Age-related Atrophy of Motor Axons in Mice Deficient in the Mid-sized Neurofilament Subunit

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Abstract. Neurofilaments are central determinants of the diameter of myelinated axons. It is less clear whether neurofilaments serve other functional roles such as maintaining the structural integrity of axons over time. Here we show that an age-dependent axonal atrophy develops in the lumbar ventral roots of mice with a null mutation in the mid-sized neurofilament subunit (NF-M) but not in animals with a null mutation in the heavy neurofilament subunit (NF-H). Mice with null mutations in both genes develop atrophy in ventral and dorsal roots as well as a hind limb paralysis with aging. The atrophic process is not accompanied by significant axonal loss or anterior horn cell pathology. In the NF-M–null mutant atrophic ventral root, axons show an age-related depletion of neurofilaments and an increased ratio of microtubules/neurofilaments. By contrast, the preserved dorsal root axons of NF-M–null mutant animals do not show a similar depletion of neurofilaments. Thus, the lack of an NF-M subunit renders some axons selectively vulnerable to an age-dependent atrophic process. These studies argue that neurofilaments are necessary for the structural maintenance of some populations of axons during aging and that the NF-M subunit is especially critical.

Key words: aging • axonal atrophy • neurofilament proteins • neuronal cytoskeleton • knockout mice

NEUROFILAMENTS (NFs)[1] are the most prominent cytoskeletal elements in large myelinated axons. Three proteins, known as the light (NF-L), mid-sized (NF-M), and heavy (NF-H) NF subunits are the principal constituents of NFs. Each subunit is the product of a separate gene (Julien et al., 1986; Myers et al., 1987; Lees et al., 1988), and in mammals the triplet proteins, as they are sometimes called, have apparent molecular weights in SDS-PAGE gels of ~68,000, 150,000, and 200,000, respectively.

The NF proteins are members of the family of intermediate filament proteins (Steinert and Roop, 1986). Like other intermediate filaments, NFs possess a relatively well conserved α helical rod domain of ~310 amino acids with variable NH_2-terminal and COOH-terminal regions. The COOH-terminal domains of NFs, however, differ from other intermediate filament proteins in being greatly extended. These extensions contain a glutamic acid rich region of unknown significance and in NF-M and NF-H a series of lysine-serine-proline (KSP) repeats (Myers et al., 1987; Lees et al., 1988) that are extensively phosphorylated in both proteins (Lee et al., 1988). All three subunits appear to be incorporated into 10-nm-diam core filaments (Hirokawa et al., 1984; Mulligan et al., 1991) through the hydrophobic interactions of their α helical rod domains. By contrast, the phosphorylated COOH-terminal tail sequences of NF-M and NF-H are found in filament sidearms that extend away from the core filaments (Hirokawa et al., 1984; Hisanga and Hirokawa, 1988; Mulligan et al., 1991; Nakagawa et al., 1995). Thus, current models of NF assembly suggest that NF-M and NF-H are the major constituents of filament sidearms and are anchored to a core of NF-L via their central rod domains.

Although much is known about NF structure and assembly many questions remain concerning NF function. NFs are most prominent in large axons (Wuerker and Kirkpatrick, 1972) whereas small unmyelinated axons and most dendrites contain few NFs. A role for NFs as a central determinant of axonal diameter has long been suspected from the correlation between NF number in axonal cross sections and axonal caliber (Friede and Sarnorajski, 1970; Hoffman et al., 1985a,b, 1988). The observation that fewer

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1. *Abbreviations used in this paper:* MT, microtubule; NF, neurofilament; NF-H, heavy neurofilament; NF-L, light neurofilament; NF-M, mid-sized neurofilament.

NFs occur at nodes of Ranvier where axonal diameter is reduced also supports this concept (Berthold, 1978).

More recently several animal models including a Japanese quail (Quiver) with a spontaneous mutation in the NF-L gene (Yamasaki et al., 1992; Ohara et al., 1993) and gene knockouts of the NF-L (Zhu et al., 1997), NF-M (Elder et al., 1998a), and NF-H (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) genes in mice have shown that radial growth of myelinated axons is inhibited in axons with a depleted NF content. Thus a role for NFs in expanding axonal caliber is now well established. However, how NFs increase axonal caliber and the exact role that individual subunits play in this process remains to be clarified. It is also not clear whether other functional consequences may result from a reduced NF content in axons. Nerve conduction velocity has been shown to be reduced in the NF-L–deficient quail (Sakaguchi et al., 1993) and axonal regeneration is delayed in NF-L–deficient mice (Zhu et al., 1997) and quails (Jiang et al., 1996). NFs have also been thought to play a role in maintaining the structural integrity of axons over time. However, no direct evidence substantiates such a role. During development, NF-L and NF-M are coexpressed initially while NF-H appears later (Shaw and Weber, 1982; Carden et al., 1987) suggesting that the NF-M and NF-H subunits may have specialized developmental functions. Whether they exert specialized functions in maintaining axons is not known.

Recently, we and others have generated mice lacking the NF-M (Elder et al., 1998a) or NF-H subunits (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) by gene targeting. Neither 4-mo-old NF-M– or NF-H–deficient mice had any overt behavioral phenotype or gross structural defects in the nervous system although the calibers of myelinated axons were diminished. NF densities were relatively unaffected in NF-H–null mutants (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) while axons of NF-M–null mutant animals contained less than half the normal number of NFs (Elder et al., 1998a). These studies demonstrated that NF-M and NF-H are required if myelinated axons are to achieve maximal diameters, and they also supported previous studies suggesting that NF-M regulates the level of NF-L (Tu et al., 1995).

To determine if axonal stability or other pathological effects develop with aging in mice lacking selective NF subunits we examined 2-yr-old NF-M–, NF-H–, and NF-M/H–null mutant mice along with 2-yr-old wild-type controls. Here we show that an age-dependent axonal atrophy develops in the lumbar roots of NF-M– and NF-M/H–deficient mice but not in animals deficient only in the NF-H subunit. Thus the lack of an NF-M subunit renders some axons selectively vulnerable to an age-related atrophic process.

Materials and Methods

Generation of Mice with NF-M– and NF-H–null Mutations

As described elsewhere, mice with singly disrupted NF-M (Elder et al., 1998a) and NF-H (Elder et al., 1998b) genes were produced by gene targeting in embryonic stem cells. To produce mice with targeted mutations in both genes, homozygous single mutants were initially crossed and the doubly heterozygous offspring were crossed to generate double mutants. Genotypes were determined by PCR or Southern blotting as described previously (Elder et al., 1998a,b).

Measurement of Axonal Diameters

Axonal diameters were measured as described previously (Elder et al., 1998a) on 1-μm-thick transverse sections of dorsal or ventral roots. Sections were stained with toluidine blue and photographed with a Zeiss Axiophot microscope. Photographic images were scanned and enlarged in Adobe Photoshop 5.0. Optimal brightness and gray scale pixel values were adjusted so as to provide the sharpest discrimination of the myelin/axon border. Axonal profiles were traced and areas were determined using the program NIH-Image. In ventral roots, all myelinated axons within each root were measured. In dorsal roots, a grid of squares was traced over each scanned image (each square equivalent to 10^3 μm^2 of actual surface area) and all axons that fell within or on the border of randomly chosen squares were measured. At least 300 axons were measured for each dorsal root. Axons were assumed to be circular for purposes of diameter calculations. Statistical analysis was performed using the program StatView (Abacus Concepts Inc.) or the SAS Statistics Package (SAS Institute).

