



## Research Report

# The high and middle molecular weight neurofilament subunits regulate the association of neurofilaments with kinesin: Inhibition by phosphorylation of the high molecular weight subunit

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## Abstract

Kinesin participates in axonal transport of neurofilaments (NFs), but the mode by which they attach to kinesin is unclear. We compared the association of NFs with kinesin in mice expressing or lacking NF-H or NF-M. In normal and M<sup>-/-</sup> mice, the leading edge of metabolically labeled NF subunits was selectively co-precipitated with kinesin. By contrast, the entire wave of radiolabeled subunits co-precipitated with kinesin in H<sup>-/-</sup> mice. Similar bulk levels of NFs co-precipitated with kinesin from normal and H<sup>-/-</sup> mice, but reduced levels co-precipitated from M<sup>-/-</sup> mice. These data suggest that both NF-H and NF-M regulate the association of NFs with kinesin. They further indicate that phosphorylation of NF-H dissociates NFs from kinesin and provides a mechanism by which NF-H phosphorylation can contribute to the slowing of NF axonal transport.

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Phosphorylation, in particular that of the C-terminal extensions of neurofilament (NF) subunits, has long been considered to regulate NF axonal transport [1,4,7,13,14,19], although the nature and extent of this regulation remain controversial [30]. One mechanism by which phosphorylation may contribute to a slowing of NF transport is by phospho-dependent interactions of NFs with their transport motors [28,29]. Since the association of NFs with their motors is likely to be reversible, site-specific C-terminal NF phosphorylation has been proposed as a potential mechanism to regulate this association; this is consistent with continued C-terminal phosphorylation and dephosphorylation of NFs during their transport along optic axons

[12,16,22]. The characteristic broadening of the wave of transporting NFs [13,14,19] has been interpreted to reflect in part differential association and dissociation of NFs with their transport motor: the fastest moving NFs would represent those spending relatively more time in association with the motor, while the slowest moving NFs represent those that have dissociated from the motor for relatively longer periods. These conclusions are supported by the more rapid axonal transport of less phosphorylated variants (and slowest transport of the most phosphorylated variants) along optic axons, by the slowing of NF transport following phosphatase inhibition *in situ* and by the relative faster rate of subunit transport within retinas prior to their entry into optic axons [9–11,16]. They are further supported by faster NF transport following elimination of NF-H and slower transport following NF-H overexpression [3,17,39].

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55 Axonal transport is divided into two broad categories,  
56 termed “slow” and “fast” axonal transport [2]. NFs undergo  
57 slow transport [2,28]. Studies from several laboratories  
58 demonstrate that the anterograde-directed motor, kinesin,  
59 mediates anterograde NF axonal transport [23,32,34–36].  
60 The ability of kinesin, which undergoes fast axonal trans-  
61 port, to translocate slower-moving NFs is explained by the  
62 observation that NFs undergo short bursts of rapid transport  
63 that are interrupted by prolonged pauses [25,33], the net  
64 result of which averages out to an overall rate consistent  
65 with slow axonal transport [2,29].

66 The association of NFs with kinesin is regulated at least  
67 in part by NF phosphorylation since extensively phosphory-  
68 lated NF subunits did not co-purify in a microtubule motor  
69 preparation from brain while less phosphorylated forms did  
70 and since phosphorylation under cell-free conditions dis-  
71 rupted the association of NF subunits with kinesin [35,36].  
72 Moreover, the front, but not the trail, of the broadening  
73 wave of radiolabeled NF subunits was co-precipitated with  
74 kinesin [36]. Since the front of the wave is enriched in  
75 poorly phosphorylated NF subunits, while the trail is  
76 enriched in extensively phosphorylated subunits  
77 [10,11,16], these findings suggest that one mechanism by  
78 which phosphorylation can regulate NF transport is by  
79 regulating the association of NFs with kinesin. To test this  
80 hypothesis, we compared herein the nature and extent of  
81 association of kinesin with NFs along optic axons in normal  
82 mice with mice lacking NF-H or NF-M [8,39].

