Extra Neurofilament NF-L Subunits Rescue Motor Neuron Disease Caused by Overexpression of the Human NF-H Gene in Mice

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Abstract. Previous studies demonstrated that transgenic mice overexpressing human neurofilament heavy (hNF-H) protein develop a progressive motor neuron disease characterized by the perikaryal accumulations of neurofilaments resembling those found in amyotrophic lateral sclerosis (ALS). To further investigate this neurofilament-induced pathology, we generated transgenic mice expressing, solely or co-constitutively, the hNF-H and the human neurofilament light (hNF-L) proteins. We report here that the motor neuron disease caused by excess hNF-H proteins can be rescued by overexpression of hNF-L in a dosage-dependent fashion. In hNF-H transgenic mice, the additional hNF-L led to reduction of perikaryal swellings, relief of axonal transport defect and restoration of axonal radial growth. A gene delivery approach based on recombinant adenoviruses bearing the hNF-L gene also demonstrated the possibility to reduce perikaryal swellings after their formation in adult mice. The finding that extra NF-L can protect against NF-H-mediated pathogenesis is of potential importance for ALS, particularly for cases with NF-H abnormalities.

Key Words: Adenovirus; Amyotrophic lateral sclerosis; Motor neuron disease; Neurofilament; Transgenic mice.

INTRODUCTION

 Neurofilaments (NFs) constitute the main type of intermediate filaments found in myelinated neurons of the adult nervous system. NFs are formed by the copolymerization of 3 intermediate filament proteins, neurofilament light (NF-L, 61 kDa), medium (NF-M, 90 kDa), and heavy (NF-H, 110 kDa) subunits (1). The 3 neurofilament subunits share with other members of the intermediate filament family a central domain of approximately 310-amino acids involved in the formation of coiled-coil dimers. Two dimers can then line up in a staggered fashion to form an antiparallel tetramer (2). There is evidence for the existence of 2 types of heterotetrameric unit in NFs, 1 containing NF-L and NF-M and the other NF-L and NF-H subunits (3). The subsequent chronology of linear and lateral associations between tetramers is difficult to discern. In vitro studies have shown that rodent NFs are obligate heteropolymers requiring NF-L together with either NF-M or NF-H for polymer formation (1, 4). More recently, studies on mice having the NF-L gene disrupted showed that NF-L in vivo is essential for NF assembly and for efficient translocation and transport of NF-M and NF-H proteins into axons (5). In contrast, neither NF-M nor NF-H proteins are an absolute requirement for NF assembly and transport as revealed by studies on NF-M knockout and NF-H knockout mice (6–9).

Abnormal accumulations of NFs, often called spheroids or Lewy bodies, have been described in a variety of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer disease and Parkinson disease (for review see 10). Paradoxically, the NF deposits in these diseases are associated with decreases in levels of mRNA coding for NF proteins. For instance, reductions in NF-L mRNA levels in affected neuronal subpopulations have been reported in ALS (11), Alzheimer disease (12) and Parkinson disease (13). However, the extent to which altered protein content, subunit stoichiometry, and abnormal NF inclusions contribute to neurodegeneration in human diseases remains unknown.

Studies on transgenic mice overexpressing normal or mutant NF proteins (14–20) and the discovery of NF-H mutations in some ALS cases (10, 21–23) provided compelling evidence for a contributory role of NFs in pathogenesis. Of relevance to the present study was the finding that overexpression of human NF-H (hNF-H) in mice provokes a motor neuron disease characterized by the formation of perikaryal neurofilamentous accumulations in spinal motor neurons and axonal transport defects (14, 24). In adulthood, these hNF-H transgenic mice exhibit neurological abnormalities such as quivering and abnormal limb contraction reflexes. The hNF-H-induced pathology progresses with the atrophy and slow degeneration of motor axons, resulting in neurogenic muscle degeneration. Surprisingly, transgenic mice expressing a 4-to-5-fold increase in the level of wild type mouse NF-H protein did not develop a motor neuron disease or overt phenotypes, despite a retardation of axonal transport of NFs (19). This discrepancy led to the suggestion that hNF-H proteins may act as mutant NF proteins in mouse neurons (19).

