Article

The neurofilament middle molecular mass subunit carboxyl-terminal tail domains is essential for the radial growth and cytoskeletal architecture of axons but not for regulating neurofilament transport rate

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The phosphorylated carboxyl-terminal "tail" domains of the neurofilament (NF) subunits, NF heavy (NF-H) and NF medium (NF-M) subunits, have been proposed to regulate axon radial growth, neurofilament spacing, and neurofilament transport rate, but direct in vivo evidence is lacking. Because deletion of the tail domain of NF-H did not alter these axonal properties (Rao, M.V., M.L. Garcia, Y. Miyazaki, T. Gotow, A. Yuan, S. Mattina, C.M. Ward, N.S. Calcutt, Y. Uchiyama, R.A. Nixon, and D.W. Cleveland. 2002. *J. Cell Biol.* 158:681–693), we investigated possible functions of the NF-M tail domain by constructing NF-M tail–deleted (NF-M$^{tail\Delta}$) mutant mice using an embryonic stem cell–mediated "gene knockin" approach that preserves normal ratios of the three neurofilament subunits. Mutant NF-M$^{tail\Delta}$ mice exhibited severely inhibited radial growth of both motor and sensory axons. Caliber reduction was accompanied by reduced spacing between neurofilaments and loss of long cross-bridges with no change in neurofilament protein content. These observations define distinctive functions of the NF-M tail in regulating axon caliber by modulating the organization of the neurofilament network within axons. Surprisingly, the average rate of axonal transport of neurofilaments was unaltered despite these substantial effects on axon morphology. These results demonstrate that NF-M tail–mediated interactions of neurofilaments, independent of NF transport rate, are critical determinants of the size and cytoskeletal architecture of axons, and are mediated, in part, by the highly phosphorylated tail domain of NF-M.

Introduction

Neurofilaments (NFs) belong to a family of intermediate filament proteins that have 10-nm diam compared with actin (4–5 nm) and microtubule (25 nm) cytoskeletal networks. NFs are the intermediate filament cytoskeletal networks of neurons and are formed as obligate heteropolymers (Ching and Liem, 1993; Lee et al., 1993) composed of neurofilament light subunit (NF-L; 68 kD), neurofilament medium subunit (NF-M; 160 kD), and neurofilament heavy subunit (NF-H; 200 kD). Neurofilaments play an important role in the establishment of proper axonal diameters (Friede and

Samorajski, 1970; Hoffman et al., 1987; Cleveland et al., 1991; Ohara et al., 1993; Zhu et al., 1997), which, in turn, act as principal determinants of the conduction velocity of electric impulses along axons (Gasser and Grundfest, 1939; Waxman, 1980; Sakaguchi et al., 1993).

With the advent of transgenics and gene deletion technologies, mice that either overexpress or are deleted for neurofilament subunit genes indicate that NFs play a pivotal role in the regulation of normal axonal diameters, and this property is sensitive to the levels of NFs in the axon (Ohara et al., 1993; Eyer and Peterson, 1994; Tu et al., 1995; Marszalek et al., 1996; Wong et al., 1996; Zhu et al., 1997; Elder et al., 1998a; Jacomy et al., 1999). Moreover, overex-

Abbreviations used in this paper: NF, neurofilament; NF-H, NF heavy subunit; NF-L, NF light subunit; NF-M, NF medium subunit.

pression of NF-L with either NF-M or NF-H results in much bigger increases in diameters (Xu et al., 1996; Meier et al., 1999) compared with inhibition of the same growth in single transgenic mice, indicating that normal subunit ratios are critical (Monteiro et al., 1990; Eyer and Peterson, 1994; Tu et al., 1995; Marszalek et al., 1996; Wong et al., 1996; Xu et al., 1996).

Newly synthesized neurofilaments are transported along axons at an average rate of 0.2–1.0 mm/d (Hoffman and Lasek, 1975; Black and Lasek, 1980), although the kinetic behavior of the entire neurofilament population within axons is complex (Nixon, 1998; Jung et al., 2000a,b; Wang et al., 2000; Rao et al., 2002b; Ackerley et al., 2003; Brown, 2003; Rao and Nixon, 2003). Together with microtubules and microfilaments, neurofilaments create a nonuniform fibrous network along the length of mature axons, which is extensively cross-linked by lateral cross-bridges formed by various linking proteins, including plectin (Errante et al., 1994; Rao et al., 1998), BPAGn/dystonin (Yang et al., 1996; Dalpe et al., 1998), gigaxonin (Bomont et al., 2000), myosin Va (Rao et al., 2002b), and, possibly, extended domains of the neurofilament triplet proteins (Willard and Simon, 1981; Hirokawa et al., 1984). How transported neurofilaments and related cytoskeletal elements accumulate locally and achieve the proper integration into this regionally specialized cytoskeletal network along axons is poorly understood.

The phosphorylation of NF-M and NF-H carboxyl-terminal domains has also been suspected of regulating axon caliber by controlling neurofilament transport (Nixon et al., 1982; Lewis and Nixon, 1988), local accumulation (Nixon, 1998; Sanchez et al., 2000), and spacing in relation to their neighbors (Gotow et al., 1992; Nixon et al., 1994; Pant and Veeranna, 1995; Sanchez et al., 1996). Phosphorylated subunits of NF-M and NF-H contain 15 and 50 mol phosphate, respectively (Jones and Williams, 1982; Julien and Mushynski, 1982; Geisler et al., 1987; Goldstein et al., 1987; Lee et al., 1988), and they are the most extensively phosphorylated proteins in neurons (Julien and Mushynski, 1982, 1983; Carden et al., 1985). The carboxyl-terminal domains of these two subunits have multiple lysine-serine-proline (KSP) repeat motifs, which are variably phosphorylated in the axon mainly after neurofilaments enter the axon (Sternberger and Sternberger, 1983; Glicksman et al., 1987; Nixon et al., 1987; Oblinger et al., 1987). This process is linked to intraaxonal local accumulation of neurofilaments (Nixon et al., 1994), which drives caliber expansion and, in turn, is regulated by signals emanating from myelinating glial cells (de Waegh et al., 1992; Hsieh et al., 1994; Nixon et al., 1994; Sanchez et al., 1996, 2000; Yin et al., 1998). NF-H overexpression in mice (Collard et al., 1995; Marszalek et al., 1996), or hyperphosphorylation of NF-H on its carboxyl-terminal region, slows axonal transport of neurofilaments (Jung et al., 2000b; Ackerley et al., 2003), whereas hypophosphorylated NF-H molecules are transported in primary neuronal cultures at much faster rates than normal (Ackerley et al., 2000, 2003; Jung et al., 2000a). However, recent studies have shown that genetic deletion of NF-H (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) or its carboxyl-terminal domain (Rao et al., 2002a; Rao and

Nixon, 2003) in mice has relatively small effects on the overall radial growth of motor axons, spacing between neurofilaments (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998), and average neurofilament transport rates (Zhu et al., 1998; Rao et al., 2002a). These observations imply that either NF-H tail phosphorylation by itself does not regulate all the properties that are previously ascribed to it, or compensatory mechanisms involving NF-M (Sanchez et al., 2000) or other molecules replace these putative functions of NF-H in mice lacking NF-H.

