

Overexpression of the Human NFM Subunit in Transgenic Mice Modifies the Level of Endogenous NFL and the Phosphorylation State of NFH Subunits

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Abstract. Neurofilaments (NFs), the major intermediate filaments of central nervous system (CNS) and peripheral nervous system (PNS) neurons, are heteropolymers formed from the high (NFH), middle (NFM), and low (NFL) molecular weight NF subunits. To gain insights into how the expression of NF subunit proteins is regulated in vivo, two transgenes harboring coding sequences for human NFM (hNFM) with or without the hNFM multiphosphorylation repeat domain were introduced into mice. Expression of both hNFM constructs was driven by the hNFM promoter and resulted in increased levels of hNFM subunits concomitant with an elevation in the levels of mouse NFL (mNFL) proteins in the CNS of both lines of transgenic mice. The increased levels of mNFL appear specific to NFM because previous studies of transgenic mice overexpressing either NFL or NFH did not result in increased ex-

pression of either of the other two NF subunits. Further, levels of the most heavily phosphorylated isoforms of mouse NFH (mNFH) were reduced in the brains of these transgenic mice, and electron microscopic studies showed a higher packing density of NFs in large-diameter CNS axons of transgenic versus wild-type mice. Thus, reduced phosphorylation of the mNFH carboxy terminal domain may be a compensatory response of CNS neurons to the increase in NFs, and reduced negative charges on mNFH sidearms may allow axons to accommodate more NFs by increasing their packing density. Taken together, these studies imply that NFM may play a dominant role in the in vivo regulation of the levels of NFL protein, the stoichiometry of NF subunits, and the phosphorylation state of NFH. NFM and NFH proteins may assume similar functions in regulation of NF packing density in vivo.

MAMMALIAN neurofilaments (NFs)¹ belong to a large family of intermediate filaments (IFs) that are found in nearly all mammalian cells, and NFs are the major IFs of central nervous system (CNS) and peripheral nervous system (PNS) neurons (Hoffman and Lasek, 1975; Liem et al., 1978; Schlaepfer and Freeman, 1978). NFs share many structural and biochemical features common to IFs formed from each of the six different classes (i.e., types I–VI) of IF proteins, but NFs possess

several unique characteristics (for recent reviews see Nixon, 1993; Fuchs and Weber, 1994). For example, unlike homopolymeric IFs that are composed of type III IF proteins (e.g., vimentin, peripherin) or the heteropolymeric IFs formed from type I and II keratins, NFs are heteropolymers of three distinct subunit proteins. Because of the unique structure of their genes, the NF triplet proteins are included in a separate class (i.e., type IV) of IF proteins, and they are known as the high (NFH), middle (NFM), and low (NFL) molecular weight NF subunits. Further, both NFM and NFH have extraordinarily long carboxy terminal domains, and their apparent molecular weights (Mr) as determined by SDS-PAGE are much higher than that deduced from their corresponding amino acid or cDNA sequences. This is thought due to the combined effects of a high content of negatively charged Glu residues and repeated motifs containing Lys, Ser, and Pro (KSP motifs) that are referred to as multiphosphorylation repeats (MPR) and are the major sites of phosphorylation

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1. *Abbreviations used in this paper:* CNS, central nervous system; hNFM, human NFM; IF, intermediate filaments; mNFH, mouse NFH; mNFL, mouse NFL; MPR, multiphosphorylation repeats; Mr, apparent molecular weight; NF, neurofilament; NFH, -M, and -L, high, middle, and low molecular weight NF; PNS, peripheral nervous system.

in NFH and NFM (Lee et al., 1988a, b). In addition, the stoichiometry of NFs varies among different CNS and PNS regions, and differs from the stereotyped 1:1 ratio of type I and II IF proteins seen in heteropolymeric keratin IFs (reviewed in Fuchs and Weber, 1994). For example, more NFH seems required in regions where the axonal diameters are large (e.g., spinal cord and sciatic nerve), compared with regions that primarily contain thin axons (e.g., neocortex). Although NFL is able to self-assemble into long, 10-nanometers-in-diameter filaments *in vitro* (Geisler and Weber, 1981; Liem and Hutchison, 1982; Mulligan et al., 1991; Balin and Lee, 1991), recent data suggest that NFL is not competent to form a filamentous network *in vivo* (Ching and Liem, 1993; Lee et al., 1993). In contrast to NFL, both NFM and NFH appear to subserve an auxiliary function, and current structural models of NFs, suggest that NFL alone forms the core of individual NFs while NFH and NFM are bound to the surface of these NFs (Liem and Hutchison, 1982; Nixon, 1993).

Despite the rapid accumulation of biochemical, structural, genetic, and pathological information on NFs and their subunit proteins, the precise function(s) of NFs still remains only partially understood (Nixon, 1993). For example, NFs have been considered major determinants of the caliber of large-diameter axons (Hoffman et al., 1984, 1987), and it is the caliber of axon that controls the conduction velocity of action potentials in large, heavily myelinated axons (Sakaguchi et al., 1993). NFs also are thought to provide mechanical support for axons and to form a stable structural matrix within axons during neurite outgrowth and elongation to facilitate axonal pathfinding in the developing nervous system (Landmesser and Swain, 1992). However, recent studies of an NF-deficient mutant quail has again raised questions about the functional importance of NFs *in vivo* (Yamasaki et al., 1992; Ohara et al., 1993).

To elucidate the structure and function of NFs as well as other aspects of the biology of NFs *in vivo*, a number of different transgenic mice have been developed and used as experimental model systems. For example, transgenic mice have been used to identify promoter sequences that direct tissue- or cell-type specific expression of NF proteins (Julien et al., 1987; Lee et al., 1992; Reeben et al., 1993; Elder et al., 1994), to probe the alternative adenylation of NFL mRNA (Beaudet et al., 1993a), to dissect out the regulatory sequences that control the levels of NF proteins (Beaudet et al., 1992), and to establish animal models of human neurodegenerative diseases (Côté et al., 1993; Xu et al., 1993; Eyer and Peterson, 1994; Vickers et al., 1994). To gain insights into the role NFM might play in regulating the level of endogenous mouse NFL (mNFL) and NFH (mNFH), two different lines of transgenic mice that constitutively overexpress human NFM (hNFM) were examined in this study. In contrast to earlier transgenic mice designed to overexpress NFL or NFH, the overexpression of hNFM in both lines of transgenic mice described here induced increased levels of mNFL proteins and diminished the levels of the most heavily phosphorylated isoforms of mNFH. Based on our studies of these transgenic mice, we conclude that NFM may play a dominant role in regulating NFL level and the phosphorylation state of NFH *in vivo*.

Materials and Methods

Transgenic Mice

The two new lines of hNFM transgenic mice studied here were generated as described earlier (Lee et al., 1992; Elder et al., 1994). The transgenic mice were identified by Southern blot analysis of tail DNA as described (Lee et al., 1992; Cole et al., 1994; Elder et al., 1994). The transgene copy number in each line was also determined by Southern blot analysis of tail DNA digested with BamHI and probed with pNF4 (Myers et al., 1987), which hybridized with both endogenous and transgenic NFM DNAs. The copy number of the transgenes was estimated by determining the ratio of the transgenic hNFM DNA to the endogenous mNFM DNA.

