



Does neurofilament phosphorylation regulate axonal transport?

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Phosphorylation of neurofilaments has long been considered to regulate their axonal transport rate and, in doing so, to provide stability to mature axons. Interpretation of data recently obtained following C-terminal deletion experiments has prompted a challenge to this hypothesis. We present evidence that these deletion studies remain consistent with, rather than refute, a role for C-terminal phosphorylation in regulation of neurofilament axonal transport.

Neurofilaments consist of three subunits, termed NF-H, NF-M and NF-L, corresponding to heavy, medium and light in reference to their molecular mass. The C-terminal regions ('sidearms') of NF-H and NF-M contain multiple phosphorylation sites [1], and protrude laterally from the filament backbone when phosphorylated [2]. Phosphorylation-dependent interactions of neurofilaments with each other and with other cytoskeletal structures are thought to regulate formation of a cytoskeletal lattice that supports the mature axon [2–4]. The importance of regulation of this process is underscored by the development of phosphorylated-neurofilament spheroids within proximal axons in motor neuron disease [1,4].

The developmental appearance of NF-H is accompanied by slowing of neurofilament axonal transport [5–7]. Hypophosphorylated neurofilaments are transported more quickly than extensively phosphorylated ones [8,9]. NF-H overexpression and increased phosphorylation retard transport of neurofilaments, and elimination of NF-H hastens it [10–13]. These studies have collectively fostered, and subsequently supported, the hypothesis that NF-H C-terminal phosphorylation decreases the rate of neurofilament axonal transport.

Deletion of the NF-H sidearm accelerates transport of the slowest-moving neurofilaments

A recent study [14] questions the above hypothesis. In this work, the investigators replaced the full-length neurofilament subunit with one lacking the sidearm – a potential improvement on prior studies in which the entire NF-H molecule was deleted, because deletion of the entire NF-H subunit increased levels of NF-M and microtubules [13,15], which could alter neurofilament transport by increasing the amount available transport machinery [16,17]. Rao and coworkers injected radiolabeled

³⁵S-methionine into the vitreous body and seven days later examined the distribution of radiolabeled proteins along optic axons. Tubulin and actin distributed identically in the presence and absence of the NF-H sidearm, indicating that overall axonal transport was unaltered. Transport of the 'front' of radiolabeled neurofilament subunits – classically defined as the location of the leading $\leq 10\%$ of radiolabeled subunits [5] and encompassing segments 5–9 of the axon at the time point presented by Rao *et al.* – was also unaltered by NF-H sidearm deletion [14] (Fig. 1). The investigators concluded that sidearm deletion did not affect transport. However, we note that transport of the 'trailing' aspect of the wave was affected by NF-H sidearm deletion; the peak of radiolabeled neurofilament subunits – defined as that segment into which $\geq 50\%$ of radiolabeled subunits have entered [5] and encompassing the first 4 segments in Rao *et al.* – was located a full segment further along axons in mice lacking the NF-H sidearm than in those expressing it (Fig. 1). Because these analyses were carried out seven days after radiolabeling, neurofilament subunits underwent average transport over this interval at 0.39 mm d^{-1} in the absence of the NF-H sidearm, but at only 0.23 mm d^{-1} in its presence. This increase is comparable to the 0.2 mm d^{-1} increase in neurofilament transport following deletion of the entire NF-H molecule [13]; deletion of the sidearm might have been the operative domain leading to accelerated neurofilament transport in these earlier studies. The differential impact of NF-H C-terminal deletion on the front and trail of the moving wave of neurofilaments is clarified by prior studies demonstrating that the front of the wave is enriched in subunits that have not undergone extensive NF-H C-terminal phosphorylation, whereas the trail contains virtually all extensively phosphorylated NF-H subunits [6,8,9] (Fig. 2). The presence or absence of the NF-H C-terminal sidearm might, therefore, be inconclusive for determining whether or not phosphorylation influences transport of the front of the neurofilament wave, because the front is relatively lacking in extensive C-terminal phosphorylation, even in normal mice. Rather, analyses of the impact of NF-H C-terminal phosphorylation on neurofilament axonal transport in the trailing portion of the moving wave are more appropriate, because the trail contains virtually all detectable extensively phosphorylated NF-H subunits. Selective acceleration of transport of the trail with no alteration of transport of the front [14], coupled with the normal concentration of

