

Subunit Composition of Neurofilaments Specifies Axonal Diameter

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Abstract. Neurofilaments (NFs), which are composed of NF-L, NF-M, and NF-H, are required for the development of normal axonal caliber, a property that in turn is a critical determinant of axonal conduction velocity. To investigate how each subunit contributes to the radial growth of axons, we used transgenic mice to alter the subunit composition of NFs. Increasing each NF subunit individually inhibits radial axonal growth, while increasing both NF-M and NF-H reduces growth

even more severely. An increase in NF-L results in an increased filament number but reduced interfilament distance. Conversely, increasing NF-M, NF-H, or both reduces filament number, but does not alter nearest neighbor interfilament distance. Only a combined increase of NF-L with either NF-M or NF-H promotes radial axonal growth. These results demonstrate that both NF-M and NF-H play complementary roles with NF-L in determining normal axonal calibers.

THE development of mature axonal morphology can be viewed as a two-phase process. In the first, the axon elongates to establish a physical contact with its target. In the second, the axon grows by up to 10-fold in diameter (100-fold in volume), a feature that is necessary for the propagation of action potentials at an appropriate speed along the axon (Arbuthnott et al., 1980). Neurofilaments (NFs)¹ play a crucial role in radial growth. The initial evidence for this was derived from a direct correlation between the axonal diameter and the number of NFs during all phases of radial axonal growth (Friede and Samajorski, 1970; Hoffman et al., 1988; reviewed by Cleveland et al., 1991). This has now been proven unequivocally by two animal models. The first of these is a quail with a spontaneous mutation in NF-L that truncates translation prematurely and leads to the absence of NF-L in homozygous animals (Ohara et al., 1993). The second is a transgenic mouse line that expresses an NF-H subunit with nearly the entire 120-kD galactosidase polypeptide attached to its carboxyl terminal tail. Expression of this mutant NF-H blocks transport of all NFs into axons (Eyer and Peterson, 1994). Axons from both animals completely lack NFs, and, as a result, radial growth of those axons is

severely inhibited (Yamasaki et al., 1991; Sakaguchi et al., 1993; Eyer and Peterson, 1994).

NFs are class IV intermediate filaments composed of three polypeptide subunits, NF-L (61 kD), NF-M (95 kD), and NF-H (115 kD). Each of these subunits share a common intermediate filament polypeptide structure: a central α -helical rod domain of 310 amino acids featuring heptad repeats capable of forming coiled-coil oligomers, a short amino-terminal head domain that is rich in arginine and serine, and a carboxyl-terminal domain of variable length and primary structure (for review see Shaw, 1991). Filaments assemble in vivo as obligate heteropolymers of NF-L and substoichiometric amounts of NF-M and/or NF-H (Lee et al., 1993; Ching and Liem, 1993; Wong et al., 1995). Although the role of NFs as intrinsic determinants of axonal caliber has been well established, the contribution of each individual polypeptide subunit is much less clear. Since NF-L is indispensable for both filament assembly under physiological conditions in vitro (Geisler and Weber, 1981; Liem and Hutchison, 1982) and in vivo (Ching and Liem, 1993; Lee et al., 1993), one primary role for it is the assembly of the filament backbone.

For NF-H, much interest has been focused on its tail domain, which is longer than the entirety of all but one other intermediate filament subunit and which contains a striking structural feature: 40 to 50 repeats containing the sequence Lys-Ser-Pro (Lees et al., 1988; Julien et al., 1988), the serines of which are heavily phosphorylated (Xu, 1990; Elhanany et al., 1994), but only within myelinated axonal segments (Sternberger and Sternberger, 1983; Lee et al., 1987, 1988). Phosphorylation correlates with wider fila-

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1. *Abbreviations used in this paper:* MSV LTR, murine sarcoma virus long terminal repeat; NF, neurofilament.

ment-filament spacing (de Waegh et al., 1992; Hsieh et al., 1994; Nixon et al., 1994; Cole et al., 1994). The tail domain is peripherally located as side arms projecting from the central core of filaments (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988; Mulligan et al., 1991). These properties have led to the proposal that the principal function for NF-H is to interact with adjacent filaments and other organelles, thereby establishing a wider filament spacing, perhaps through repulsion between the adjacent filaments as a result of the highly negatively charged phosphates carried by its tail (Carden et al., 1987; Matus, 1988). The functional role for NF-M is unclear, although roles in promoting filament elongation (Nakagawa et al., 1995) and specifying nearest neighbor distance between filaments have been proposed (Nakagawa et al., 1995; Wong et al., 1995).

By transgenic technology, we now examine the mechanism underlying NF-dependent radial growth by changing the subunit composition of NFs through overexpressing each of the three NF subunits alone or in combination with each other. We find that increasing each NF subunit alone leads to the inhibition of radial growth. Only a combined increase of either NF-H or NF-M with NF-L produces significantly larger axonal diameters. Neither inhibition nor enhancement of growth correlates with changes in the nearest neighbor filament-filament spacing. Thus, the tail domains of NF-M and NF-H structure axoplasm in a manner that promotes radial growth, but essential interactions must include interactions between NF and other axonal components or between NF that are not nearest neighbors.

