments are transported along axons in a rapid intermittent and bidirectional manner but there is a long-standing controversy about whether this applies to all axonal neurofilaments. Some have proposed that only a small proportion of axonal neurofilaments are mobile and that most are deposited into a persistently stationary and extensively cross-linked cytoskeleton that remains fixed in place for many months without movement, turning over very slowly. In contrast, others have proposed that this hypothesis is based on a misinterpretation of the experimental data and that, in fact, all axonal neurofilaments move. These contrary perspectives have distinct implications for our understanding of how neurofilaments are organized and reorganized in axons both in health and disease. Here, we discuss the history and substance of this controversy. We show that the published data on the kinetics and distribution of neurofilaments along axons favor a simple "stop and go" transport model in which axons contain a single population of neurofilaments that all move in a stochastic, bidirectional and intermittent manner. Based on these considerations, we propose a dynamic view of the neuronal cytoskeleton in which all neurofilaments cycle repeatedly between moving and pausing states throughout their journey along the axon. The filaments move infrequently, but the average pause duration is on the order of hours rather than weeks or months. Against this fluid backdrop, the action of molecular motors on neurofilaments can have dramatic effects on neurofilament organization that would not be possible if the neurofilaments were extensively cross-linked into a truly stationary network. © 2012 Wiley Periodicals, Inc

Key Words: neurofilament, axonal transport, cytoskeleton, neuron

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Introduction

"Stop and Go" Movement of Neurofilaments in Axons

Neurofilaments, which are the intermediate filaments of neurons, are space-filling cytoskeletal polymers that accumulate in axons during postnatal development. The principal known function of neurofilaments is to increase axonal cross-sectional area, thereby increasing the rate of propagation of the nerve impulse [Perrot et al., 2008]. Radioisotopic pulse-labeling studies in vivo have demonstrated that neurofilament proteins are transported along axons and that they are among the slowest of axonally transported proteins, moving anterogradely at average rates of approximately 0.1–1 mm/day [Hoffman and Lasek, 1975; Lasek et al., 1984] (see Figs. 1A and 1B). In contrast, time-lapse fluorescence imaging of cultured neurons has demonstrated that neurofilament proteins actually move at fast rates in both anterograde and retrograde directions, and that the movements are also intermittent [Wang et al., 2000]. To reconcile these observations, we have proposed that slow axonal transport is caused by rapid bidirectional movements interrupted by prolonged pauses [Brown, 2000, 2003, 2009]. According to this "stop and go" model, the cargoes of slow axonal transport are propelled by fast motors, but the average velocity is slow because they spend some of their time moving backwards and most of their time pausing.

Neurofilaments Cycle Between Distinct Short and Long-Term Pausing States

Our kinetic analysis of neurofilament transport in axons of cultured neurons has revealed that neurofilaments cycle between two distinct kinetic states which we have termed "on-track" and "off-track" [Trivedi et al., 2007]. Neurofilaments in the on-track state alternate between short bouts of movement interrupted by short pauses, whereas neurofilaments in the off-track state pause for longer periods of time without movement (Fig. 1C). The average pause

times are about 20–30 s in the on-track state and about 1 h in the off track state [Trivedi et al., 2007], and computational modeling predicts similar pause times in vivo [Jung and Brown, 2009]. The overall slow rate of neurofilament transport is due largely to the fact that these cargoes spend most of their time in the off-track pausing state. As the names of these states suggest, we have speculated that neurofilaments in the on-track and off-track states may differ in their proximity to microtubules, which are the tracks along which they move, but it is also possible that they differ in some other way that could influence their pausing behavior such as in their interaction with molecular motors or in the activity of those motors.

The Polymer Transport Controversy

The axonal transport of neurofilament proteins has been no stranger to controversy. In the 1980s and 1990s there was considerable debate about the form in which these proteins move. Lasek and colleagues proposed that neurofilament proteins move in the form of assembled polymers [Tytell et al. 1981; Lasek et al. 1984; Lasek 1986] whereas Hirokawa and colleagues proposed that neurofilament polymers do not move and that these proteins are transported in the form of unassembled subunits [Okabe et al., 1993; Takeda et al., 1994; Terada et al., 1996]. These hypotheses became known as the polymer and subunit transport models and they were hotly debated [Baas and Brown, 1997; Hirokawa et al., 1997]. The controversy was ultimately resolved by the direct observation of neurofilament polymer movement in axons of cultured neurons