Quantitative Western Blots

Transgenic and age-matched wild-type mice were sacrificed by carbon dioxide asphyxiation. Different regions of the CNS were dissected out immediately and homogenized in BUST buffer (8 M urea, 0.5% SDS, 2% β -mercaptoethanol, and 50 mM Tris, pH 7.4) at a ratio of 1 ml/100 mg wet tissue, according to previously described methods (Lee et al., 1992; Cole et al., 1994). The tissue suspensions were sonicated well and centrifuged in a TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA) at 100,000 g for 1 h at 25°C, and the protein concentrations in the supernatants were determined. Proteins were separated on 7.5% SDS-PAGE gels, and different amounts of total protein from different tissues were loaded in each lane of a gel according to the following scheme: neocortex and hippocampus, 40 μ g; brain stem, 20 μ g; and spinal cord and peripheral nerves, 10 μ g. For quantitative experiments, identical amounts of proteins from transgenic and control samples were loaded in groups of six separate lanes of the same gel. Gels were transferred to nitrocellulose membranes for qualitative and quantitative Western blot studies as described (Balin et al., 1991; de Waegh et al., 1992; Lee et al., 1992; Bramblett et al., 1993; Cole et al., 1994). The membranes were blocked for 1 h in TBS, pH 7.6, containing 5% nonfat milk. Primary antibodies specific for each of the NF subunits, some of which recognize NFH or NFM in different states of phosphorylation (see Table I), were applied to the nitrocellulose membranes overnight. After five washes in GB buffer (50 mM triethanolamine, 100 mM NaCl, 2 mM EDTA, 1 mM sodium azide, 0.1% SDS, and 0.5% Triton X-100, pH 7.5) and a final wash in PTX buffer (10 mM phosphate buffer, pH 7.3, 300 mM NaCl, 2 mM EGTA, 2 mM sodium azide), the membranes were probed with either 10 μ Ci 125 I-labeled goat anti-mouse IgG (ICN Radiochemicals, Irvine, CA) for mouse primary antibodies or 10 μ Ci 125 I-labeled Protein A (ICN) for rabbit primary antibodies for 1 h. After 5 washes in GB buffer, the blots were dried and placed on Phosphor-Imager plates (Molecular Dynamics, Sunnyvale, CA) for 4 h. The plates were then scanned, and the abundance of bound antibody overlying the labeled bands was quantified with ImageQuant software (Molecular Dynamics).

Table I. Result of Immunohistochemistry Staining NFMBam – versus Wild Type

Antibody	Dilution	Epitope	Staining intensity
RMO3	Neat	NFM, Pind/tail outside MPR	++
RMO44	100 \times	NFM, Pind/core	++
RMO189	100 \times	NFM, Pind/core	+
RMO254	500 \times	NFM, Pind/tail within MPR	=
RMO255	200 \times	NFM, Pind/tail the last 20 aa	++
Rabbit Anti-NFL	2000 \times	NFL, Pind/tail	++
RMO24	10 \times	NFH, P ³⁺ /tail within MPR	+/-
RMDO9	1000 \times	NFH, P ⁻ /tail without MPR	+/=
RMO217	10 \times	NFH, P ²⁺ /tail within MPR	+/=

Three pairs of 3-mo-old and two pairs of 1-mo-old mice were compared. Neocortex and alveus of hippocampus were used for comparison. P³⁺, heavily phosphorylated epitope in NFH; P²⁺, moderately phosphorylated epitope in NFH; P⁻, non- or poorly phosphorylated epitope in NFH; Pind, phosphorylation-independent epitope in NFM or NFL; MPR, multiphosphorylation repeats in human NFM or mouse NFH; (+), slightly stronger staining intensity in NFMBam- mice than in wild-type mice; (++) , prominently stronger staining intensity in NFMBam- mice than in wild-type mice; =, equal staining intensity in NFMBam- and wild-type mice; +/- and +/=, variable staining intensity results.

RNase Protection Assays

Isolation of total cellular RNA from whole-brain and RNase protection assays was performed on 1-mo-old to 3-mo-old transgenic mice and their corresponding wild-type age-matched controls, as previously described (Lee et al., 1992), with [α - 32 P] UTP-labeled probes. The murine NFM and murine β -actin probes have been described previously (Lee et al., 1992). To construct a probe for murine NFL, a cDNA clone containing amino acid 22 to the poly A site cloned as a SmaI/EcoRI in pUC8 (gift of Dr. N. Cowan), was cloned as a SalI/EcoRI fragment into pBluescript II SK+. NFL sequence 3' to the SacI site at amino acid 415 (Lewis and Cowan, 1986) was removed by digesting with SacI and religating the ends. For use as a probe template, this final plasmid was digested at a KpnI site found at amino acid 368, made blunt ended with T4 DNA polymerase, and a probe was synthesized with T3 RNA polymerase. This probe protected a 140-bp product. A murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in pBSIIKS (pCCD32) was obtained from Dr. D. Werner (Lu et al., 1990). After digestion with AvaII, a T7 RNA polymerase-generated probe protected a 240-bp fragment.

Each reaction contained 5- μ g brain RNA and 5- μ g tRNA. Quantitation was done by densitometry. To ensure linearity, a set of RNA standards containing 0–10 μ g wild-type brain RNA supplemented with tRNA as needed to give 10 μ g in each sample was run in all experiments. Experimental samples were always run in duplicate. Murine NFL and NFM levels were normalized to the levels of actin or GAPDH expression, respectively.

Immunohistochemistry

The properties of the anti-NF-subunit-specific antibodies used here are summarized in Table I. Tissue fixation by immersion in 70% ethanol/150 mM NaCl, as well as tissue processing and sectioning and the immunohistochemical procedures used here are described in a number of publications (e.g., Lee et al., 1986, 1987; Trojanowski et al., 1986, 1989; Carden et al., 1987; Schmidt et al., 1987, 1991). Briefly, the immunohistochemical procedures involved application of the primary anti-NF protein antibody overnight at 4°C, followed by the detection of bound antibody using a peroxidase anti-peroxidase or avidin biotin complex reporter system. To demonstrate quantitative differences in the immunohistochemical-staining intensity obtained with some of these antibodies, 6- μ m-thick sections from the same brain levels of transgenic as well as control mice were cut and placed side by side on the same slides for comparative immunohistochemical studies with the same antibody.

Electron Microscopic Studies

Adult transgenic and age-matched control mice were sacrificed by carbon dioxide asphyxiation and perfusion-fixed through the heart, as described earlier (Trojanowski and Gonatas, 1983; Cole et al., 1994). After flushing the intravascular compartment with 50 ml of 0.1 M cacodylate buffer pH 7.4, the mice were perfused with 50 ml of fixative (2% paraformaldehyde and 2% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4). The brains were dissected out and immersion-fixed for an additional 24 h. Samples, including the alveus and adjacent hippocampus, from one of the transgenic mouse lines (i.e., the NFMBam⁻ mice described in Results) and from the wild-type mice were trimmed to 1 mm³ under a dissecting microscope; the samples were postfixed in osmium tetroxide for 20 min, dehydrated, and then embedded in Epon-Araldite resin. For morphometric studies of the density of NFs in axons running in the alveus, ultrathin sections through the alveus and adjacent hippocampus were examined under an electron microscope (Hitachi, H-600, Nissei Sangyo, America Ltd., Gaithersburg, MD). 60 axons from the alveus of each animal were photographed at a nominal magnification of $\times 20,000$. Axons in the white matter of the alveus cut exactly in cross-section were randomly selected, and all of the NFs in each of these axons were counted using methods similar to those described earlier (Cole et al., 1994). For quantitation, we arbitrarily classified these axons into the following six size categories: axons with diameters 0.1–0.3, 0.3–0.5, 0.5–0.7, 0.7–0.9, 0.9–1.2, and 1.2–1.5 μ m. We then counted the number of NFs per axon and, for both transgenic and wild-type mice, grouped these data together for axons belonging to each of the size categories.

Statistical Analyses

Statistical analyses of the quantitative immunoblot data and of the morphometric data on the density of NFs were performed using a *t* test and Microsoft Excel software as described (Cole et al., 1994).

Results

Production of Transgenic Mice

Three lines of transgenic mice carrying hNFM transgene were generated. The NFM27 line was characterized and reported earlier (Lee et al., 1992), and studies of this line are not presented here except to compare the transgene copy number in the NFM27 mice with the other two lines of transgenic mice, because our previous study (Lee et al., 1992) showed low levels of hNFM protein expression in this line (Fig. 1). The second line (designated NFM37) carried a transgene containing full-length genomic DNA encoding hNFM with 2.8 kb of upstream sequences (Elder et al., 1994). The third line (designated NFMBam⁻) carried a transgene that harbored a truncated version of the hNFM gene devoid of a 726-bp-long BamHI-BamHI fragment of DNA containing the coding sequence for the hNFM MPR domain in the third exon of the hNFM gene (Myers et al., 1987). Both transgenes were transmitted in a Mendelian fashion. The NFMBam⁻ line was bred to homozygosity, but the NFM37 line remained hemizygous. The NFM27 line was estimated to have ~ 6 copies of transgene, the homozygous NFMBam⁻, 12 copies, and the hemizygous NFM37 ~ 12 copies. None of the 3-mo-old hemizygous NFM37, homozygous NFMBam⁻, or 1-yr-old homozygous NFMBam⁻ transgenic mice showed evidence of intraneuronal NF inclusions, even though the 1-yr-old NFM27 mice develop neurofibrillary accumulations in their cortical neurons (Vickers et al., 1994).

