Neurofilament-dependent Radial Growth of Motor Axons and Axonal Organization of Neurofilaments Does Not Require the Neurofilament Heavy Subunit (NF-H) or Its Phosphorylation

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Abstract. Neurofilaments are essential for establishment and maintenance of axonal diameter of large myelinated axons, a property that determines the velocity of electrical signal conduction. One prominent model for how neurofilaments specify axonal growth is that the 660–amino acid, heavily phosphorylated tail domain of neurofilament heavy subunit (NF-H) is responsible for neurofilament-dependent structuring of axoplasm through intra-axonal crossbridging between adjacent neurofilaments or to other axonal structures. To test such a role, homologous recombination was used to generate NF-H–null mice. In peripheral motor and sensory axons, absence of NF-H does not significantly affect the number of neurofilaments or axonal elongation or targeting, but it does affect the efficiency of survival of motor and sensory axons. Loss of NF-H caused only a slight reduction in nearest neighbor spacing of neurofilaments and did not affect neurofilament distribution in either large- or small-diameter motor axons. Since postnatal growth of motor axon caliber continues largely unabated in the absence of NF-H, neither interactions mediated by NF-H nor the extensive phosphorylation of it within myelinated axonal segments are essential features of this growth.

Key words: neurofilaments • radial growth • axoplasm • motor neurons • sensory neurons

A series of preceding efforts (Friede and Samorajski, 1970; Hoffman et al., 1987; Cleveland et al., 1991; Lee and Cleveland, 1994) have proven that neurofilaments are essential elements for establishing the correct diameters (and hence volume) of large myelinated motor and sensory axons. These axons elongate during development with diameters of ~1–2 μm, but after stable synapse formation in early postnatal life and concomitant with myelination, they grow markedly in diameter. This growth continues at a slow rate throughout adulthood, ultimately yielding axons that in humans reach diameters of up to 14 μm (Kawamura et al., 1981), corresponding to a >100-fold increase from their initial volumes. Establishment of axon caliber is of importance for normal functioning of the nervous system since caliber is a principal determinant of the conduction velocity at which nerve impulses are propagated along the axon (Gasser and Grundfest, 1939; Arbuthnott et al., 1980; Sakaguchi et al., 1993). Overwhelming evidence has demonstrated that neurofilaments—assembled as obligate heteropolymers (Ching and Liem, 1993; Lee et al., 1993) of three polypeptide subunits, light neurofilament (NF-L; 68 kD),1 mid-sized neurofilament (NF-M; 95 kD), and heavy neurofilament (NF-H; 115 kD)—are essential for establishing the caliber of large myelinated axons. The initial suggestion of this arose from the linear relationship between neurofilament number and axonal cross-sectional area during the phase of rapid growth in diameter (Friede and Samorajski, 1970) and during regrowth after axonal injury (Hoffman et al., 1987). The importance of neurofilaments in specifying normal axonal caliber was proven unequivocally by analysis of a recessive mutation (quv) in a Japanese quail that

1. Abbreviations used in this paper: ES, embryonic stem; NF-H, NF-M, and NF-L, heavy, mid-sized, and light neurofilament subunits.
lacks neurofilaments as the result of a premature translation terminator in the NF-L gene. Radial growth of axons fails completely in these animals (Yamasaki et al., 1992; Ohara et al., 1993), with a consequent reduction in axonal conduction velocity and generalized quivering (Sakaguchi et al., 1993). This requirement for neurofilaments has been confirmed in mice both by expression of a NF-H–β-galactosidase fusion protein that completely inhibits neurofilament transport into axons (Eyer and Peterson, 1994) and by targeted deletion of the NF-L gene (Zhu et al., 1997). In both cases, loss of axonal neurofilaments results in failure of normal radial growth. Moreover, since neurofilaments are obligate heteropolymers of NF-L and substoichiometric levels of NF-M or NF-H (Ching and Liem, 1993; Lee et al., 1993), absence of NF-M caused by gene disruption leads to markedly fewer axonal neurofilaments and a suppression of radial growth (Elder et al., 1998).