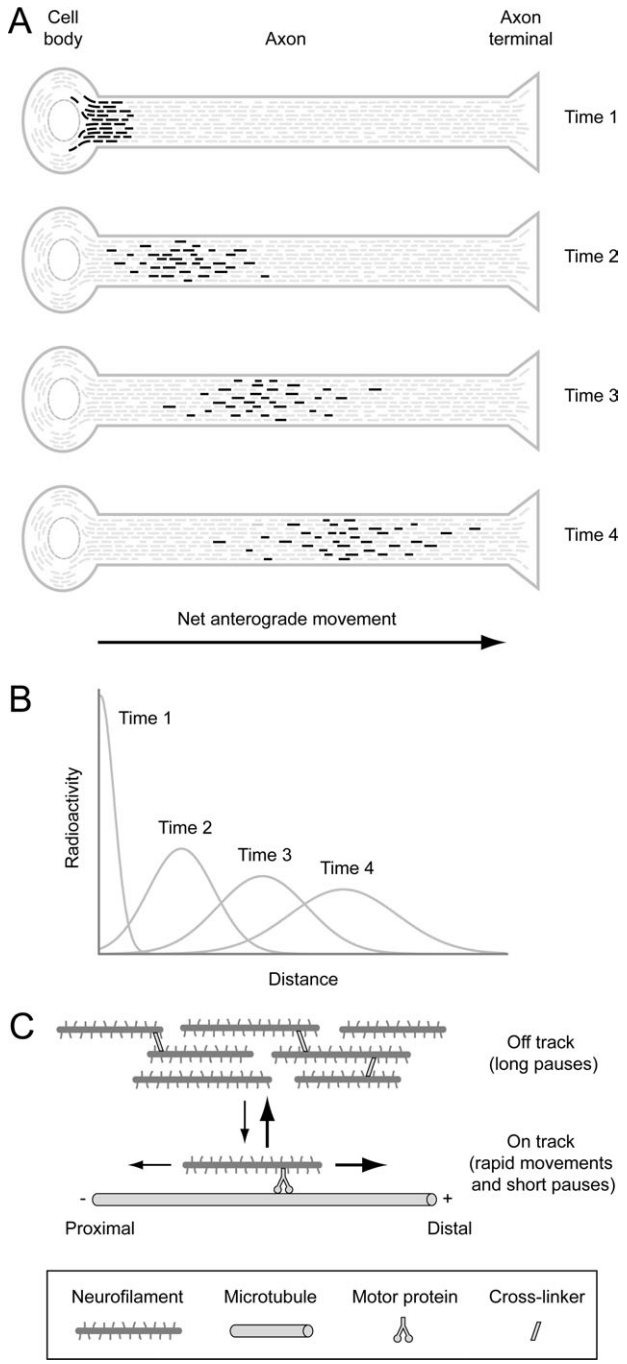


Fig. 1. Axonal transport of neurofilaments. A. Schematic diagram illustrating the movement of a pulse of radiolabeled neurofilaments along an axon in a radio-isotopic pulse labeling experiment, meant to be representative of axons in a long nerve such as the sciatic. To perform such an experiment, radioactive amino acids are injected into the vicinity of neuronal cell bodies. The radiolabeled amino acids become incorporated into newly synthesized proteins, generating a pulse of radiolabeled proteins that move out along the axons by the mechanisms of axonal transport. The most commonly used injection sites are the dorsal root ganglia, which labels sensory neurons, the anterior horn of the spinal cord, which labels motor neurons, and the eye, which labels retinal ganglion cells. This diagram shows a single axon but in reality these experiments are performed on nerves that contain thousands of axons that run in parallel, so the actual kinetics are a summation of the kinetics of many individual axons. The pulse of radiolabeled neurofilaments is sharp initially but spreads considerably over time due to the stochastic, intermittent and bidirectional nature of the movement. **B**. Schematic illustrating the distribution of the pulse of radiolabeled neurofilaments at the four time points shown in A. The neurofilaments form a symmetrical and slowly moving Gaussian wave that spreads as it propagates distally [Hoffman and Lasek, 1975; Hoffman et al., 1983, 1985; Xu and Tung, 2001; Brown et al., 2005; Craciun et al., 2005; Jung and Brown, 2009]. The average velocity is usually in the range 0.2–1.0 mm/day and depends on the age of the animal and the particular nerve being studied. **C**. Schematic diagram of the "stop and go" model of neurofilament transport. Neurofilaments are transported by motor proteins along microtubule tracks in a rapid intermittent manner, cycling between kinetically distinct on-track and off-track states. Neurofilaments in the on-track state engage in rapid bouts of movement interrupted by short pauses, whereas neurofilaments in the off-track state pause for prolonged periods without any movement. Off-track neurofilaments may be interconnected temporarily by cytoskeletal cross-linkers such as spectraplakins. The neurofilaments switch between forwards (anterograde) and backwards (retrograde) movement, but anterograde movements predominate, resulting in a net anterograde directionality.

[Brown, 2000; Wang et al., 2000; Yan and Brown, 2005]. This do not rule out the possibility that neurofilament proteins may also be transported in the form of unassembled subunits, but it is now generally accepted that neurofilament polymers are at least the principal cargo structures of neurofilament protein transport.

The Stationary Axonal Cytoskeleton Controversy

While it is now clear that neurofilament polymers move in axons, there is disagreement about whether they all move. This controversy originated more than 25 years ago, when Nixon and colleagues proposed that a significant proportion of axonally transported neurofilaments are deposited along axons into a persistently stationary and extensively cross-linked cytoskeleton [Nixon and Logvinenko, 1986]. According to this stationary axonal cytoskeleton hypothesis, the deposited neurofilaments remain fixed in place for many months without movement and turn over only very slowly. This hypothesis was challenged by Lasek et al. [1992], who proposed that it was based on a misinterpretation of the experimental data and that axons contain a single population of mobile neurofilaments which all move relentlessly at a slow average rate. Many years later, Nixon and colleagues responded to this criticism by repeating their earlier kinetic analysis in an improved manner and they claimed that their new findings supported their earlier conclusions [Yuan et al., 2009]. In the following sections we explain the experimental basis for this controversy and then we summarize how we have used computational modeling to resolve it.

The Original Evidence for the Stationary Axonal Cytoskeleton Hypothesis

The stationary axonal cytoskeleton hypothesis was introduced to explain the distribution and axonal transport of neurofilaments in mouse optic nerve [Nixon and Logvinenko, 1986], and has been explained in several review articles [Nixon, 1991, 1992, 1998]. In essence, the hypothesis was based on three principal lines of evidence:

1. *The neurofilament transport waves are markedly asymmetric with a pronounced trailing edge*. Radio-isotopic pulse labeling in mouse optic nerve generated a pulse of radiolabeled neurofilaments which formed a markedly asymmetric wave with a substantial trailing component, giving the impression that many radiolabeled neurofilaments were left behind as the wave propagated along the axon [Nixon and Logvinenko, 1986]. Nixon and colleagues proposed that this trailing component represented the progressive deposition of radiolabeled neurofilaments into a persistently stationary cytoskeleton during their transit along the axon.
2. *The kinetics of neurofilament departure from a window of the optic nerve are biphasic.* Nixon and colleagues analyzed the transit of a pulse of radiolabeled neurofilaments through a 9 mm "window" of the mouse optic nerve, extending from the eye towards the brain [Nixon and Logvinenko, 1986]. The total amount of radioactivity in the nerve window increased during the first week as the pulse of radiolabeled neurofilaments entered the optic nerve from the eye, and then declined slowly over a period of many months as the neurofilaments gradually moved through the nerve window towards the nerve terminals. Importantly, the kinetics of this departure were found to be biphasic, with an initial rapidly declining phase followed by a later more slowly declining phase. The authors concluded that there were two distinct populations of axonal neurofilaments. They proposed that the initial more rapidly declining phase was due to the departure of a population of mobile neurofilaments from the nerve window and that the later more slowly declining phase was due to extremely slow degradation or mobilization of a population of persistently stationary neurofilaments which represented a substantial fraction of the total axonal neurofilaments.
3. *Neurofilaments are distributed nonuniformly along axons.* Nixon and colleagues reported that the neurofilament content of mouse retinal ganglion cell axons is not constant along the length of the optic nerve, but actually increases approximately twofold in a proximal-to-distal manner [Nixon and Logvinenko, 1986]. Thus these axons contain about twice as many neurofilaments per cross-section in the distal optic nerve compared to more proximal locations. The authors proposed that this gradient was formed by the progressive deposition of mobile filaments into a stationary network during their transport along the axon, and that the existence of this gradient was evidence in support of their deposition model.

A Challenge to the Stationary Axonal Cytoskeleton Hypothesis

The rebuttal of the stationary axonal cytoskeleton hypothesis by Lasek et al. [1992] was based on three key points:

1. *The proteins of slow axonal transport can be resolved into two groups, termed Slow Components "a" and "b"* [Black and Lasek, 1979, 1980]. Slow component "a" in mouse optic nerve is comprised predominantly of neurofilament proteins and tubulin, which move at average rates of less than 0.2 mm/day [Jung and Shea, 1999], whereas Slow Component "b" comprises several hundred different proteins, including actin, which move at average rates of about 1–2 mm/day [Lewis and Nixon, 1988; Paggi et al., 1989]. Due to the short length of the mouse optic nerve (about 11 mm from the eye to the superior colliculus) and the considerable

amount of spreading of the transport waves, these two groups of proteins overlap and do not resolve fully before the faster moving Slow Component "b" proteins reach the nerve terminals.

2. *Some Slow Component "b" proteins co-migrate with neurofilament proteins by one-dimensional (1D) gel electrophoresis due to their similar molecular mass.* Most of the Slow Component "b" proteins are cytosolic and can be separated from neurofilaments by detergent extraction because cytosolic proteins are generally soluble in nonionic detergents whereas neurofilaments are insoluble. Alternatively, these proteins can be separated from the neurofilament proteins by 2D electrophoresis. Lasek and colleagues noted that Nixon and Logvinenko [1986] used total nerve protein, which contains both soluble and insoluble proteins, and 1D electrophoresis, which fails to separate neurofilament proteins from Slow Component "b" proteins of similar size.
3. *Pure neurofilament transport kinetics exhibit fairly symmetrical waves with no pronounced trailing edge.* By analyzing neurofilament transport kinetics in mouse optic nerve using 2D electrophoresis, which separates neurofilament proteins from Slow Component "b" proteins of similar size based on their charge, Lasek and colleagues concluded that there was no evidence for the selective retention of a subpopulation of neurofilaments behind the wave peak. These authors also noted that this is similar to the transport behavior of neurofilaments in longer nerves, where Slow Components "a" and "b" are better resolved [Hoffman et al., 1985; Xu and Tung, 2001].

Based on these observations, Lasek and colleagues proposed that the pronounced asymmetry of the neurofilament transport waves in the data of Nixon and Logvinenko [1986] was actually due to contamination of the neurofilament transport kinetics with faster moving Slow Component "b" proteins that comigrate with neurofilament proteins by 1D electrophoresis. Furthermore, they proposed that the initial more rapidly declining phase of the biphasic decay kinetics in the data of Nixon and Logvinenko [1986] was actually due to Slow Component "b" proteins, not neurofilament proteins. According to this hypothesis, the data of Nixon and Logvinenko [1986] did not represent pure neurofilament kinetics until most of the faster moving Slow Component "b" proteins had moved through the nerve window, i.e., after about 45 days (Fig. 2A, B and C). However, Lasek and colleagues did not reproduce the exact experimental conditions of Nixon and Logvinenko [1986]. For example, they used different radioisotopically labeled amino acids, which can result in differential labeling of proteins depending on their amino acid composition, and they used different injection-sacrifice intervals. In addition, Lasek and colleagues used different approaches to analyze, quantify and display the data, making it hard to perform a direct side-by-side comparison of their data with the data of Nixon and colleagues.

A Resolution to the Controversy

To address the criticisms of Lasek and colleagues, Nixon and colleagues repeated their original experiments with some technical improvements [Yuan et al., 2009]. First, they used a Triton-insoluble fraction of the optic nerve tissue for their analyses, effectively depleting soluble Slow Component "b" proteins and enriching for insoluble protein complexes such as neurofilaments. Second, the authors confirmed the purity of the neurofilament protein bands on their polyacrylamide gels by using 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mapping [Yuan et al., 2009]. The authors reported that the resulting kinetics were comparable to those obtained in their earlier study [Nixon and Logvinenko, 1986], so they concluded that their new data confirmed the stationary axonal cytoskeleton model. Notably, the authors estimated that fewer than 10% of axonal neurofilaments are transported in a "stop and go" manner in mouse optic nerve and that greater than 90% are fixed in place for months without movement.