Materials and Methods

Construction of Transgenic Mice

Construction of transgenic mice carrying the murine NF-L gene (Fig. 1 A), in which the authentic 5' promoter was replaced by the murine sarcoma virus (MSV) long terminal repeat (LTR), has been described previously (Monteiro et al., 1990). Construction of mice carrying a murine NF-M transgene (Fig. 1 B) in which the authentic 5' promoter was replaced by the MSV LTR and the carboxy-terminal 50 amino acids were substituted with a 12-amino acid myc epitope tag has also been described (Wong et al., 1990, 1995). Both NF-L and NF-M lines were maintained in a C57B6 genetic background. To construct NF-H transgenic mice, a 23-kb *Sal*I fragment of a murine NF-H genomic clone (Julien et al., 1988; see Fig. 1 C) was injected into fertilized mouse eggs and transgenic mice were produced as described by Monteiro et al. (1990). The initial founders were of C57B6/C3H F2 hybrid backgrounds. Both NF-L and NF-H constructs encode entirely wild-type mouse proteins, while the NF-M construct encodes a slightly truncated NF-M molecule that is epitope tagged at its carboxyl terminus (see above).

Protein Blot and Quantification

Protein immunoblotting and quantification has been described in detail previously (Xu et al., 1993). Briefly, mouse sciatic nerves were homogenized in buffer containing 25 mM sodium phosphate (pH 7.2), 1 mM EGTA, 1% SDS, and 1 mM PMSF, and protein concentration measured with the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). 2–5 μ g of total protein were then loaded onto 7% polyacrylamide gels, electrophoresed (Laemmli, 1970), and transferred onto nitrocellulose filters as described (Lopata and Cleveland, 1987) using a minigel apparatus (Bio Rad Laboratories, Hercules, CA). The blot was then incubated with a mixture of antibodies recognizing each of the NF subunits: two polyclonal antibodies raised against the carboxyl-terminal 12 amino acids of NF-H and NF-L, respectively (Xu et al., 1993), and an mAb against NF-M (Boehringer Mannheim Biochemicals, Indianapolis, IN). Detection of each polypeptide band was carried out by first incubating the filter with a goat anti-rabbit IgG or rabbit anti-mouse IgG polyclonal antibodies (Sigma

Immunochemicals, St. Louis, MO), followed by incubation with ¹²⁵I-labeled protein A (Amersham Corp., Arlington Heights, IL). The amount of protein in each band was quantified by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) and comparing a dilution series of standards of known amounts of partially purified mouse NF proteins that had been immunoblotted in parallel.

Tissue Processing and EM

Mouse tissues were initially fixed by intracardiac perfusion with a solution of 0.1 M sodium phosphate, pH 7.6, 4% paraformaldehyde, and 2.5% glutaraldehyde followed by further fixation by immersion in the same fixative for 24 h at 4°C. Ventral roots were isolated according to Sidman et al. (1971), postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h, dehydrated in graded alcohols, and embedded in Epon-Araldite resin (EM Sciences, Fort Washington, PA). 1- μ m sections were stained with toluidine blue and examined by light microscopy; thin sections were then cut, stained with uranyl acetate and lead citrate, examined, and photographed in a Hitachi H-600 EM.

Morphometric Analysis

For measuring axonal diameters, microscopic video images of transverse sections of L5 ventral root (1 μ m thick, stained with toluidine blue) at a magnification of 100 were digitized using a frame grabber board (Targa M8) and image analysis software (Bioquant; R&M Biometrics, Memphis, TN). The cross-sectional areas of myelinated axons >1.5 μ m² were measured in continuous nonoverlapping fields with center-of-gravity exclusion to avoid double counting. The illumination and optimum grey-scale pixel value for discrimination of the myelin/axon border was independently chosen for each field to minimize systematic bias. For each axon, the result is reported as the diameter of a circle of equivalent area.

To measure nearest neighbor distances between neurofilaments, cross-sections of axons >3.0 μ m in diameter were photographed at a magnification of 20,000 and were further enlarged fivefold during printing. Neurofilaments were identified in these end on views as dots of \sim 10 nm in diameter. Positions of neurofilaments were marked by puncturing the photographic print with a push pin. By laying the final prints on a light box, neurofilament positions could easily be imaged with a CCD camera and digitized using the Bioquant image analysis software.

Results

Mice Transgenic for Each of the Three NF Subunits Express Significantly Elevated Levels of the Respective NF Subunit

To examine the role that each NF subunit contributes to promoting radial axonal growth, independent transgenic mouse lines that express significantly elevated levels of each of the three NF subunits in motor and sensory neurons were constructed using the gene constructs illustrated in Fig. 1, A–C. By crossing different transgenic lines, we also generated mice that express high levels of any pair of the three subunits.

Transgenes encoding the mouse NF-L and NF-M subunits were previously constructed by replacing the corresponding 5' promoter sequences of the murine NF-L or NF-M genes with the strong promoter from the MSV LTR (Fig. 1, A and B). An additional modification for NF-M was that the last 50 carboxyl-terminal amino acids were deleted and replaced by a 12-amino acid tag sequence (Wong et al., 1990, 1995). This epitope-tagged NF-M is fully competent for coassembly with vimentin or NF-L in transfected cells (Wong et al., 1990), and in nonneuronal cells, it mediates a \sim 30-nm filament-to-filament spacing indistinguishable from that specified by full-length NF-M (Nakagawa et al., 1995). As described earlier, in the NF-L transgenic line 58, the transgene is expressed highly in nervous tissues, although accumulation is also found in some