Although it is clear that neurofilaments are essential for specifying axonal diameter, the mechanism through which neurofilaments mediate increases in axonal size remains unsettled. The linear correlation between neurofilament number and axonal cross-sectional area initially suggested that the axon expanded or contracted to maintain a constant density of neurofilaments (Hoffman et al., 1987). That radial growth was not simply a function of the number of neurofilaments was proven by elevation of wild-type NF-L levels in transgenic mice. This revealed that a twofold increase in the number of neurofilaments (with an elevated proportion of the NF-L subunit) actually decreases axonal diameter slightly (Monteiro et al., 1990; Xu et al., 1993). This led to one attractive and plausible model for how neurofilaments mediate initial growth and then maintain it: the long, COOH-terminal tail domains of NF-M and NF-H, which extend from the core of the 10-nm-diameter filament (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988; Troncoso et al., 1990), support axonal growth by crossbridging between adjacent neurofilaments or to other axonal constituents, thereby forming a three-dimensional lattice that determines volume. Consistent with this, transgenic methods that increased filament number (by producing higher levels of NF-L) combined with higher levels of NF-M or NF-H yielded increased axonal volumes by 30–45% (Xu et al., 1996).

Superimposed on contributions to radial growth provided by the number and subunit composition of neurofilaments, it is also now clear that the general relationship of neurofilament content and caliber is apparently regulated by the relative degree of phosphorylation of NF-M and NF-H. The tail domain of NF-H is largely composed of a KSP tripeptide, the serine of which is nearly stoichiometrically phosphorylated in myelinated axonal segments (Julien and Mushynski, 1982; Carden et al., 1985; Lee et al., 1988). It has been proposed that phosphorylation of NF-H, and to a lesser extent NF-M, increases the total negative charge and lateral extension of their side arms (Glicksman et al., 1987; Myers et al., 1987), thereby mediating the increased neurofilament spacing found in myelinated segments and/or increased crossbridging to other axonal components such as microtubules (Hirokawa, 1982). Phosphorylation of both NF-H and NF-M tail domains is strongly correlated with radial growth, but the more extensive repeat domain in NF-H (which in mice contains 51 KSP repeats versus only 4 for murine NF-M) has focused most attention on NF-H. In the normal setting, unmyelinated initial axonal segments contain dephosphorylated NF-H and display higher filament density and much smaller diameters than the adjacent myelinated segments (Hsieh et al., 1994; Nixon et al., 1994). Consistent with this is the finding that a primary defect in myelination (in the Trembler mouse) decreases phosphorylation of NF-H, increases neurofilament density, and inhibits normal radial growth of axons (de Waegh et al., 1992). Similarly, deletion of the peripheral myelin-associated glycoprotein, which has been proposed to signal from the myelinating Schwann cell to the axon, results in reduced neurofilament phosphorylation, decreased neurofilament spacing, and reduced axonal calibers (Yin et al., 1998). These examples show the direct relationship between neurofilament phosphorylation and axonal diameter within myelinated axonal segments.

That NF-H is a primary component of radial growth has been supported by strong correlative evidence: increases in the level of NF-H mRNA is most pronounced during the earliest phase of radial growth (0–4 wk postnatal) (Schlaepfer and Bruce, 1990), suggesting its importance in the process. Doubling NF-M content in transgenic mice yields a 50% reduction in axonal NF-H and strongly inhibits its radial growth, despite a constant level of NF-L (Wong et al., 1995). Furthermore, modest increases in NF-H mildly enhance radial growth in transgenic mice (although higher levels severely retard growth by slowing transport and trapping neurofilaments in neuronal cell bodies) (Marszalek et al., 1996). These three examples lend support to the idea that NF-H levels modulate radial growth of axons.

To examine directly the role of NF-H and its phosphorylation in structuring axoplasm and on neurofilament-dependent radial growth of large myelinated axons, we now report the use of homologous recombination to produce mice devoid of NF-H and to document the consequences of chronic absence of NF-H (and its phosphorylation) on elongation and survival of both large and small motor and sensory neurons, organization of neurofilaments and other organelles in those axons, and establishment and maintenance of axonal caliber.