To evaluate this conclusion, we performed a direct comparison of the decay kinetics published by Nixon and Logvinenko [1986] and Yuan et al. [2009] [Li et al., 2012]. Contrary to the conclusion of Yuan et al. [2009], this comparison indicates that the kinetics are actually very different. The two decay curves are not superimposable: the initial rapidly declining phase in the data of Nixon and Logvinenko [1986] is absent from the pure neurofilament kinetics of Yuan et al. [2009] (Fig. 2D). While the kinetics of Nixon and Logvinenko [1986] are biphasic, the kinetics of Yuan et al. [2009] are distinctly monophasic. Moreover, the pure neurofilament kinetics of Yuan et al. [2009] align well with the second phase of the Nixon and Logvinenko [1986] kinetics. These observations are consistent with the proposal of Lasek et al. [1992] that only the second phase of the biphasic decay kinetics of Nixon and Logvinenko [1986] corresponds to pure neurofilament kinetics and that the first (more rapidly declining) phase can be explained by contamination of the neurofilament kinetics with soluble Slow Component "b" proteins that are similar in size to the neurofilament proteins and therefore not resolved by 1D electrophoresis.

Computational Modeling of Neurofilament Transport in the Mouse Optic Nerve

If the above critique of the stationary axonal cytoskeleton hypothesis is correct then it should be possible to explain the pure neurofilament kinetics of Yuan et al. [2009] in terms of a single population of neurofilaments moving in a "stop and go" manner. To test this, we have performed

computational modeling of neurofilament transport in the mouse optic pathway [Li et al., 2012]. The power of computational modeling is that it forces us to make our hypotheses explicit and enables us to ask in a quantitative way whether or not those hypotheses can explain the experimental data.

To simulate neurofilament movement, we used a kinetic model based on the "stop and go" motile behavior of neurofilaments in cultured neurons [Brown et al., 2005; Jung and Brown, 2009; Trivedi et al., 2007]. In this model the neurofilaments move linearly and independently along axons, cycling between distinct on-track and off-track states as described above (Fig. 1C). The model is described in detail in the methods section of Li et al. [2012]. The movement of the filaments is treated as a stochastic process, which means that the transitions between the moving and pausing states are described by probabilities. The probabilities were obtained directly from our kinetic measurements in cultured neurons, with no assumptions about the underlying molecular mechanism

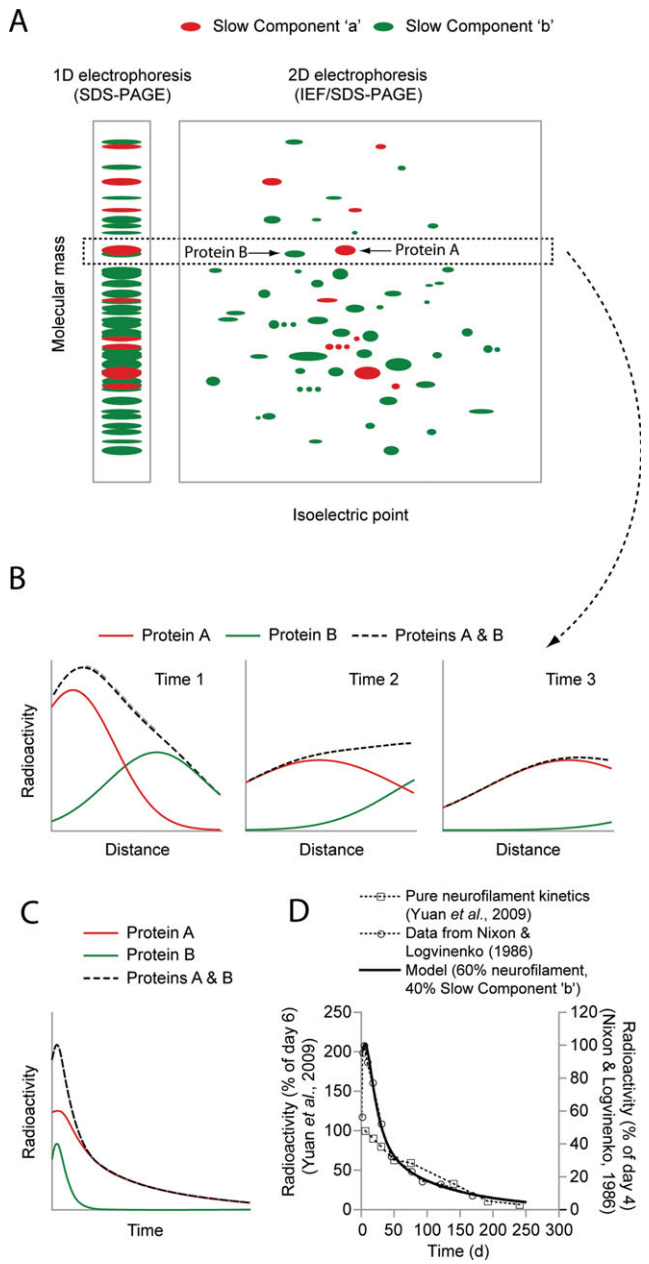
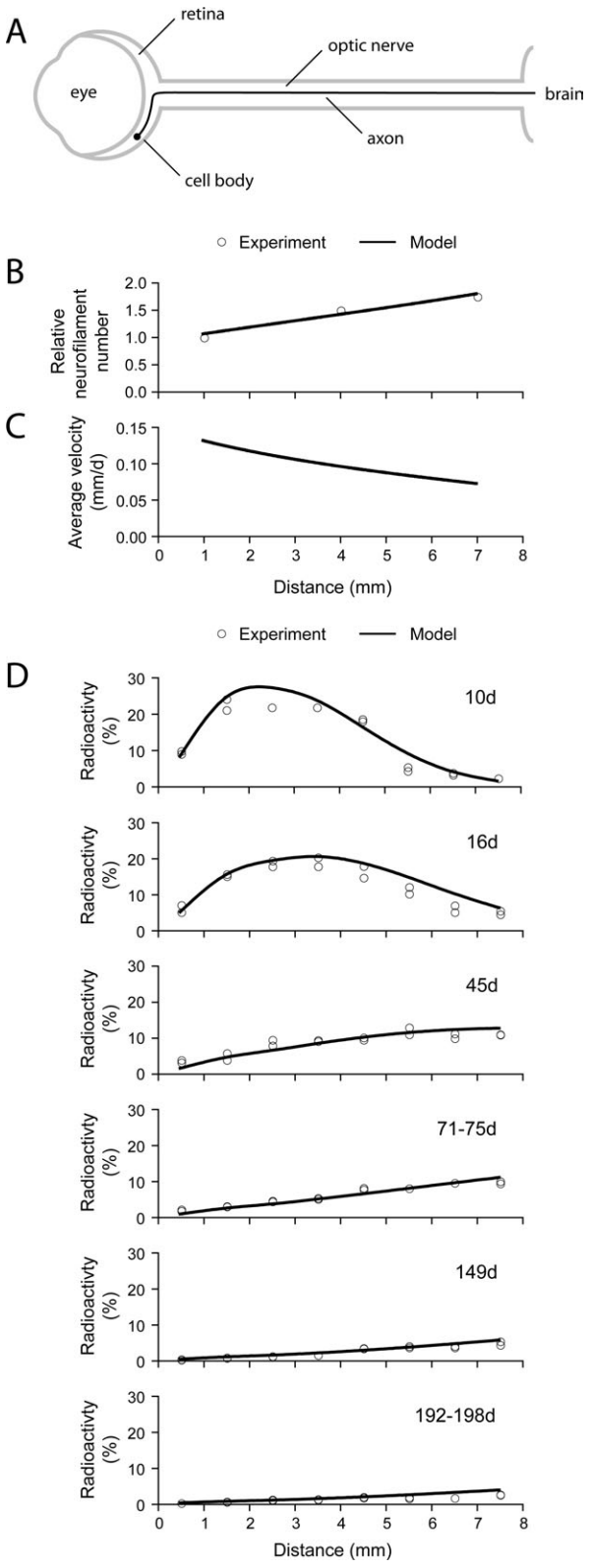