Electron Microscopy

Tissues were processed for electron microscopy by standard methods as described previously (Friedrich and Mugnaini, 1981; Elder et al., 1998a). Mice were fixed by vascular perfusion with 2% formaldehyde (from paraformaldehyde), 1% glutaraldehyde, and 0.12 M sodium phosphate buffer, pH 7.4. Samples were postfixed in buffered osmium tetroxide, embedded in Epon, and examined using a JEOL 100CX electron microscope (Akashima, Japan).

NFs and microtubules (MTs) were counted in cross-sectional images of axons photographed at a magnification of 20,000 and enlarged an additional 2.5-fold during printing. NF densities were determined as described previously (Elder et al., 1998a) using methods similar to those described by Price et al. (1988). A template of hexagons (each equivalent to an actual area of 0.10 square microns) was placed on each print and the number of NFs that fell within alternate hexagons were counted.

Preparation of Specimens for Immunoperoxidase Staining

Control and mutant mice were perfused with buffered 4% paraformaldehyde and 50-μm-thick sections of spinal cord were cut with a vibratome. Immunoperoxidase staining was performed with the monoclonal antibodies SMI-31 or SMI-32 (Sternberger Monoclonals Inc.) or with a rabbit anti–NF-L polyclonal antiserum provided by Dr. Virginia Lee (University of Pennsylvania, Philadelphia, PA). Primary antibodies were diluted 1:1,000 in PGBA (0.12 M phosphate buffered saline, 0.1% gelatin, 1% BSA, 0.05% sodium azide) and were visualized with species-specific biotinylated secondary antibodies (Amersham Pharmacia Biotech) followed by peroxidase conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.). Peroxidase reactions were developed with diaminobenzadine. Preparations were examined and photographed with a Zeiss Axiophot microscope.

Results

Ventral Root Axons Atrophy with Aging in Mice Lacking a Mid-sized Neurofilament Subunit

Previously we produced mice with null mutations in the NF-M (Elder et al., 1998a) and NF-H (Elder et al., 1998b) genes using gene targeting in embryonic stem cells. By interbreeding the homozygous single mutant lines we produced mice with null mutations in both genes. The calibers of myelinated axons are diminished in 4-mo-old NF-M (Elder et al., 1998a), NF-H– (Elder et al., 1998b), and NF-M/H–deficient mice (Elder et al., 1999) although the mice otherwise lack any overt structural defects in the nervous system and have no obvious neurological abnormalities. To determine if axonal stability or other pathological ef-

fects develop with aging in mice lacking these NF subunits we studied four 2-yr-old NF-M, three 2-yr-old NF-H, and four 2-yr-old NF-M/H–null mutants along with three 2-yr-old wild-type controls. In each animal the brain, spinal cord, optic nerves, the lumbar dorsal and ventral roots, the dorsal root ganglia, and the sciatic nerve were examined.

At the light microscopic level no abnormalities were noted in any of the control animals. No abnormalities were noted in any of the NF null mutant animals in the brain, spinal cord, or optic nerves except that as in 4-mo-old NF-null mutants, the myelinated axons in all regions were visibly smaller.

By contrast, many of the ventral lumbar roots in 2-yr-old NF-M and essentially all of the lumbar ventral roots in the 2-yr-old NF-M/H–null animals showed pathological changes. Examples of lumbar ventral roots from wild-type, NF-M–, NF-H–, and NF-M/H–null mutant animals are shown in Fig. 1. Myelinated axons in the NF-M and NF-M/H mice were frequently irregular in shape and appeared shrunken and collapsed, resulting in axonal profiles that were dramatically smaller than wild-type axons. In the NF-M/H–null mutants occasional dystrophic axons with accumulations of cellular organelles and multilamellar membranous profiles could also be seen. Occasionally, giant ballooned axons could also be seen in NF-M/H–deficient roots. More rarely, degenerating profiles could also be seen in the NF-M–null mutants. However, such changes occurred in <1% of the axonal populations in either mutant, although they were never observed in the controls. There was no evidence in either mutant of macrophage infiltration or other features of Wallerian degeneration. Accompanying the axonal shrinkage and collapse there was frequently an expansion of the endoneurial space.

Similar changes were seen in 9 of 20 ventral roots examined in NF-M–null mutant animals and examples of atrophic roots could be seen at all lumbar levels examined (L3 through L5). All 19 roots examined in the NF-M/H showed dramatic shrinkage of axonal diameter. By contrast none of 15 ventral roots from the three 2-yr-old wild-type animals and none of 18 ventral roots from the 2-yr-old NF-H–null mutants showed any changes similar to those in the NF-M and NF-M/H animals. Thus, lumbar ventral roots in NF-M– and NF-M/H–deficient animals but not NF-H–deficient animals develop an axonal atrophy with aging.

Interestingly, in these same animals the pattern of selectivity was different in the lumbar dorsal roots. Examples of dorsal roots from 2-yr-old wild-type, NF-M–, NF-H–, and NF-M/H–deficient animals are shown in Fig. 1. Whereas lumbar dorsal root axons in the NF-M/H–deficient animals exhibited similar changes to those in the ventral roots, none of 16 lumbar dorsal roots from the 2-yr-old NF-M–deficient animals showed any obvious changes. Dorsal roots were also unremarkable in appearance in the NF-H–null mutant animals. Thus, dorsal root axons appear to be less sensitive to the loss of the NF-M subunit, although removal of both the NF-M and NF-H subunits renders these axons vulnerable to the atrophic process.

Previously we found that in 4-mo-old NF-M–null mutant animals axonal diameters in the ventral roots are decreased by ~20% (Elder et al., 1998a) whereas in 4-mo-old NF-M/H–null mutants axonal diameters decrease by >30% (Elder et al., 1999). To quantify the effect on axonal diameter in ventral roots of 2-yr-old NF-null mutant animals we measured axonal diameters from the wild-type and mutant roots shown in Fig. 1 (see Fig. 2 and Table I). Axonal diameters were reduced by >50% in the NF-M and NF-M/H roots with axonal areas falling to <25% of control. By contrast, average axonal diameter in the 2-yr-old NF-H–null mutant was only mildly decreased being within 10% of control. Similar mild effects on axonal diameter are seen in 4-mo-old NF-H–null mutant roots where axonal diameters decrease by ~18% (Elder et al., 1998b). As noted above, not all roots in 2-yr-old NF-M–null mutants showed obvious atrophic changes. Interestingly, in those roots that were not obviously affected by the pathological process quantitative morphometry showed that axonal diameters were decreased by ~20% as in young NF-M–null mutant animals (data not shown).