Materials and Methods

Production and Screening of Mice with an NF-H Gene Disruption

A targeting vector for disrupting the mouse NF-H gene using homologous recombination was constructed (from a mouse NF-H gene cloned from a 129 SVJ library) by inserting a 1.6-kb KpnI-SacI fragment of NF-H (which lies 1.5-kb 5′ to NF-H transcriptional initiation site; see Fig. 1 A) adjacent to the phosphoglycerol kinase–promoted neomycin gene of plasmid pSK (Tybulewicz et al., 1991). Next, the 6.7-kb Smal-EcoRV fragment of the NF-H gene (containing all three introns and coding sequences from amino acid 34 through most of the KSP repeats in the tail) was ligated just 3′ to the neomycin gene. Finally, a phosphoglycerol kinase–promoted thymidine kinase gene cassette was ligated 3′ to this latter NF-H gene segment to yield a final targeting construct. After linearization with KpnI, the targeting DNA was electroporated into embryonic stem (ES) cells (RI cells kindly provided by Andreas Nagy, University of Toronto, Toronto, Can-

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ing an affinity-purified rabbit polyclonal antibody (pAb-NF-L ampli- 
cated, and DNA was prepared and digested with HindIII or with 
ecoRV, separated on 0.8% agarose gels, and transferred to Hybond N+ 
filters (Amersham Corp., Arlington Heights, IL). NF-H fragments were 
identified by hybridization either with the 1.4 kb EcoRV/AatII 
fragment of NF-H gene, which encodes some of the KSP multiphosphorylation re- 
petals, or with NF-H 3'-flanking region, with the 5' fragment (see Fig. 1). DNA labeling was performed by random primer ex- 
tension using [α-32P]dATP.

Targeted ES cell clones were injected into C57BL/6J blastocysts to pro- 
duce chimeric animals. Chimeric animals from two independent ES cell 
clones were bred to C57BL/6J mice, and mice heterozygous for the dis- 
ruptions were identified by analysis of mouse tail DNAs (prepared as be- 
fore [Monteiro et al., 1990]).

Analysis of Neurofilament RNA Levels in 
Nervous Tissues

Brains and spinal cords of 5-wk-old animals were dissected from NF-H de- 
letion, heterozygous, and wild-type mice and immediately frozen at 
−80°C. Total cellular RNA was purified as described (Chomczynski 
and Sacchi, 1987). In brief, tissues were homogenized in 4 M guanidinium thi- 
ocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M 2-mercap- 
toethanol. Protein was removed by addition of an equal volume of phenol 
in 0.2 M sodium citrate, pH 4) and one-fifth volume of chloroform/ 
isoamyl alcohol (49:1). After mixing well, samples were centrifuged at 
10,000 × g for 20 min at 4°C. The RNA in the aqueous phase was precipi- 
tated by adding 2.5 M ammonium acetate. Finally, the RNA pel- 
licate was collected by centrifugation, dissolved in the homogeniza-

tional area of each axon was calculated and reported as a diameter of a 
circle of equivalent area. Axon diameters were grouped into 0.5-μm bins.

Analysis of Filament Spacing: Nearest 
Neighbor Analysis

To measure nearest neighbor distances between neurofilaments, cross- 
sections of axons larger than 3.0 μm in diameter were photographed at a 
magnification of 20,000 and enlarged an additional 4.25-fold by printing. 
Neurofilaments were identified in these end-on views as dots ~10 nm in 
diameter. Positions of neurofilaments were marked by puncturing the 
print with a push-pin. By laying the final prints on a light box, neurofila-
ments positions could easily be imaged, and nearest neighbor distances 
were calculated for each filament using a digital imaging program (Bio-
quant, Nashville, TN).

Results

Mice Producing no NF-H mRNA or Protein Are Viable, 
with Elevated Levels of NF-M and Tubulin

To generate mice homozygously deleted for the NF-H gene, a targeting vector was constructed from the mouse 
NF-H gene (cloned from a mouse 129 SVJ library) by re- 
placing the 1.6-kb segment containing the proximal pro-
moter, methionine initiation codon, and 33 additional 
codonts of the amino-terminal region of NF-H with a neo-
mycin resistance gene (Fig. 1 A; see also Materials and 
Methods). After electroporation into ES cells and selec-
tion for integration by resistance to the neomycin homo-
gene G418, cells in which one NF-H allele was targeted 
were identified by genomic DNA blotting. With probes 
corresponding to the gene segments just 5' and 3' to that 
contained in the gene targeting vector, this revealed that 2 
of 70 lines examined had been correctly targeted at both 5' 
(Fig. 1 C) and 3' (Fig. 1 B) sites of integration. Both clones 
were injected into C57BL/6J blastocysts, and both lines 
produced chimeric animals that when mated to C57BL/6J

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mice transmitted the disrupted allele to their progeny, as revealed by blotting of genomic tail DNAs from such animals (Fig. 1 D).