We report here an analysis of doubly hNF-L,hNF-H transgenic mice derived by the crossbreeding of mice expressing the human NF-H gene (14) with mice expressing...
the human NF-L gene (25). Our results reveal that extra NF-L proteins can rescue the motor neuron disease caused by hNF-H overexpression in transgenic mice. In addition, we demonstrate that a gene therapy approach based on recombinant viruses bearing the hNF-L gene can be used to reduce neurofilamentous swellings in this mouse model of motor neuron disease.

MATERIALS AND METHODS

Generation of Transgenic Animals

The parental transgenic mouse lines were obtained by the microinjection of genomic fragments into 1-cell stage embryos as described in Julien et al (25) and Côté et al (14) for the hNF-L line 29 and hNF-H line 20 transgenic lines respectively. Homozygous mice of each parental line were crossed to obtain mice heterozygous for both transgenes. Further cross-breeding of the first generation yielded normal mice and mice heterozygous or homozygous for each transgene. The animals used in this study were not pure inbred mice, but were dominantly of C57BL/6 genetic background. The genotypes of transgenic mice were identified by Southern blotting of tail genomic DNA. Briefly, approximately 1 cm of mouse tail was digested in 10 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.5% SDS, 2 mM EDTA with 0.6 mg/mL of proteinase K (Roche Diagnostics, Laval, Canada) at 55°C for 4 hours (h). The digested tissue was then extracted with phenol-chloroform and the aqueous phase precipitated in ethanol. The resulting genomic DNA pellet was resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 10 µg of DNA was digested overnight with a selected restriction enzyme. The digestion product was run on an agarose gel, transferred to a charged nylon membrane (GeneScreen Plus, NEN Life Science Products, Boston, MA) and hybridized as described by Sambrook et al (26). The probe used to specifically detect the hNF-H gene was a PCR product spanning the fourth exon of the hNF-H gene. For detection of the hNF-L transgene, the probe corresponded to a PsI fragment from the first exon of the mouse NF-L gene that hybridizes with the gene of both species. Filters were exposed on BioMax MR films (Eastman-Kodak, Rochester, NY), using Cronix intensifying screens (DuPont, Guelph, Canada).

Northern Blotting

Total RNA was prepared from freshly isolated or flash-frozen spinal cords from transgenic and normal mice. Homogenization was carried out in 5 mL of Trizol (Gibco-BRL, Burlington, Canada) per gram of tissue and total RNA isolation performed according to manufacturing's guidelines. Five or 10 µg of total RNA was loaded on a 1% agarose-formaldehyde gel and processed for northern blotting as described by Sambrook et al (26). The radiolabeled probes used for the detection of hNF-L and hNF-H transgenes were the same as those used for genomic screening. The loading was standardized using a mouse actin cDNA as a probe. Filters were exposed to BioMax MR films (Eastman-Kodak), using Cronix intensifying screens (DuPont).

Protein Analysis

Mice were sacrificed and the relevant tissues were dissected for immediate processing or flash-frozen in liquid nitrogen. To