NF-M domain function has been less extensively investigated than NF-H, although existing data suggest that NF-M may be the more influential subunit in regulating known neurofilament behaviors. NF-M, unlike NF-H, is essential for filament assembly (Elder et al., 1998a; Jacomy et al., 1999). Deletion of NF-M protein results in a fourfold acceleration of the rate of slow axonal transport of NFs in sciatic nerves (Jacomy et al., 1999). Overexpression of NF-M has complex effects on neurofilament number, transport, spacing, and axon caliber (Tu et al., 1995; Wong et al., 1996; Xu et al., 1996; Xu and Tung, 2001). To investigate the in vivo role of NF-M tails specifically, we constructed NF-M tail deletion mice by the ES cell–mediated "gene knockin" approach, which does not alter subunit stoichiometry, and therefore, represents a relatively selective in vivo perturbation of NF-M tail function. This work has revealed a distinct role of NF-M tails in establishing inter-neurofilamentous cross-bridges that control the normal spacing between neurofilaments and modulate axon radial growth. The findings also demonstrate that NF-M and NF-H tails, despite structural similarities, have distinct functions.

Results

Loss of NF-M tails does not change the subunit ratios of neurofilaments

NF-M tail–deleted mice (NF-M$^{tail\Delta}$) are produced by construction of a targeting vector from the mouse NF-M gene (Jacomy et al., 1999) in which the carboxyl-terminal 426 amino acids of NF-M were replaced with a Myc epitope tag and a neomycin phosphotransferase gene (Fig. 1 A). Transfection of targeting vector and selection yielded 2 out of 282 ES cell clones that were correctly recombined into one of the NF-M alleles. Both of the ES cell clones were injected into C57BL/6J blastocysts to produce chimeric animals that, when bred to C57BL/6J mice, transmitted the NF-M$^{tail\Delta}$ allele to the progeny. Mating pairs of heterozygous male and female mice for NF-M$^{tail\Delta}$ produced homozygous animals (Fig. 1, B and C) at the expected Mendelian frequency. These mice are viable, reproduce normally, and have no overt phenotype up to 2 yr of age.

Total tissue extracts of equal amounts from sciatic and optic nerves on Coomassie blue–stained gel indicated that full-length NF-M is reduced in heterozygous mice, and completely absent in homozygous mice, and is replaced by a novel 50-kD polypeptide only in heterozygous and homozygous animals (Fig. 1 D, lanes 2, 3, 5, 6, 8, and 10, arrows; Fig. 1, E and F, and see Fig. 5 B). This was confirmed by probing the protein blots of the same extracts with an mAb directed against the rod domain of NF-M
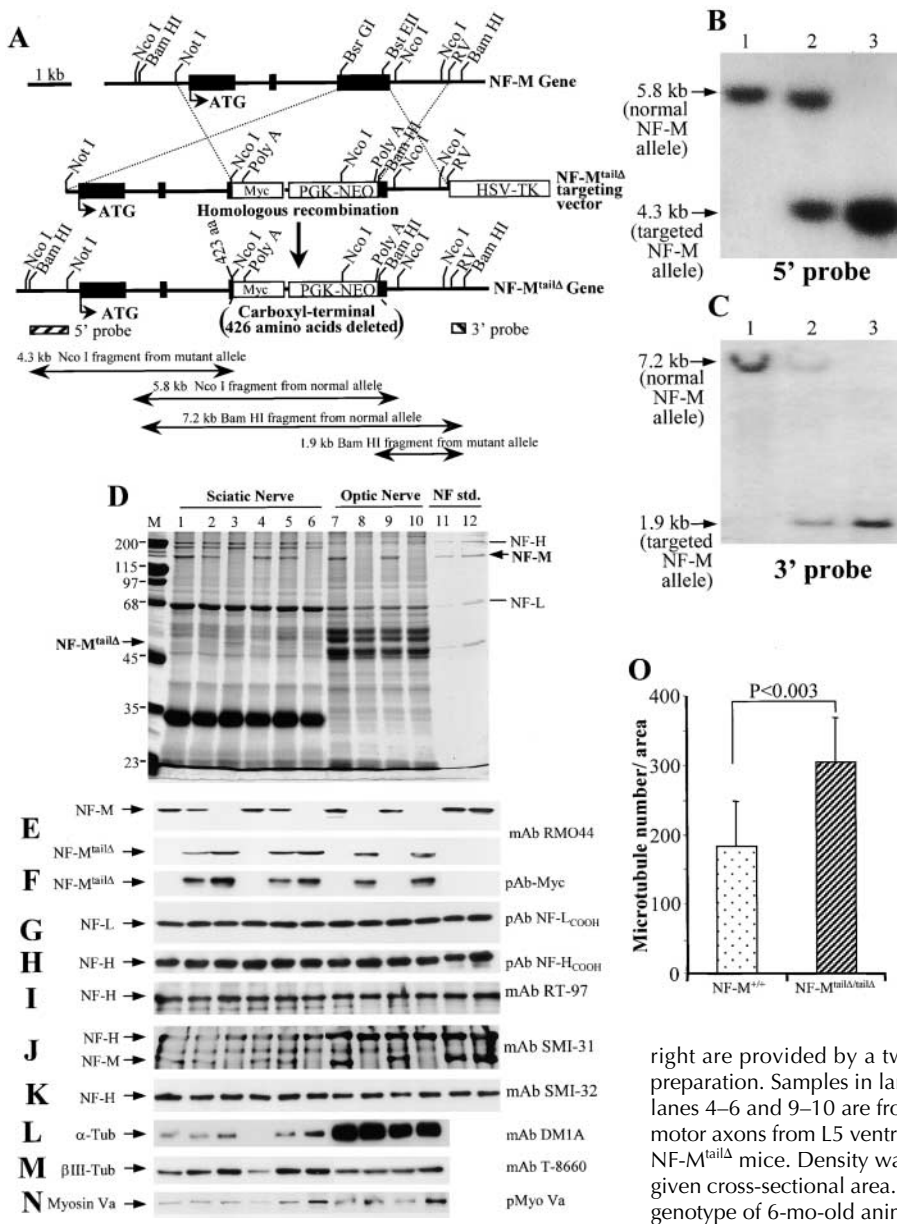
Figure 1. **NF-M[tailΔ] does not alter the subunit ratio of neurofilament subunits.** (A) Construction of NF-M[tailΔ] results in a loss of 426 of the 449–amino acid tail of NF-M protein, and this region is replaced with a Myc epitope tag. The three exons of NF-M gene are indicated by the black boxes and interrupted by two introns. ATG denotes the translation initiation codons. Dotted lines indicate the regions that underwent homologous recombination between the targeting vector and the endogenous NF-M allele. (B and C) Identification of NF-M[tailΔ] mice. (B) Genomic DNA blots of mouse tails hybridized to the region denoted as 5' probe in A. (C) A similar blot as in B but hybridized with the sequence in A marked 3' probe. DNA are from littermate mice with two wild-type NF-M alleles (lane 1), heterozygous (lane 2), or homozygous (lane 3) for the NF-M[tailΔ] allele. (D–N) NF-M tail deletion does not change NF subunit ratio but increases axonal tubulin. Sciatic and optic nerve extracts from wild-type (lanes 1, 4, 7, and 9), NF-M[tailΔ] heterozygous (lanes 2 and 5), and NF-M[tailΔ] homozygous (lanes 3, 6, 8, and 10) mice were fractionated on 7% SDS–polyacrylamide gels and stained with Coomassie blue (D) or immunoblotted with antibodies that recognize (E) NF-M in a phosphoindependent manner; (F) NF-M[tailΔ] with Myc antibodies; (G) NF-L; (H) NF-H; (I) NF-H phosphospecific, RT-97; (J) NF-H phosphospecific, SMI-31; (K) NF-H dephosphospecific, SMI-32; (L) α-tubulin; (M) neuron-specific βIII-tubulin; and (N) Myosin Va. (D) In lanes 11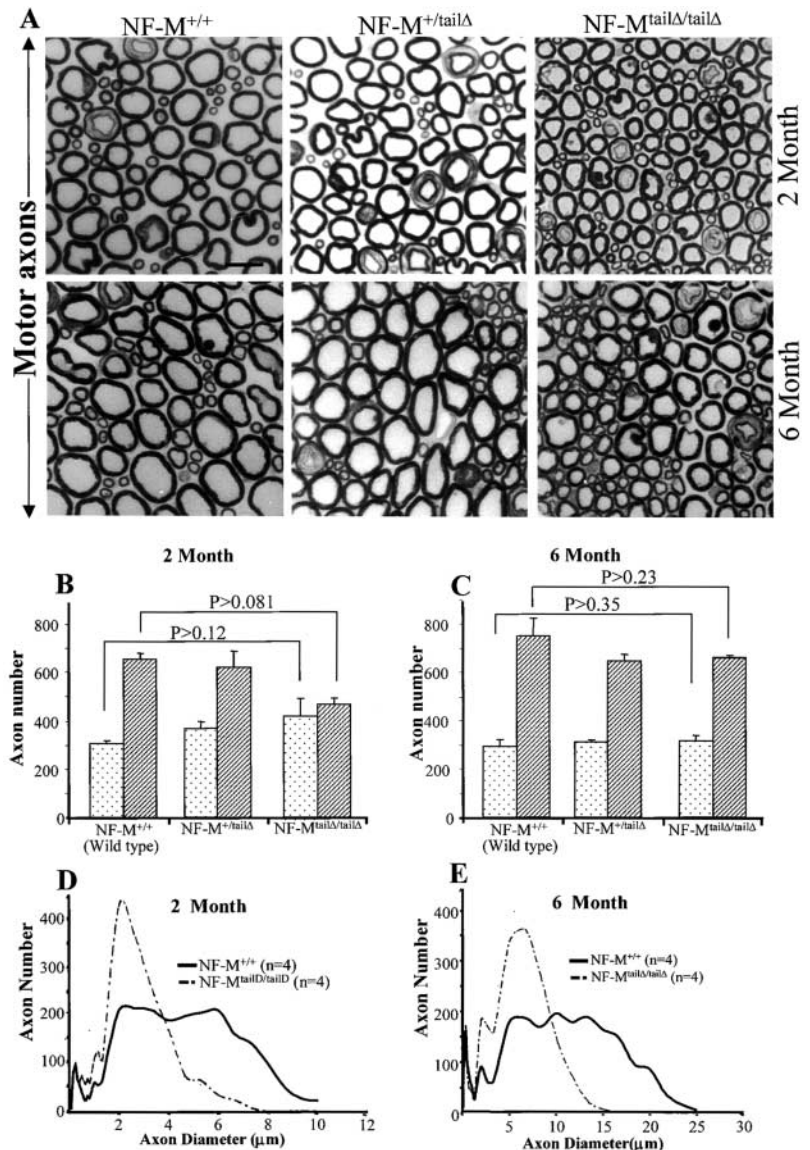 and 12, quantification standards at right are provided by a twofold dilution series of a neurofilament preparation. Samples in lanes 1–3 and 7–8 are from 2-mo-old mice, and lanes 4–6 and 9–10 are from 6-mo-old mice. (O) Microtubule density in motor axons from L5 ventral roots of wild-type or littermate homozygous NF-M[tailΔ] mice. Density was measured by counting all microtubules in a given cross-sectional area. Counts are an average from 11 axons for each genotype of 6-mo-old animals. Error bars represent SD.