Mating of heterozygotes to each other produced animals with both NF-H genes disrupted, as well as littermates that retained one or both normal NF-H alleles in the 1:2:1 ratios expected for Mendelian inheritance. Mice with both disrupted genes were viable and fertile, displaying no overt phenotype, at least up to 6 mo of age. To establish that the disruption eliminated NF-H expression, RNA was prepared from brain and spinal cords of animals with NF-H alleles that were normal or heterozygously or homozygously disrupted. Blotting with a probe corresponding to codons 798 to 1087 of mouse NF-H revealed the complete absence of stable mRNAs carrying NF-H coding sequences in the NF-H–deleted mice (Fig. 1 E, lanes 5 and 6). Relative to ribosomal RNA, phosphorimaging demonstrated that mRNAs coding for NF-L and NF-M were not markedly affected by presence or absence of NF-H mRNA (Fig. 1 E, lanes 1–6).

Immunoblotting with an antibody (Xu et al., 1993) raised to the carboxy terminus of NF-H (Fig. 2 B) or antibodies that recognize either the unphosphorylated NF-H tail domain (SMI-32; Fig. 2 D) or the same domain when phosphorylated (SMI-31; Fig. 2 C) confirmed the complete absence of NF-H in extracts from brain, spinal cords, and sciatic nerves of mice with two disrupted alleles (Fig. 2, B–D, lanes 3, 6, and 9). Animals heterozygous for the disruption had intermediate levels of NF-H, which phosphorimaging revealed to be between ~60–70% of the level of control samples in brain and sciatic nerves. Parallel immunoblots demonstrated that NF-L levels were unaffected by the presence or absence of NF-H in spinal cord and sciatic nerve extracts from all three genotypes (Fig. 2 F). In contrast, use of an antibody insensitive to the phosphorylation state of NF-M (RMO44; Tu et al. 1995) demonstrated that NF-M levels were elevated after diminution or elimination of NF-H, with phosphorimaging revealing a twofold increase, relative to normal mice, in spinal cords (compare Fig. 2 E, lanes 4 and 6) and sciatic nerves (compare Fig. 2 E, lanes 7 and 9) but not brain of NF-H–disrupted animals. These findings provide additional evi-

**Figure 1.** Disruption of the mouse NF-H gene by homologous recombination. (A) Strategy for disruption of the mouse NF-H gene. A targeting construct for disruption of the NF-H gene was constructed by inserting a 1.7-kb gene encoding resistance to neomycin in place of 1.6 kb NF-H putative promoter and the first 34 codons of the gene. The four NF-H exons are indicated by filled boxes interrupted by three introns. ATG denotes the NF-H translation initiation codon. Unique HindIII (H3) and EcoRV (RV) sites were introduced into the disrupted gene allele after homologous recombination. RI, EcoRI; WT, wild type; MT, mutant; PGK, phosphoglycerate kinase promoter; NEO, neomycin phosphotransferase gene; TK, thymidine kinase gene. (B–D) Screening of (B and C) ES and (D) mouse tail DNAs for targeted inactivation of the NF-H gene. (B) Genomic DNA blot of ES cell DNA after digestion with HindIII was probed with a segment 3' to the targeted domain (the highlighted EcoRV-AatII fragment in A). The normal NF-H allele produces an 18-kb fragment; the targeted allele produces a 10-kb fragment. (C) Genomic DNA blot of ES cell DNA after digestion with EcoRV was probed with a 5' probe (the EcoRI-NdeI fragment denoted in A). The normal allele produces a 15-kb fragment; the targeted allele produces a 7-kb fragment. (B and C) Lane 1, wild-type ES cell DNA; lanes 2 and 3, DNA from two targeted ES cells. (D) EcoRV-digested mouse tail DNA probed with the 5' probe. DNAs are from a mouse with (lane 1) two wild-type alleles or (lane 2) heterozygous or (lane 3) homozygous for disruption of the NF-H gene. (E) NF-L, NF-M, NF-H, and βIII-tubulin mRNA levels in mice with zero, one, or two copies of a disrupted NF-H gene. 20 μg of total RNA isolated from 5-wk-old brains and spinal cords of control mice and mice heterozygous or homozygous for disruption of the NF-H gene were fractionated on 1% formaldehyde agarose gels, blotted on to nylon membranes, and probed with radiolabeled cDNA sequences for each subunit (see Materials and Methods). Lanes 1, 3, and 5, brain RNAs from wild-type, heterozygous, and homozygous mice. Lanes 2, 4, and 6, spinal cord RNAs from wild-type, heterozygous, and homozygous mice.
and Sternberger, 1983) to detect a phosphorylated deter-