Distribution of hNFM mRNA in the CNS of Transgenic Mice

Northern blot analysis of different CNS regions was performed to determine the expression levels of the transgenic hNFMBam⁻ mRNA in the NFMBam⁻ mice. The expression of the full-length hNFM transgene driven by its own promoter was previously shown to be restricted mainly to neural tissues (Lee et al., 1992). Since less NF mRNA transcripts were present in the hippocampus and neocortex than in brain stem and spinal cord, 50 μ g of total RNA from hippocampus and neocortex and 20 μ g of total RNA from brain stem and spinal cord were loaded in each lane. The pNF4 probe detected an mRNA band

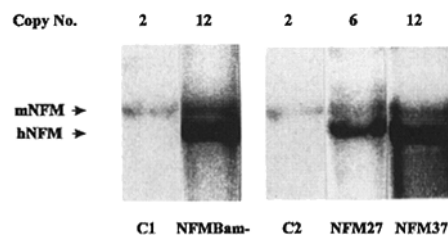


Figure 1. Transgene copy numbers in three hNFM transgenic lines by Southern blot analyses. 15- μ g tail DNA from mice of each of the three human NFM transgenic lines were digested with BamHI, fractionated, and hybridized with [α - 32 P]dCTP-labeled pNF4 (NFM) probe to document the copy numbers of the transgenes. The estimated copy number was obtained by doubling the ratio of transgenic hNFM to endogenous mNFM (listed above the Southern blot). C1 and C2 represent BALB-c and C57Bl/6J \times DBA2J hybrid mice, respectively.

shorter than mNFM mRNA in all four regions of the CNS of NFMBam⁻ mice (Fig. 2 A). The same blots were stripped and reprobed with the BamHI-BamHI DNA fragment that is deleted in the NFMBam⁻ transgene, and only the endogenous mNFM mRNA was seen in transgenic mice (data not shown), which confirmed that this shorter band was the transgenic hNFMBam⁻ mRNA.

The ratio of hNFMBam⁻ mRNA to mNFM mRNA was calculated as described earlier. Quantitative Northern blot analyses demonstrated that the relative expression levels of transgenic mRNA varied among different regions of the CNS (Fig. 2 B). Hippocampus and neocortex expressed as much hNFM mRNA as mNFM mRNA. However, in brain stem and spinal cord, the transgene transcripts were about half of the endogenous mNFM mRNA. If the amount of RNA loaded for each region is taken into consideration, the absolute amounts of hNFMBam⁻ mRNA transcripts in the brain stem and spinal cord are equal to or greater than those in neocortex and hippocampus. The RNase protection assay of NFM37 mice demonstrated that

the hNFM mRNA was expressed at relatively high levels both in brain and spinal cord (Elder et al., 1994).

Transgenic hNFM Protein Expression Correlates with hNFM mRNA Levels

Well-characterized monoclonal (mAbs) and polyclonal antibodies (Table I) specific for NF proteins (including mAbs that distinguish between differentially phosphorylated isoforms of NFH and NFM) were used for immunoblot studies of transgenic and wild-type mice. Quantitative Western blots revealed an hNFMBam⁻ band in the NFMBam⁻ mice of ~100 kD, whereas the M_r of hNFM in the NFM37 line was ~170 kD (Fig. 3, A and C). The large molecular weight difference resulted not only from the deletion of 242 amino acids in hNFMBam⁻ protein, but also from the inability of the NFMBam⁻ to be phosphorylated because the major phosphorylation sites are contained within the deleted region. The full-length transgenic hNFM protein had the same M_r as that of native, heavily phosphorylated hNFM protein, and it was recognized by mAb HO14, which binds specifically to heavily phosphorylated hNFM protein (data not shown, but see Lee et al., 1992; Schmidt et al., 1987). These data suggest that this full-length transgenic hNFM protein was phosphorylated in murine neurons to the same extent as in human neurons of the normal adult CNS.

The ratio of hNFM and hNFMBam⁻ protein to endogenous mNFM protein was used to estimate the relative abundance of the transgenic protein in the CNS. Neocortex and hippocampus expressed as much hNFMBam⁻ protein as mNFM, but the brain stem and spinal cord expressed much lower levels of hNFMBam⁻ protein, that is, ~35% of those for mNFM protein (Fig. 3 B). Peripheral nerves such as the sciatic and trigeminal nerves also contained relatively lower levels of transgenic hNFMBam⁻ protein (data not shown). However, when the amount of protein loaded was taken into consideration, the absolute amount of hNFMBam⁻ protein in brain stem and spinal cord of the NFMBam⁻ mice was very similar to that seen in the neocortex and hippocampus of these same mice.

The levels of the transgenic hNFMBam⁻ protein in the four nervous regions correlated with their hNFMBam⁻ mRNA levels. The high levels of hNFM protein observed in all CNS regions tested in line NFM37 (Fig. 3 D) also correlated with a relatively high level of hNFM RNA expression in this line (Elder et al., 1994). We have also observed a close relationship between human NFM mRNA and its protein levels in a previously described transgenic line NFM27 (Lee et al., 1992). Thus, it seems likely that in all these lines, the level of hNFM protein is controlled primarily at the transcriptional level.

Transgenic hNFMBam⁻ Proteins Are Incorporated into Intermediate Filaments In Vivo

To monitor the copolymerization of hNFMBam⁻ with other endogenous mouse NF subunits into IFs in vivo, neocortical samples of the NFMBam⁻ and control mice were extracted with a high-salt buffer containing Triton X-100. This buffer separates unincorporated proteins into the supernatant while retaining the proteins assembled as IFs in the pellets (Fig. 4 A). Quantitative Western blotting

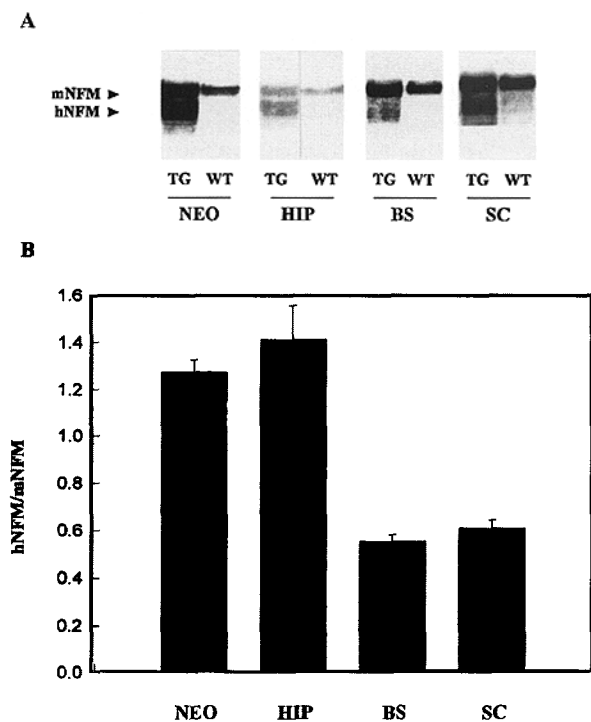


Figure 2. The relative expression levels of hNFM mRNA in different regions of the nervous system of 3-mo-old NFMBam⁻ transgenic mice. (A) Northern blots of four different regions of the nervous system were generated with the pNF4 (NFM) cDNA probe, which recognized both hNFMBam⁻ and mNFM mRNA bands. The amount of total RNA in each lane is as follows: neocortex, 50 μ g; hippocampus, 50 μ g; brain stem, 20 μ g; spinal cord, 20 μ g. (B) Bar graphs of the ratios of the hNFMBam⁻ to mNFM mRNA signals. Each bar represents at least six observations obtained from quantitative Northern blots of 12 age-matched animals. In one blot, each sample was identically loaded into three lanes of a given blot, and the amount of radioactivity in the three lanes from the same sample was averaged and considered as one observation. The neocortex and hippocampus expressed hNFMBam⁻ mRNA at higher relative levels than the brain stem and spinal cord. BS, brain stem; HIP, hippocampus; NEO, neocortex; SC, spinal cord; TG, transgenic; WT, wild type.