Fig. 1. Northern and western blot analysis of double transgenic mice. (A) Detection of NF-L and hNF-H mRNAs in spinal cord of transgenic mice. The probe used for the detection of NF-L hybridizes with both human and mouse NF-L transcripts. An actin cDNA probe was used for standardization. Lane 1, hNF-H+/+; lane 2, hNF-H+/-; lane 3, hNF-L+/+; lane 4, hNF-L+/-; lane 5, hNF-L+/-; hNF-H+/-; lane 6, hNF-L+/+; hNF-H+/-; lane 7, normal; lane 8, hNF-L+/-; hNF-H+/-; lane 9, hNF-L+/-; hNF-H+/- mice. (B) Coomassie stained SDS-PAGE and western blot analysis of Triton-insoluble spinal cord protein extracts from 6-month-old mice. Western blots were carried out on replica filters. Lane identification is identical to Figure 1A.
obtain Triton-insoluble cytoskeletal fractions, tissues were homogenized in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 10 mg/mL aprotinin, 2 mg/mL leupeptin, 2 mg/mL pepstatin, and 1% Triton X-100. Homogenates were centrifuged for 20 minutes (min) at 4°C at 13,000 × g in a microfuge. The Triton-insoluble pellet was rehomogenized in SÜB (0.5% SDS, 8 M urea, 2% β-mercaptoethanol). The re-suspended material was centrifuged at room temperature for 15 min in a microcentrifuge. Protein concentration of the resulting supernatants was measured using Bio-Rad Protein Assay (Bio-Rad, Mississauga, Canada), a Bradford-based protein assay. For immunoblotting, protein samples in 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 0.7 M β-mercaptoethanol were loaded on 7.5% SDS-PAGE and subsequently transferred to nitrocellulose. Filters were blocked for 3 h in gelatin-buffer (20 mM Tris-HCl pH 7.3, 150 mM NaCl, 1% fish skin gelatin (Sigma, Oakville, Canada) and 0.1% Tween 20) and then incubated with primary antibodies for 4 h at room temperature or overnight at 4°C. The primary antibodies were diluted in the gelatin-buffer at the following titers: monoclonal mouse anti-human NF-L, DP5-112 1:2,000 (N.T.L., France); monoclonal mouse anti-NF-L RPN1105 1:1,000 (Amersham, Baie d’Urfé, Canada); monoclonal rat anti-human NF-H (OC95) 1:200 (kindly provided by V. M.-Y. Lee, University of Pennsylvania); polyclonal rabbit anti-NF-H 1:1,000 (Sigma); monoclonal mouse anti-NF-M MN18 1:1,000 (Roche Diagnostics); monoclonal mouse anti-β-tubulin KMX-1 1:200 (Roche Diagnostics); monoclonal mouse anti-actin C4 1:1,000 (Roche Diagnostics). After several washes in gelatin-buffer, membranes were incubated for 1 h with peroxidase-conjugated secondary antibodies (anti-mouse, anti-rat or anti-rabbit, Jackson ImmunoResearch Laboratories Inc., Mississauga, Canada) diluted 1:1,000 in gelatin-buffer. The membranes were washed once in gelatin-buffer and 3 times in 20 mM Tris-HCl pH 7.3, 150 mM NaCl. Detection of the immune complex was performed with the chemo-luminescent ECL detection kit (Amersham).

Morphometric Analysis

In order to measure axonal calibers, thin sections (1 μm) of Epon-embedded (Marivac, Halifax, Canada) L5 ventral roots from transgenic and normal mice were stained with 2% Toluidine blue (Canemco, Dorval, Canada). The images of sections were then digitalized using a Hamamatsu CCD camera (Hamamatsu Photonic, Hamamatsu city, Japan) mounted on a Leitz Diaplan microscope (Leitz, Deerfield, USA) with a 100× objective. The digitalized images were subsequently analyzed using morphometric software (Image 1, Universal Imaging Corp., USA). Axonal diameters were calculated from cross-sectional areas.

Generation of Adenoviral Vectors

The Ad5-CMV-LacZ vector is a replication-defective dl 309 adenovirus serotype 5 virus (27) in which the E1 region of the genome between nucleotides 452 and 3328 has been replaced with an expression cassette consisting of a bacterial lacZ gene open reading frame fitted with a SV-40 polyadenylation signal, and placed under the control of the human cytomegalovirus (CMV) promoter/enhancer. The construction, production, titration, and purification of this vector has been described previously (28). The Ad5-hNFL adenoviral vector has the same genotype and has been constructed in the same way as the Ad5-CMV-LacZ, except that the E1 region has been replaced with a DNA fragment bearing the human NF-L gene under the control of its own minimal 0.3 kb promoter (29).

Mixed Spinal Cord Cultures

Cultures of dissociated spinal cord-DRG were prepared from E13 hNF-H overexpressors or normal mouse embryos.