(RMO44; Tu et al., 1995), which detects both full-length and NF-M[tailΔ] proteins (Fig. 1 E). Quantification of the immunoreactive bands on these blots also demonstrated that similar levels of full-length or NF-M[tailΔ] proteins were present in control or homozygous NF-M[tailΔ] mice, respectively (Fig. 1 E). Immunoblotting with a polyclonal Myc antibody (Gill et al., 1990) showed that NF-M[tailΔ] protein was present only in heterozygous and homozygous mice (Fig. 1 F, lanes 2, 3, 5, 6, 8, and 10). Immunoblotting with antibodies directed toward the carboxyl-terminal 12 amino acids of both NF-L and NF-H demonstrated that the levels of these two subunits were not significantly affected by the loss of NF-M tails (Fig. 1, G and H). To study whether loss of NF-M tails had an effect on the phosphorylation status of the NF-H in NF-M tail–deficient axons, we probed the blots with the phosphorylation-specific antibodies RT-97 and SMI-31. The results indicate that both RT-97 (Fig. 1 I) and SMI-31 (Fig. 1 J) epitopes are slightly increased in NF-M[tailΔ] axons. The levels of dephosphorylation-specific epitopes did not significantly alter in NF-M tailless axons (Fig. 1 K). We have recently identified a molecular motor protein, myosin Va, as a neurofilament binding protein that is essential for the normal organization of neurofilament networks in the axon (Rao et al., 2002b). To our surprise, levels of myosin Va were up-regulated in both sciatic and optic axons of the NF-M tail–deficient mice (Fig. 1 N).

NF-M tails influence the dynamics of microtubules in the axon

Alteration of any of the three NF subunits in the axon results in an increased density of microtubules in the axon (Zhu et al., 1997, 1998; Rao et al., 1998). It was postulated that dephosphorylated NF-H tails bind microtubules

Figure 2. **NF-M tail is important for radial growth of motor axons.** (A) Cross sections of L5 motor (ventral root) axonal profiles from wild-type, NF-M$^{tail\Delta}$ heterozygous, and NF-M$^{tail\Delta}$ homozygous mice at (top) 2 mo or (bottom) 6 mo of age. Bar, 20 μm. (B and C) Numbers of small (<4 μm diam; dotted bars) and large (>4 μm diam; hatched bars) axons in L5 motor roots of (B) 2- or (C) 6-mo-old wild-type and NF-M$^{tail\Delta}$ heterozygous and homozygous mice. Counts are an average from three to four animals for each genotype. (D and E) Distributions of axonal diameters in motor axons of (D) 2- or (E) 6-mo-old wild-type and homozygous animals. Points represent the average distribution of axon diameters form the entire roots of four mice for each genotype and age group. Error bars represent SD.

more effectively (Hisanaga and Hirokawa, 1990), and this binding is inhibited by the phosphorylation of NF-H tails by CDC2 kinase (Hisanaga et al., 1991). Recently, we have shown that NF-H tails do not influence the tubulin levels or the microtubule numbers in NF-H tail–deleted motor or sensory axons, suggesting that NF-H tails do not regulate the microtubule dynamics (Rao et al., 2002a). To study the effect of NF-M tail deletion on microtubule content in the axons, we quantified the tubulin subunits in wild-type and their littermate heterozygous and homozygous mice. The immunoblot results indicate that both α-tubulin (Fig. 1 L) and neuron-specific βIII-tubulin (Fig. 1 M) levels are increased in NF-M tail–deleted axons. To investigate the polymerization state of tubulin, we measured the density of microtubules in NF-M$^{tail\Delta}$ motor axons. Our quantitative data revealed an approximately two-fold increase in total number of microtubules in NF-M tail–deleted axons (Fig. 1 O; P < 0.003). These observations suggest that NF-M tails influence the dynamics of microtubules in the axon.