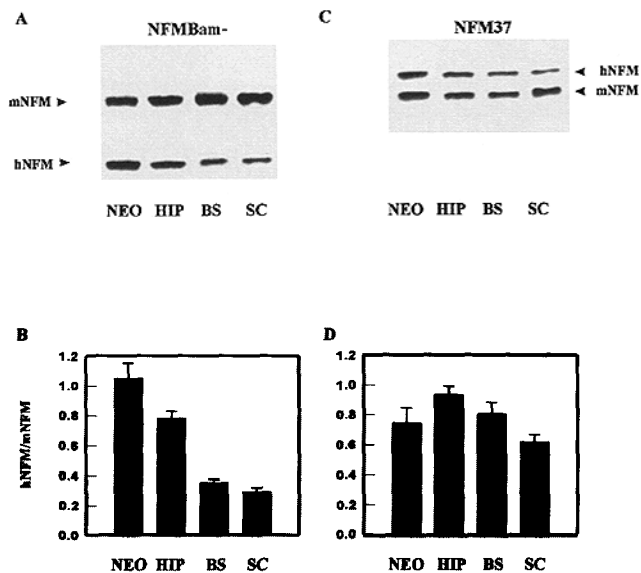


Figure 3. The relative expression levels of the transgenic NFM protein in different regions of the nervous system. *A* and *C* represent quantitative Western blots of different CNS regions from the NFM37 and NFM37 transgenic mice, respectively. The blots were probed with RMO189 (a phosphorylation-independent NFM mAb), which recognizes both the endogenous mNFM and transgenic hNFM37 and hNFM protein. *B* and *D* are bar graphs showing the relative expression levels of hNFM37 and hNFM to endogenous mNFM protein in different CNS regions, respectively. Each bar represents at least six observations obtained from quantitative Western blots of three pairs of sex-matched 3-mo-old adult mice. Note that in NFM37 mice, neocortex and hippocampus regions express higher levels of hNFM37 protein than brain stem and spinal cord regions, and in NFM37 mice, all four CNS regions express high levels of hNFM transgenic mice. (For definitions, see Fig. 2.)

showed that ~90% of hNFM37 transgenic protein was retained in the pellets and only 10% was recovered in the soluble fraction. This percentage of soluble hNFM37 protein was very close to that of soluble mNFM protein in both the transgenic and wild-type mice (Fig. 4 *B*). These results demonstrated that hNFM37 transgenic proteins were incorporated into filaments *in vivo* to the same extent as the endogenous mNFM proteins, regardless of the deleted MPR in the tail domain of the transgenic NFM protein. Furthermore, the transgenic protein did not appear to displace endogenous NF proteins from NFs since the percentage of soluble mNFM, mNFL, and mNFH in the NFM37 mice was similar to that in the wild-type mice (Fig. 4, *C* and *D*).

The Expression of hNFM in Transgenic Mice Is Associated with Increased Accumulation of mNFL

Because NFs are obligate heteropolymers of the NF triplet, and these subunits are assembled into NFs with a defined stoichiometry *in vivo*, we sought to determine the consequences of the expression of the hNFM protein on the relative levels of the endogenous mouse NF subunits in the transgenic mice. Representative immunoblots of the NF proteins from NFM37 and NFM37 transgenic mice versus wild-type mice that were used for quantitation

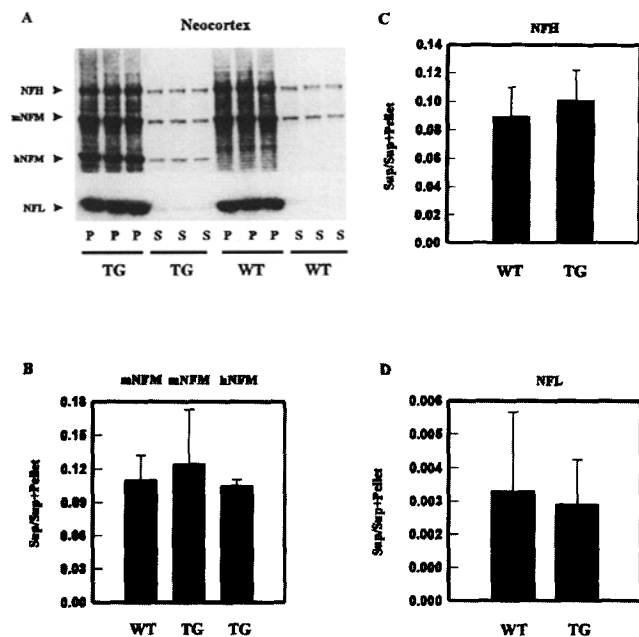


Figure 4. Incorporation of hNFM37 protein into NFs *in vivo*. (*A*) A representative quantitative Western blot of neocortex extracted with Triton X-100-containing buffer. Triplicate samples of 40- μ g total protein from each pellet (*P*) and supernatant (*S*) of neocortical tissue from transgenic NFM37 and wild-type mice were loaded. The blots were overexposed to show the faint NF bands in supernatants. *B–D* show quantitative bar graphs of soluble portions of NFM, NFH, and NFL proteins in transgenic and wild-type mice. Each bar represents six observations as in *A* of three pairs of 3-mo-old animals. There is no significant difference between the soluble portion of hNFM37 protein and that of mNFM protein in transgenic or wild-type mice. The soluble portions of NFH and NFL proteins in transgenic mice are also similar to those in wild-type mice. (For definitions, see Fig. 2.)

are shown in Fig. 5. Remarkably, quantitative data of these blots demonstrated that mNFL protein increased in the CNS of both lines of transgenic mice relative to those of wild-type mice. In NFM37 mice, there was a consistent twofold increase in the relative abundance of mNFL in the neocortex and hippocampus, where the relative levels of transgenic hNFM37 proteins were high (Figs. 5 *A*, and 6, *A* and *C*). In contrast, in the spinal cord, where the level of hNFM37 proteins was only ~35% of mNFM proteins, the relative abundance of mNFL of these mice was similar to that of the wild-type mice (Fig. 5 *B*). However, mNFL was also increased in the brainstem of the NFM37 mice (i.e., similar to neocortex and hippocampus), despite the fact that the hNFM37 proteins were expressed at a level of only 40% of that of the mNFM protein. Similar studies of the NFM37 transgenic mice revealed a similar increase of mNFL protein in all of the CNS regions where the relative levels of hNFM proteins were high (Figs. 5, *C* and *D*, and 6, *D* and *F*).

Increased mNFL in Transgenic Mice Is Regulated at the Posttranscriptional Levels

To determine if the increase in mNFL protein in transgenic mice resulted from an increase in mNFL message levels, RNase protection assays were conducted on both

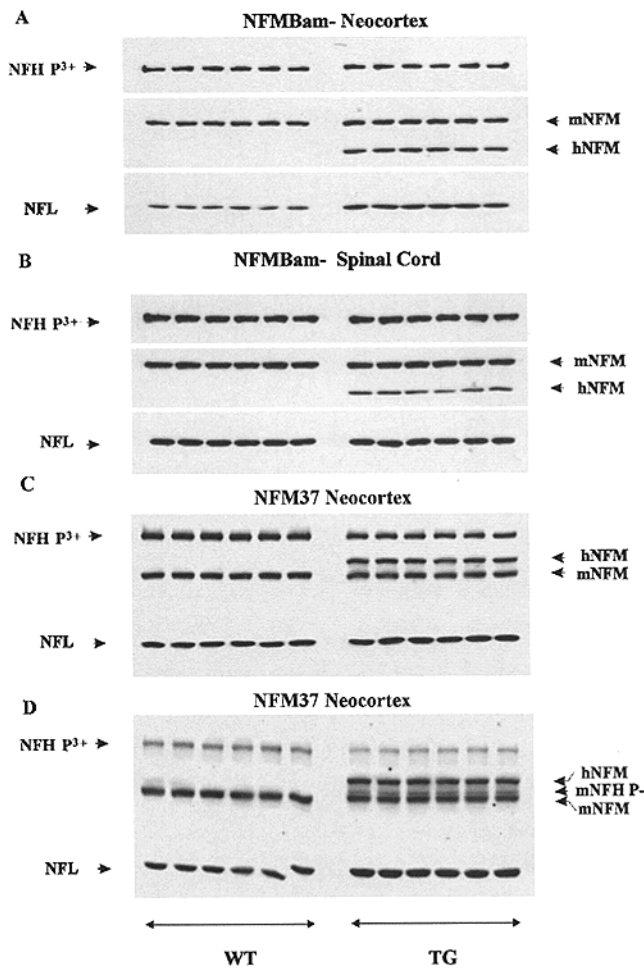


Figure 5. Representative Western blots of 3-mo-old NFMBam- mice versus wild-type mice and NFM37 versus wild-type littermate mice that were used for quantitation. Six identical lanes from either neocortex or spinal cord of transgenic and wild-type mice were loaded in one gel and transferred to nitrocellulose membranes. The blots of NFMBam- versus wild-type mice were cut into three parts. The NFH bands were revealed with the mAb RMO24, which recognized murine heavily phosphorylated NFH (*mNFH P³⁺*) epitope; the NFM bands were revealed with the mAb RMO189, which recognized the rod domains of hNFMBam- and mNFM proteins, and the NFL bands were revealed with a polyclonal rabbit anti-NFL antiserum. The blots of NFM37 versus wild-type mice were cut into two parts. The top part was incubated with the antibody mixture of either RMO24 and RMO189 (C) or RMdO9 and RMO189 to reveal the non-phosphorylated NFH isoform (*mNFH P⁻*) (D) in neocortex of NFM37 mice. The bottom part was incubated with the polyclonal rabbit anti-NFL antiserum.