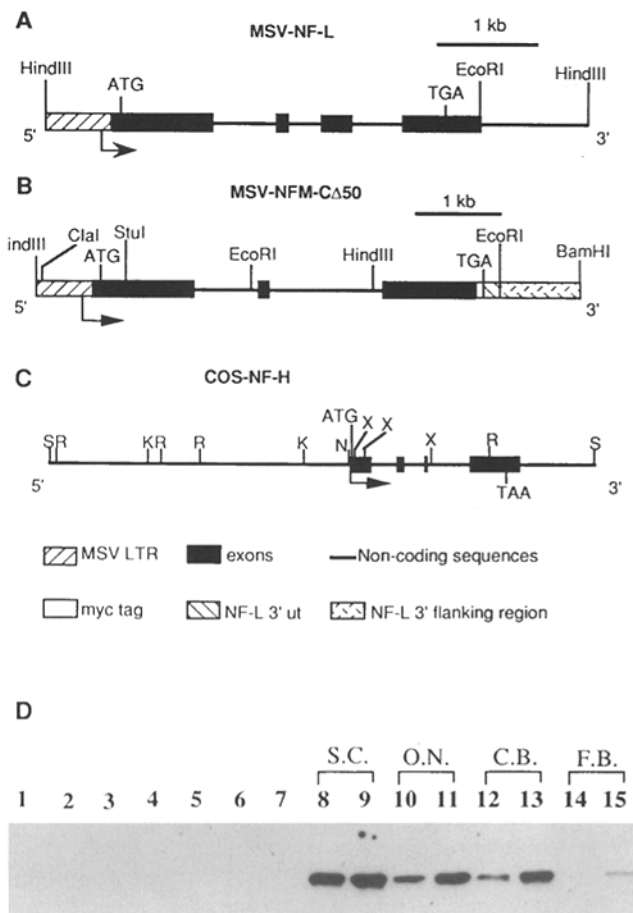


Figure 1. Gene constructs for NF transgenic lines. (A) Transgene for NF-L transgenic mice (L mice): MSV LTR (marine sarcoma virus long terminal repeat) was used as a promoter at the 5' end to transcribe the authentic fragment \sim 4 kb that encompass all the coding sequence of mouse NF-L (see Monteiro et al., 1990). (B) Transgene for NF-M transgenic mice (M mice): Similar to L mice, the MSV-LTR was used as a promoter to transcribe the mouse NF-M gene. This gene was also modified at its 3' end by replacing the sequence coding for the last 50 amino acids and noncoding region with the sequence coding for a 12-amino acid myc peptide followed by NF-L 3' untranslated region and flanking region (see Wong et al., 1995). (C) A 23-kb *Sal*I fragment derived from a mouse NF-H cosmid clone was used to make NF-H transgenic mice (H mice). Arrows in A–C mark the transcription initiation sites. (D) NF-H accumulation, detected using an anti-NF-H polyclonal antibody (Xu et al., 1993) on a protein immunoblot of 10 μ g total tissue protein. Lanes 1–7, NF-H expression in the eye, lung, heart, liver, spleen, kidney, and muscle. Lanes 8, 10, 12, and 14, NF-H levels in the sciatic nerve, optic nerve, cerebellum, and forebrain of a wild-type mouse. Lanes 9, 11, 13, and 15, NF-H levels in the same tissue from an H mouse.

nonneuronal cells (Monteiro et al., 1990). Transgenic NF-M accumulates almost exclusively in neuronal tissues, particularly in lower motor and sensory neurons of the peripheral nervous system (Wong et al., 1995).

For NF-H, a 23-kb fragment encompassing the entire mouse NF-H coding sequences, 7.5 kb of the 5' promoter sequence, and 1.8 kb of the 3' flanking sequence (Fig. 1 C) were injected into mouse embryos, and six founder lines were obtained and examined for the level of NF-H accumulation in the sciatic nerve. One line (No. 35) contained

twice the normal level of NF-H mRNA in neuronal tissue (not shown). Using an NF-H-specific polyclonal antibody (Xu et al., 1993), protein immunoblotting of the total protein from various tissues revealed an elevated level of NF-H in nervous tissues, but no NF-H in the nonnervous tissue, indicating that the accumulation of transgenic NF-H is nervous tissue specific (Fig. 1 D). This is consistent with nervous system-specific expression seen earlier for a similar human NF-H transgene (Cote et al., 1993).

To examine the extent of the increase in NF subunits in axons, we quantified the levels of all three NF subunits in sciatic nerves. Shown in Fig. 2 are the NF subunit levels in 6-wk-old mice generated by crossing NF-L transgenic (L mice) with NF-M transgenic (M mice), which yielded four genotypes in the littermates: normal (nontransgenic) mice, NF-L transgenic, NF-M transgenic, and NF-L/NF-M doubly transgenic (LM mice). Both transgenic lines, initially produced as C57B6/A or C57B6/C3H hybrids, were backcrossed for several generations into a C57B6 background. In both L and M mice, the respective subunits are increased by approximately twofold (Fig. 2; each data point represents the average between six and nine measurements from a minimum of three animals, and standard deviations are given in Table I), whereas in LM mice, the respective levels of the two subunits increased further, reaching threefold the wild-type levels (Fig. 2 B). In M mice, axonal NF-H levels fall 50%, a feature that is almost completely reversed by the simultaneous increase of NF-L (Fig. 2), consistent with the proposal that NF-M may compete with NF-H for coassembly or cotransport with NF-L (Wong et al., 1995). Pairwise *t* tests confirmed that these differences in subunit content were highly significant ($P < 0.01$).