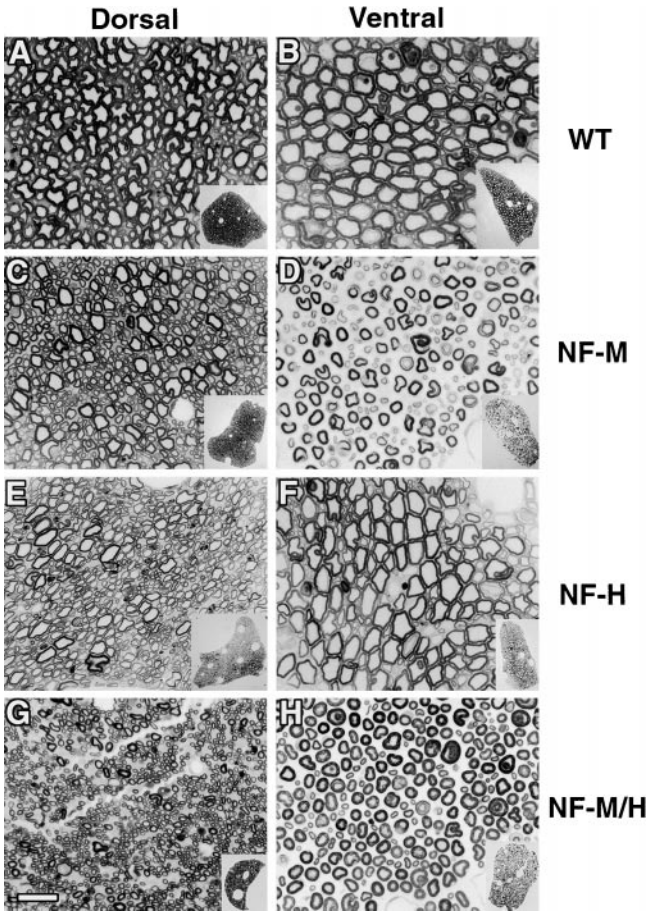

Figure 1. Axonal morphology in dorsal and ventral roots of old NF-null mutant animals. Light microscopy of toluidine blue–stained cross sections of L4 dorsal (A, C, E, and G) or L4 ventral (B, D, F, and H) roots from 2-yr-old wild-type (A and B), NF-M–null mutant (C and D), NF-H–null mutant (E and F), or NF-M/H–null mutant (G and H) mice. Insets show cross sections of whole roots at lower magnification. The ventral root axons in the NF-M– and NF-M/H–null mutants appear shrunken and frequently irregular in shape. In the dorsal roots only axons in the NF-M/H–null mutant are affected by the atrophic process. Bar, 10 μm.

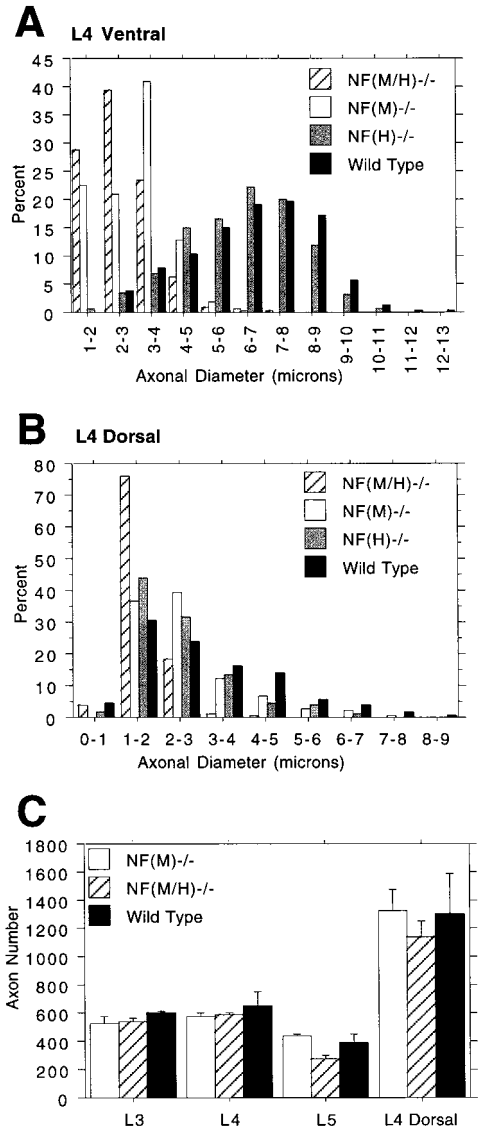

Figure 2. Axonal calibers and axon number in lumbar roots of aging NF-null mutant animals. (A) Diameters of all myelinated axons were measured in the L4 ventral roots of the 2-yr-old animals shown in Fig. 1. Note the marked reduction of axons >5 μm in diameter in the NF-M and NF-M/H mutants accompanied by an increase in smaller diameter fibers. (B) Diameters of myelinated axons in the dorsal roots shown in Fig. 1 were obtained by randomly sampling at least 300 myelinated axons in each root as described in Materials and Methods. Note that in contrast to the ventral roots, the distribution of axonal diameters in the NF-M–null mutant is more similar to the NF-H and wild-type than to the NF-M/H. (C) Myelinated axons were counted in the L3 (n = 4 wild-type, n = 5 NF-M −/−, n = 3 NF-M/H −/−), L4 (n = 4 wild-type, n = 8 NF-M −/−, n = 4 NF-M/H −/−), and L5 (n = 4 wild-type, n = 5 NF-M −/−, n = 4 NF-M/H −/−) ventral lumbar roots or the L4 dorsal roots (n = 3 each genotype) of 2-yr-old wild-type and mutant animals. There were no statistically significant differences between the mutants and controls in any root.

Examination of the frequency distribution of axons in the ventral roots (Fig. 2 A) shows the dramatic depletion of large axons in the NF-M– and NF-M/H–null mutant roots. Whereas >70% of axons in the NF-H–null mutant and control were >5 μm, few (<3%) were >5 μm in the NF-M– or NF-M/H–null mutants. By contrast, in the dorsal roots a different pattern was seen (Fig. 2 B). Although both the NF-M– and NF-H–null mutant roots contain fewer large diameter axons than the control, the distribution of axonal sizes in the NF-M root appeared more similar to the NF-H–null mutant and wild-type roots than to the NF-M/H. In contrast to the NF-M/H–null mutant in which <5% of dorsal root axons were >2.5 μm, >35% of axons in the wild-type, NF-M, and NF-H mutant roots were >2.5 μm. Table I shows that, as expected from this distribution, average axonal diameter and area are also relatively preserved in the NF-M– compared to the NF-M/H–null mutant dorsal roots.

Table I.

	Ventral		Dorsal	
	Diameter	Area	Diameter	Area
	μm	μm^2	μm	μm^2
Wild-type	6.6 ± 1.9	36.5 ± 19.0	2.9 ± 1.6	8.8 ± 9.5
NF-M −/−	3.0 ± 1.1*	8.0 ± 5.5*	2.6 ± 1.1*	6.2 ± 6.4*
NF-H −/−	6.2 ± 1.7*	32.7 ± 16.5*	2.4 ± 1.2*	5.6 ± 6.1*
NF-M/H −/−	2.6 ± 1.0*	6.2 ± 5.1*	1.7 ± 0.5*	2.4 ± 1.6*

Axonal diameters were calculated from the axonal areas measured in the dorsal and ventral roots shown in Fig. 1. In ventral roots, all myelinated axons contained in the root were measured. Values for dorsal roots were obtained by randomly sampling at least 300 myelinated axons in each root as described in Materials and Methods. Values are presented ± SD.
* indicates a value that is statistically different ($P < 0.005$) from the relevant control.

To determine if axons were being lost in the NF-M– or NF-M/H–null mutants the number of axons remaining in the ventral roots of 2-yr-old mutant and control roots were counted. There was no significant difference in axonal counts in the L3, L4, or L5 ventral roots or the L4 dorsal root between wild-type, NF-M–, and NF-M/H–null mutant animals (Fig. 2 C). In the NF-M mutant there also did not appear to be a significant difference in the number of surviving axons when comparing roots that were clearly atrophic with roots that were less affected by the process (data not shown). Thus, we conclude that permanent axonal loss is not a major feature of the pathological process and that the depletion of large axons in the ventral roots of NF-M– and NF-M/H–null mutants and the dorsal roots of NF-M/H–null mutants is the result of an atrophic process.