NF-M tails are essential for radial growth of motor axons but not for axon survival

To understand the function of NF-M tails and their phosphorylation in the radial growth of motor axons, L5 ventral roots of NF-M$^{tail\Delta}$ homozygous mice and their littermate controls were examined. Axonal profiles at either 2 or 6 mo of age demonstrate that the loss of NF-M tails in homozygous mice has severe inhibition of radial growth of motor axons (Fig. 2 A, compare NF-M$^{+/+}$ with NF-M$^{tail\Delta/tail\Delta}$ at 2 and 6 mo of age, respectively). To study the effects of the loss of NF-M tails on the survival of motor neurons, we counted the total number of axons from wild-type, heterozygous, and homozygous NF- M$^{tail\Delta}$ ventral roots at 2 and 6 mo of age. The total number of axons between wild-type and homozygous NF- M$^{tail\Delta}$ axons did not change significantly, but the relative proportions of axons (of sizes > or <4 μm) changed significantly (Fig. 2, B and C). Although the total number of axons did not change at either 2 or 6 mo of age (Fig. 2 C), the growth of NF-M tail–deficient axons is severely compromised (Fig. 2, D and E). To measure the
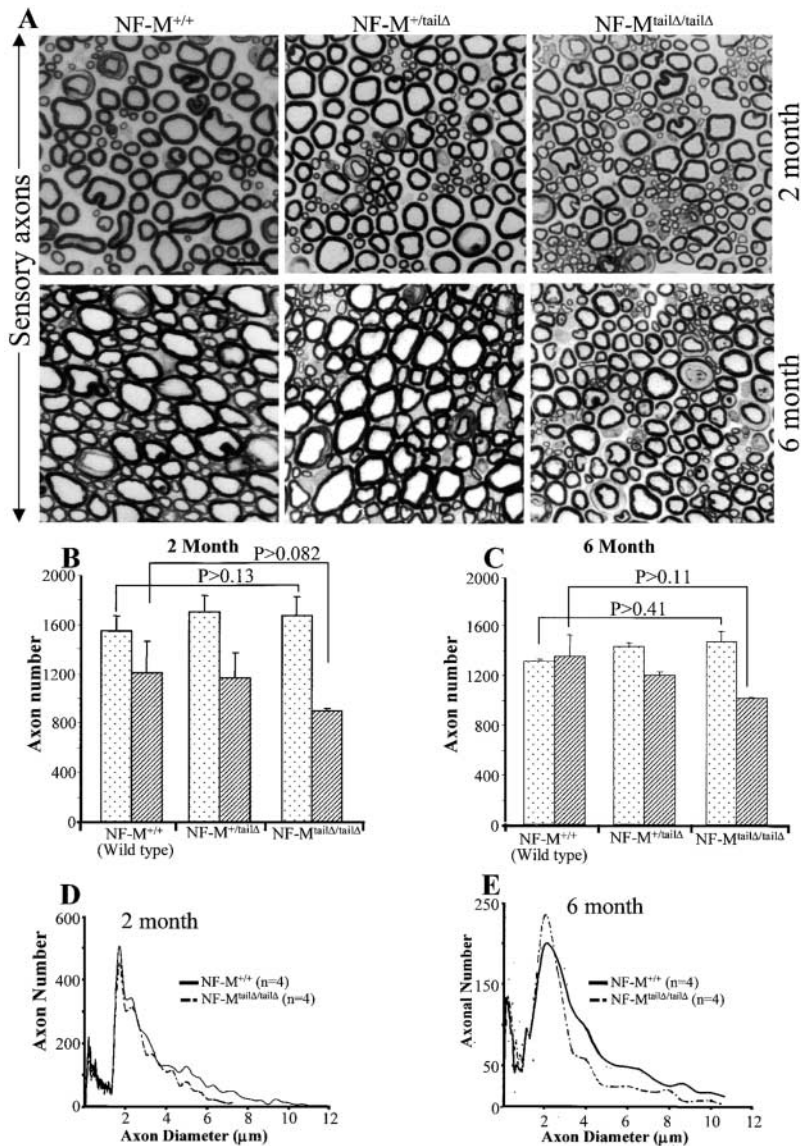
Figure 3. **NF-M tail is essential for calibers of sensory axons.** (A) Cross sections of L5 sensory (dorsal root) axonal profiles from wild-type, NF-M$^{tail\Delta}$ heterozygous, and NF-M$^{tail\Delta}$ homozygous mice at (top) 2 or (bottom) 6 mo of age. Bar, 20 μm. (B and C) Numbers of small (<4 μm diam; dotted bars) and large (>4 μm diam; hatched bars) axons in L5 sensory roots of (B) 2- or (C) 6-mo-old wild-type and NF-M$^{tail\Delta}$ heterozygous and homozygous mice. Counts are an average from three to four animals for each genotype. (D and E) Distributions of axonal diameters in sensory axons of (D) 2- or (E) 6-mo-old wild-type and NF-M$^{tail\Delta}$ homozygous animals. Points represent the average distribution of axon diameters form the entire roots of four mice for each genotype and age group. Error bars represent SD.

changes associated with the loss of NF-M tails more precisely in motor axons, all the axons within each ventral root (Fig. 2 A) were measured, 2- and 6-mo-old wild-type and NF-M tail–deficient homozygous mice and their effective diameters were calculated. Axons in wild-type mice grew bimodally from 2 to 6 mo with peak values of 5-μm and 13-μm diam, respectively (Fig. 2, D and E). In NF-M tail–deleted motor axons, the small diameter axons grew normally at both 2 and 6 mo of age (Fig. 2, B and C; <4-μm-diam axons), but the growth of large diameter axons was severely inhibited by as much as 50% at 2 mo (Fig. 2 D, the peak value was 5 μm for the control and 2.5 μm for NF-M$^{tail\Delta}$ mice) and 6 mo of age (Fig. 2 E, the peak value for large diameter axons was ~6 μm in NF-M$^{tail\Delta}$ compared with 13 μm in wild-type mice). These results indicate that NF-M tails are important for the radial growth of motor axons.