NFMBam- and NFM37 transgenic mice using a murine NFL probe that protected a 140-bp segment of mNFL mRNA (Fig. 7 A). These studies showed that the levels of mNFL mRNA in both lines of transgenic mice were not elevated compared with age-matched wild-type mice (Table II). These data suggest that the induction of mNFL protein in the transgenic mice is regulated by posttranscriptional mechanisms. Further, RNase protection assays of mNFM (Fig. 7 B) showed that the levels of mNFM mRNA in both lines of transgenic mice also remained unchanged when

compared with the control mice (Table II), and this was consistent with the finding that the levels of mNFM protein in the transgenic mice were the same as those in the control mice (Figs. 5 and 6).

mNFH Subunits Are Phosphorylated to a Lesser Extent in Transgenic Mice Than in Wild-Type Mice

In view of the shared structural and biochemical properties of mNFH and hNFM that may render similar domains in these NF proteins (e.g., the MPR domains) functionally redundant, we sought to determine if the overexpression of hNFM might influence posttranslational modifications (e.g., phosphorylation) of mNFH that are thought to regulate the dynamic functions of these proteins (de Waegh et al., 1992; Nixon, 1993; Cole et al., 1994). Using the RMO24 mAb (which recognizes a heavily phosphorylated or mNFHP³⁺ epitope) and the mAb RMdO9 (which recognizes a nonphosphorylated or mNFHP⁻ epitope), we noted that the neocortex, hippocampus, and brainstem of the NFMBam- mice had 20% less mNFH P³⁺ isoforms than the same regions in the wild-type mice (Fig. 6, A and B). A similar decrease in the abundance of the highly phosphorylated mNFH P³⁺ was also observed in the brains of the NF37 transgenic line (Figs. 5, C and D, and 6, D and E). Furthermore, a protein band with a molecular weight similar to that of the mNFHP⁻ isoform was detected in the neocortex (Fig. 5 D) and hippocampus (data not shown) of NFM37 mice by RMdO9 mixed with RMO189, but not by RMO24 mixed with RMO189 (Fig. 5 C). Since the same band also was detected by RMdO9 alone (data not shown), this band undoubtedly was the poorly phosphorylated mNFHP⁻ protein. Interestingly, we did not see this band in the same brain regions of the NFMBam- mice (data not shown). There was no significant diminution in the levels of the highly phosphorylated NFHP³⁺ isoforms in the spinal cord of either the NFMBam- (Fig. 6, A and B) or the NFM37 mice (Fig. 6, D and E). Thus, our data suggest that the phosphorylation state of mNFH protein decreased in the telencephalic neurons of both transgenic lines, and that this occurred to a greater extent in the NFM37 mice than in the NFMBam- mice.

Antibodies to NF Subunits Differentially Stain Axons of Transgenic versus Wild-Type Mice

Immunohistochemistry was used to confirm the Western blot data and to visualize the differential expression of NF proteins in the 1.5- and 3-mo-old transgenic versus the wild-type mice in situ. We focused our observations on the neocortex and hippocampus because these two regions had the highest relative levels of the transgenic hNFM protein. Fig. 8 shows representative data from the NFMBam- mice, which were obtained with three different antibodies to NF subunits, and Table I summarizes the results of the immunohistochemical studies conducted with all of the anti-NF protein antibodies in this line of transgenic mice. There was no obvious difference in the staining intensity of axons produced with the mAb RMO254 in the NFMBam- versus the wild-type mice, and this mAb (which recognizes the NFM MPR epitope deleted in the transgenic hNFMBam- protein) was used as a control antibody (compare Fig. 8, A and D). However, other anti-

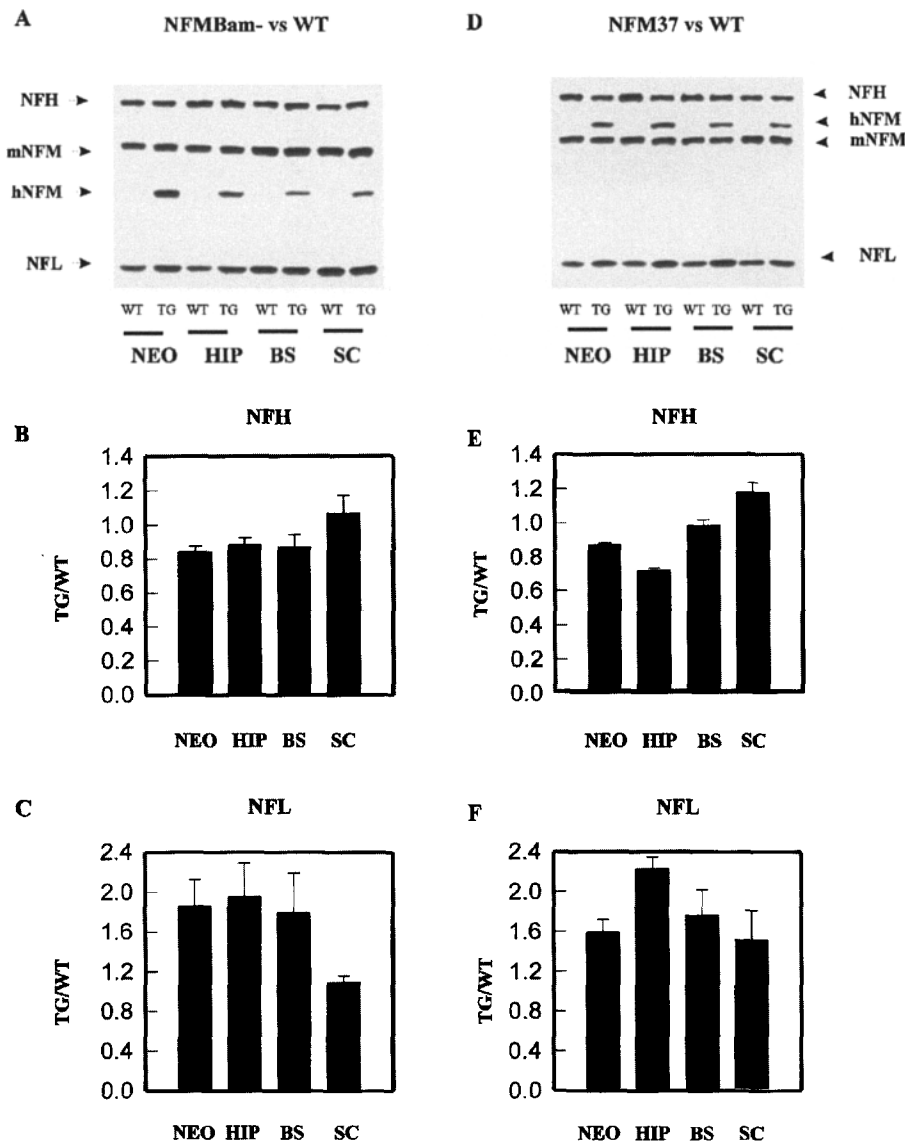


Figure 6. The induction of the mNFL protein and a reduction of the mNFHP³⁺ isoform in transgenic mice. *A* and *D* are Western blots of four CNS regions from 3-mo-old NFMBam⁻ versus wild-type, and NFM37 versus wild-type mice, respectively. The blots were cut into three parts each, which were incubated with RMO24 (mNFHP³⁺), RMO189 (NFM), and rabbit anti-NFL polyclonal antiserum, respectively. *B* and *C* are quantitative ratios of the mNFHP³⁺ isoform and the mNFL protein of NFMBam⁻ to wild-type mice, *E* and *F* show similar ratios of NFM37 to wild-type mice. Each bar represents the average of at least 6 separate observations from quantitative Western blots shown in Fig. 5 of three pairs of animals. One observation was defined as the average of the radioactivity counts of the six identical lanes from one sample in one gel. Note that there is a 20% reduction in the mNFHP³⁺ isoform and a twofold increase in mNFL protein in the regions overexpressing high relative levels of hNFM protein both in NFMBam⁻ (e.g., *NEO*, *HIP*, and *BS*) and NFM37 (e.g., *NEO*, *HIP*, and *BS*). Interestingly, there is no significant reduction in the mNFHP³⁺ isoform in the spinal cord of the NFM37 mice, even though there is a moderate induction of NFL protein.