Fig. 2. Contamination of the neurofilament transport kinetics in mouse optic nerve by Slow Component "b" proteins. A. Schematic diagram of hypothetical radiolabeled axonal proteins resolved by 1D gel electrophoresis, i.e., SDS-PAGE, or 2D gel electrophoresis, i.e., isoelectric focusing in the first (horizontal) dimension and SDS-PAGE in the second (vertical) dimension. Slow Component "a" proteins (which are relatively few) are shown in red and Slow Component "b" proteins (which are relatively numerous) are shown in green. The box demarcated with the dashed line shows a hypothetical Slow Component "a" protein, Protein A, and a hypothetical Slow Component "b" protein of similar molecular mass, Protein B. These proteins comigrate using 1D electrophoresis, but can be resolved using 2D electrophoresis because they have different isolectric points. **B**. Schematic representation of the distribution of a pulse of these radiolabeled proteins along a hypothetical short nerve (meant to be representative of the optic nerve) at three different time points. Radioactivity associated with Protein A, which moves more slowly (in Slow Component "a"), is shown in red. Radioactivity associated with Protein B, which is transported more rapidly (in Slow Component "b"), is shown in green. The dashed black line represents the sum of the radioactivity profiles for the two proteins and is representative of the profile that would be observed if the analysis was performed using 1D electrophoresis of total nerve protein, as in Nixon and Logvinenko [1986]. **C**. Schematic representation of the time course of appearance and disappearance of radioactive protein in the entire nerve window over time. Note that Protein B, which moves in Slow Component "b," enters and exits the nerve window more rapidly than Protein A. The decay kinetics of the individual proteins are monophasic, but the decay kinetics of the two proteins combined (dashed black line) is biphasic. The initially more rapidly declining phase corresponds to Protein B, which departs the nerve more rapidly, and the later more slowly declining phase corresponds to Protein A, which departs more slowly. **D**. The experimental data of Nixon and colleagues, as plotted in Li et al. [2012]. The symbols represent the data for neurofilament protein M from Yuan et al. [2009] (open squares) and from Nixon and Logvinenko [1986] (open circles). Yuan et al. [2009] used a Triton-insoluble protein fraction, yielding pure neurofilament protein and monophasic decay kinetics. In contrast, Nixon and Logvinenko [1986] used total nerve protein (soluble and insoluble), yielding biphasic decay kinetics. The solid black line represents the output of the model, assuming contamination of the neurofilament protein kinetics with a hypothetical Slow Component "b" protein (relative weighting of 60% neurofilament protein and 40% Slow Component "b" protein). Note that the model shows good agreement with the biphasic decay kinetics of Nixon and Logvinenko [1986] at all times, and also with the monophasic decay kinetics of Yuan et al. [2009] at later times (>50 days), after the Slow Component "b" proteins in the model have left the nerve window. Thus the biphasic decay kinetics of Nixon and Logvinenko [1986] can be explained by contamination of the neurofilament transport kinetics with faster moving Slow Component "b" proteins.

of movement. Neurofilaments which reached the end of the axon were assumed to be either degraded with a half life of 2 days, as observed in vivo [Paggi and Lasek, 1987; Garner, 1988], or to reverse direction, as we have observed in cultured neurons [Uchida and Brown 2004].



Importantly, this model assumes that the "stop and go" motile behavior of neurofilaments in cultured neurons also occurs in vivo. We believe that this is a reasonable assumption that is supported by studies on other axonally transported cargoes, such as mitochondria and vesicles, which move with similar kinetics in vivo as they do in cultured neurons [Misgeld et al., 2007; Reis et al., 2012]. Moreover, our computational modeling studies have demonstrated that the stop-and-go kinetics of neurofilament transport in cultured neurons can explain the kinetics of neurofilament transport in vivo in a variety of nerve cell types [Brown et al., 2005; Craciun et al., 2005; Jung and Brown, 2009]. Nevertheless, it will be important to verify this assumption experimentally when it becomes technically possible to do so.