NF-M tails influence the radial growth of sensory axons

Loss of NF-H tails did not affect the growth of sensory axons in NF-H tail–deficient mice (Rao et al., 2002a). To study the effects associated with the loss of NF-M tails on sensory axonal diameters, L5 dorsal root axonal profiles were examined at 2 and 6 mo of age in wild-type, heterozygous, and homozygous NF-M tail–deficient mice. The axonal profiles indicate that the loss of NF-M tails results in a substantial inhibition of growth of sensory axons (Fig. 3 A, compare NF-M$^{+/+}$ with NF-M$^{tail\Delta/tail\Delta}$ at 2 and 6 mo of age, respectively). We counted the total number of axons in wild-type and NF-M tail–deleted dorsal roots to study whether loss of NF-M tails has an effect on the survival of these axons. The counts (Fig. 3, B and C) demonstrate that loss of NF-M tails does not have a significant effect on the total number of axons between wild-type and homozygous NF-M$^{tail\Delta}$ mice, and the relative proportions of axons into small (<4 μm) and large diameter (>4 μm) is affected (Fig. 2 B). To examine the diameter changes associated with the loss of NF-M tails, the diameter of every axon in each dorsal root was measured at 2 and 6 mo of age. Sensory axons in wild-type mice grew from 2 to 6 mo of age. The effect of NF-M tail deletion on axon calibers was evident on large diameter axons
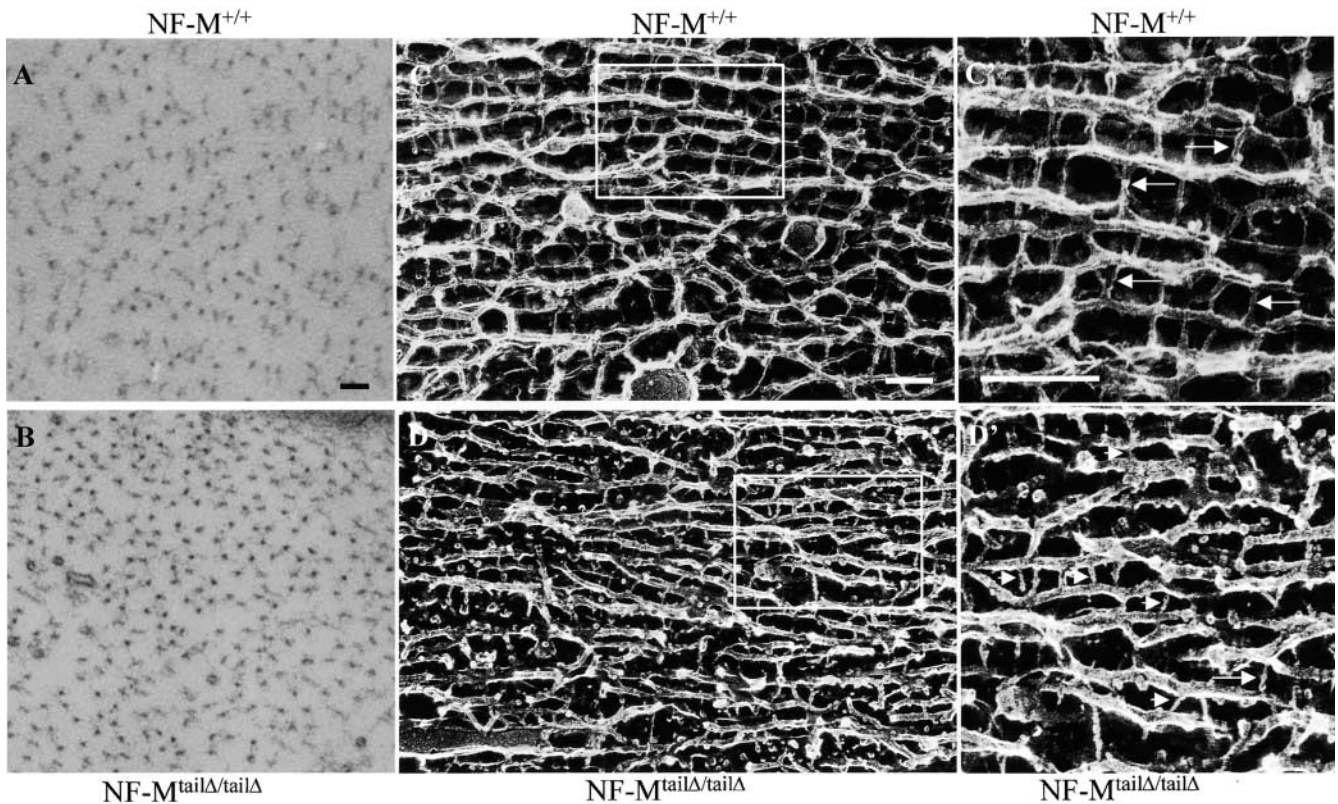
Figure 4.   **Altered neurofilament organization in NF-M^{tailΔ} axons.** Thin-section electron micrographs of 6-mo-old motor axons of the L5 ventral roots of normal (A) or NF-M^{tailΔ} (B) animals. Quick freeze deep etch micrographs of sciatic nerves from (C) wild-type and (D) NF-M^{tailΔ} mice were imaged by quick freeze deep etch microscopy. (C′ and D′) Higher magnification views of areas boxed in C and D. (long arrows) Fine long cross-bridges; (short arrows) short cross-bridges. The majority of the fine long cross-bridges are missing in NF-M^{tailΔ} axons. Bars: (A) 500 nm; (C and C′) 200 nm.

(Fig. 3, D and E). These observations demonstrate that NF-M tails are important for the radial growth of sensory axons.

NF-M tails influence the organization of neurofilaments in the axons

To study the effect of NF-M tail deletion on axoplasm and the effects of organization of neurofilaments in the axon, motor axon cross sections were analyzed by electron microscopy. Although the immunoblots analyses of sciatic and optic nerve extracts indicated that the loss of NF-M tails does not affect the levels of NF subunits in the axon (Fig. 1, D–H), axonal diameters were markedly reduced. As expected from these results, NF density in NF-M tail–deficient axons was clearly increased (Fig. 4, compare A with B). We also observed that the nearest neighbor distances of NFs were reduced in NF-M tailless axons because the filaments are much closer to each other (unpublished data).

To examine the structural and long-range effects on the organization of neurofilaments in more detail, longitudinal sections of the sciatic nerves from wild-type and NF-M tail–deficient nerves were examined by quick freeze deep etch analysis. The 10-nm-diam neurofilaments were longitudinally organized in parallel orientation in the axoplasm and contain fine long cross-bridges that appear to interconnect filaments in wild-type axons (Fig. 4, C and C′, arrows). In contrast, in NF-M tailless axons, filaments were interconnected with mostly short cross-bridges (Fig. 4 D, small arrows) with relatively fewer fine long cross-bridges (Fig. 4, D and D′, large arrow). Therefore, filaments in NF-M tailless mice are very close to each other and display an increased packing density compared with wild-type axons, this is consistent with the aforementioned biochemical and EM data. These findings also suggest that the fine long cross-bridges formed between filaments in wild-type mice are formed by both NF-H (Chen et al., 2000; Rao et al., 2002a) and NF-M tail domains (this paper). Although the majority of these fine cross-bridges were lost in NF-H tail–deleted mice, overall filament organization was not affected as in NF-M tailless mice (Rao et al., 2002a), suggesting that NF-M tails have unique properties in maintaining NF spacing in the axons.