NFM antibodies (e.g., RMO44) to epitopes that were shared between transgenic and endogenous NFM proteins stained axons in the hippocampus and neocortex of the NFMBam⁻ mice more strongly than did the wild-type counterparts (compare Fig. 8, *B* and *E*). Further, the staining intensity of axons in the NFMBam⁻ mice with a rabbit anti-NFL polyclonal antiserum also was stronger than that seen in the wild-type mice (compare Fig. 8, *C* and *F*). However, the three anti-NFH antibodies yielded results that did not differ in the NFMBam⁻ versus the wild-type mice, and we ascribe this to the fact that the 20% decrease in the levels of the mNFHP³⁺ isoforms observed by Western blot methods in these transgenic mice was not large enough to be visualized by immunohistochemistry. Notwithstanding, the immunohistochemical studies described here served to confirm and extend the key immunochemical data. Less detailed studies of the 3-mo-old NFM37 mice yielded similar observations (data not shown). Finally, although perikaryal and/or axonal aggregates of NFs have been detected in other lines of NF transgenic mice (Côté et al., 1993; Xu et al., 1993; Eyer and Peterson, 1994;

Vickers et al., 1994), none were observed in the transgenic mice studied here or in the 1-yr-old NFMBam⁻ mice (data not shown). These data suggest that the overexpression of NFM protein in these two lines of transgenic mice is not incompatible with the efficient export of NFs from perikarya into axons and the subsequent orthograde transport of NFs down the axon.

Packing Density of Neurofilaments Is Increased in Axons of Transgenic Mice versus Those of Wild-Type Mice

Electron microscopic studies were performed to compare the packing density of NFs in telencephalic white-matter axons of the transgenic mice versus those of the wild-type mice. To do this, we focused our quantitative studies of axonal NFs on the alveus because this is a reproducibly identifiable white-matter tract of the hippocampus wherein we noted large differences in the intensity of the NF protein immunoreactivity between the NFMBam⁻ and the wild-type mice. The axons in this area are in the 0.1–1.5 μm in

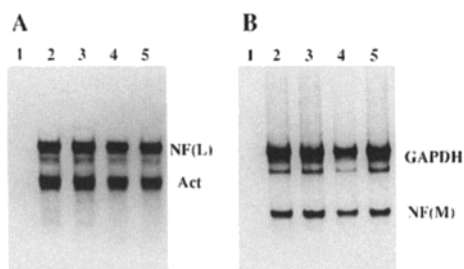


Figure 7. Relative levels of expression of mNFL and mNFM RNAs in transgenic lines. RNase protection assays were performed as described in experimental procedures. In *A*, 20,000 cpm of an mNFL-specific probe and 10,000 cpm of a murine β -actin probe were hybridized with yeast tRNA (lane 1), total brain RNA from a transgenic NFM37 mouse (lane 2), a wild-type NFM37 littermate (lane 3), a transgenic NFM Bam⁻ mouse (lane 4), or a wild-type NFM Bam⁻ mouse (lane 5). Protected fragments were separated as double-stranded RNA on a 6% native polyacrylamide gel. Positions of the actin and mNFL protected fragments are indicated. In *B*, hybridizations were performed with 40,000 cpm of an mNFM-specific probe and 10,000 cpm of a murine GAPDH probe. Lane order is the same as in (*A*). Exposures in both *A* and *B* were for 18 h.

diameter range, with most in the 0.3–0.8 μ m size class. For quantitative study, alvear axons were arbitrarily divided into six size categories, with axonal diameters of 0.1–0.3, 0.3–0.5, 0.5–0.7, 0.7–0.9, 0.9–1.2, and 1.2–1.5 μ m. Fig. 9 shows the representative axons (*A*) and their NF density distribution histogram (*B*) from the second size category. These data reveal a shift to the right in the NF packing density for the NFM Bam⁻ mice. The number of NFs in 180 axons of this category from three NFM Bam⁻ mice and 180 axons of the same size class from three wild-type mice were counted, and the results are shown in Table III. The total NF number of the NFM Bam⁻ mice was 8,100 and that of the wild-type mice was 5,580. The average number of NFs per axon was 45 in the NFM Bam⁻ mice and 31 per axon in the wild-type mice ($P < 0.01$). Thus, the axons had a higher NF packing density in the transgenic versus the wild-type mice; the same trend was also demonstrated in all other axonal categories (Table III).

Discussion

The studies described here sought to gain insights into the

Table II. RNase Protection Assays of NFM37 and NFM Bam⁻ versus Control

	NFL	NFM
NFM37	112.7 \pm 9.7	107.3 \pm 9.2
NFM37 control	100.2 \pm 3.1	100.2 \pm 12.2
NFM Bam ⁻	109.8 \pm 11.5	99.1 \pm 11.0
NFM Bam ⁻ control	100.0 \pm 3.4	101.2 \pm 8.5

Quantitation of mouse NFL and NFM mRNA in transgenic lines. RNase protection assays were performed as described in experimental procedures and illustrated in Fig. 7. Two transgenic and two control adult animals (aged 1–3 mo) were analyzed from each line. Expression in control animals was set at 100 and values are given with standard deviations. Results are the average of at least six observations on whole-brain RNA in each animal. Values for paired *t* tests are: NFL in NFM37 vs. control, $P = 0.048$, and NFM Bam⁻ vs. control, $P = 0.059$; NFM in NFM37 vs. control, $P = 0.013$, and NFM Bam⁻ vs. control, $P = 0.678$.

regulation of NF protein metabolism and the stoichiometry of NF subunits using transgenic technology. We have shown that overexpressing hNFM in the CNS of both types of transgenic mice induces increased levels of mNFL proteins in addition to reducing levels of highly phosphorylated isoforms of mNFH and increasing the packing density of NFs in large-diameter CNS axons. We speculate that these effects are specific to NFM because previous studies of transgenic mice that express NFL or NFH did not evidence similar changes. The observations on the spacing of NFs in CNS axons suggest that neurons try to accommodate increased NFs in their axons by reducing their interfilamentous spaces. The concomitant decrease in mNFH P³⁺ isoform is compatible with the view that the decrease of phosphorylation state of the carboxy terminal MPR domain of NFH increases the packing density of axonal NFs by reducing the negative charges on NFH sidearms, which limits lateral extension of the tail domain of NFH away from individual NFs (Carden et al., 1987; de Waegh et al., 1992; Cole et al., 1994; Nixon et al., 1994). Taken together, the data from these two lines of transgenic mice have two important implications for understanding how the NF network is regulated in neurons, that is, these data suggest that (1) NFM may play a dominant role in the *in vivo* regulation of NFL levels, the stoichiometry of NF subunits and the phosphorylation state of NFH, and (2) the packing density of NFs in CNS axons is determined by both the NF numbers and the phosphorylation state of NFH.

Native NFs have been estimated to assemble at molar ratios for NFH, NFM, and NFL, ranging from 1:2:2 to 1:2:9 depending on the species and neuronal populations investigated (Mori and Kurokawa, 1980; Shekhet and Lasek, 1980; Brown et al., 1981; Moon et al., 1981; Chiu and Norton, 1982; Scott et al., 1985). Although all three NF subunit proteins are competent to self-assemble into long IFs when combined together *in vitro* (Balin and Lee, 1991; Liem and Hutchison, 1982), NFL forms a filamentous network of IFs *in vivo* only in the presence of either NFM or NFH (Ching and Liem, 1993; Lee et al., 1993). Further, an imbalance in the molar ratio of NF proteins has been implicated as a mechanism responsible for certain neurodegenerative conditions such as motor neuron diseases (Côté et al., 1993; Xu et al., 1993). This is consistent with the notion that changes in the molar ratio of NF proteins may alter the transport of NFs down axons, thereby resulting in the accumulation of perikaryal and axonal aggregates of NFs. Thus, disruption of mechanisms that regulate the stoichiometry of the subunit proteins of NFs may impair the function of neurons and lead to lethal consequences.