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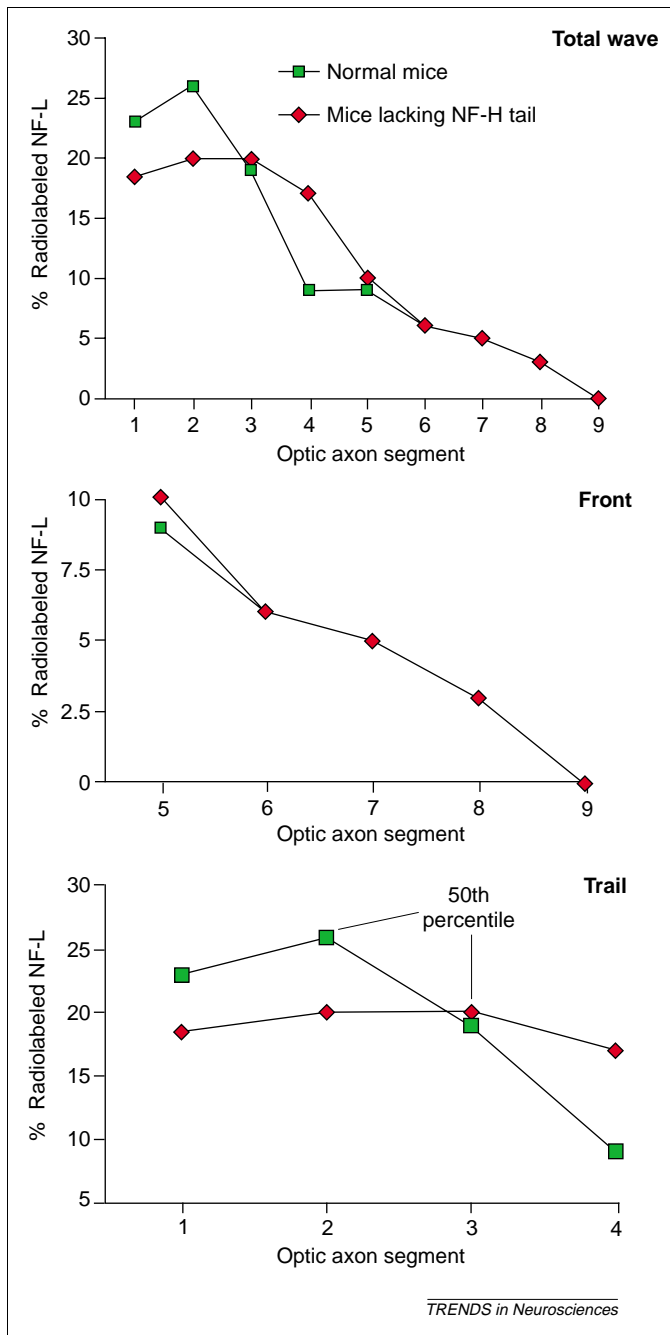


Fig. 1. Deletion of the C-terminal sidearm of the heavy neurofilament subunit (NF-H) accelerates neurofilament axonal transport. Panels present the percentage distribution of radiolabeled light neurofilament subunit (NF-L) along successive 1.1 mm segments of the total optic axon ('Total wave') or within the front or trail (presented separately to highlight these regions). Using data from Ref. [14]. Although data for NF-L are presented, all three subunits display identical distribution [14]. Note that the front ($\leq 10\%$ of radiolabel [5]) is identical in the presence or absence of the NF-H sidearm, whereas the peak ($\geq 50\%$) is recovered within segment 2 for normal mice but within segment 3 for mice lacking the NF-H sidearm.

extensive C-terminal phosphorylation within the trail and its absence from the front [8], support the prior hypotheses that NF-H C-terminal phosphorylation regulates neurofilament transport. Accordingly, although the data of Rao *et al.* indeed demonstrate that deletion of the NF-H sidearm does not affect overall axonal transport, these data remain consistent with the notion that the NF-H sidearm plays a role in the progressive slowing of neurofilament axonal transport, and that this role is