Neurofilaments Are Depleted and the Ratio of Microtubules/Neurofilaments Is Increased in Ventral Root Axons of NF-M–deficient Animals

To look for an ultrastructural basis for the atrophic collapse of axons in the aging NF-M– and NF-M/H–null mutants we examined electron micrographs of lumbar ventral roots from mutant and control animals as well as NF-H–null mutant animals. Previously we found that NFs were depleted in ventral root axons of 4-mo-old NF-M–null mutants, although the filaments were otherwise of normal configuration (Elder et al., 1998a). In these young NF-M–deficient animals NF density was reduced from 174/μm^2 in control axons to 75/μm^2 in null mutants and there was an increase in the ratio of MTs to NFs. By contrast, NF numbers are slightly depleted (~10%) in 4-mo-old

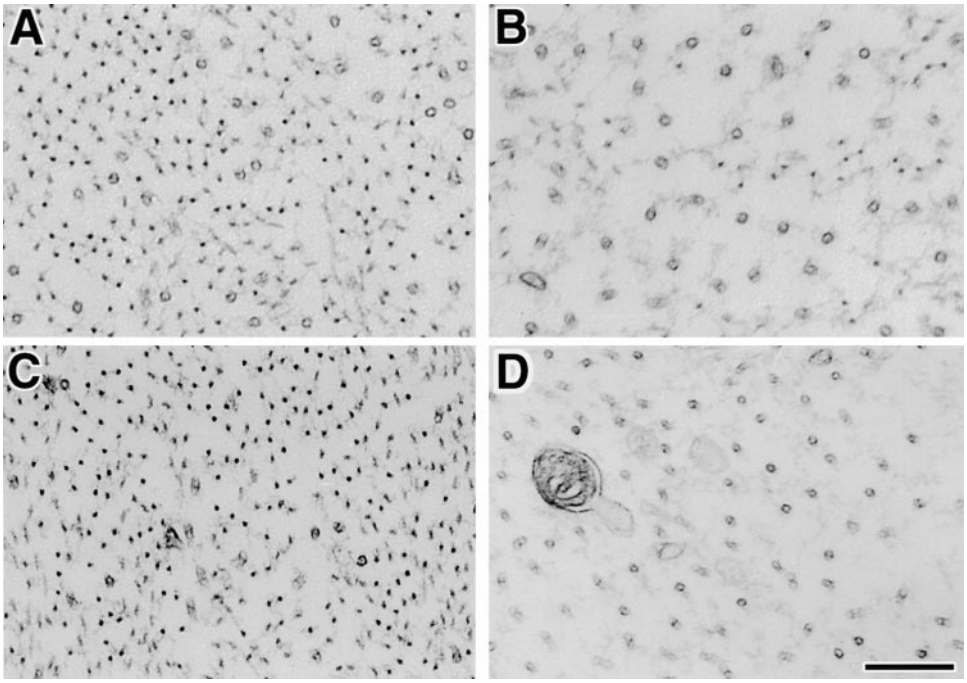

Figure 3. Fine structure of axons in old mice with NF-null mutations. Lumbar root axons from 2-yr-old wild-type (A), NF-M– (B), NF-H– (C), and NF-M/H– (D) null mutant mice are shown. In ventral roots of the NF-M–null mutant (B), NFs are sparse and MTs are plentiful, whereas axoplasm of the NF-M/H mutant (D) contains only MTs. Wild-type (A) and NF-H–null mutant (C) show numerous NFs and fewer MTs. Bar, 200 nm.

NF-H–null mutants (Elder et al., 1998b) and axons in 4-mo-old NF-M/H–null mutants are essentially devoid of NFs (Elder et al., 1999). The latter observation in NF-M/H–deficient axons is consistent with in vitro studies suggesting that rodent NFs are obligate heteropolymers requiring NF-L plus either NF-M or NF-H for filament formation to occur (Ching and Liem, 1993; Lee et al., 1993).

NFs were plentiful in the control and NF-H–null mutant axons (Fig. 3). Also as expected, axons in the 2-yr-old NF-M/H animals were essentially devoid of NFs. Axons in atrophic roots of old NF-M–null mutant animals contained relatively normal appearing NFs. However, NF numbers appeared even more dramatically depleted than in axons of young NF-M–null mutants. To quantify the effect on NF number in the old NF-M–null mutant, NFs were counted in the internodal regions of axons over a range of sizes and NF counts were plotted against axonal area. As shown in Fig. 4 A, axons in the null mutant consistently contained vastly fewer NFs than axons in controls with the mutant axons having only ~20% as many NFs as a comparably sized wild-type axons.

NF densities were determined directly as described in Fig. 4 B (also see Table II). NF density was reduced from 180/μm^2 in 2-yr-old control axons to 62/μm^2 in the 2-yr-old mutant ($P < 0.0001$). Thus, compared to 4-mo-old NF-M–null mutants, NFs are even further depleted in axons of old NF-M–null mutant animals (34% of control in 2-yr-old vs. 43% in 4-mo-old, $P < 0.0001$ for 1 yr vs. 2 yr).

By contrast, these same axons contained relatively more MTs. Axons in the NF-M–null mutant contained nearly double the number of MTs found in comparably sized wild-type axons (Fig. 5 A), increasing the average ratio of MTs to NFs from 0.18 ± 0.9 (SD) in wild-type to 1.57 ± 1.13 in the mutant axons ($P < 0.0001$, see Fig. 5 B and Table II). By comparison, MT to NF ratios in 4-mo-old NF-M–null mutants increase from 0.22 in wild-type to 0.83 in mutant axons (Elder et al., 1998a).

Thus, aging in the NF-M–null mutant is associated with a loss of NFs from axons that already possess a depleted NF content and is accompanied by a major reorganization of the axoplasm towards a MT-based cytoskeleton. It has long been known that NF number correlates better with axonal diameter than MT number, particularly in larger axons (Friede and Sarnorajski, 1970). Interestingly, in ventral root axons of the old NF-M–null mutant, MT number correlated better with axonal diameter ($r^2 = 0.713$) than did NF number ($r^2 = 0.503$), whereas as expected in wild-type control the correlation was better with NF number (0.879 for NFs vs. 0.725 for MTs).

Table II.

	NF density		Ratio MTs/NFs	
	Dorsal	Ventral	Dorsal	Ventral
	per μm^2			
Wild-type				
4 mo	ND	174	ND	0.22
1 yr	ND	178	ND	ND
2 yr	162	180	0.15	0.18
NF-M				
4 mo	89	75*	ND	0.83*
1 yr	104	61*	ND	1.09
2 yr	93*	62*	0.51	1.57*

NF densities (per μm^2) and the ratio of MTs/NFs are shown for the indicated genotypes and animal ages. ND indicates not determined. * indicates a value that is of a statistically significant difference ($P < 0.0001$) from the relevant age-matched control. NF densities in ventral roots of 1-yr-old and 2-yr-old NF-M animals are also significantly different from 4-mo-old NF-M animals ($P < 0.0001$) and ventral roots are different from dorsal roots in 2-yr-old NF-M animals ($P < 0.0001$). Values for 4-mo-old animals are taken from Elder et al. (1998a).