NF subunit levels were also determined (Fig. 3) from 8-wk-old mice generated by crossing L with H mice and M with H mice. As indicated before, multiple (six to nine) measurements from three different animals of each genotype were obtained by immunoblotting. Relative to nontransgenic littermates, in L and H mice, the respective subunits are increased by approximately twofold (Fig. 3 and Table I), while in mice transgenic for both NF-L and NF-H (LH mice), the respective levels for the two subunits increased further (Fig. 3 B and Table I), similar to the situation for LM mice. (It should be also noted that the older age of the mice used in this analysis, as well as the different genetic backgrounds of the H mice [see above], combined to yield an increase in overall neurofilament content in the nontransgenic animals [compared to the 6-wk-old animals in the L and M mating shown in Fig. 2].) Reminiscent of the depression of NF-H after the increase in M mice (Fig. 2), increased NF-H in H mice resulted in a lower level of NF-M (Fig. 3 and Table I), supporting the conclusion that the two subunits compete for assembly and/or transport. Most surprising was the change in all three NF subunit levels in the mice doubly transgenic for NF-M and NF-H: total NF content was reduced by nearly half (45%) (Fig. 3). Again, pairwise *t* tests confirmed that these differences in subunit content were highly significant ($P < 0.05$). The reduction of the total NF content in the sciatic nerve in both H and MH mice reflects a reduction of NFs in axons (see below) and accumulation of NFs in motor neuron cell bodies (seen in morphological examination of the spinal cords of both mice [not shown]).

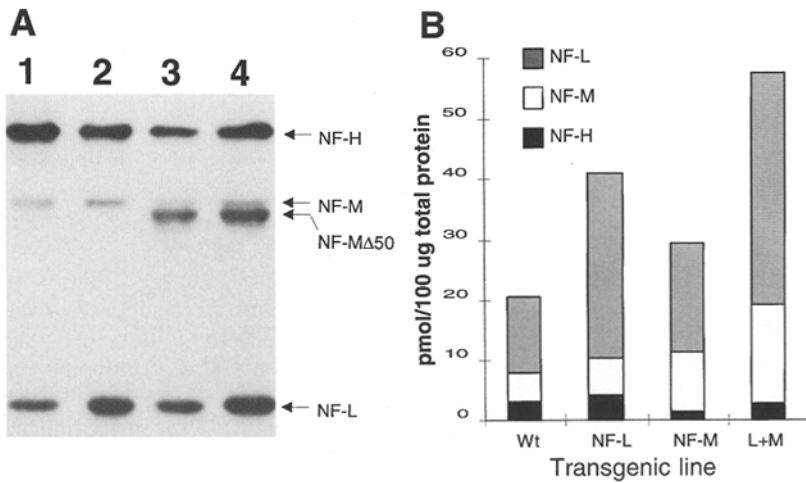


Figure 2. Changes in NF subunit content generated by crossing L and M mice. (A) Immunoblot of 2.5 μ g of total proteins extracted from sciatic nerve of 6-wk-old mice using antibodies specific for each of the three NF subunits (see Materials and Methods). Lane 1, wild-type mice; lane 2, L mice; lane 3, M mice; lane 4, LM mice. Notice that the transgene NF-M is smaller than the endogenous NF-M (see LM lane). (B) The average NF content quantified by phosphorimaging (each point is the average of at least six independent determinations).

An Increase in Each of the Three NF Subunits Alone Inhibits Radial Axonal Growth

To test what effect altered levels of each NF subunit had on radial growth, axonal calibers were measured in the L5 ventral roots of all the transgenic lines expressing higher levels of any single subunit or any pair of subunits. Examination of ventral roots was chosen for three reasons. First, spinal motor neurons have the highest level of transgene expression, as indicated by the extent of NF accumulation in neuronal cell bodies in L, M, and H animals (Xu et al., 1993; Wong et al., 1995; data not shown). Axons in ventral roots originate from the spinal motor neurons and thus are the optimal choice for evaluating the effect of transgene expression. Second, all of these axons are myelinated and their numbers are nearly constant among animals (960 ± 49 per L5 ventral root), thus making the measurement feasible and statistically significant. Third, measurement of NF levels in wild-type and NF-M transgenic mice have already shown similar changes in levels of endogenous and transgene encoded subunits comparing ventral roots and sciatic nerves (Wong et al., 1995).

To examine the effect of altered NF levels on radial growth, axonal calibers in 8-wk-old mice that express different levels of NF-L were analyzed: wild-type, L mice

that express twice the level of NF-L in the wild type (Monteiro et al., 1990), and doubly NF-L transgenic mice (LL mice) that express approximately four times the level of the wild type (Xu et al., 1993). (The LL mice were generated by crossing two independent NF-L transgenic lines; while most of these die from skeletal muscle atrophy at or before 3 wk of age [Xu et al., 1993], occasional animals survive this critical period as transgene expression declines at older ages.) This analysis revealed that the size of axons is the largest in wild-type animals, smaller in L mice, and smallest in the LL mice (Fig. 4 A). Thus, at essentially constant (or even slightly elevated) levels of NF-M and NF-H (Figs. 2 B and 3 B and Table I), increases in NF-L result in reduced sizes of axons $>3 \mu$ m in diameter (Fig. 4 A). Statistical comparison between these distributions using the Wolcoxon rank sum test revealed $P = 0.001$ for doubly or singly transgenic axons vs. wild type and $P = 0.03$ for doubly transgenic vs. singly transgenic. These values indicate that the differences seen are highly significant. Smaller axonal sizes are the consequence of growth inhibition, since reduction in axonal growth was also seen in 4-wk (not shown) and 6-wk-old mice (see below). Phosphorylation