83 Murine retinal ganglion cells of normal mice and mice  
84 lacking NF-H (H<sup>-/-</sup>; 39) or NF-M (M<sup>-/-</sup>; 8) were

radiolabeled in situ by injection of 70  $\mu\text{Ci}$   $^{35}\text{S}$ -methionine in 85  
a total volume of 0.2  $\mu\text{L}$  via a pulled glass capillary pipette 86  
into the vitreous of anesthetized mice [65]. Mice were 87  
sacrificed by cervical dislocation at 6 days following 88  
injection, at which time  $\geq 90\%$  of radiolabeled NF subunits 89  
have entered optic pathways but have not reached the final 90  
segment (indicating no appreciable exit from optic axons) 91  
[36]. Retinas were dissected away from the rest of the eye, 92  
and optic axons dissected into  $9 \times 1.1$  mm segments on a 93  
glass slide on dry ice. Retinas and segments from 5 to 11 94  
mice were pooled and homogenized in 1% Triton X-100 in 95  
50 mM Tris (pH 6.9) containing 2 mM EDTA, 1 mM PMSF 96  
and 50  $\mu\text{g/ml}$  leupeptin at 4  $^{\circ}\text{C}$  by 50 strokes in a tight-fitting 97  
glass-Teflon homogenizer [20]. The Triton-insoluble cytos- 98  
keleton was sedimented by centrifugation at  $15,000 \times g$  for 99  
15 min. Data presented in Fig. 1 for normal mice appeared 100  
previously [36] and are reproduced herein (with permission) 101  
to facilitate comparison of subunit distribution of normal 102  
optic axons with that of H<sup>-/-</sup> and M<sup>-/-</sup> mice. Precipitation 103  
was carried out using a 1:150 dilutions of a polyclonal 104  
antibody that quantitatively immunoprecipitates all 3 NF 105  
subunits (R39) or a pan-specific polyclonal anti-kinesin 106  
antibody raised against bovine brain kinesin (Cytoskeleton, 107  
Inc., Denver, CO) that does not sediment NFs in the absence 108  
of kinesin followed by protein A–sepharose (10 mg; Sigma) 109  
as described [35]. Precipitated material was subjected to 110  
SDS-gel electrophoresis and placed against film to generate 111  
autoradiographs, which were digitized and analyzed via NIH 112  
Image software by encircling the entire band. Since the NF 113  
triplet co-migrated along optic axons, we subsequently 114

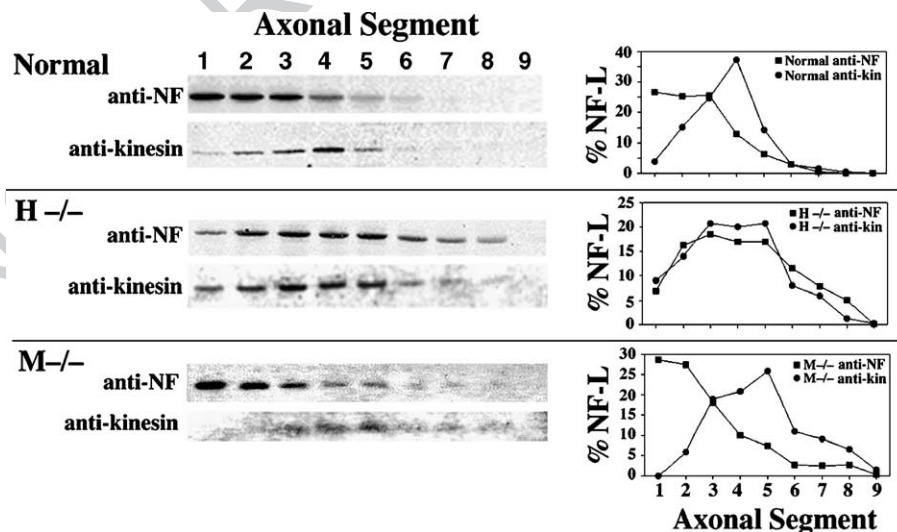


Fig. 1. Differential transport rates and association of NFs with kinesin in normal, H<sup>-/-</sup> and M<sup>-/-</sup> mice. Optic axons from normal, H<sup>-/-</sup> and M<sup>-/-</sup> mice were radiolabeled for 6 days with  $^{35}\text{S}$ -methionine, dissected into  $9 \times 1.1$  mm segments and subjected to immunoprecipitation with R39 (“anti-NF”) or co-precipitation with anti-kinesin. Precipitated material was subjected to electrophoresis and autoradiography. The 70 kDa region (corresponding to NF-L) is presented. The accompanying graphs present the percent distribution of radiolabeled NF-L obtained via densitometric analyses of autoradiographs following immunoprecipitation with R39 and co-precipitation with anti-kinesin for each mouse strain. Note the similarity in the profile of NF-L recovered by both antibodies from H<sup>-/-</sup> mice, while the profiles obtained for these antibodies differed in normal and M<sup>-/-</sup> mice (see text and Table 1). As shown previously, anti-kinesin selectively co-precipitated the front of the radiolabeled wave in normal mice [reproduced with permission from Yabe et al. (2000) and presented to facilitate comparison]. Similarly, anti-kinesin also selectively co-precipitated the front of the radiolabeled wave in M<sup>-/-</sup> mice. By contrast, anti-kinesin co-precipitated the entire wave of radiolabeled subunits from H<sup>-/-</sup> mice.