NF-M tail deletion does not alter average rates of neurofilament transport

To study the role of NF-M tail and its phosphorylation in the regulation of slow axonal transport, optic nerve proteins of the wild-type and NF-M tail–deficient mice were labeled with [^{35}S]methionine, and the rates of movement of components in the slow phase of axoplasmic transport were measured. NF-M^{tailΔ} in mice results in the appearance of a novel 50-kD NF-M tail–deleted polypeptide, and eliminates full-length NF-M protein (Fig. 5 B). In cytoskeletal fractions of optic axons, the movement of full-length NF-M in wild mice was indistinguishable from that of truncated NF-M tailless protein in NF- M^{tailΔ} optic axons (Fig. 5, A and B,
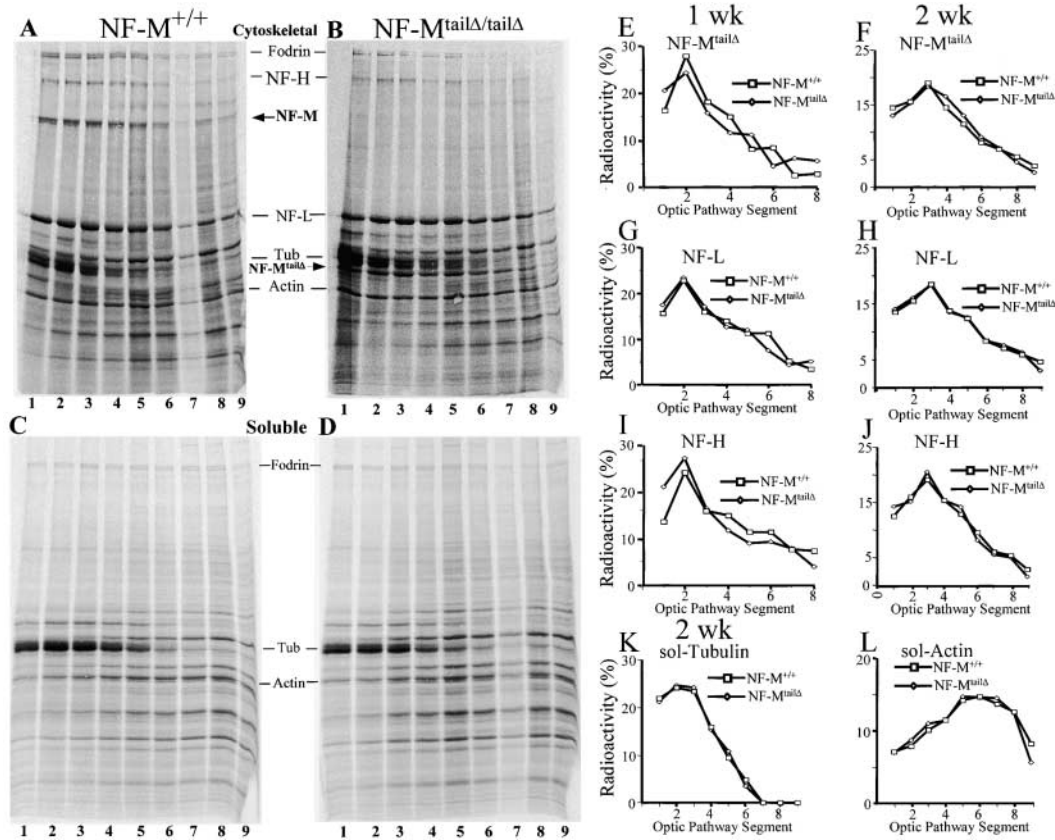
Figure 5.   **The deletion of phosphorylated tail domain of NF-M does not alter the rate of slow axonal transport of neurofilaments.**
(A–D) The speed and composition of slow axonal transport was determined by intravitreal injection of radiolabeled [^{35}S]methionine of 4-mo-old (A and C) control and (B and D) NF-M–deleted mice. Optic nerve proteins were fractionated into (A and B) cytoskeleton and (C and D) soluble fractions with a Triton X-100–containing buffer. Fractionated proteins were separated on 5–15% SDS–polyacrylamide gels, transferred to nitrocellulose, and visualized by X-ray film and phosphorimaging. (E–L) Quantification of NF subunits and tubulin and actin transport in optic nerves. (E and F) NF-M$^{tail\Delta}$; (G and H) NF-L; (I and J) NF-H; (K) soluble tubulin; and (L) soluble actin. 1-wk (E, G, and I) and 2-wk (F, H, and J–L) slow axonal transport studies.

full-length and truncated NF-M are indicated by arrows; and Fig. 5, E and F). Confirming this observation is the finding that NF-L (Fig. 5, G and H) and NF-H (Fig. 5, I and J) from NF-M tail–deficient axons also moved at the same speed as the corresponding subunits in wild-type axons. The movement of cytoskeleton-associated (unpublished data), soluble tubulins (Fig. 5 K), and actin (Fig. 5 L) also was not significantly affected by the loss of NF-M tails. Additional transport studies performed at different time points, 3 d (unpublished data) and 1 wk (Fig. 5, E, G, and I), after pulse labeling also revealed no change in the transport pattern as seen at the 2-wk time point (Fig. 5, H–L). These results demonstrate that loss of the NF-M tails does not influence the average rate of slow axonal transport of NFs. Coupled with previous studies in NF-H tail–deleted mice (Rao et al., 2002a), these findings indicate that deletion of the tail domain of either NF-H or NF-M alone does not alter the average rate of NF axonal transport even though NF-M tail deletion has much more profound effects on radial growth and cytoarchitecture of the axon than NF-H tail deletion. It is possible that the presence of either one of these tails is enough to slow the NF transport. To address this question, axonal transport studies have to be performed in axons of NF-M/H double tailless mice.

Discussion

Although the long carboxyl-terminal tail domains of NF-M and NF-H share important structural features, we have identified a distinctive in vivo role of the NF-M tail in neurofilament behavior and axon caliber expansion. The importance of axonal transport of neurofilaments to radial growth is well established; however, the novel function of the NF-M tail identified here reveals that axon radial growth requires the modulation of neurofilament spacing as well as adequate delivery of neurofilaments to axons. This work provides direct in vivo evidence for a long-suspected role of the carboxyl-terminal domain of the NF-M of increasing the space-occupying capability of neurofilaments during axon growth.