Elder et al., 1998). Using antibody SMI-31 (Sternberger

amount of NF-L (Wong et al., 1995; Marszalek et al., 1996;

dence that in lower motor and sensory neurons, NF-M and

NF-H in mice with zero, one, or two copies of a disrupted NF-H gene. (A) Total tissue extracts from 5-wk-old brain, spinal cord, and sciatic nerves were fractionated on 7% SDS–polyacrylamide gels and stained with (A) Coomassie blue or (B–I) electroblotted to nitrocellulose. (B) NF-H detected with a peptide antibody recognizing the extreme COOH terminus of NF-H (Xu et al., 1993); (C) phosphorylated NF-H and NF-M detected with monoclonal antibody SMI-31; (D) nonphosphorylated NF-H detected with monoclonal antibody SMI-32; (E) NF-M detected with monoclonal antibody RM 044 (Tu et al., 1995); (F) NF-L detected with a polyclonal peptide antibody recognizing the COOH terminus of NF-L (Xu et al., 1993); (G) α-tubulin detected with monoclonal antibody DM1A; (H) the neuron-specific class III, β-tubulin iso-
type with mAb TuJ1 (Lee et al., 1990); and (I) plectin detected with polyclonal antiserum P21 (Wiche and Baker, 1982). (Plectin migrates with a mobility of ~500 kD in brain and spinal cord but at ~160 kD in nerve samples using both this antibody and monoclonal antibody 10F6 [Foisner et al., 1991]; not shown.) Lanes 10–14 represent four times longer exposures than lanes 1–6.)

Absence of NF-H Has Surprisingly Little Effect on Radial Growth of Motor Axons

Since neurofilaments are known to be a principal determi-
nant of axonal caliber (Hoffman et al., 1987; Yamasaki et al., 1992; Ohara et al., 1993; Zhu et al., 1997) and NF-H phosphorylation has been tightly linked to neurofilament-dependent control of axon diameter (de Waegh et al., 1992; Hsieh et al., 1994; Nixon et al., 1994), we examined how the changes in NF-H content were reflected in changes in axonal caliber of large myelinated motor axons. Light microscopic inspection of the L5 ventral roots (Fig. 3A) revealed the surprising finding that myelinated axons from wild-type mice, mice heterozygous for NF-H, or mice with both gene copies disrupted were qualitatively similar in caliber to those of wild-type mice (compare Fig. 3A, left, middle, and right panels).

To quantify potential caliber changes associated with loss of NF-H expression, cross-sectional areas of every axon within each ventral root (Fig. 4) were measured from 4- and 9-wk-old animals (two animals of each genotype at each time point). Each area was then converted to a diam-
eter corresponding to a circle of the equivalent area. As