115 present densitometric data only for NF-L for simplicity  
 116 only [14]. Total subunit radiolabeled recovered from the  
 117 retina, and all axonal segments were defined as 100%,  
 118 and the relative amount of radiolabel in each segment was  
 119 then expressed as a percentage of the total radiolabel.  
 120 Densitometric values from individual autoradiographs  
 121 from each of two experiments were pooled, resultant  
 122 ratios for each experiment were averaged, and the mean  
 123 values were statistically compared using a one-tailed  
 124 Student's *t* test.

125 The distribution of radiolabeled NF subunits along the  
 126 optic pathway obtained following co-precipitation with a  
 127 pan-specific polyclonal anti-kinesin antibody was compared  
 128 with that obtained by immunoprecipitation of NF subunits  
 129 with a phospho-independent antibody (“R39”) directed  
 130 against all 3 NF subunits. Immunoprecipitation with R39  
 131 confirmed that, as reported previously in peripheral nerve  
 132 [8,39], NF transport in optic axons was accelerated in both  
 133 H<sup>-/-</sup> and M<sup>-/-</sup> mice (Fig. 1; Table 1). In normal mice,  
 134 the profile of radiolabeled subunits immunoprecipitated  
 135 with R39 differed markedly from that co-precipitated with  
 136 anti-kinesin. R39 immunoprecipitated the entire wave of  
 137 radiolabeled subunits, yielding a distribution of NF subunits  
 138 along axons identical to that of total axonal segments  
 139 without precipitation (e.g., [16,20]). By contrast, co-  
 140 precipitation with anti-kinesin selectively co-precipitated  
 141 the front of the moving wave in normal mice (Fig. 1; see  
 142 also [35]). In normal mice, the peak of radiolabeled NF-L  
 143 immunoprecipitated with R39 lagged a segment behind the  
 144 peak co-precipitated with anti-kinesin, further indicating  
 145 that the faster-moving subunits were selectively associated  
 146 with kinesin in situ [36]. A similar pattern was observed for  
 147 M<sup>-/-</sup> mice, although the peak co-precipitated with anti-  
 148 kinesin was one segment ahead of that co-precipitated from  
 149 normal mice. By contrast, the distribution of radiolabeled  
 150 subunits in H<sup>-/-</sup> mice differed dramatically from that of  
 151 normal or M<sup>-/-</sup> mice. The distribution of radiolabeled  
 152 subunits in H<sup>-/-</sup> mice following immunoprecipitation with  
 153 R39 or co-precipitation with anti-kinesin was virtually  
 154 identical; the entire wave of radiolabeled subunits was  
 155 recovered from H<sup>-/-</sup> mice whether immunoprecipitated

with R39 or co-precipitated with anti-kinesin (Fig. 1).  
 Moreover, the peak of radiolabeled subunits was recovered  
 in the same segments with either antibody (Table 1). These  
 data indicated that a larger proportion of radiolabeled  
 subunits along the entire optic pathway segments of H<sup>-/-</sup>  
 mice remained in association with kinesin in situ, as opposed  
 to the selective association of only the front of the moving  
 wave with kinesin in normal and M<sup>-/-</sup> mice.

The findings of the present study, taken together with  
 prior reports, allow several conclusions and speculations  
 regarding the nature of association of NFs with kinesin. One  
 conclusion is that the binding site(s) for kinesin cannot  
 reside exclusively in NF-H; if this were the case, we would  
 have observed a reduction, rather than an increase, in the  
 level of radiolabeled NFs co-precipitated with kinesin in  
 H<sup>-/-</sup> mice. By contrast, reduction in NFs co-precipitating  
 with kinesin from M<sup>-/-</sup> mice suggests that NF-M may  
 mediate the association of NFs with kinesin [38], which  
 would be consistent with the prior observation of transport  
 of NF-M in the absence of NFs [31]. However, NF-M  
 cannot be the only subunit that mediates NF-kinesin  
 associations since some NFs still do associate with kinesin  
 in mice lacking NF-M. The continued association of some  
 NFs with kinesin after elimination of NF-H or NF-M  
 suggests by default that NF-L mediates association of NFs  
 with kinesin. However, this contrasts with the failure of  
 purified NF-L to co-precipitate with anti-kinesin in cell-free  
 analyses under conditions in which both NF-H and NF-M  
 were co-precipitated [36]. In addition, few NFs are detected  
 within axons of mice lacking both NF-H and NF-M  
 [8,38,39]. These data collectively suggest that both NF-M  
 and NF-H are capable of mediating the association of NFs  
 with kinesin and that NF-L is apparently not capable of  
 efficient association with kinesin in cell-free analyses or  
 in situ, at least in the absence of either NF-H or NF-M.