Fig. 2. Reduction of perikaryal swellings by extra hNF-L. Light micrographs of spinal motor neurons in the lumbar region of 6-month-old, (a), normal; (b), hNF-L+/+; (c), hNF-H+/+; (d), hNF-L+/+;hNF-H+/+; (e), hNF-H−/−; (f), hNF-L+/−;hNF-H+/+; (g), hNF-L+−;hNF-H−/−; (h), hNF-L+/+;hNF-H−/−. NF swellings appear as lightly stained areas. Thin arrows point to normal appearing motor neurons, large arrows in (h) point to a giant axon found in hNF-L+/−;hNF-H+/− mice.
to reduce proliferation of non-neuronal cells. Spinal cord-DRG cultures were used in infection experiments with adenoviral vectors 3 weeks after dissociation.

In Vitro Infection of Cultured Motor Neurons

In vitro infection studies were carried out using dissociated spinal cord-DRG cultures from hNF-H transgenic or normal mice. Replication-incompetent adenoviruses were added to the cultures at a final titer of $5 \times 10^9$ PFU/mL of Ad5-hNF-H virus, or alternatively Ad5-CMV-LacZ (data not shown). Cells were exposed to adenoviral vectors for 5 h at 37°C and were then washed in culture medium and further cultured in modified N3 medium. At 24 h, 48 h, or 72 h following exposure to adenoviral vectors, cells were fixed in phosphate buffered 4% paraformaldehyde solution and incubated with 0.1% Triton X-100 in Tris-buffered saline for 10 min at room temperature. The cells were treated with the primary antibodies following a blocking of 3 h at room temperature in IP buffer, 20 mM HEPES pH 7.9, 250 mM KCl, 1% BSA, 0.2% fish skin gelatin (Sigma), 0.1% Triton X-100, as described in the immunohistochemistry section.

Delivery of Recombinant Adenoviruses to Motor Neurons

Injections of adenoviruses were performed in 2-month-old hNF-H +/+ transgenic and normal mice. All surgical procedures were carried out under general anesthesia and in accordance to The Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The recombinant adenoviruses were in 10 mM Tris-HCl buffer solution pH 7.6 at of $3 \times 10^7$ PFU/mL. Ten injections of 2 µL each were performed in the right tibialis muscle. Control injections were done using a solution of 5mg/ml bovine serum albumin (Sigma) in 10 mM Tris-HCl pH 7.6. The mice were killed at 7 days, 14 days, 21 days, or 9 months postinjection and analysed for β-galactosidase activity or for human NF-L expression.

Detection of β-galactosidase Activity

Anesthetized mice were perfused with 50 mL of PBS pH 7.4 followed by 50 mL of phosphate buffered 2% paraformaldehyde pH 7.4. Tissues were dissected and further fixed for 45 min at room temperature in fresh fixative. After 2 washes in PBS pH 7.4, the samples were incubated overnight in a staining solution (1mg/mL X-Gal [Sigma], 5 mM K$_4$Fe(CN)$_6$, 5 mM K$_3$Fe(CN)$_6$3H$_2$O, 2 mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% NP40 in PBS) at 37°C. After 3 brief washes with 3% DMSO in PBS, the samples were immersed in 15% sucrose in PBS for cryosectioning. Cryosections of 15 µm were mounted onto gelatin-coated slides, dehydrated, and counterstained with neutral red.

Immunohistochemistry

Anesthetized animals were perfused with 50 mL of PBS pH 7.4, followed by 50 mL of phosphate buffered 4% paraformaldehyde pH 7.4. The tissues were dissected and further fixed for 2 h to overnight in fresh fixative at 4°C. Samples were sectioned using a vibratome and 25-µm-thick sections were mounted on gelatin-coated slides and permeabilized with 0.3% Triton X-100.