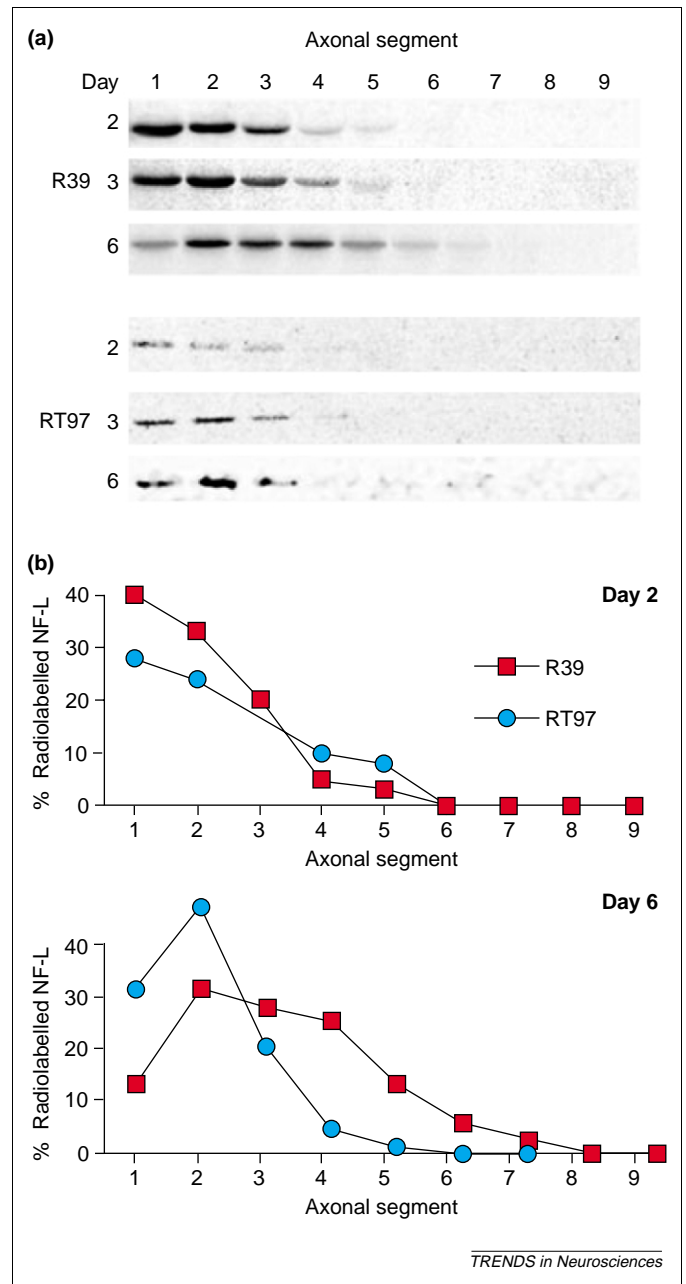


Fig. 2. Extensively phosphorylated heavy neurofilament subunits (NF-H) are transported more slowly than less phosphorylated subunits. At 2–6 d following intravitreal injection of ^{35}S -methionine, neurofilament subunits were immunoprecipitated from optic axon segments with an NF-H C-terminal anti-phospho-antibody (RT97) or with a phosphorylation-independent polyclonal antibody (R39) [11], then subjected to electrophoresis and autoradiography (a). (b) Densitometric analyses of the distribution of radiolabeled NF-H precipitated by each antibody as a percentage of total radiolabeled NF-H. Note that NF-H subunits expressing the RT97 phosphorylated epitope are absent from the leading segments, and are instead concentrated within the trail. Reproduced, with permission, from Ref. [8].

modulated by phosphorylation. Of interest would be to examine the impact of NF-H C-terminal deletion on the association of neurofilament isoforms with the slowest-moving neurofilament population (the so-called stationary cytoskeleton) that is retained for extended periods along optic axons in long-term radiolabeling analyses [9].

Several additional points should be considered. Although it is possible to attribute the increase in neurofilament transport rate following deletion of the

entire NF-H molecule to the compensatory increase in microtubules [13,15], this explanation does not account for the decrease in transport rate that accompanies increased NF-H expression [12]. A second consideration is that NF-M sidearm phosphorylation undergoes a compensatory increase to the extent that it displays the phosphorylation-dependent RT97 epitope (which is normally detected only on NF-H) in mice in which the entire NF-H molecule or only its sidearm have been deleted [13,14,18,19]. Such compensatory changes in NF-M after modulation of NF-H expression might well effect neurofilament transport following deletion of NF-H and/or the NF-H sidearm.

Phosphatase inhibition alters neurofilament phosphorylation and transport

However, the above analyses only indirectly support a role for phosphorylation of the NF-H C-terminal in regulation of neurofilament transport. This has been more directly addressed by correlation of regional alterations transport rate with regional increases in neurofilament phosphorylation before and following *in situ* manipulation of phosphatase activity [11]. Neurofilaments normally exhibit a relatively fast transport rate upon exiting the retina, and undergo progressive slowing concomitant with extensive phosphorylation along axons. *In situ* phosphatase inhibition dramatically increased NF-H C-terminal phosphorylation in retinas and proximal axonal segments (where such phosphorylation is normally weak or absent), and decreased axonal transport of the peak in this region by 40%; overall axonal transport was not similarly decreased. These data demonstrate an inverse relationship between neurofilament phosphorylation and transport.