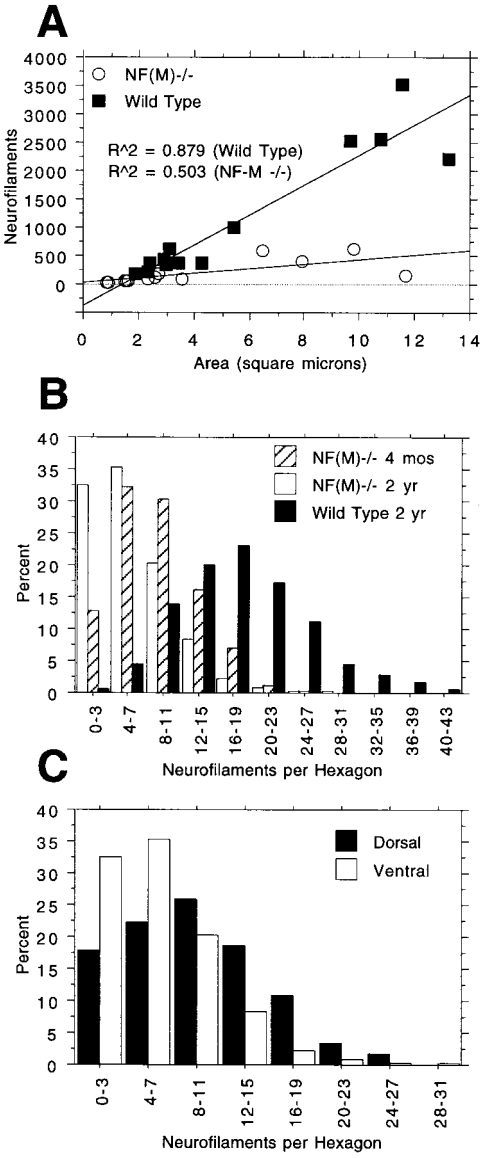

Figure 4. Neurofilament content in aging NF-M–deficient animals. (A) NFs were counted in myelinated axons from L5 ventral roots of 2-yr-old wild-type and control animals. The number of NFs in each axon was plotted against axonal size (area in square microns). Note that in myelinated axons of similar size the wild-type has more NFs than the NF-M–null mutant. Regression equations: $y = -368 + 264x$ for wild-type ($r^2 = 0.879$) and $y = 27 + 41x$ for NF-M $-/-$ ($r^2 = 0.503$). $P = 0.0001$ for effect of genotype on combined slope plus intercept. (B and C) NF densities were determined using methods similar to those described by Price et al. (1988). A template of hexagons was applied over each electron micrograph and the number of NFs per hexagon counted in alternate hexagons. Hexagons were excluded only if vesicular organelles filled more than ~10% of the hexagon. At least 300 hexagons each equivalent to an area of 0.10 square microns were counted for each group and a frequency distribution plot was generated showing the number of NFs per hexagon. In B, NF densities in ventral root axons are shown for 4-mo- and 2-yr-old NF-M–null mutants and 2-yr-old wild-type animals. The average number of NFs per hexagon was 18.0 ± 7.3 (SD) in 2-yr-old control axons, 6.2 ± 4.5 in 2-yr-old NF-M–null mutant and 8.5 ± 4.5 in 4-mo-old NF-M–null mutant axons ($P < 0.0001$ for both mutants vs. control and for 4-mo-old vs. 2-yr-old mutants). In C, NF densities are compared in dorsal and ventral root axons of 2-yr-old NF-M–null mutants. Values were 9.3 ± 5.8 NFs per hexagon in the 2-yr-old dorsal roots and 6.2 ± 4.5 in 2-yr-old ventral roots ($P < 0.0001$).

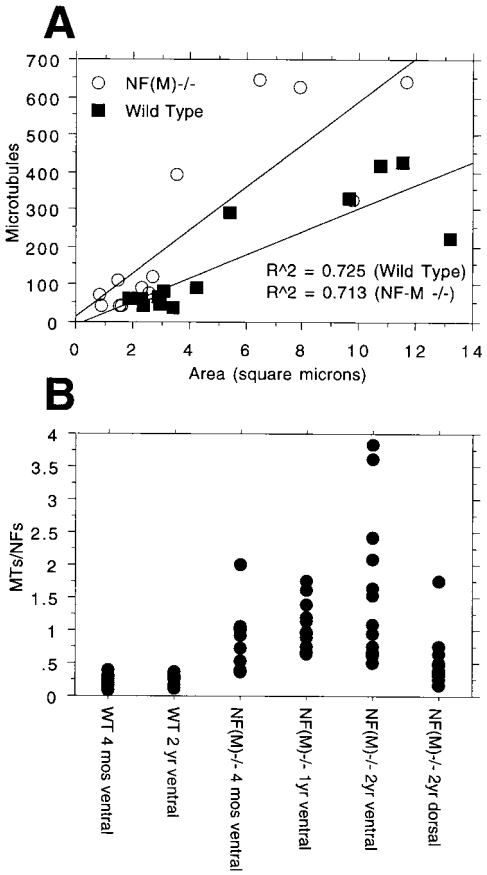

Figure 5. Microtubule content in aging NF-M–deficient animals. (A) MTs were counted in the same axons as in Figure 4 A. Note the relatively increased numbers of MTs in the NF-M–null mutant. Regression equations: $y = -9 + 31x$ for wild-type ($r^2 = 0.725$) and $y = 13 + 57x$ for NF-M $-/-$ ($r^2 = 0.713$). $P = 0.0005$ for effect of genotype on combined slope plus intercept. (B) The ratio of MTs/NFs is shown for axons of the indicated genotypes and ages. Note the increasing ratio of MTs/NFs with age in the ventral roots of NF-M–null mutant animals. Data for ventral roots from 4-mo-old wild-type and NF-M mutant are taken from Elder et al. (1998a).

Relative Preservation of Neurofilament Numbers in Unaffected Dorsal Root Axons of NF-M–deficient Animals

We also examined wild-type and NF-M–mutant dorsal root axons from 2-yr-old animals to determine if the depletion of NFs was a selective effect seen only in vulnerable ventral root axons. Interestingly, NF depletion in the dorsal roots did not occur to the same extent as in ventral roots. NF densities were 92/μm^2 in 2-yr-old NF-M–null mutant roots compared to 162/μm^2 in the 2-yr-old controls ($P < 0.0001$) and the ratio of MTs/NFs was 0.51 ± 0.39 in mutant and 0.15 ± 0.08 in 2-yr-old control ($P < 0.0001$). As shown in Fig. 4 C (see also Table II), whereas little dif-