Fig. 3. Restoration of axon calibers by hNF-L coexpression. Light micrographs show transverse sections of L5 ventral roots of the various transgenic mice at 6 months of age. (a), normal; (b), hNF-L+/+; (c), hNF-H++; (d), hNF-L+/+;hNF- H+++; (e), hNF-H++; (f), hNF-L+/+;hNF-H+/-; (g), hNF- L++;hNF-H+/-; (h), hNF-L+/+;hNF-H+/-; Mice overexpressing the hNF-H transgene alone show dramatic decrease in axonal caliber and some degeneration profile (c and e). Coexpression of hNF-L restores axonal calibers (d and f).
in PBS for 5 min at room temperature. Sections were then blocked 2 h in IF buffer (20 mM Hapes pH 7.9, 250 mM KCl, 1% BSA, 0.2% fish skin gelatin (Sigma), 0.1% Triton X-100) and incubated with the primary antibody (same dilutions described in the western blotting section) overnight at 4°C in a humid chamber. Following several washes with IF buffer, the sections were incubated with the secondary antibodies. For indirect fluorescence detection, we used a fluorescein-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc.) and slides were mounted using Slow Fade (Molecular Probes Inc., Eugene, OR). Alternatively, we used secondary biotin-conjugated antibodies and streptavidin-peroxidase system (Vectastain, Vector Laboratories, Burlingame, CA) to obtain a brown precipitate from DAB (3,3'-diaminobenzidine, Sigma).

Electron Microscopy

Anesthetized animals were perfused with 50 mL of PBS pH 7.4, followed by 50 mL of Jones's fixative pH 7.4 (65 mM NaCl, 2.68 mM KCl, 3.26 mM NaH$_2$PO$_4$, 14.42 mM Na$_2$HPO$_4$). The tissues were dissected and further fixed overnight in fresh fixative at 4°C. The samples were postfixed in 2% osmium tetroxide for 2 h and dehydrated in a graded series of ethanol solutions and Epon (Marivac) embedded according to standard protocols. Ultrathin sections were stained with uranyl acetate and lead citrate prior to observation on a Philips CM10 electron microscope (Philips, Eindhoven, Netherlands).

Axonal Transport Study

Two-month-old hNF-H$^+/+$, hNF-L$^+/+$, hNF-H$^+/+$ and normal mice were anesthetized using sodium pentobarbital and 2 mL of PBS containing 300 μCi of 35S-methionine (Mandel, Guelph, Canada) was injected into the ventral horn of the spinal cord at the level of the first lumbar segments. Twenty-eight days after injection, the injected region of the spinal cord, the L5 ventral roots, L5 DRGs, and both sciatic nerves were removed. The ventral roots and DRGs were pooled into 1 fraction, designated vr, corresponding to an axonal length of 12 mm. The sciatic nerves were then cut into 8 segments of 3 mm each, and corresponding segments of the 2 nerves were pooled. Each fraction was homogenized in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 10 mg/mL aprotinin, 2 mg/mL leupeptin, 2 mg/mL pepstatin, and 1% Triton X-100 and Triton-insoluble preparation were obtained as described in protein isolation section. Cytoskeleton-enriched preparations and supernatants were separated on 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue (Bio-Rad). After destaining in 30% methanol, 10% acetic acid, the gels were incubated 30 min at room temperature in Amplify (Amersham). Dried gels were exposed to BioMax MR films (Eastman-Kodak).
RESULTS

Generation of Doubly Transgenic Mice Expressing Human NF-L and NF-H Proteins

By mating mice expressing hNF-H (14) with mice expressing hNF-L (25) proteins, we derived doubly hNF-L/hNF-H transgenic mice heterozygous for each transgene. Further inbreeding of those mice yielded mice heterozygous or homozygous for each transgene. The parental hNF-H (line 200) and hNF-L (line 29) transgenic lines were obtained by the microinjection of genomic fragments encoding the hNF-H or hNF-L gene, flanked by 5' promoter region and 3' sequences. Although the mice were not pure inbred lines, their genetic background was enriched in C57BL/6. The hNF-H overexpression did not result into significant loss of spinal motor neurons and did not affect life span.