Explaining the Gradient in Neurofilament Content Along Optic Nerve Axons

As noted above, Nixon and Logvinenko [1986] reported that there is a proximal-to-distal gradient in neurofilament number along the optic nerve (Figs. 3A and 3B). According to the stationary axonal cytoskeleton hypothesis, this gradient is generated by a gradual deposition of neurofilaments into a stationary cytoskeleton during their movement along the axon, but our simulations indicate that such models actually generate a gradient of *decreasing* neurofilament number along the axon, exactly the opposite of what is observed experimentally. A simple alternative mechanism that does generate a gradient of increasing neurofilament content along these axons would be a gradient of decreasing transport velocity (Fig. 3C). In fact, a slowing of neurofilament transport along axons has been

Fig. 3. Computational modeling of neurofilament transport in the mouse optic nerve. A. Schematic of the optic pathway from one eye. The axons of retinal ganglion cells course through the retina and emerge from the eye at the optic disk to form the optic nerve, which extends to the contralateral side of the brain. **B**. The gradient in neurofilament content along the mouse optic nerve. The experimental data (open circles) are from Nixon and Logvinenko [1986]. The solid line represents the predictions of our computational model [from Li et al., 2012]. **C**. The gradient in average neurofilament transport velocity that is required to explain the gradient in neurofilament content shown in B [from Li et al., 2012]. **D**. The kinetics of neurofilament transport in the mouse optic nerve [from Li et al., 2012]. The open symbols represent the experimental data from Yuan et al. [2009] for NFL and NFM, recalculated as described in Li et al. [2012], and the solid line represents the output of the "stop and go" model. The radioactivity at each time point is expressed as a percentage of the total radioactivity in the nerve window at day 10, as described in Li et al. [2012]. Note that the model generates a symmetrical wave when the kinetic parameters are uniform [Brown et al., 2005; Craciun et al., 2005; Jung and Brown, 2009], but the wave here is not perfectly symmetrical because of the gradient in the transport velocity.

reported in the optic nerve [Jung and Shea, 1999] and also in the sciatic nerve [Hoffman et al., 1983, 1985; Watson et al., 1989; Xu and Tung, 2001]. Remarkably, we found that such a gradient in neurofilament transport velocity can be explained by only a small increase in the proportion of the time that the filaments spend pausing (from ≈96% proximally to ≈98% distally), or alternatively by only a small decrease in the proportion of the time that they spend moving anterogradely (from ≈63% proximally to ≈58% distally) [Li et al., 2012]. Thus subtle changes in pausing or directionality can have significant effects on neurofilament distribution and shallow gradients in these parameters can explain the proximal-to-distal gradient in neurofilament number along mouse optic nerve axons.

Explaining the Shape and Velocity of the Neurofilament Transport Wave

To analyze the transport kinetics, we simulated the movement of a pulse of radiolabeled neurofilaments along axons of retinal ganglion cells [Li et al., 2012]. These axons course through the retina for an average distance of 1 mm before emerging from the eye to form the optic nerve. We assumed that the pulse of radiolabeled amino acid lasted for 5 h but we found that the precise duration had no significant effect on the shape or velocity of the wave by the time that it entered the optic nerve. As expected for a stochastic transport model with a net anterograde bias, the population adopted a Gaussian waveform that spread as it propagated along the axon, and this was independent of the shape of the starting pulse [Jung and Brown, 2009]. By analyzing the shape and location of the waves in the data of Yuan et al. [2009], we found that the average velocity of neurofilament transport slows by about fivefold within the retina, and then by a further twofold along the length of the optic nerve. The magnitude of this slowing is broadly consistent with the experimental data of Jung and Shea [1999]. Factoring in this slowing, the computational model displayed remarkably good agreement with the experimental data of Yuan et al. [2009] at all time points (Fig. 3D). Thus it is possible to explain the pure kinetics of neurofilament transport in mouse optic nerve by a simple "stop and go" model in which axons contain a single population of neurofilaments that all move stochastically in a rapid, intermittent and bidirectional manner.

Explaining the Apparent Retention of Neurofilaments in the Axons Long After the Wave Peak Has Passed

Nixon and colleagues reported that a significant fraction of the radiolabeled neurofilaments in their radioisotopic pulse labeling experiments appeared to be retained in the optic nerve window behind the wave peak, giving rise to an apparently stable distribution of radiolabeled neurofilaments that remained constant between 71–75 and 192–198 days after injection [Yuan et al., 2009]. However, our analyses of the pure neurofilament transport kinetics of Yuan et al. [2009] indicate that this interpretation is incorrect; the radiolabeled neurofilaments remaining in the axon between 71–75 and 192–198 days after injection are indeed distributed along the axon in a proximal-to-distal gradient, but the slope decreases continuously throughout this time period as the radiolabeled neurofilaments continue to exit the nerve window at its distal end [Fig. 3D; see also Fig. 6 in Li et al., 2012]. The radiolabeled filaments depart slowly but continuously throughout this time period. If we consider the amount of radiolabeled neurofilament protein in the nerve window at day 10 to be 100%, then only 6% remains after 240 days. The explanation for this slow departure is that there is enormous spreading of the pulse of radiolabeled neurofilaments during the time course of the experiment. We have shown previously that this spreading is due in part to the bidirectional excursions of the neurofilaments, with a significant fraction of the neurofilaments moving backwards at any point in time [Brown et al., 2005; Jung & Brown, 2009]. According to the stationary axonal cytoskeleton model, the radiolabeled neurofilament proteins that remain in the axon at later times represent a distinct population of stationary neurofilaments that are deposited behind the moving wave front [Yuan et al., 2009], but our modeling indicates that these neurofilaments are simply the tail end of a single population of moving neurofilaments that spreads considerably due to the stochastic, intermittent and bidirectional motile behavior.