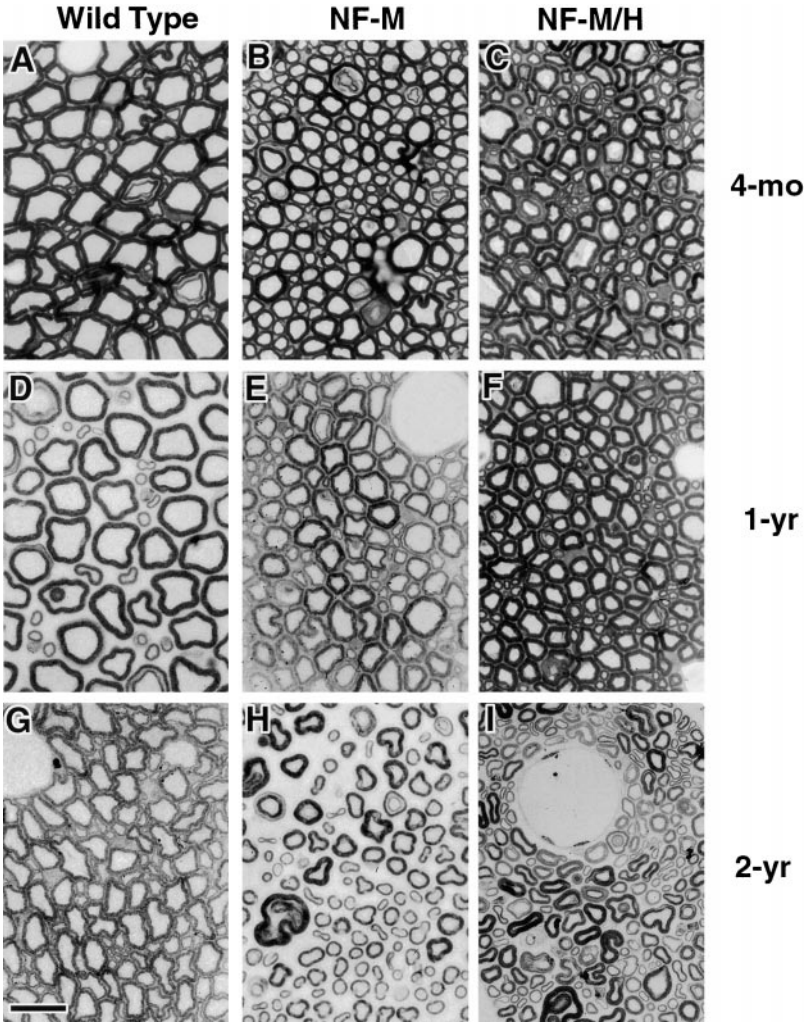

Figure 6. Time course of changes in ventral roots of old NF-null mutant animals. Light microscopy of toluidine blue–stained cross sections of L5 ventral roots from wild-type (A, D, and G), NF-M–null mutant (B, E, and H), and NF-M/H–null mutant (C, F, and I) at 4 mo (A–C), 1 yr (D–F), or 2 yr (G–I) of age. Myelinated axons in the ventral roots of the 4-mo- and 1-yr-old NF-M– and NF-M/H–deficient animals, although reduced in size, are otherwise normal in appearance. By contrast, axons in the ventral roots of the 2-yr-old NF-M– and NF-M/H–null mutants appear shrunken and frequently irregular in shape. Bar, 10 μm.

ference exists between NF densities in control dorsal and ventral root axons, NFs are significantly more depleted in mutant ventral than dorsal roots ($P < 0.0001$). We also measured NF densities in dorsal root axons of 4-mo-old and 1-yr-old NF-M–deficient animals and found NF densities in these axons to be 89/μm^2 and 104/μm^2, respectively. Thus, NFs are significantly less depleted in dorsal compared to ventral root axons and dorsal root axons do not undergo the age-related decrease in NF densities seen in the ventral root axons.

Neurofilament Depletion without Atrophic Changes in Ventral Roots of One-Year-Old NF-M–null Mutant Animals

To determine the time course of the axonal atrophy in the ventral roots we examined six 1-yr-old NF-M– and four 1-yr-old NF-M/H–null mutants. Fig. 6 shows a comparison of the appearance of ventral root axons from 4-mo-, 1-yr-, and 2-yr-old wild-type and mutant animals. As expected, myelinated axons in 1-yr-old NF-M and NF-M/H animals appeared smaller than 1-yr-old control (Fig. 6, D–F). However, the axons appeared relatively normal in shape and we did not observe any definite pathological changes like those seen in 2-yr-old ventral roots (Fig. 6, G–I) in any of 42 ventral roots collected from 1-yr-old NF-M or in 24 roots from the NF-M/H animals. Thus, the atrophy is predominantly occurring after 1 yr of age. Quantitative longitudinal data on L5 ventral roots from wild-type, NF-M–, and NF-M/H–null mutant animals is presented in Table III. Most remarkably, these data show that whereas a significant expansion of axonal caliber occurs between 4 mo

Table III.

	Diameter	Area
	μm	μm^2
Wild-type		
4 mo	4.6 ± 2.2	20.4 ± 17.6
1 yr	6.1 ± 2.1	32.8 ± 17.8
2 yr	4.1 ± 2.0	16.0 ± 14.2
NF-M		
4 mo	3.3 ± 1.3	9.7 ± 7.6
1 yr	3.5 ± 1.3	11.0 ± 7.9
2 yr	2.9 ± 1.0	7.5 ± 5.1
NF-M/H		
4 mo	2.8 ± 1.1	7.1 ± 5.8
1 yr	2.7 ± 1.0	6.5 ± 4.8
2 yr	2.1 ± 0.8	4.1 ± 3.3

Axonal diameters were calculated from the axonal areas measured in L5 ventral roots for the indicated ages and genotypes. Values are presented ± SD. Mutants at all ages were statistically different ($P < 0.0001$) from the relevant age-matched control.

and 1 yr of age in wild-type animals, axons in the NF-M–null mutant expand only slightly and axons in NF-M/H–null mutant roots do not expand at all. Wild-type as well as NF-M– and NF-M/H–mutant axons then all undergo varying degrees of age related atrophy between one and two years of age. Interestingly, on a percentage basis the atrophy in the L5 roots between 1 and 2 yr of age in the NF-M– and NF-M/H–null mutants is actually slightly less than wild-type. However, the lower base from which the mutants start at one year causes these smaller percentage changes to have a significant absolute effect on axonal caliber at 2 yr.

Despite the relatively maintained axonal calibers at 1 yr of age, ultrastructural analysis of ventral root axons from 1-yr-old NF-M animals revealed that the depletion of NFs had already occurred at this age. NF density (see Table II) was 61/μm^2 in 1-yr-old ventral root axons compared to the 62/μm^2 noted above that was observed in 2-yr-old animals (P = 0.68). Thus, the NF depletion appears to be established before gross atrophic changes occur.

Lack of Pathology in Anterior Horn Cells or Muscle in Aging NF-M– and NF-M/H–null Mutant Mice

The lumbar ventral roots contain axons that arise from motor neurons in the lumbar spinal cord. To determine if changes in anterior horn cells might be responsible for the axonal atrophy, spinal cord sections from the lumbar and cervical levels were examined to assess anterior horn cell morphology. Light microscopy revealed no evidence for anterior horn cell degeneration in either region in 2-yr-old NF-M– or NF-M/H–null mutants (Fig. 7, A–C). Examination of lumbar spinal cord sections by electron microscopy also found no perikaryal, dendritic, or axonal abnormalities in the mutants (data not shown).

To determine if NF-L or NF-H might be accumulating in anterior horn cell perikarya we immunostained spinal cord sections from NF-M– and NF-M/H–null mutants as well as controls with an anti–NF-L antibody or with the antibodies SMI-31 or SMI-32, which detect phosphorylated (SMI-31) or unphosphorylated epitopes (SMI-32) on the NF-M and NF-H subunits (Lee et al., 1988). Phosphorylated epitopes such as those stained by SMI-31 are normally largely restricted to axons and are absent from neuronal cell bodies and dendrites, whereas unphosphorylated epitopes such as those stained by SMI-32 are frequently found in cell bodies and dendrites (Vickers et al., 1994). Both the distribution and abundance of NF-L staining was similar in anterior horn cell perikarya in the NF-M– and NF-M/H–null mutants and control (Fig. 7, D–F). Likewise, SMI-32 staining gave a similar pattern and intensity in both the NF-M mutant and control (Fig. 7, G and H). SMI-31 only faintly stained the perikarya of anterior horn cells in the NF-M mutant and control indicating that phosphorylated epitopes of NF-H are not abnormally accumulating in the cell body (data not shown). As expected, no staining of the NF-M/H–null mutant spinal cord was found with either SMI-31 or SMI-32 (data not shown).

To determine if the axonal atrophy was producing changes in muscle we examined toludine blue–stained sections from the tibialis anterior muscles of 2-yr-old mutant and control animals. Consistent with the lack of axonal loss noted above, muscle fibers in the NF-M– and NF-M/H–null mutants appeared normal without any evidence of group atrophy or other changes suggestive of functional denervation (data not shown).