In the parental hNF-L and hNF-H lines, we previously reported that levels of transgenes mRNAs were ~3-fold the levels of respective endogenous genes (14, 31). As shown in Figure 1a, the levels of mRNA in the spinal cord of doubly hNF-L/hNF-H transgenic mice (3-month-old) corresponded to those found in singly transgenic mice bearing the hNF-L or hNF-H transgenes alone. The levels of hNF-L or hNF-H transcripts were doubled in mice homozygous for the transgenes, as compared with heterozygous mice.

The increases in hNF-L and hNF-H mRNA levels did not result in comparable increases in protein levels, as shown in Figure 1b and in the Table. Densitometric analyses were performed on the Coomassie stained SDS-PAGE of cytoskeletal protein-enriched preparations from the spinal cord. A 3-fold increase from exogenous mRNAs in mice homozygous for hNF-H (hNF-H+/+) or hNF-L (hNF-L+/+) resulted in protein levels increased to 208 ± 12% for NF-H and 115 ± 3% for NF-L, respectively. We reported previously that in hNF-L transgenic mice, the human NF-L protein species constitutes nearly 80% of the total NF-L protein content in the spinal cord (31). It is noteworthy that in doubly transgenic hNF-L+/+;hNF-H+/+ mice, the levels of foreign proteins were further enhanced with a content in NF-L and NF-H proteins increased to 130 ± 5% and 251 ± 9%, respectively, the levels found in normal mice (Table). This additional increase is likely due to a reciprocal stabilization of additional NF-L and NF-H proteins that are able to form heterodimers (32). The expression of hNF-L and hNF-H species was further confirmed by western blotting, using specific antibodies directed against the human NF-H protein and the human NF-L proteins (Fig. 1b). Whereas the levels of NF-M were down-regulated in transgenic mice expressing the hNF-H proteins, the levels of tubulin and actin remained similar to those of normal mice.

### Table

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EXTRA NF-L PROTECTS AGAINST NF-H MEDIATED PATHOGENESIS

Fig. 6. Restoration of slow axonal transport by hNF-L coexpression. $^{35}$S-Methionine was injected into the spinal cord of 3-month-old animals at the entry point of the L5 ventral root. After 28 days, the L5 ventral roots, L5 DRGs and 8 successive 3 mm segments of sciatic nerves were isolated. The pooled ventral roots and DRGs (lane vr) represent 12 mm of axonal length. As previously reported, the rate of axonal transport is diminished in hNF-H+/+ mice. The axonal transport of cytoskeletal components in doubly transgenic mice is significantly enhanced as compared with hNF-H+/+ mice. Note the transport rates for mouse NF-M (arrow) and tubulin (asterisk) which are restored in the doubly transgenic mice.

Reduction of Perikaryal NF Accumulations in Doubly Transgenic Mice

The spinal cord from 6-month-old transgenic mice was examined by light microscopy (Fig. 2). Mice homozygous or heterozygous for the hNF-H transgene developed abnormal accumulations of NFls in the perikarya and proximal axons of spinal motor neurons (Fig. 2c, e), previously reported in Côté et al. (14). In contrast, the 3-fold increase of hNF-L mRNA in the hNF-L+/+ mice did not lead to abnormal neurofilamentous accumulations in motor neurons (Fig. 2b). Remarkably, the coexpression of hNF-L proteins in the doubly hNF-L/hNF-H transgenic mice reduced dramatically the number and size of perikaryal swellings (Fig. 2d, f, h). These beneficial effects of extra hNF-L proteins are particularly striking when hNF-H+/− mice (Fig. 2c) are compared with hNF-L+/−;hNF-H+/− mice (Fig. 2f) whose spinal cords are virtually devoid of perikaryal swellings, revealing a gene dosage effect. Mice heterozygous for hNF-L and homozygous for hNF-H (hNF-L+/−;hNF-H+/+) developed large perikaryal swellings (Fig. 2g), reminiscent of mice expressing hNF-H alone (Fig. 2c, e). In contrast, no NF inclusions were detected in perikarya of motor neurons from mouse homozygous for hNF-L and heterozygous for hNF-H (hNF-L+/+;hNF-H+/−) (Fig. 2h, small arrows). Yet, hNF-L+/+;hNF-H+/− mice exhibited some giant proximal axons (large arrows). Similar results were obtained with 1-year-old mice of the various genotypes (data not shown).