It is possible that one or more of the NF triplet proteins serve to regulate and maintain the stoichiometry of these subunits in intact NFs. Since transgenic mice that overexpress different NF subunits seem to tolerate a wider range in the levels of NFL compared to NFH and NFM (Monteiro et al., 1990; Cote et al., 1993; Xu et al., 1993; Vickers et al., 1994), NFL probably is unlikely to regulate the stoichiometry of NF proteins. Further, the delayed appearance of NFH during development suggests that NFH also is unlikely to regulate NF stoichiometry. However, the present studies show that an exogenous hNFM protein becomes incorporated into the cytoskeleton *in vivo* and reg-

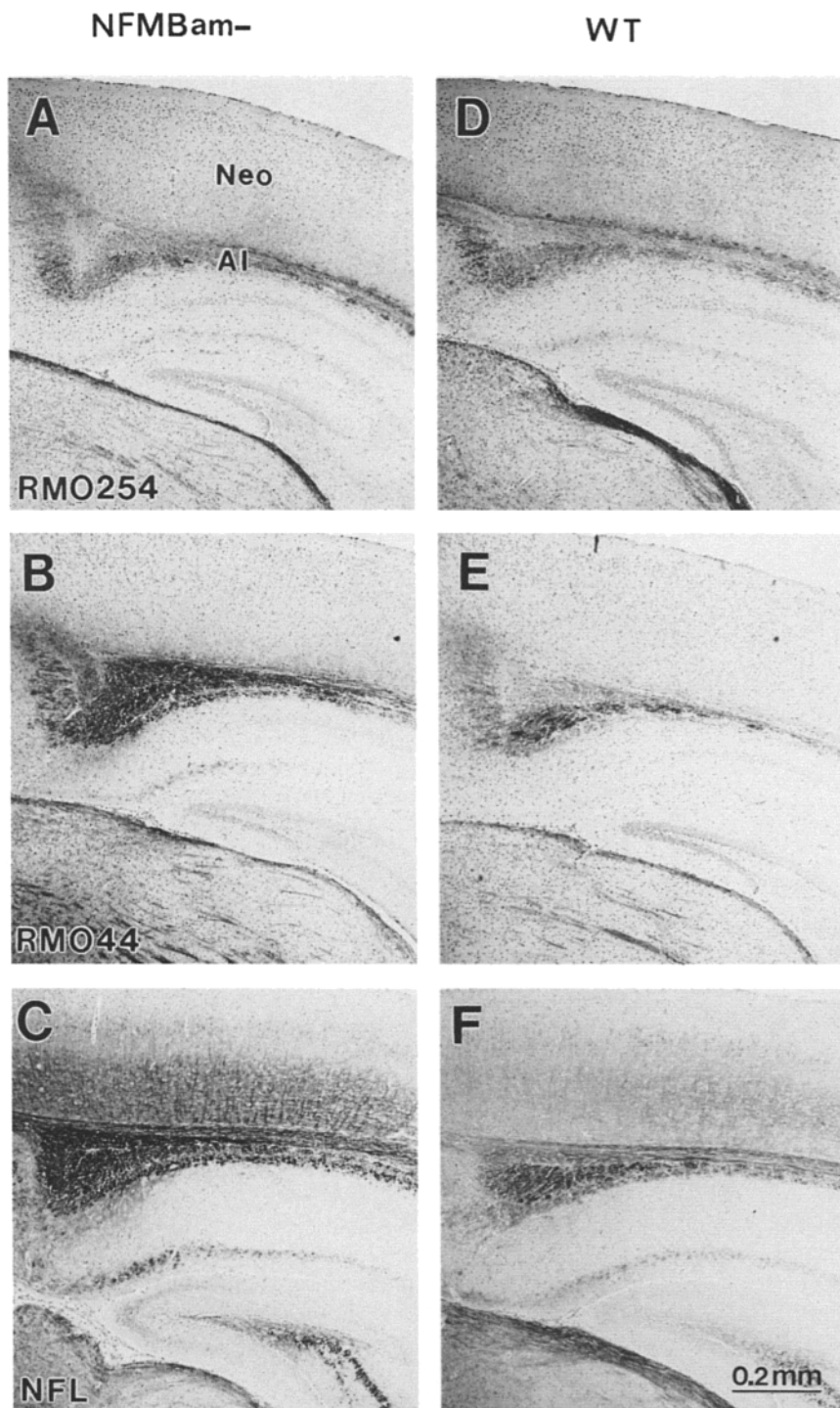


Figure 8. Comparison of immunohistochemical preparations of cortex and the alveus of the hippocampus probed with three different anti-NF antibodies. *A–C* are NFMBam⁻ mice, and *D–F* are wild-type mice. RMO254 (which recognizes the MPR of human NFM protein that is absent in the hNFMBam⁻ protein) was used as a control antibody to show the similar staining intensity between transgenic and wild-type mice (*A* and *D*, respectively). The mAb RMO44 (which recognizes rod domains of both hNFMBam⁻ and mNFM proteins) stains transgenic tissue more strongly than the wild-type tissue (*B* and *E*, respectively). The induction of mNFL protein in the transgenic NFMBam⁻ mice is evidenced by the stronger staining than in the wild-type mice using the rabbit anti-NFL antibody (*C* and *F*, respectively).

ulates the stoichiometry of the endogenous mouse NF proteins.

The ratio of hNFM to mNFM proteins was high in the neocortex and the hippocampus in NFMBam⁻ mice, but low in the brain stem and the spinal cord. However, the absolute amounts of transgenic hNFM proteins in the latter two regions were equal to or greater than those in the former two regions, which suggests that the transgene was expressed as efficiently in the spinal cord and brainstem as in the neocortex and hippocampus. The detection of relatively low levels of transgenic hNFM in the spinal cord and brain stem may in part reflect the fact that many more en-

dogenous NF proteins are present in these two regions. Quantitative Western and Northern blot data correlated the expression of hNFM protein with the level of hNFM mRNA, which suggested that the expression of the transgene is basically controlled at the transcriptional level and that it is subjected to the same transcriptional regulation in all of the CNS regions. The NFM27 mice not examined in this study expressed relatively low levels of hNFM mRNA, resulting in low expression of hNFM and no evidence of increased levels of NFL induction by Western blot analysis (Lee et al., 1992). Although the NFM37 mice expressed higher levels of hNFM proteins in all four CNS regions

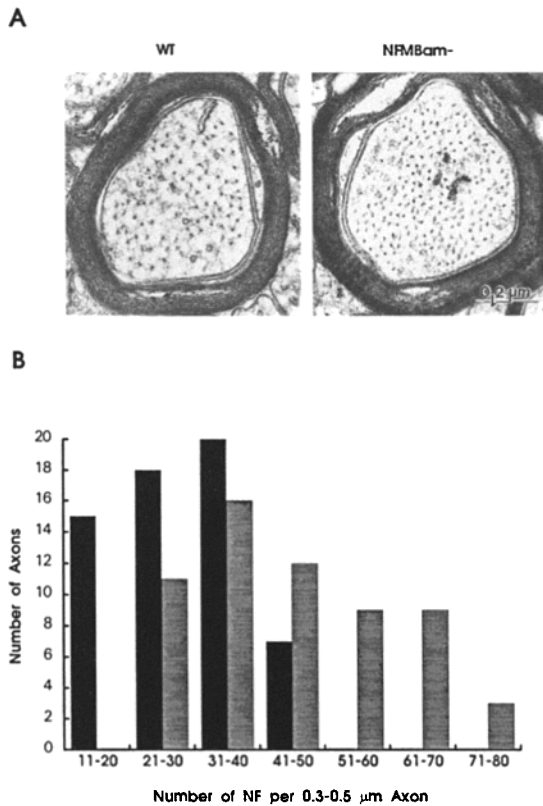


Figure 9. Electron microscopic analyses of the white-matter axons in the alveus of the hippocampus in the NFMBam⁻ transgenic mice show more NFs and a higher packing density than similar-sized axons in the wild-type mice. **A** shows electron micrographs of representative alveal axons of the NFMBam⁻ and wild-type mice. Magnification: $\times 60,000$. **B** shows a histogram of the numbers of NFs per 0.3–0.5 μm axon in the alveus of the hippocampus in the NFMBam⁻ and wild-type mice. The shaded bars represent wild-type mice; the horizontal bars, NFMBam⁻ mice. The distribution pattern of the numbers of NF per 0.3–0.5 μm axons is broader and shifts to the right in the NFMBam⁻ mice when compared with wild-type mice.

examined here, the reasons for this are not obvious. However, it is possible that the site of integration of the genomic DNA for hNFM in the NFM37 mice influences the level of expression of this protein in the CNS.