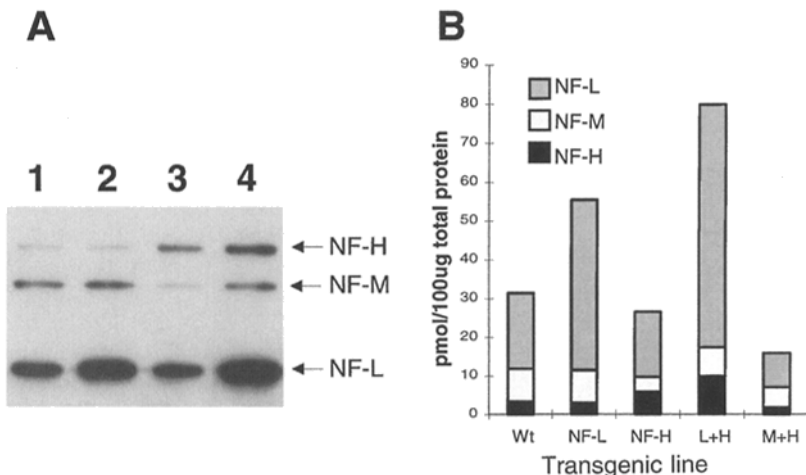


Figure 3. Changes in NF subunit content in 8-wk-old mice generated by crossing L with H mice, as well as in MH mice (NF-M and NF-H doubly transgenic). (A) Immunoblot carried out the same way as described in Fig. 2. Lane 1, wild-type mice; lane 2, L mice; lane 3, H mice; lane 4, LH mice. (B) The average NF content was quantified by phosphorimaging.

Table 1. Changes in Average Axonal Cross-sectional Area Caused by Changes in NF Subunit Composition

Genotype	Subunit overexpressed	Level of each NF subunit relative to its level in wild-type mice			Average change in cross-sectional area (percent of wild type)	Molar ratio of NF-M + NF-H
		NF-L	NF-M	NF-H		
Wild type	—	100	100	100	—	0.61
L	NF-L	230 (± 44)	115 (± 34)	110 (± 34)	- 4	0.30
M	NF-M	140 (± 17)	210 (± 55)	50 (± 6)	- 40	0.60
H	NF-H	90 (± 9)	45 (± 5)	180 (± 0)	- 34	0.58
LM	NF-L + NF-M	300 (± 50)	360 (± 30)	90 (± 13)	+ 46	0.50
LH	NF-L + NF-H	320 (± 6)	90 (± 0)	300 (± 14)	+ 30	0.26
MH	NF-M + NF-H	45 (± 16)	60 (± 5)	60 (± 11)	- 55	0.80

*Summary of the data shown in Figs. 2–8.

levels of NF-H are unaffected as judged either by immunoreactivity to phosphorylation specific antibody SMI 31 (not shown) or by gel mobility.

To investigate the effects of high levels of NF-M or NF-H on axonal caliber, similar measurements were made in M and H mice. Both lines showed more striking reductions in the axonal caliber (Fig. 4, B and C, and Table I), compared to that seen for L mice (Fig. 4 A and Table I). Average growth in axonal volume was reduced 35–40%, respectively. As in the L mice, reduction in caliber was most obvious in axons >3 μm. Similar findings have previously

been reported for M mice (Wong et al., 1995) and for mice expressing human NF-H to a level 2–3-fold above mouse NF-H (Cote et al., 1993). In the latter case, reduction in axonal size is accompanied by reduction in filament content arising, at least in part from slowing of neurofilament transport (Collard et al., 1995).

A Combined Increase in NF-L with Either NF-M or NF-H Stimulates Radial Axonal Growth

To determine what effect high levels of two NF subunits would have on axonal growth, caliber was measured in mice doubly transgenic for L and M (LM mice) or L and H (LH mice). Although small axons (i.e., axons <3 μm) were unchanged in number and size, increasing NF-M together with NF-L significantly enlarged calibers of those axons that normally undergo radial growth, resulting in an expansion of axonal volume by ~50% (Fig. 5 A and Table I).

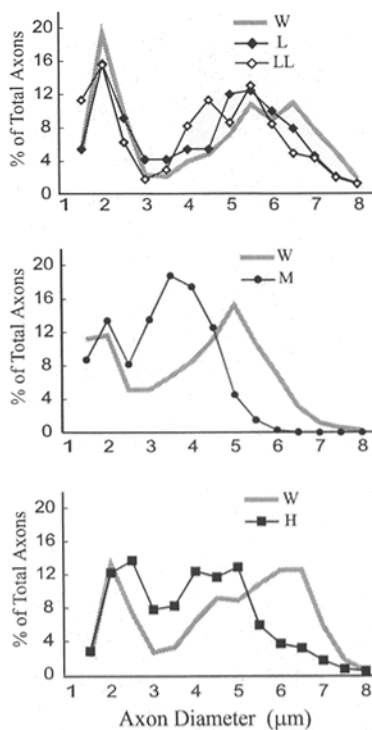


Figure 4. Reduced axonal caliber as a result of increasing each of the three NF subunits individually. (A) An increase in NF-L inhibits radial growth of axons. Distribution of axonal diameters in L5 ventral roots of doubly (LL), singly (L) NF-L transgenic mice, or wild-type littermate mice (8 wk old). The mean diameters for axons >3 μm from the LL, L, and wild-type are 5.1, 5.3, and 5.6 μm, respectively. (B) An increase in NF-M inhibits radial growth of axons. The average of measurements from three animals (6 wk old) for both wild-type and M mice are shown. (C) An increase of NF-H inhibits radial growth of axons. Measurements are from 8-wk-old wild-type and H mice.