Figure 2. Levels of neurofilament subunits NF-L, NF-M, and NF-H in nerve samples from NF-H–free mice compared with normal littermates (Fig. 2I, compare lanes 4 and 6, 7 and 9). Regardless of the genotype, full-length plectin (~500 kD) was seen in brain and spinal cord samples, while two plectin antibodies revealed a stable accumulation product that migrated with an apparent size of less than 200 kD in all peripheral nerve samples. (Note that lanes 7–9 of Fig. 2I represent a lower molecular weight portion of the immuno- blot than lanes 1–6.) Since it is retained in neurofila-
ment-enriched nerve fractions, this plectin fragment (or isoform or plectin-related molecule) most probably contains a neurofilament-binding site (such as that found near the carboxy terminus of typical plectin [Elliott et al., 1997]).
expected, motor axons of wild-type animals showed a typical bimodal distribution of diameters representing the small and large myelinated axons, with initial diameters centered on 1.5 and 5 μm. Both small and large axons grow considerably in size between 4 and 9 wk of age, achieving diameters of 2.5–3 and 8.5 μm, respectively. Summing the areas of all axons after this 5-wk growth period revealed a threefold increase in axonal volume of the larger axons and a doubling of size for the smaller size class. This bimodal axon size distribution and growth phase was also observed in mice heterozygous or homozygous for the NF-H deletion, although at both time points reducing or deleting NF-H clearly decreased or eliminated the very largest diameter axons (e.g., those larger than 9 μm in Fig. 4, E and F) and shifted the distributions toward smaller sizes (Fig. 4, E and F, arrows). However, by 9 wk of age, loss of NF-H resulted in axonal populations of both large and small motor axons that were only slightly (25 and 20%, respectively) reduced in total axon area relative to wild-type animals. Similar findings were also seen in 15-wk-old animals. Additional radial growth of ~20% continued to equivalent extents in both normal and NF-H–null animals, with a similar loss of the largest population of motor axons found in 15-wk-old NF-H–deleted animals (not shown). Thus, for motor axons, NF-H content (and any interactions provided by it or its long tail domain) are dispensable for the majority of radial growth, despite the fact that it is necessary for achieving the largest diameters.

Radial Growth of Sensory Axons Requires NF-H

To examine how NF-H contributes to radial growth of sensory axons, calibers were measured in L5 dorsal roots of wild type and animals heterozygous or homozygous for NF-H disruption. While there are many small sensory axons that do not undergo radial growth, light microscopic examination revealed an apparent inhibition of growth of the largest caliber axons (Fig. 3 A). Comparing size distributions of 4- and 9-wk-old wild-type animals demonstrated that radial growth of both larger and smaller axons occurs (Fig. 5 A). For animals with reduced (Fig. 5 B) or no (Fig. 5 C) NF-H content, growth of the smallest axons was eliminated, while growth of the largest class (initially >4 μm) continued, but at a reduced level compared with normal mice, especially for the largest axons (>8 μm in normal mice). Similar findings emerged from analysis of 15-wk-old animals (not shown).

NF-H Is Required for Survival of the Normal Number of Motor and Sensory Axons

The complete absence of neurofilaments (arising from disruption of the NF-L gene) during initial axon elongation, targeting, and myelination has been demonstrated to yield a 13% reduction in the survival of initial motor axons (Zhu et al., 1997). To determine whether NF-H content affects this developmental survival of motor axons, axons were counted in the L5 ventral and sensory roots of wild-

Figure 3. Absence of NF-H does not markedly affect growth in motor axon caliber. (A) Cross sections of L5 motor (ventral root) and sensory (dorsal root) axons from wild type (+/+), NF-H heterozygous (+/−), and NF-H homozygous (−/−) mutant mice. Sections are from 4- and 9-wk-old mice as indicated. (B and C) Absence of NF-H yields a partial loss of motor and sensory axons in early postnatal life. Counts of small (<4 μm diameter; black bars) and large (≥4 μm diameter; crosshatched bars) axons in L5 motor and (C) sensory root axons from 9-wk-old mice with zero, one, or two disrupted NF-H alleles. Counts are averages from three to four animals for each genotype and age. Bar, 10 μm.
type mice and mice with one or both copies of the NF-H gene disrupted. After the initial burst of radial growth, this quantitation revealed that by 9 wk of age, the total number of surviving motor axons was ~13% lower ($n = 6$, $P = 0.03$) in the absence of NF-H than in its presence (Fig. 3B). This reflects a preferential loss of large (>4 μm in diameter) axons with no significant change in the number of small axons. A similar trend was seen when the sensory axons were counted (Fig. 3C). Again, the number of large- but not small-caliber axons was reduced by ~19% ($n = 6$, $P = 0.03$).