Not previously considered was the impact of phosphatase inhibition on transport of the front. Re-examination of these data demonstrate a 44% decrease in transport of the front (matching the 40% decrease of the peak) within regions displaying *de novo* increased phosphorylation (Fig. 3) – further evidence for a cause-and-effect relationship between NF-H C-terminal phosphorylation and neurofilament transport. Of interest would be to examine the influence of phosphatase inhibition on neurofilament transport in mice lacking the NF-H sidearm.

Finally, NF-H constructs with sidearms engineered to mimic the dephosphorylated state underwent faster transport in cortical neurons than did similar constructs engineered to mimic the phosphorylated state [20]; this latter result is consistent with prior studies of the distribution of endogenous neurofilaments that collectively support a role for neurofilament phosphorylation in regulation of axonal transport. Further studies will be necessary to resolve the discrepancy raised by the studies of Rao *et al.* regarding the nature and extent to which phosphorylation of NF-H, and perhaps NF-M, sidearms regulates neurofilament transport in axons. Such resolution will be essential to provide direction for continued studies of the role of the axonal cytoskeleton in health and in motor neuron disease.

Concluding remarks

We consider that the data of Rao *et al.* are consistent with, and do not refute, a role for C-terminal phosphorylation in

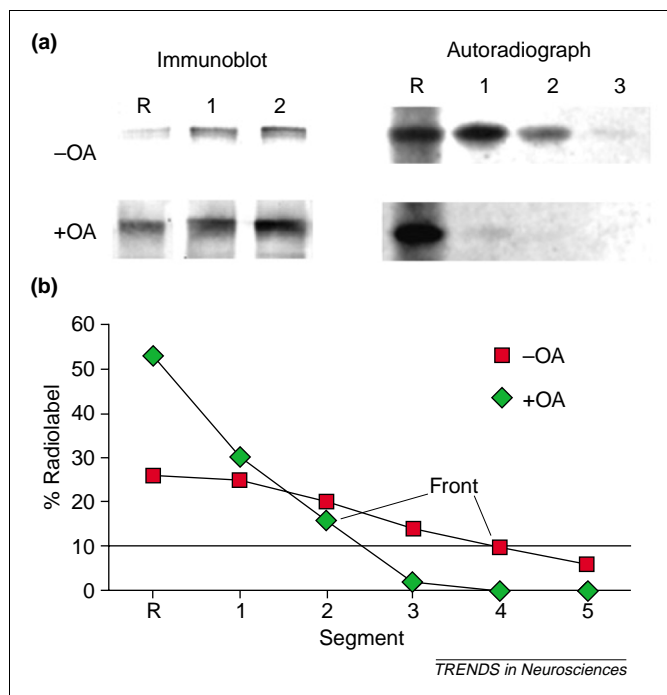


Fig. 3. Phosphatase inhibition increases neurofilament phosphorylation and decreases neurofilament axonal transport rate. (a) Immunoblot and autoradiographic analyses of retinas (R) and proximal segments (1–3) of optic axons harvested from mice one day after intravitreal injection of ^{35}S -methionine (to radiolabel neurofilaments), with and without the phosphatase inhibitor okadaic acid (OA). Immunoblot analyses present the 200 kDa region probed with the monoclonal antibody RT97; the autoradiograph presents NF-L (the 70 kDa neurofilament subunit) as an index of total radiolabeled neurofilaments. (b) The percentage distribution of radiolabeled NF-L. Note that okadaic acid increases regional C-terminal phospho-immunoreactivity of the heavy neurofilament subunit (NF-H) and decreases transport of the front of radiolabeled subunits. The horizontal line at the 10% level denotes the difference in migration of the front under each condition. Reproduced, with permission, from Ref. [11].

regulation of neurofilament axonal transport. However, we stress that their studies make the important distinction that this role is confined to the trailing aspect of the moving wave, rather than affecting overall neurofilament transport. Because it is the trail that exhibits protracted residence time in axons [6,8,9], their novel approach provides important insight into mechanisms by which neurofilament phosphorylation contributes to increased residence time along axons, potentially reducing the need for neurofilament replacement.

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