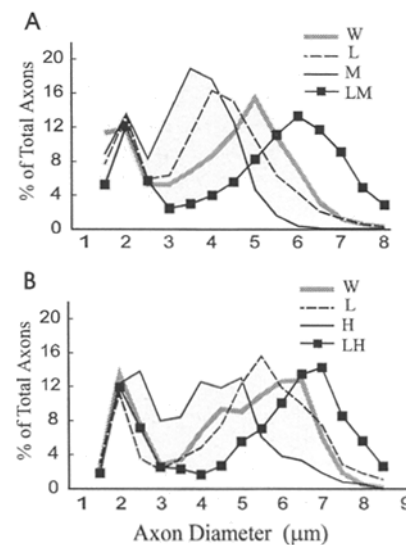


Figure 5. Increased axonal caliber as a result of a combined increase of NF-L with either NF-M or NF-H. (A) A combined increase in NF-L and NF-M stimulates radial axonal growth. The average size distribution of axons in L5 ventral roots from three wild-type, three L, three M, and two LM mice (6 wk old) are shown. All the animals were littermates generated from crossing L with M mice. (B) A combined increase in NF-L and NF-H stimulates radial axonal growth. The size distribution of axons in L5 ventral roots from wild-type, L, H, and LH mice (8 wk old) are shown. All the animals were littermates generated from crossing NF-L with NF-H transgenic mice.

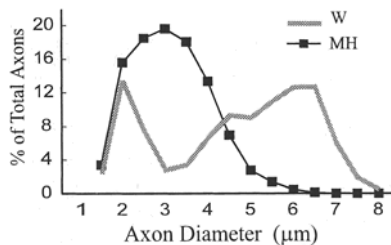


Figure 6. A combined increase of NF-M with NF-H strongly inhibits radial axonal growth. The average size distribution in L5 ventral roots from analysis of one wild-type mouse and three MH mice are depicted. All mice were 8 wk old.

A similar situation was seen for LH mice (Fig. 5 B). At 8 wk of age, LH mice displayed a 30% increase in axonal volume (Fig. 5 B and Table I), while increasing NF-H alone reduced axonal volume to only ~65% of normal. The same kind of changes shown in Fig. 5 B were also observed in 4-wk-old mice (not shown). We conclude that radial

growth of axons can be enhanced by simultaneous increase in NF-L and either NF-M or NF-H.

Increasing Both NF-M and NF-H Most Severely Inhibits Radial Axonal Growth

An attractive model for how NFs might promote radial growth and why increases in NF-L and NF-M or NF-L and NF-H yield larger axons is that cross-bridging mediated by the NF-M and NF-H tails establishes adjacent filament spacing. To test this, axonal caliber was measured in MH mice (Fig. 6). Not only did increasing NF-M or NF-H alone inhibit radial growth (Fig. 4, B and C), increasing both further inhibited growth, yielding the smallest axons among all the transgenic lines we examined (Fig. 6), with an average axonal diameter reduced by 24%. This corresponds to an overall reduction in axonal volume of 55%, compared with wild-type animals (Fig. 6 and Table I). These estimates of axon volume changes are minimum estimates of the actual changes, since all axons (including