The data of the present study also suggest that phospho-  
 NF-H inhibits the association of transporting NFs with  
 kinesin since the trail of radiolabeled NFs, normally not  
 co-precipitated with kinesin, was co-precipitated with  
 kinesin in the absence of NF-H. This may be an indirect  
 effect; for example, NFs bearing extensively phosphorylated  
 C-termini exhibit increased NF–NF associations [6,16,37],  
 and such associations may preclude binding to kinesin.  
 Since phospho-NF-H is concentrated within the trail of the  
 transporting wave [10,11,16] and phosphorylation of  
 NF-H is thought to mediate NF–NF associations [6,15,37],  
 these data prompt the speculation that NFs lacking NF-H  
 may not exhibit the protracted residence time along axons  
 characteristic of NFs containing NF-H [20]. The present  
 data are consistent with that possibility since less radiolabel  
 persisted within the initial optic pathway segments in  
 H<sup>-/-</sup> versus normal or M<sup>-/-</sup> mice (e.g., Fig. 1). Alternatively,  
 since NF-M undergoes increased C-terminal phosphorylation  
 in the absence of NF-H [26,39] and since these more  
 extensively phosphorylated forms are selectively not  
 associated with kinesin, one speculation arising from these

t1.1 Table 1  
 t1.2 Differential association of radiolabeled NF-L with kinesin in normal, H<sup>-/-</sup>  
 and M<sup>-/-</sup> mice

	Location of peak of NF-L radioactivity		
	Normal	H <sup>-/-</sup>	M <sup>-/-</sup>
t1.5 Anti-NF	3	5	3
t1.6 Anti-kinesin	4	5	5

Values present the segment (out of 9 × 1.1 mm segments) of optic axons in which the peak was recovered following co-precipitation with R39 (anti-NF) or anti-kinesin as indicated 6 days after injection of radiolabel. The peak (defined as the 50th percentile) was determined from the densitometric data presented in Fig. 1 as described in Materials and methods. Note the difference in migration of the peak of radiolabeled subunits precipitated by anti-NF versus anti-kinesin from normal and M<sup>-/-</sup> mice, while these values are instead identical for H<sup>-/-</sup> mice.

t1.7

findings is that extensively phosphorylated NF-M may mediate NF–NF associations in the absence of NF-H. The data of this and prior studies collectively leave open the possibility that NF-H and NF-M may mediate complementary/overlapping roles in NF transport and residence time within axons and that these roles are regulated by phosphorylation. That is, NF-M may be primarily responsible for association of NFs with their anterograde transport vector, while NF-H may be primarily responsible for mediating NF–NF associations; however, either subunit can apparently perform either function. Overlapping roles for phosphorylation of NF-H and NF-M could also explain why the transport rate of NFs is increased following deletion of either subunit [8,39] since the lack of either subunit would both minimize phospho-dependent NF–NF associations and allow NFs to spend more time with their motor, yet the NF transport rate does not achieve a rate consistent with fast axonal transport since phosphorylation of either remaining subunit would negatively regulate sustained association of NFs with their motor. The fact that both NF-H and NF-M contain many phosphorylation sites that are targets of multiple kinases [22], coupled with compensatory increase and alteration in NF-M phosphorylation in the absence of NF-H [26,39], and the fact that sidearm presence and phosphorylation each influences axonal caliber [5,17,18,21] and NF–NF interactions [15,16,27,37], all of which impede transport, represent factors that have confounded interpretation of the regulation of phosphorylation on NF axonal transport in general. These factors further confound interpretation of the regulation of subunit expression and phosphorylation on the association of NFs with their transport vector.

Despite these complexities, the data of the present study provide further insight into mechanisms by which NF phosphorylation might negatively regulate NF axonal transport velocity. The resultant increase in residence time of NFs within axons would serve to reduce the frequency of turnover of axonal NF subunits, which would decrease the metabolic burden of maintenance of the axonal cytoskeleton on the neuron. The aberrant accumulation of NFs, including abnormally phosphorylated isoforms, within perikarya and proximal neurons in multiple human disorders [24] underscores the importance of elucidating the mechanisms regulating NF transport.

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