We found that the NF-M tail domain regulates axon caliber, in part, by increasing the spacing between neurofilaments. Our data showing that the deletion of NF-M tail domains eliminates most fine long neurofilament cross-bridges are consistent with the concept that the NF-M tail increases interneurofilament spacing by extending these cross-bridges laterally between filaments (Fig. 6) as predicted from earlier antibody decoration studies (Willard and Simon, 1981; Hirokawa et al., 1984). Transfection of carboxyl-terminal mutants of NF-M along with wild-type NF-L into Sf9 cells in-
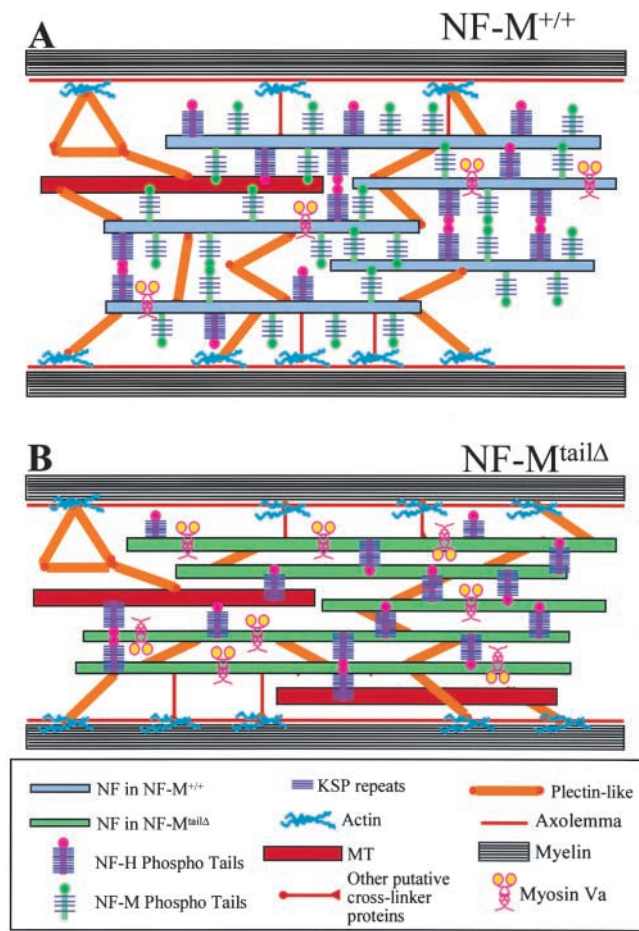
Figure 6. **Neurofilament-dependent organization of axoplasm.** In normal axons (A), axoplasm is organized into a volume-determining three-dimensional array by a series of linkages that span between adjacent neurofilaments (blue-gray) and between neurofilament and microtubules (red) or cortical actin (blue) filaments. NF-M (turquoise) and NF-H (purple) tails form cross-bridges between neurofilaments (Nakagawa et al., 1995; Chen et al., 2000, Rao et al., 2002b). Neurofilaments, microtubules, and cortical actin are interlinked by plectinlike linkers (orange; Errante et al., 1994; Svitkina et al., 1996). The neuronal cytoskeleton is also stabilized by putative cytoskeletal cross-linker proteins between neurofilaments, microtubules, actin in motor and sensory axons (Yang et al., 1996; Dalpe et al., 1998; Leung et al., 1999), and neurofilament binding motor protein myosin Va (Rao et al., 2002b). (B) In the absence of NF-M tails, NF-H, gigaxonin and other putative linker proteins, and elevated levels of myosin Va organize a three-dimensional array with reduced axonal diameters to support and maintain altered axonal volume.

dicates that the carboxyl-terminal region of NF-M is capable of forming these fine long cross-bridges to organize neurofilaments (Nakagawa et al., 1995). This function of NF-M tails may be shared to some extent with the NF-H tail because deleting this region causes loss of fine cross-bridges (Chen et al., 2000; Rao et al., 2002a). However, NF-H tail deletion has a much smaller effect on the organization of neurofilaments in the axons or on axon caliber than deleting NF-M tails. This may suggest a role of the NF-H tail in stabilizing neurofilament cross-bridging rather than in regulating axonal diameters and cytoskeletal architecture.

The NF-M tail domain, corresponding to a 426–amino acid region of the NF-M, contains multiple KSP repeats that

are under complex regulation by a number of protein kinases and phosphatases (Strack et al., 1997; Ackerley et al., 2000; Grant and Pant, 2000; Veeranna et al., 2000). Our results provide in vivo support for the notion that the tail domains of NF-M extend laterally from the neurofilament when they are phosphorylated. These findings support earlier studies showing that axonal caliber expansion during development not only involves local neurofilament accumulation in axons (Sanchez et al., 1996, 2000) but also increases interneurofilament spacing that strongly correlates with the extensive phosphorylation of NF-M tail domains (de Waegh et al., 1992; Hsieh et al., 1994; Sanchez et al., 1996, 2000). Phosphorylation of the NF-M tail to dynamically regulate neurofilament spacing represents a possible mechanism by which axons might acutely modulate their calibers and electrophysiologic properties without altering the expression or axonal transport rate of neurofilament proteins.

Neurofilaments and microtubules interconnect in the axonal cytoskeleton (Hirokawa et al., 1984). Microtubule numbers in the axon are sensitive to changes in the subunit composition of neurofilaments (Rao et al., 1998; Zhu et al., 1998; Jacomy et al., 1999), supporting a likely interaction between these two structural elements. NF-H tails, in particular, have been implicated in modulating this microtubule binding activity (Hisanaga and Hirokawa, 1990; Miyasaka et al., 1993), although the deletion of NF-H tails did not affect either total tubulin or microtubules in either motor or sensory axons (Rao et al., 2002a). However, we found here, that the NF-M tail is essential for maintaining the proper number of microtubules in the axon. In the absence of NF-M tails, neurofilaments and other axonal structures may still remain associated by means of additional putative cytoskeletal linker proteins (Errante et al., 1994; Yang et al., 1996; Dalpe et al., 1998; Rao et al., 1998), gigaxonin (Bomont et al., 2000), and the neurofilament-binding motor protein myosin Va (Rao et al., 2002b; Fig. 6).

In this work, we also observed that despite major influences of NF-M tails on axon caliber and neurofilament organization, eliminating this phosphorylated tail region without altering subunit ratios had no significant effect on the average rate of transport of NF-M$^{tail\Delta}$, NF-L, NF-H, tubulin, and actin in optic axons. These observations demonstrate that appropriate expression and axonal transport of neurofilament proteins are crucial for radial growth, independent neurofilament interactions mediated by NF-M are also essential. The independence from the axonal transport process of key aspects of neurofilament organization is consistent with evidence that most neurofilaments in mature myelinated axons are extensively cross-linked to each other and to various stationary cytoskeletal structures (Fig. 6). The recent in vitro studies indicate that slow axonal transport of neurofilaments is due to rapid movements and frequently long "pauses" seen in growing axons in neuronal cultures (Roy et al., 2000; Wang et al., 2000). It has been argued over the years that NFs are transported as filaments, oligomers, and subunits (Terada et al., 1996; Bass and Brown, 1997; Hirokawa et al., 1997; Yabe et al., 1999; Prahlad et al., 2000; Roy et al., 2000; Shah et al., 2000; Wang et al., 2000; Shah and Cleveland, 2002). However, the motor proteins that modulate NF axonal transport are not known. The correla-

tive evidence suggests that kinesin, dynein/dynactin, and myosin Va motor proteins may be regulating this process (Yabe et al., 1999; Prahlad et al., 2000; Shah et al., 2000; Rao et al., 2002b; Xia et al., 2003).