Hind Limb Paralysis Develops in Two-Year-Old NF-M/H–null Mutant Animals

In contrast to the lack of neurological findings in young NF-deficient animals, four of five NF-M/H animals that have lived to 2 yr of age have developed a grossly apparent hind limb paralysis (Fig. 8). Thus, the axonal atrophy in

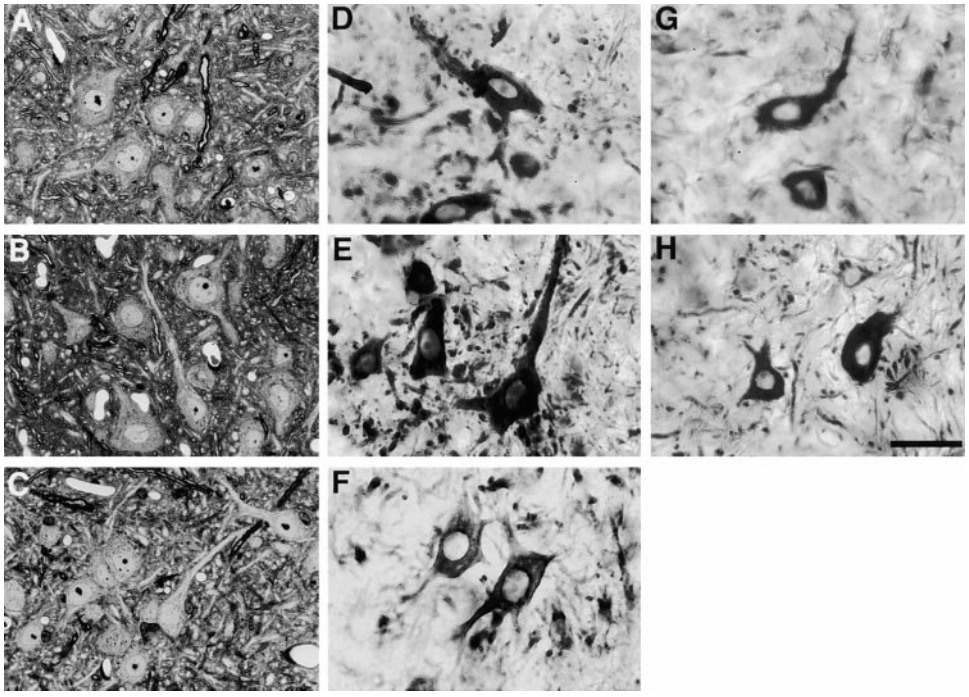

Figure 7. Lack of anterior horn cell changes in old NF-deficient mice. In A–C, phase contrast images are shown of toluidine blue–stained sections of lumbar spinal cord from 2-yr-old wild-type (A), NF-M– (B), or NF-M/H– (C) null mutant animals. In D–H immunoperoxidase-stained sections of lumbar spinal cord are illustrated from wild-type (D and G), NF-M– (E and H), or NF-M/H– (F) null mutant animals stained with an NF-L antibody (D–F) or SMI-32 (G and H). No significant differences between NF-M or NF-M/H mutants and wild-type were noted. Bar, 20 μm.

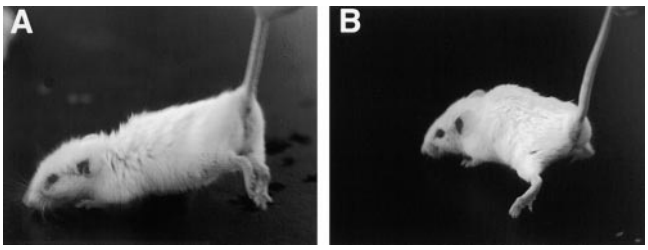

Figure 8. Hind limb paralysis in 2-yr-old NF-M/H–null mutant animal. In A, a 2-yr-old NF-M/H–null mutant animal is shown which exhibits a hind limb paralysis. Note the abnormal posture of the hind limbs resulting from an inability to extend the hind limbs in comparison to a wild-type mouse shown in B.

the lumbar roots appears to be functionally significant even though no significant axonal loss or muscle atrophy is occurring.

Discussion

NFs have long been suspected to be critical determinants of axonal diameter based on the close correlation between NF number and axonal caliber (Friede and Sarnorajski, 1970; Hoffman et al., 1985a; Hoffman et al., 1985b, 1988). This role has now been well established in several animal models including a Japanese quail (Quiver) with a mutation in the NF-L gene (Yamasaki et al., 1991; Ohara et al., 1993) and gene knockouts in mice of the NF-L (Zhu et al., 1997), NF-M (Elder et al., 1998a), and NF-H (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) genes. In all these examples radial growth of myelinated axons was inhibited in axons with a depleted NF content. How NFs expand axonal caliber remains incompletely understood. Additionally, the consequences for axonal function of an altered NF content and the roles that individual subunits play in NF function are only beginning to be understood.

The most important function of NF-L may well be its ability to stimulate filament formation. Axonal NFs are absent in both Japanese quail (Quiver) with a nonsense mutation in the NF-L gene (Ohara et al., 1993) and in mice whose NF-L gene was disrupted by gene targeting (Zhu et al., 1997). Axons in mice with disruptions of both the NF-M and NF-H genes are also essentially devoid of NFs (Elder et al., 1999). These animal studies support previous work in transfected cells suggesting that in vivo rodent NFs are obligate heteropolymers requiring NF-L plus either NF-M or NF-H to form filamentous networks (Ching and Liem, 1993; Lee et al., 1993).

Previous studies have also pointed to an essential role for NF-M in driving the formation or maintenance of normal numbers of NFs (Elder et al., 1998a). Here we provide direct evidence that the NF-M subunit is also required for the structural stability of axons with aging by showing that myelinated axons atrophy with aging in the peripheral nerve roots of NF-M– and NF-M/H–deficient animals. In both animals the most prominent feature of the pathological process was a collapse of axonal caliber. Myelinated axons in affected roots frequently had irregularly shaped profiles and appeared shrunken and collapsed compared to axons in aged wild-type animals. Reduced axonal calibers are also seen in ventral root axons of 4-mo-old NF-M– (Elder et al., 1998a) and NF-M/H–null mutants (Elder et al., 1999). However, compared to the reduction in axonal area seen in young animals ($\sim$35% in NF-M and $\sim$45% in NF-M/H) axonal areas in the affected roots of old animals were reduced in some nerves by $>$70% in old NF-M–null mutants and $>$80% in NF-M/H–null mutants.

The process is best termed atrophic since it represents an abnormal collapse of axonal structure that is not seen with normal aging. The net effect is the near complete elimination of all large myelinated axons in affected nerves. The process is selective in affecting peripheral nervous system but not central nervous system axons, and in the peripheral nervous system in affecting axons in ventral but not dorsal roots in the NF-M–null mutant. Among ventral roots in the NF-M–null mutant the process is also selective in not affecting all ventral roots equally. The lack of any significant reduction in axonal number in either ventral or dorsal roots and the rarity of degenerating profiles suggests that the process does not result in permanent axonal loss and that the loss of large myelinated axons is due to their shrinkage to become small myelinated axons.

Some degree of perikaryal and axonal atrophy is considered a normal aspect of aging (Peters and Vaughn, 1981; Spencer and Ochoa, 1981) and normal lumbar ventral roots do undergo an age-related reduction in axonal caliber (see Fig. 6). What distinguishes the process in the NF-M and NF-M/H roots from the effects of normal aging is the severity of the reductions compared to age-matched control animals and the apparent functional consequences of the reductions in at least the NF-M/H–null mutant. The process is specific to loss of the NF-M subunit to the degree that ventral roots of NF-H–null mutant animals do not show a reduction in axonal size beyond that seen with normal aging even though they also start with smaller axons at younger ages.