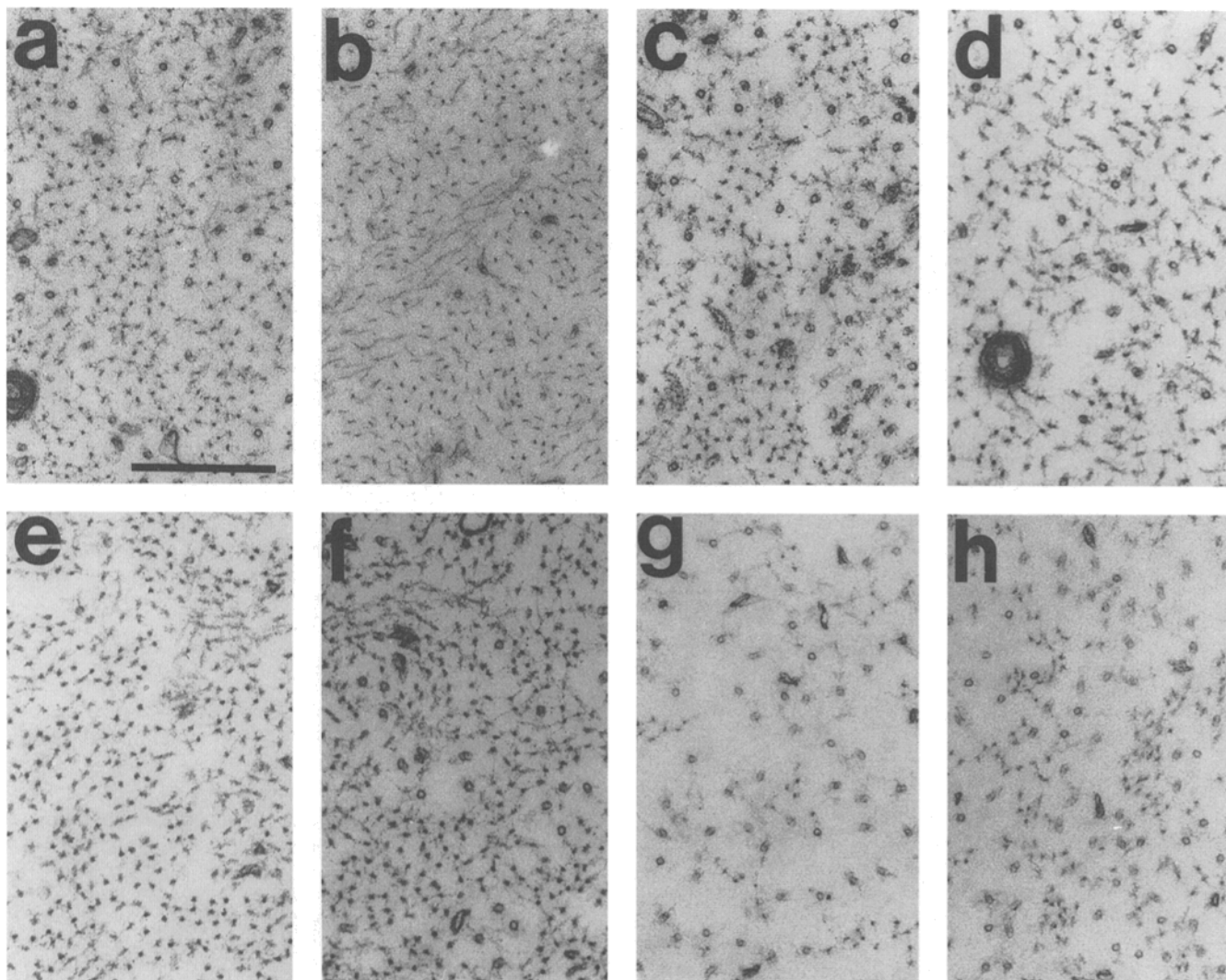


Figure 7. Changes in neurofilament density in the axons from different lines of transgenic mice. Examples of axoplasm viewed from axonal cross-sections are shown: (a) wild type; (b) NF-L transgenic; (c) NF-M transgenic; (d) NF-H transgenic; (e) NF-L and NF-M doubly transgenic; (f) NF-L and NF-H doubly transgenic; (g and h) NF-M and NF-H doubly transgenic. Bar, 0.5 μm.

those $<3 \mu\text{m}$ in diameter that are not affected in these transgenic mice) have been included in this analysis.

An Increase in NF-L Decreases Interfilament Distance, but Increase in Either or Both NF-M and NF-H Does Not Affect Interfilament Distance

To examine how organization and interfilament spacing is affected by changes in subunit composition, we examined the filament distribution in cross-sections of ventral root axons (Fig. 7). Compared with the distribution in wild-type axons that displayed an average density of 178 ± 18 filaments/ μm^2 , filaments were packed nearly twofold more densely in axons from L mice (Fig. 7 *b*; 318 ± 26 filaments/ μm^2). In contrast, filament densities in M mice (Fig. 7 *c*) and H mice (Fig. 7 *d*) were essentially unchanged in comparison with wild-type mice (Fig. 7 *a*). The most striking change was seen in axons from MH mice (Fig. 7, *g* and *h*); axons were almost depleted in NFs, and the axoplasmic cytoskeleton was dominated by microtubules, not NFs. Also noteworthy is a clustered distribution of the NFs that are present in MH mice (Fig. 7 *h*), indicating that there must be an associative interaction between NFs.

To test whether altered subunit composition affects interfilament spacing, we recorded the positions of each filament in individual axons and calculated nearest neighbor distances for each NF. Fig. 8 displays the outcome for wild-type, L, M, H, LM, LH or MH mice. Increasing NF-L lowered the average nearest neighbor distance from a little more than 50 to 44 nm, consistent with the increased density of NF coupled to inhibition of radial growth. Statistical comparison using two-sample *t* tests revealed these differences to be significant ($P = 0.0016$). Increasing NF-M alone or both NF-M and NF-H also lowered average nearest neighbor distance, but more modestly (yielding average spacings of 47 and 48 nm, respectively). (Increases in NF-M and NF-H produced a higher variation between axons, probably the result of a smaller sampling of filament distances caused by the lack of filaments in these axons [see Fig. 7, *g* and *h*]). Although increasing NF-L and NF-M together significantly enhanced radial growth, the expres-

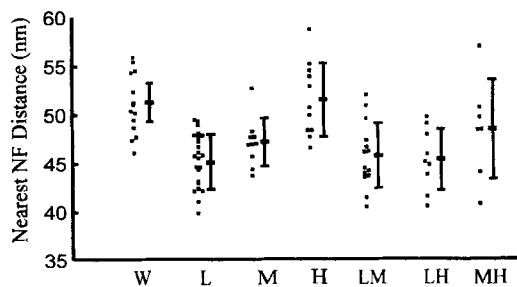


Figure 8. Changes in the nearest neighbor distance between neurofilaments in the axons from different lines of transgenic mice. Distribution of nearest neighbor distance between filaments in mice of various genotypes. Each dot represents the average nearest neighbor distance from a single axon. The bars mark the average and SD calculated from values of these dots. Statistical comparison using two-sample *t* tests reveal significant differences only for wild-type vs. L mice ($P = 0.0016$), wild-type vs. LM mice ($P = 0.034$), M vs. L mice ($P = 0.019$), and wild-type vs. LH mice, H vs. L mice, and H vs. LH mice (all P values <0.001).

sion of additional NF-M in L mice (i.e., producing LM mice) did not restore wild-type interfilament spacing (comparison of wild-type and LM mice demonstrated significant differences [$P = 0.034$]).

Perhaps even more surprising was that increasing NF-H (Fig. 8) did not significantly affect interfilament distance either in mice with normal or elevated NF-L content. Rather, the decrease in filament spacing found in NF-L singly transgenic animals was unaffected by simultaneous elevation in LH mice of both NF-L and NF-H despite enhanced radial growth and a normal ratio of NF-L to NF-H (Table I). These data demonstrate that NF-H content alone does not specify the nearest neighbor interfilament distances in axons.