Axonal Atrophy in hNF-H Mice Is Alleviated by Extra hNF-L

The L5 ventral roots from 6-month-old mice were examined at light microscopy. A dramatic atrophy of motor axons in hNF-H+/+ and hNF-H+/− transgenic mice could be observed (Fig. 3c, e). The axonal atrophy was more pronounced in the hNF-H+/+ (Fig. 3c) than hNF-H+/− (Fig. 3e) animals, emphasizing again the gene dosage effect of the transgenes. In doubly homozygous hNF-L/hNF-H transgenic mice, coexpression of hNF-L restored the radial growth of axons (Fig. 3d, f, h). Remarkably, in the doubly heterozygous hNF-L+/−;hNF-H+/− mice (Fig. 3e) and in the hNF-L+/+;hNF-H+/− mice (Fig. 3h), some ventral root axons of larger than normal caliber were observed. To quantify the changes of axonal calibers, cross-sectional areas of L5 ventral root axons were analyzed using morphometric software (Image 1, Universal Imaging Corp.). Normal mice and hNF-L mice showed a bimodal distribution of axonal calibers with peaks at 2–3 μm and 7–8 μm (Fig. 4a). In contrast, no bimodal distribution and a significant increase in the percentage of small axons were observed in mice expressing hNF-H alone (Fig. 4b, note Y-scale differs from a). The coexpression of hNF-L together with hNF-H restored the radial growth of axons. Thus, the bimodal distribution of axonal calibers was completely reestablished in the doubly heterozygous hNF-L+/−;hNF-H+/− mice.

Fig. 7. Rescue of abnormal hindlimb contracture. (a) Normal mice extend their legs when lifted by their tail whereas hNF-H overexpressing mice contract their hindlimbs. (b) In contrast, mice overexpressing both human NF transgenes extend their limbs like normal mice.
In addition, a rescue of axonal atrophy in the hNF-L+/+;hNF-H+/+ mice (Fig. 4b) and the hNF-L+/−;hNF-H+/+ mice (Fig. 4d) could be detected, albeit the bimodal distribution was not fully recovered (Fig. 4b). Interestingly, the frequency of axons with diameters exceeding 13 μm was significantly increased for some doubly transgenic mice (Fig. 4c, d).

Integrity of Axonal Cytoskeleton Recovered in Doubly Transgenic Mice

The L5 ventral roots of 6-month-old animals were analyzed by electron microscopy (EM). Transverse sections of large motor axons from normal mice revealed an abundance of NF profiles (Fig. 5a). In motor axons of hNF-L+/+ mice, an increased density of NFs as compared with normal could be observed (Fig. 5b). In contrast, in homozygous or heterozygous hNF-H transgenic mice, (Fig. 5c, e), the cytoskeleton was markedly perturbed and the number of intact NF structures was reduced dramatically. In hNF-H+/+ mice at 12 and 24 months of age, EM revealed in these shrunken axons a general disruption of the NF network and fewer microtubules, as compared with younger animals (24). Consistent with the above morphometric data, the coexpression of hNF-L with hNF-H led to the re-establishment of a normal cytoskeleton in axons from young and old mice. The expression of hNF-L in hNF-L+/−;hNF-H+/− mice (Fig. 5f) and in hNF-L+/+;hNF-H−/− mice (Fig. 5h) restored a normal NF density and distribution across the axoplasm (Fig. 5d, f). It is remarkable that a relatively low protein ratio of NF-L to NF-H in hNF-L+/−;hNF-H+/+ transgenic mice was sufficient to dramatically improve the cytoskeletal integrity in motor axons (Fig. 5g). Analysis of 12-month-old animals yielded similar results (data not shown).