The ultrastructural correlate of the collapsed axonal caliber was a depletion of axonal NFs. NF densities in ventral root axons of 4-mo-old NF-M–null mutants are reduced to 43% of the wild-type level although the filaments are otherwise of normal configuration (Elder et al., 1998a). In 2-yr-old NF-M–null mutant axons, NFs, although still normal in appearance, were visibly depleted in number compared to 4-mo-old animals, and quantitative studies revealed that NF densities were reduced to 34% of 2-yr-old control values. In both young and old NF-M/H–null mutants, axons are essentially devoid of NFs due to the obligate heteropolymer nature of rodent NFs.

By contrast, these same axons appeared to contain more MTs. NF-M–mutant axons contained nearly double the number of MTs found in comparably sized control axons resulting in myelinated axons in the NF-M–null mutant in which MTs generally outnumbered NFs. The prominence of MTs in NF-deficient axons could reflect a true increase in MT content per axon, perhaps as a reaction to the loss of NFs. Alternatively, axons might contain their normal complement of MTs but MTs might appear increased due to a concentration effect caused by a decreasing axonal caliber. We have investigated previously these possibilities in 4-mo-old NF-M/H–null mutant animals (Elder et al., 1999) that also contain an apparent increase in MTs. By

measuring tubulin levels in a constant length of nerve we found that tubulin levels were actually moderately decreased in nerves of the double mutant animals. We further found that if the thickened myelin sheaths were used as a marker of axonal size, MT numbers relative to number of myelin lamellae were normal in the NF-M/H–null mutant axons. Thus, MTs are not increased in absolute numbers in these nerves and rather individual axons likely possess a complement of MTs that is nearly normal for the size that the axons should have become. These results argue against the existence of a compensatory mechanism that increases tubulins or MTs in response to a loss of NFs and rather suggest that a concentration effect accounts for their apparent increase in NF-deficient nerves. Relatively similar observations have been made concerning MT numbers in relation to number of myelin lamellae in axons of NF-L–deficient quail (Zhao et al., 1995).

The relative roles of MTs and NFs in determining axonal diameter have long been discussed (Lasek et al., 1983). In large caliber axons, MT content does not correlate with axonal diameter as closely as does NF content (Friede and Sarnorajski, 1970; Hoffman et al., 1984). MTs likely have more significance in maintaining the diameter of small myelinated and unmyelinated axons where they are frequently the major cytoskeletal component. Interestingly, in ventral root axons of the old NF-M–null mutants, MT number correlated better with axonal diameter than did NF number emphasizing that NFs probably play a diminished role in maintaining the caliber of these axons. The atrophic changes in these axons may also suggest that a MT-based cytoskeleton is less effective in maintaining axonal structure with aging than is a NF-based one.

We do not know what underlies the basis for the selective vulnerability of ventral root axons in the NF-M–null mutant animals. The lumbar ventral roots contain axons that arise from motor neurons in the lumbar spinal cord, whereas the lumbar dorsal roots are composed of axons emerging from the sensory neurons of the dorsal root ganglia. Overexpression of either normal or mutant NF proteins in transgenic mice can cause a motor neuron disease that resembles the human disease amyotrophic lateral sclerosis (Cote et al., 1993; Xu et al., 1993; Lee et al., 1994). The pathological basis for motor neuron dysfunction in these animals is likely the accumulation of NF aggregates in anterior horn cells that also resemble those seen in the human disease. Interestingly, sensory neurons in these overexpression models may contain neurofilamentous accumulations but do not exhibit signs of degeneration. Indeed, the selective vulnerability of ventral compared to dorsal root axons that we observe here in the NF-M–null mutants is quite similar to that observed by Lee et al. (1994) who overexpressed an NF-L transgene containing a leucine to proline mutation in the α helical rod domain of NF-L. However, the NF-M–null mutant differs fundamentally from the NF-L(Pro) mutation in lacking perikaryal aggregates of NFs.

The most conspicuous difference between the dorsal and ventral root axons in old NF-M animals was the ultrastructural finding that NFs are less depleted and the ratio of MTs/NFs remains closer to normal in the dorsal root axons. Interestingly, dorsal roots do atrophy in NF-M/H–deficient axons where NFs are essentially absent. This finding may again point to a NF-based cytoskeleton being more resistant to collapse during aging than a MT-dominated one. Besides the differences in NF number, relative differences in the stresses that the dorsal and ventral roots are normally subjected to might potentiate the selective vulnerability. Additionally, levels of or posttranslational modifications to the NF-H subunit may differ in dorsal and ventral roots and leave axons with L/H filaments in the ventral roots more vulnerable to degeneration. It is well known that different neuronal populations can express different NF profiles, especially with regard to phosphorylation (Szaro et al., 1990) and indeed in bovine nerve roots the KSP repeat region of NF-H is significantly more phosphorylated in the ventral than the dorsal roots (Soussan et al., 1996).

It is clear, however, that NF loss cannot be directly correlated with axonal collapse since axons in NF-M/H–null mutants lack NFs throughout life, yet axonal collapse occurs only in old animals. NF loss is also already present by 1 yr of age in the NF-M–null mutant although the gross collapse of axonal calibers was only seen in 2-yr-old animals. This argues that the collapse is not simply a direct result of a depleted NF number but rather a depleted NF content renders these axons more susceptible to atrophy.

The reduced NF densities must reflect fewer NFs being transported into or a decreased stability of axonal filaments that are formed. As noted above, some perikaryal and axonal atrophy is considered a normal aspect of aging (Peters and Vaughn, 1981; Spencer and Ochoa, 1981). Although the molecular alterations underlying these changes remain incompletely described, an age related decrease in NF mRNA levels has been observed in rodents during normal aging (Parhad et al., 1995; Kuchel et al., 1997). These studies have also reported a corresponding decrease in NF subunit proteins and a decrease in NF numbers within axons. Age-related losses of NF-L protein also occur in rabbit hippocampus (Van der Zee et al., 1997); whether these changes reflect transcriptional or posttranscriptional events has not been established. Axons in the lumbar ventral roots of 4-mo-old NF-M–null animals already show a substantial depletion of NFs (Elder et al., 1998a). A further age related decrease in NF-L and NF-H mRNA on top of an already depleted NF supply could contribute to the dramatic depletion of NFs that occurs in old NF-M–null mutants.

The process described here is functionally significant with old NF-M/H animals developing a gross hind limb paralysis. We have not observed any gross paralysis in old NF-M–null mutants. However, we have tested a group of 1-yr-old NF-M–null mutants in the rotarod test and in preliminary studies found that their performance appears to be impaired (our unpublished observations). Thus, we suspect that future studies will document motor impairments in NF-M–null mutants as well. One expected consequence of a reduced axonal diameter is a reduced nerve conduction velocity and this has been demonstrated to occur in the NF-L–deficient quail (Sakaguchi et al., 1993). A reduced nerve conduction velocity could in turn impair motor function. Alternatively, the motor impairments observed here could reflect interference with axonal transport of synaptic proteins or other elements critical in synaptic transmission.

Collectively, these results indicate that the NF-M subunit plays a previously unknown role in maintaining axonal structure with aging. These findings may have implications for neurodegenerative diseases. For example, a depleted NF content might render other neuronal populations more susceptible to excitotoxic or other insults thought to be involved in human neurodegenerative diseases. Reports have described decreased levels of NF-L mRNA beyond that seen in normal aging in Alzheimer's disease brain (McLachlan et al., 1988; Clark et al., 1989; Somerville et al., 1991; Robinson et al., 1994). Future studies in these animals promise to yield additional insights into the mechanisms that underlie this degenerative process as well as lead to further clarification of the normal function of NFs and their role in neurodegenerative diseases.

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