Comparison to Neurofilament Transport Kinetics in Other Nerves

It is important to note that radio-isotopic pulse labeling experiments in the sciatic nerve have also yielded broadly symmetrical neurofilament transport waves with no evidence of neurofilament deposition behind the advancing wave [Hoffman et al., 1985; Xu and Tung 2001; Jung and Brown 2009], and this also has been reported for Slow Component "a" in phrenic and hypoglossal nerves [Lasek, 1980; Lasek et al., 1984]. Thus neurofilament transport in mouse optic nerve is not fundamentally different from other nerve cell types, and in all these cases the movement is consistent with the "stop and go" model. However, the optic nerve is distinct in two important ways. First, the average velocity of neurofilament transport is about 0.1–0.2 mm/day proximally, which is about five times slower than in the sciatic, phrenic and hypoglossal nerves. Second, the sciatic, phrenic and hypoglossal nerves are significantly longer than the optic nerve. In long nerves the pulse of radiolabeled neurofilaments spreads enormously, reaching many centimeters in width

[Hoffman et al., 1985; Xu and Tung, 2001]. However, due to the short length of the optic nerve the entire wave exceeds the entire length of the nerve window within a week (see Fig. 3D), whereas the entire wave is fully contained within the excised portion of the mouse sciatic nerve for at least 2 months [Xu and Tung, 2001]. Thus the short length of the mouse optic nerve presents a particular challenge for radioisotopic pulse labeling studies.

Pausing or Stationary: What's the Difference?

At first glance it may seem that the stationary axonal cytoskeleton hypothesis is not fundamentally different from the "stop and go" hypothesis. Both hypotheses consider that there are two distinct kinetic states for neurofilaments, one mobile and the other stationary, and both hypotheses recognize that the neurofilaments spend only a small proportion of their time in the mobile state. However, as Yuan et al. [2009] have observed, these models are actually fundamentally different. In the stationary axonal cytoskeleton model, <10% of the axonal neurofilaments are engaged in axonal transport and >90% are deposited in a persistently stationary cytoskeleton that remains fixed in place for many months without movement [Yuan et al., 2009]. In contrast, our computational analyses have shown that all neurofilaments are engaged in axonal transport and that the experimental data are not consistent with the existence of a persistently stationary population. For example, our simulations of neurofilament transport in the optic nerve predict that the average neurofilament cycles on and off track more than 200 times over a period of 10 days, with average on and off track pause durations of about 20 s and 60 min, respectively [Li et al., 2012]. Using the analytical solutions described in Jung and Brown [2009] we calculate that the probability of a neurofilament remaining paused off track for more than 12 h is <0.001%. Thus the two models have very different implications for the mechanism of neurofilament transport and the dynamics of the axonal neurofilament array.

The Importance of Analyzing Neurofilament Transport on Both Fast and Slow Time Scales

If neurofilaments cycle continuously between distinct on-track and off-track kinetic states, then it is reasonable to ask why these distinct kinetic states do not yield biphasic decay kinetics in the radioisotopic pulse labeling experiments of Yuan et al. [2009] (Fig. 2D). The explanation for this is that the rate of cycling is fast relative to the time scale of the pulse labeling experiments. The neurofilaments switch between on-track and off-track states on a time scale of minutes or hours, but this behavior averages out during their transport along axons and consequently they behave as a single population on a time scale of days and weeks. To resolve the on and off track pausing states, it is necessary to analyze the decay kinetics on a time scale of minutes or hours [Trivedi et al., 2007]. While there is general agreement that axonal neurofilaments spend a large proportion of their time pausing, our analyses indicate that there is no persistently stationary population and that the vast majority of neurofilaments move over the course of several hours. Thus it is important to note that the "stop and go" model does not predict that all axonal neurofilaments are identical, but simply that any heterogeneities among the neurofilament population (for example, in terms of pausing behavior) are not persistent on the long time scales that are typical of most radioisotopic pulse labeling experiments.

The Importance of Resolving Slow Components "a" and "b"

Our contention that the neurofilament transport kinetics of Nixon and Logvinenko [1986] were contaminated with faster moving Slow Component "b" proteins which comigrate by 1D SDS-PAGE depends on the assumption that there are Slow Component "b" proteins in mouse optic nerve that have similar electrophoretic mobility to neurofilament proteins. In fact, it is known that several hundred Slow Component "b" proteins are radiolabeled in pulse-labeling studies on retinal ganglion cells [Tytell et al. 1981; Brady and Lasek 1982; Garner and Lasek 1982], and some of these proteins do comigrate with neurofilament polypeptides on 1D gels [Lasek et al., 1984; Lewis and Nixon, 1988; Shea et al., 1988; Lasek et al., 1992]. For example, in mouse optic nerve there are two structurally related Triton-soluble Slow Component "b" proteins with apparent molecular weights of 197,000 and 200,000 which comigrate with the highest molecular weight neurofilament protein (neurofilament protein H) by 1D SDS-PAGE. Radioisotopic pulse labeling studies have shown that these proteins form broad waves that move faster than neurofilament protein H, yet overlap considerably [Lewis and Nixon, 1988]. Moreover, these proteins could be separated by Triton-extraction, taking advantage of the insolubility of neurofilaments in the presence of nonionic detergents. Lasek and colleagues have also described a Triton-soluble Slow Component "b" protein with an apparent molecular weight of 70,000 which comigrates with neurofilament protein L [Lasek et al., 1992]. Clearly, these Slow Component "b" proteins would be expected to contaminate the neurofilament transport kinetics in pulse-labeling experiments if total nerve protein were separated by 1D SDS-PAGE. By simulating the movement of Slow Component "b" proteins in optic nerve, we confirmed that this can explain the discrepancy between the biphasic decay kinetics of Nixon and Logvinenko [1986] and the monophasic decay kinetics of Yuan et al. [2009] (Fig. 2D) [Li et al., 2012].