**Nearest Neighbor Spacing between Neurofilaments Is Unaffected by NF-H Content**

To examine whether reduction in NF-H content, and the corresponding increase in axonal NF-M, affects neurofilament organization in axons, the nearest neighbor spacing between neurofilaments in cross sections of ventral roots was compared in 9-wk-old wild-type animals and littermates that were heterozygously and homozygously de-
leted for the NF-H gene. Qualitative inspection of electron micrographs from wild-type versus NF-H–deleted animals revealed no consistent differences in neurofilament spacing (compare Fig. 6, A–C), a point reinforced by marking neurofilament positions in ventral root axons (n = 9, including axons ranging in diameters from 4 to 8 μm) and calculating nearest neighbor spacings. As seen previously (Hsieh et al., 1994; Wong et al., 1995; Marszalek et al., 1996; Xu et al., 1996), measurement of nearest neighbor distances revealed a broad distribution centered on a 45-nm spacing in wild-type mice. Examination of all distances from 10 axons revealed that neither reduction of NF-H nor its complete absence markedly affected the distributions of nearest neighbor distances (Fig. 6 D). A different measure, comparing the mean nearest neighbor distance calculated axon by axon, again revealed very similar average filament spacing, with only a possible shift (P = 0.05 using a two-tail t test) toward very slightly smaller distances in the NF-H–null axons compared with normal axons (Fig. 6 E). This pattern of filament–filament distances was confirmed by examination of overall axonal organization in longitudinal sections of motor axons. Although less organized axoplasm in the NF-H deficient might have been anticipated, especially in light of the demonstration that dephosphorylated NF-H directly binds to microtubules in vitro (Hisanaga and Hirokawa, 1990; Hisanaga et al., 1991; Miyasaka et al., 1993), this was not the case. The absence of NF-H–dependent interactions between adjacent neurofilaments or between neurofilaments and microtubules did not diminish the “straightness” of neurofilaments or their overall orientation along the long axis of the axon.

Despite the unchanged spacing and organization of neurofilaments, one difference in axonal organization was apparent by inspection: microtubule density was higher in the animals with diminished or no NF-H. Counting microtubules in axons of each genotype revealed a 60% increased density of microtubules in axons from NF-H–deleted animals (Fig. 6 F). A two-tailed paired t test revealed this difference to be highly significant (P < 0.001). The increase in microtubule number quantitatively corresponds to the similar increase in tubulin content seen in both the spinal cord and sciatic nerve homogenates of NF-H–free animals (Fig. 2, G and H, lanes 6 and 9). In light of the known induction of tubulin expression by nearly an order of magnitude during axonal regeneration (Hoffman et al., 1987) and the reduction in initial survival of motor and sensory axons in the NF-H–null mice, this increase in tubulin levels may reflect partial activation of a regenerative program.

Discussion

Several lines of preceding evidence had implicated a primary role for NF-H in crossbridging between adjacent neurofilaments or between neurofilaments and other axonal structures (Hirokawa et al., 1984; Marszalek et al.,
As had been anticipated, it should be emphasized that the collective evidence still supports a role for NF-H in radial growth, certainly an important one in sensory axons and probably a redundant one in motor axons. Indeed, prior work with transgenic mice expressing elevated levels of wild-type NF-L (Monteiro et al., 1990), NF-H (Marszalek et al., 1996; Xu et al., 1996), or epitope-tagged NF-M (Wong et al., 1995) has demonstrated that an excess of any subunit inhibits radial growth to a greater degree than does absence of NF-H, while increasing both NF-L (thereby assembling more filaments) together with either NF-M or NF-H stimulates radial growth (Xu et al., 1996). This lead to the view that radial growth requires contributions from all three subunits: NF-L to support filament assembly and a scaffolding of NF-M and NF-H tails to crossbridge between neurofilaments and/or other axonal components (Hisanaga and Hirokawa, 1990; Xu et al., 1996). What is clear now, however, is that changing the content of NF-H (with a corresponding elevation in NF-M) does not affect nearest neighbor spacing of neurofilaments, a finding that goes hand in hand with the identical situation after doubling axonal NF-M and a corresponding loss in NF-H (Wong et al., 1995).