Discussion

To earlier evidence that NFs are essential for the large volume increase that begins during radial growth of axons, our current efforts have proven that different NF subunits play different yet complementary roles in supporting axonal expansion. Hence, disturbing the normal ratio among the three NF subunits by increasing any individual subunit compromises the ability of NF to stimulate growth. Moreover, a combined increase in NF-M and NF-H produces the smallest axons (with a diminished number of NF), while axonal calibers are significantly expanded only when increased NF-L is combined with either NF-M or NF-H (Fig. 5 and Table I). Thus, where previous genetic efforts have proven that NF investment is necessary for growth in caliber (Sakaguchi et al., 1993; Eyer and Peterson, 1994), the present studies demonstrate that NF-L in combination with either NF-M or NF-H is sufficient to promote radial axonal growth.

In principle, the dependence of radial growth on a combination of NF subunits could arise from an influence on filament number and/or on interactions between filaments and other axonal components, including the axonal transport machinery. Filament number could be altered by changes in filament assembly properties. In the mice transgenic for NF-L, a twofold increase in the level of NF-L results in a nearly twofold increase in NF density and NF number, but a slight decrease in axonal caliber. Clearly, in these mice, the twofold lowered ratio of NF-M and NF-H to NF-L (see Table I) does not impair the assembly of filaments, a finding that is consistent with the earlier observations using DNA transfection that had demonstrated that NF were obligate heteropolymers of NF-L and NF-M or NF-H, but with assembly tolerating a wide range of subunit stoichiometries (Lee et al., 1993; Ching and Liem, 1993; Nakagawa et al., 1995). On the other hand, the lower number of axonal filaments in M, H, and MH mice is consistent with the possibility that filament assembly may be impaired by raising the level of NF-M and/or NF-H relative to NF-L. Further, the biochemical quantity of total NF subunits in axons of M mice exceeds that in the wild type (Fig. 2), but the number of filaments is lower (Fig. 7), raising the possibility of a larger nonfilamentous pool. In fact, despite the increased synthesis of NF-H and NF-M in H and M mice, the ratio of the sum of NF-H and NF-M to NF-L remains the same as in the wild type, suggesting that this ratio is probably near the saturation level for coassembly, even in wild-type mice, and that to accommodate an

increase in either NF-M or NF-H one subunit, there must be a reciprocal loss of the other subunit.

Concerning how NF interact with each other and how that relates to radial growth, the current findings have proven that subunit composition does affect interfilament distance, albeit more modestly than one might have predicted. The increase in the number of NFs in L mice is accompanied by a reduced interfilament spacing and a slight reduction in caliber (Figs. 7, 8, and 4 A, respectively). This is consistent with a widely cited model postulating that NF-H and/or NF-M determine interfilament spacing (e.g., Carden et al., 1987; Matus, 1988), probably by projecting their long carboxyl terminal tails (side arms) peripherally (Hirokawa et al., 1984; Nakagawa et al., 1995). In accord with this model, an increase in NF-L would lower the ratio of NF-H and NF-M to NF-L, thus lowering the density of side arms and possibly resulting in a corresponding reduction in inter-filament spacing. On the other hand, doubling the number of NF-M or NF-H tails (in M or H mice) does not increase interfilament distance and in the case of NF-M, actually lowers it (Figs. 7 and 8).

One factor that could profoundly influence interfilament spacing is the level of phosphorylation in the tail domain of NF-H and NF-M. Wider interfilament distance correlates with high levels of phosphorylation (de Waegh et al., 1992; Hsieh et al., 1994; Nixon et al., 1994; Tu et al., 1995). In a transgenic line that accumulates human NF-M to a level equivalent to endogenous mouse NF-M in small diameter (<1.5- μ m) axons of the neocortex (but not in the peripheral nervous system), Tu et al. (1995) found a reduction of phosphorylated NF-H and an increase of nonphosphorylated NF-H (the total level of NF-H was not measured). In our study, changes in the level of NF subunits do not appear to affect the level of phosphorylation of NF-H, since in both M and H mice, the relative proportion of immunoreactivity to SMI 31 (an antibody that reacts with highly phosphorylated NF-H) and to a phosphorylation-independent polyclonal anti-NF-H antibody remains the same as in the wild type (Wong et al., 1995; not shown). Thus, at least in the sciatic nerve, neither a decrease (as a result of increased NF-M) nor an increase in NF-H appears to affect its level of phosphorylation.

Finally, regarding the kinds of interactions that may exist between NF, the complete absence of the nearest neighbor distances <20 nm in all transgenic mice measured in this study supports the presence of short range, repulsive interactions that prevent very close packing of filaments. Superimposed on this, however, the clustered distribution of NFs in axons from MH mice that are relatively depleted in NFs (Fig. 7 h) demonstrates that, as had long been predicted by the filament cross-bridging model (Hirokawa et al., 1984; Nakagawa et al., 1995), at least some NF interactions are mediated by an associative interaction between filaments. Equally clear, however, is that interactions between nearest neighbor filaments do not specify radial growth. Nearest neighbor distances in LM mice and M mice are comparable, but average axonal volume differs by 240%. Thus, while maximal radial growth is mediated by a combination of NF subunits, the key property needed to stimulate caliber growth must include longer range interactions of the tails of NF-M and/or NF-H with other ax-

onal components, or between NFs that are not nearest neighbors.

We thank Dr. Jean-Pierre Julien for providing the cosmid containing the mouse NF-H gene.

This work was supported by grants from the NIH to D.W. Cleveland (NS27036) and to J.W. Griffin and D.W. Cleveland (NS22849). D.W. Cleveland is the recipient of a Jacob Javits Neuroscience Investigator award from the NIH. Z.S. Xu and P.C. Wong were supported by postdoctoral fellowships from the Muscular Dystrophy Association. M.K. Lee was supported by a postdoctoral fellowship from the NIH.

Received for publication 7 February 1996 and in revised form 25 March 1996.

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