Neurofilament Pausing in Cultured Neurons

In addition to their radio-isotopic pulse-labeling experiments, Yuan et al. [2009] also performed an analysis of neurofilament transport in cultured embryonic cortical neurons using live-cell time-lapse fluorescence microscopy. By tracking the movement of single neurofilaments in photobleached gaps along the axons, the authors confirmed the rapid, intermittent and bidirectional motility that we and others have reported previously [Roy et al., 2000; Wang et al., 2000; Wang and Brown, 2001; Ackerley et al., 2003; Uchida and Brown, 2004; Wang and Brown, 2010]. To analyze the neurofilament transport kinetics on longer time scales, the authors employed a variant of the fluorescence photoactivation pulse-escape technique of Trivedi et al. [2007]. This method uses photoactivation or photoconversion to generate a pulse of fluorescent neurofilaments in the axons. Since the neurofilaments exit the activated region very rapidly when they move, the kinetics of departure are dictated largely by the pause times of the neurofilaments. Thus the pausing kinetics can be determined from the kinetics of departure of neurofilaments from the activated regions.

Instead of using a photoactivatable or photoswitchable fluorescent fusion protein, Yuan et al. [2009] used a conventional fluorescent fusion protein and created a pseudo-activated region by photobleaching the flanking regions. Using this approach, these authors found that the kinetics of neurofilament departure were slower in more neurofilament-rich proximal axonal regions and faster in more neurofilament-poor distal axonal regions. Thus the extent of neurofilament pausing varied inversely with axonal neurofilament content. In proximal axons, where the neurofilament content was highest, the authors claimed that there was no appreciable loss fluorescence from the pseudo-activated regions, implying that the neurofilaments were "unequivocally stationary". To understand these data, it is important to note that the experiments were all performed by co-expressing GFP-tagged neurofilament protein L and untagged neurofilament protein M, which resulted in a marked increase in axonal neurofilament content that may have artificially increased the proportion of neurofilaments that are off track. Nevertheless, close inspection of the data shows that there was loss of neurofilament fluorescence even in the most neurofilament-rich axons. In fact, in two of the figures the fluorescence in proximal or highly expressing axons decreased by 30–40% within the first 30 min [see Figs. 3D and 6D in Yuan et al., 2009], which is comparable to the average rate of neurofilament departure that we have reported in cultured sympathetic neurons from superior cervical ganglia [35% lost within the first 30 min; Trivedi et al., 2007]. In a third figure the fluorescence in a proximal axon decreased by about 30% over a period of 5 h [see Fig. 5C in Yuan et al., 2009]. This is within the range that we have observed for cultured neurons from dorsal root ganglia [Alami et al., 2009], which are neurofilament-rich axons that exhibit much greater off-track pausing than neurons from superior cervical ganglia. Based on these considerations, the kinetic analysis of neurofilament pausing by Yuan et al. [2009] appears to be broadly similar to previous reports in other nerve cell types, and we have shown that those kinetics can be fully explained in terms of the "stop and go" model [Trivedi et al. 2007; Alami et al. 2009].

Implications for Neurofilament Organization in Axons

Over the past 25 years, the concept of a stationary axonal cytoskeleton has influenced how many researchers think about axonal cytoskeletons [e.g., LoPachin et al., 2004, 2005; Barry et al., 2007; Millecamps et al., 2007; Yuan et al., 2009; Sunil et al., 2012]. Perhaps the willingness of researchers to adopt this concept has been influenced by electron micrographs of axons prepared by the rapid-freeze deep-etch method, which have given the impression that the axonal cytoskeleton is an extensively cross-linked network [Hirokawa, 1982; Schnapp and Reese, 1982]. Indeed, the stationary axonal cytoskeleton hypothesis likens neurofilaments to static structural elements in a building, as if welded or cemented together [Yuan et al., 2009]. However, biochemical and morphometric studies on neurofilaments have failed to demonstrate extensive or strong cross-linking interactions between neurofilaments or their sidearms [Price et al., 1988; Brown and Lasek, 1993], and biophysical experiments suggest that neurofilament sidearms interact through long-range repulsive forces, and function more to space adjacent filaments apart rather than to link them together [Price et al., 1988; Brown and Lasek, 1993; Brown and Hoh, 1997; Kumar et al., 2002]. Moreover, it is hard to reconcile the notion of an extensively cross-linked neurofilament network with the extraordinarily dynamic nature of axonal cytoplasm in which organelles and other axonally transported cargoes must continually push neurofilaments aside and apart as they move through the forest of cytoskeletal polymers that makes up axonal cytoplasm. Thus we must be cautious about inferring strength and mechanisms of interaction based on the appearance of neurofilament sidearm projections in electron micrographs. Certainly there are sure to be cross-links between neurofilaments, but there is no evidence that neurofilaments are extensively or stably interconnected in a manner that would preclude intermittent movement.

In conclusion, we believe that the experimental data favor a dynamic model of the axonal cytoskeleton in which the interactions between neurofilaments are mostly weak or reversible, allowing these cytoskeletal polymers to cycle freely between mobile and immobile states during their journey down the axon. Certainly the filaments

spend most of their time not moving, but the average pause duration is on the order of hours rather than weeks or months. Against this more fluid backdrop, the regulated action of molecular motors or temporary cross-linkers on neurofilaments can have dramatic effects on neurofilament organization in ways that would not be possible if the neurofilaments were extensively cross-linked into a truly stationary network, and this may have important implications for the mechanisms by which neurofilaments are organized and reorganized in along axons both in health and disease.

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