The current evidence, along with two earlier efforts (Wong et al., 1995; Xu et al., 1996), implicates NF-M (see schematic in Fig. 7) as the subunit that specifies nearest neighbor spacing mediated by its 439-amino acid tail that can extend 30–40 nm from the surface of the filament (Hisanaga and Hirokawa, 1988). Indeed, such crossbridges have been observed after simultaneous expression of NF-L and NF-M (using baculovirus) in insect cells (Nakagawa et al., 1995). Schwann cell–dependent phosphorylation of the KSP repeats in the NF-M tail may lengthen the effective cross-link, so as to force wider filament spacing, a feature potentially necessary, but not sufficient, for radial growth. It must be noted, however, that since nearest neighbor neurofilament spacing is also almost unchanged by the complete absence of NF-M (Elder et al., 1998), the simplest interpretation is that multiple, redundant elements (including NF-M) establish this spacing.

Whatever the interaction between neurofilaments, it must be an attractive one, not simply a repulsion of the negatively charged tails, since even in mice with few axonal neurofilaments (as a consequence of disruption of the NF-M gene [Elder et al., 1998] or trapping most neurofilaments in the cell bodies by expressing high levels of NF-H [Marszalek et al., 1996] or NF-M [Wong et al., 1995]), the few axonal filaments are found in clusters with spacings
identical to those in normal mice (Xu et al., 1996). Moreover, since radial growth can be markedly inhibited despite no change in interfilament distance, this gives further weight to the argument that interactions between nearest neighbor filaments do not specify radial growth.

Although it appears unlikely that nearest neighbor filament spacing is a key determinant of radial growth, a great deal of previous evidence still argues for the involvement of neurofilaments in some other manner. The key properties needed to stimulate this growth must include long-range interactions between neurofilaments that are not nearest neighbors or between neurofilaments and other axonal components. Attractive candidates for these additional interactions include the family of proteins such as BPAG1n/dystonin, proven recently to be a crossbridger between neurofilaments and actin filaments (Yang et al., 1996) that is essential for survival of sensory neurons (Brown et al., 1995; Guo et al., 1995). BPAG1n/dystonin, while expressed at highest levels in sensory neurons, is also found in embryonic and postnatal motor axons (Dowling et al., 1997), and absence of it leads both to rampant sensory axon degeneration with more modest numbers of motor axon abnormalities, including degeneration (Dowling et al., 1997). Another known cross-linker with binding sites for intermediate filaments, actin filaments, and microtubules is plectin (Wiche, 1989), a very large (466-kD), essential (Andra et al., 1997) protein expressed in many neurons, including motor neurons (Errante et al., 1994). While we have not been able to measure BPAG1n/dystonin levels as a function of NF-H content, plectin levels do not change in the absence of NF-H, a finding consistent with plectin as one probable linking component in motor axons that acts in place of NF-H in mediating the bulk of neurofilament-dependent radial growth. However, we found only a plectin fragment (or much smaller isoform or plectin-related polypeptide) to accumulate in peripheral nerves. Definition of precisely what aspects of cross-linking are contributed by this shortened plectin will have to await determination of whether it oligomerizes and whether it retains the actin-binding domain normally situated near plectin’s amino terminus and/or a microtubule-binding domain (whose position is not yet mapped within plectin).

Seen from this perspective, a combination of cross-linkers, including NF-H and NF-M, along with members of the plectin and BPAG1n families, interlink between neurofilaments, microtubules, and cortical actin arrays to determine a three-dimensional, space-filling array of connected structural elements that expand axonal volume. Contacts mediated by NF-H are largely, but not completely, dispensable in motor neurons and may, in fact, be compensated by additional interactions mediated by the increased number of microtubules and additional NF-M. This leads to a model (Fig. 7) for structuring axoplasm using elements with overlapping function, with each neuronal type relying upon a different balance of individual components. These findings, along with the discovery of mutations in plectin as the primary cause of one form of muscular dystrophy accompanied by defects in skin and cardiac cell organization (Gaché et al., 1996; Andra et al., 1997; Smith et al., 1997), reinforce the essential nature of correct structuring of cytoplasm through establishment and maintenance of a flexible, deformable scaffold of interlinked cytoskeletal elements.

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