NF-H carboxyl-terminal domain phosphorylation has been proposed as a key determinant of slow axonal transport rate of neurofilaments (Nixon et al., 1982; Lewis and Nixon, 1988; Jung et al., 2000a,b; Ackerley et al., 2003). In vitro cell culture studies and in vivo NF-H overexpression results indicate that the NF-H modulates the rate of NF axonal transport (Collard et al., 1995; Marszalek et al., 1996). Moreover, reducing the phosphorylation state of some of the KSP repeats of NF-H, in cells overexpressing this subunit, accelerates the rate of slow axonal transport of NFs (Ackerley et al., 2003). Axonal transport studies in sciatic nerves of NF-H null mice show increased NF transport rates (Zhu et al., 1998). However, transport studies in optic nerves of NF-H null and NF-H tail domain deletion mice do not exhibit altered rates of slow axonal transport of NFs (Rao et al., 2002a), indicating the NF transport rate is not modulated by the phosphorylated tail of NF-H in axons under these conditions (Rao et al., 2002a; Rao and Nixon, 2003). The results also indicate that loss of NF-M–phosphorylated tails do not influence the rate of NF transport. In this work, it is possible that the presence of the phosphorylated tail on either the NF-H or NF-M is sufficient to mask effects on neurofilament transport of deleting the tail on the other subunit. This possibility can be tested in mice in which both NF-H and NF-M tails are eliminated. However, independent of the resolution of this issue, the results of this paper clearly define a role for the carboxyl-terminal tail of NF-M in neurofilament organization, which does not require a change in rate of neurofilament transport.

Materials and methods

Construction of NF-M$^{tail\Delta}$ mice by gene-targeted knockin approach
A genomic clone isolated from mouse 129 SVJ library was used for construction of NF-M$^{tail\Delta}$ (provided by J.P. Julian, McGill University, Montreal, Canada; Jacomy et al., 1999). A 7-kb BamHI fragment of NF-M genomic clone was subcloned and cut with BsrGI to create truncation at codon 423 of the NF-M gene in the third exon. The resulting construct was fused in frame to a carboxyl-terminal Myc tag and pGK-NEO cassette for positive selection of the ES cell clones. During the construction of targeting vector, a novel BamHI site was engineered to screen the ES clones at the end of the NEO cassette. BstEII and EcoRV fragment of the NF-M sequence was used as the 3′ arm to promote homologous recombination. At the end of the 3′ arm, an HSV-TK cassette was cloned for negative selection of ES cell clones to create a final targeting vector (Fig. 1 A). NF-M tailless targeting construct was linearized with NotI, electroporated into RI ES cells, and selected with G418 and Gancyclovir at 200 μg/ml and 50 μM/ml, respectively (Joyner, 1994). Drug-resistant colonies were amplified, and DNA was digested with either NcoI or BamHI from the colonies, separated on 0.8% agarose gels, transferred to Hybond N+ filters (Amersham Biosciences), and hybridized with random prime-labeled 0.8-kb BamHI–NotI (5′-probe) or 0.3-kb EcoRV–BamHI (3′-probe) fragments (Fig. 1 A). 2 out of 282 ES clones underwent perfect homologous recombination at both the 5′ and 3′ ends of the gene to produce NF-M tail–deleted gene as indicated in Fig. 1 (B and C, respectively).

Immunoblotting of neurofilament and tubulin subunits
Sciatic and optic nerve extracts were made from 2 to 6-mo-old animals as described previously (Rao et al., 1998). Protein concentration was determined using bicinchoninic acid assay kit (Pierce Chemical Co.). Protein extracts, as well as known amounts of neurofilament standards, were separated on 7% polyacrylamide gels with SDS and transferred to nitrocellu-

lose membranes. Full-length and NF-M$^{tail\Delta}$ proteins were detected with an mAb raised against the rod domain of NF-M (Tu et al., 1995). The NF-H (pAb-NF-H$_{COOH}$; Xu et al., 1993), the NF-L (pAb-NF-L$_{COOH}$; Xu et al., 1993), the Myc-tagged NF-M$^{tail\Delta}$ subunit with a polyclonal Myc antibody (Gill et al., 1990), and the myosin Va with a polyclonal antibody (pMyo Va, Evans et al., 1997) were probed and followed by secondary anti–rabbit IgG. mAbs to NF-M (RMO44; Tu et al., 1995), NF-H (SMI-31, 32; Sternberger and Sternberger, 1983; RT-97; Sanchez et al., 2000), α-tubulin (DM1A; Sigma-Aldrich), and neuron-specific β$_{III}$-tubulin (T-8660; Sigma-Aldrich) were used to identify each subunit, followed by goat anti–mouse IgG (Sigma-Aldrich). After developing the blots with appropriate reagents, the immunoreactive bands were visualized by autoradiography and quantified by phosphorimaging (Molecular Dynamics) using known amounts of purified mouse spinal cord neurofilament standards.

Axonal transport studies
Retinal ganglion cells from 4-mo-old NF-M$^{tail\Delta}$ or their control littermate mice were radiolabeled in situ with 100 μCi [^{35}S]methionine by intravitreal injection with a calibrated micropipette apparatus into anesthetized mice (Nixon, 1980). After injection, mice were killed by cervical dislocation and optic pathways were dissected after 3 d, 1 wk, and 2 wk. Three to four animals were analyzed for each genotype and time point. The optic pathways were frozen and cut into eight or nine consecutive 1.1-mm segments. Each was homogenized with a buffer containing 1% Triton X-100, 50 mM Tris, pH 6.8, 2 mM EDTA, 1 mM PMSF, and 50 μg/ml of protease inhibitor cocktail (Boehringer). After centrifugation, the Triton-insoluble cytoskeleton and soluble protein fractions were analyzed on 5–15% polyacrylamide gradient gels, transferred to nitrocellulose membranes, and quantified by phosphorimaging.

Morphometric analysis
2–6-mo-old NF-M$^{tail\Delta}$ and their littermate control mice were perfused transcardially with 4% PFA, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed overnight in the same buffer. Samples were treated with 2% osmium tetroxide, washed, dehydrated, and embedded in Epon-Araldite resin. 0.75-μm-thick sections for light microscopy were stained with toluidine blue, and 700-nm-thin sections for electron microscopy were stained with uranyl acetate and lead acetate. Axons were counted in L5 root cross sections from three to four mice of each genotype and each age group. Axon diameters from four animals of each genotype and age were measured using the Bioquant Software.

Quick freeze deep etch analysis of neurofilaments in sciatic nerves
Sciatic nerves of 6-mo-old NF-M$^{tail\Delta}$ and their control littermate animals were dissected and incubated in oxygenated artificial cerebrospinal fluid, pH 7.3, containing the following (mM): 126 NaCl, 22 NaHCO$_3$, 1 Na$_2$HPO$_4$, 2.8 KCl, 0.88 MgCl$_2$, 1.45 CaCl$_2$, and 3.5 glucose. After sectioning with a razor blade, the tissue was frozen by slamming against a liquid helium-cooled copper block (model E7200; Polaron), as described previously (Gotow et al., 1999). The frozen tissue was mounted onto the freeze fracture apparatus (model BAF 400D; Balzers), fractured, and deep etched and rotary replicated with platinum/carbon at an angle of 25°. The replicas were examined with an electron microscope (model H-300; Hitachi) at 75 kV.

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