Interestingly, we noted a concomitant increase in the levels of mNFL proteins wherever the ratio of hNFM to mNFM was high in both of the transgenic lines. These data suggest that the relative levels of hNFM, rather than the absolute amounts of hNFM, are responsible for inducing the increased expression of mNFL. A similar phenomenon had been observed previously for other IFs in the cultured cells. For example, the expression of a keratin type II (i.e., K6b) protein was shown to induce the synthesis of a keratin type I protein in 3T3 fibroblasts transfected with a construct encoding the K6 IF protein (Giudice and Fuchs, 1987). Similarly, when desmin was transfected into T24 cells, it also induced the synthesis of endogenous vimentin in these cells (Pieper et al., 1992). The present study is the first to show an increase in one subunit (i.e., NFL) of a heteropolymeric IF by another subunit (i.e., NFM) that normally is found in the same IF in vivo. Because this was

Table III. Neurofilament Number per Axon in Alveus of Hippocampus

Axon category	Control ($n = 180$)	NFMBam ⁻ ($n = 180$)	<i>t</i> Test
0.1–0.3 μm	15 \pm 5	30 \pm 14	$P = 0.0015$
0.3–0.5 μm	29 \pm 10	45 \pm 15	$P < 0.001$
0.5–0.7 μm	56 \pm 16	86 \pm 28	$P < 0.001$
0.7–0.9 μm	79 \pm 16	119 \pm 38	$P = 0.0013$
0.9–1.2 μm	110 \pm 20	161 \pm 24	$P = 0.0031$
1.2–1.5 μm	151 \pm 36	196 \pm 24	$P = 0.036$

seen in two lines of hNFM transgenic mice and correlated well with the expression levels of hNFM protein, it is unlikely these findings reflect effects due to the site of integration of the transgene. Indeed, the increase in mNFL was unique in that there was no change in the levels of mNFM and mNFH proteins; it also appeared to be due specifically to the overexpression of NFM, because transgenic mice that overexpressed NFL or NFH did not induce changes in the levels of the other NF subunits (Côté et al., 1993; Xu et al., 1993; Tu and Lee, unpublished). Thus, we infer that a unique function of NFM protein may be to regulate and maintain the stoichiometry of NF subunits in vivo. This view is also supported by several other lines of evidence. First, in lower animal species such as *Myxicola* or lamprey, the major NF subunit has characteristics similar to mammalian NFM protein (Chin et al., 1989; Pleasure et al., 1989), suggesting that NFM protein is the archetype NF protein of the CNS. Second, during development of the CNS, NFM coexpresses with NFL (Carden et al., 1987; Tohyama et al., 1991) or appears somewhat earlier than NFL (Tapscott et al., 1981; Bennett and Dilullo, 1985; Szaro et al., 1989; Yachnis et al., 1993). Thus, this temporal relationship places NFM in a critical position to regulate the synthesis of the other two NF subunits.

Multileveled regulatory mechanisms have been proposed to control the synthesis of the NFL subunit (Lindenaum et al., 1988; Ikenaka et al., 1990; Monteiro et al., 1990; Nakahira et al., 1990; Beaudet et al., 1993a,b; Shneidman et al., 1993). Our RNase protection assays revealed no significant increase in mNFL mRNA in either of these two lines of transgenic mice, suggesting that the increased levels of mNFL protein are due to posttranscriptional regulatory mechanisms. Although the nature of these mechanisms is unclear at this time, there are several plausible considerations. For example, since NFM proteins bind with high affinity to single-stranded RNA and DNA in vitro (Traub et al., 1985), it is possible that such interactions are involved in regulating NF protein levels in vivo. Alternatively, the assembly of IF proteins into filaments may increase the levels of an IF subunit in transfected cells by retarding the degradation of the artificially expressed protein (Giudice and Fuchs, 1987; Knapp and Franke, 1989; Kulesh et al., 1989). Thus, the overexpression of NFM protein could increase the levels of mNFL protein by copolymerizing with and stabilizing all available mNFL. In this regard, it is interesting that the expression of K14 cyokeratin without its partner K6b in transfected 3T3 fibroblasts led to the formation of aggregates of K14, while no aggregates formed when both K14 and K6b were coexpressed in transfected cells, where they formed a network (Giudice and Fuchs, 1987). This finding may ex-

plain why no NF inclusions are found in the NFMBam– mice for up to 1 yr (data not shown).

Notably, mNFH protein was less extensively phosphorylated in both lines of transgenic mice described here. Although the decrease in the highly phosphorylated NFHP³⁺ isoform in the NFMBam– mice might be interpreted to reflect a decrease in total mNFH protein, the appearance of a poorly phosphorylated mNFHP– isoform in the NFM37 mice clearly demonstrates that the extent of phosphorylation of mNFH indeed decreases in these transgenic mice. Furthermore, the larger decrease in the phosphorylation state of mNFH in the NFM37 mice, compared with the NFMBam– mice may be ascribed to the presence of MPR in the hNFM, which is fully phosphorylated in the NFM37 mice. Thus, we infer that the phosphorylation state of mNFH protein is regulated by the amount and phosphorylation state of hNFM proteins.

Electron microscopy showed that the axons of NFMBam– mice had more NFs packed at a higher density than control mice. Because the packing density of myelinated axons is determined by both the number of NFs and the phosphorylation state of these NFs (Monteiro et al., 1990; de Waegh et al., 1992; Gotow et al., 1994; Nixon et al., 1994), our data suggest that hippocampal neurons of the NFMBam– mice compensate for the increased number of NFs by reducing the extent to which the mNFH is phosphorylated. This in turn allows NFs to pack at higher densities without increasing the caliber of axons. The more obvious reduction of mNFH phosphorylation state in NFM37 mice compared with the NFMBam– mice further substantiates the view that both NFM and NFH proteins contribute to regulating the NF packing density and that their phosphorylation state is regulated in a coordinate manner. With more potential phosphorylation sites in its tail domain, the NFH protein may be a better interfilamentous spacer than NFM. The neocortical and hippocampal neurons in transgenic mice may prefer NFM as the spacer rather than NFH because their myelinated axons are small. That the extent of phosphorylation of mNFH protein in spinal cord remains unchanged in transgenic mice may reflect the different capacities of the myelinated axons in brain or spinal cord to accommodate NF sidearms. Accordingly, the myelinated axons in the spinal cord are much larger in diameter than those in the brain. The random distribution pattern of interneurofilamentous spaces (Price et al., 1988) suggests that the myelinated axons in the spinal cord could accommodate variable number of NFs. Thus, the increase in NFM and NFL proteins may have less of an impact on spinal cord versus brain axons. Accordingly, this increase does not change the extent of phosphorylation of mNFH in the spinal cord. This interpretation is supported by the electron microscopy studies of the sciatic nerves of NFM37 and control mice, which did not reveal any significant change in the packing density of NFs or the diameter of these axons in the NFM37 mice (data not shown).

While these and several of the other issues discussed earlier concerning the mechanisms that regulate the metabolism and function of NFs remain to be specified in detail, the studies of transgenic mice described here suggest that NFM might play a dominant role in the *in vivo* regulation of NFL expression, the stoichiometry of NF subunits,

and the phosphorylation state of NFH in the brain. Further, our studies imply that the phosphorylation state of both NFM and NFH proteins plays a role in regulating the packing density of NFs in CNS axons. Additional insights into the validity of these inferences should help clarify how alterations in the metabolism of NF proteins contribute to the pathogenesis of human neurodegenerative diseases characterized by a disruption of the neuronal cytoskeleton.

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