Improved Axonal Transport in Doubly Transgenic Mice

Defects in axonal transport have been proposed to underlie the pathogenic mechanism in hNF-H transgenic mice (Collard et al, 1995). Therefore, the rate of transport of cytoskeletal proteins into axons of 2-month-old doubly transgenic mice was studied by monitoring radiolabeled, slowly transported polypeptides, 28 days after the injection of 35S-methionine into the spinal cord. In each segment along the length of the sciatic nerve (c.f. Materials and Methods), the transported radiolabeled polypeptides present in the Triton-insoluble fraction were loaded onto SDS-PAGE and analyzed by fluorography. Whereas normal mice showed a leading peak corresponding to a transport rate of ~0.75 mm/day for the 3 NF subunits (Fig. 6), axonal transport was impaired in hNF-H+/+ mice with a leading peak for NF-L and NF-M corresponding to an axonal transport rate of ~0.64 mm/day (see arrows). The transport of tubulin was also altered in hNF-H+/+ mice with leading edge at ~0.96 mm/day instead of ~1.18 mm/day in normal mice (Fig. 6, asterisk). The coexpression of hNF-L enhanced the anterograde axonal transport rate, not only for NF proteins, but also for tubulin, with transport rate of ~0.86 mm/day and ~1.18 mm/day, respectively (Fig. 6, bottom panel).

No Overt Phenotypes in Mice Coexpressing Human NF-L and NF-H

Whereas hNF-H+/+ and hNF-H−/− mice acquired progressive motor dysfunction and weaknesses, mice coexpressing hNF-L and hNF-H did not develop overt clinical symptoms. Moreover, they rarely exhibited the hind limb contraction reflex, characteristic of motoneuronal disorders, observed in mice expressing the hNF-H transgene alone (Fig. 7). We also noted that the hNF-H+/+ mice loose body weight during aging. At 1 year of age, the hNF-H+/+ mice have a weight of 31.5 ± 3.6 g (n = 12) instead of 48.5 ± 5.2 g (n = 10) for normal mice. At the same age, the hNF-L−/+;hNF-H+/+ mice had an average body weight of 39.0 ± 4.6 g (n = 9), which is closer to normal.

Reduction of Perikaryal Swellings Using Adenoviral Vectors Expressing hNF-L

To further examine whether neurofilamentous inclusions can be reduced after their formation in adult mice, we tested a gene delivery protocol with a recombinant adenovirus coding for hNF-L in the hNF-H+/+ transgenic mice. Adenoviral vectors have previously been used with success for gene delivery to motor neurons (33, 34). A replication-defective adenoviral vector containing an expression cassette for the hNF-L gene under the control of its own promoter (Ad5-hNFL) was created. Cultures of dissociated spinal cord from hNF-H+/+ embryos were used to demonstrate the cell-specificity of the Ad5-hNFL virus. In this in vitro assay, expression of the hNF-L gene was restricted to neuronal cells following infection with Ad5-hNFL (Fig. 8c). In contrast, following infection with another viral vector encoding the bacterial
expression in hNF-H+/+ mice spinal cord ipsilateral to the injection. 21 days after injection of Ad5-hNFL into the right tibialis muscle. (e), No hNFL expression could be detected in the spinal cord contralateral to the injection side. (f), Toluidine blue stained Epon sections show a reduction in the number of perikaryal swellings in the ipsilateral spinal cord (arrows) when compared with (g) the noninjected contralateral side. The hNFL proteins in spinal motor neurons could be detected 9 months after muscular virus injection. At this time point, the motor neurons expressing hNFL had no neurofilamentous swelling (d, insert), unlike motor neurons on the contralateral side (e, insert). Bars = 50 μm.
LacZ gene under the control of the cytomegalovirus promoter (Ad5-CMV-LacZ) resulted in the production of β-galactosidase in the various cell types present, including non-neuronal cells (data not shown).

We then tested different approaches for the in vivo gene transfer to motor neurons. We first tested the direct injection of the viruses into the spinal cord. Although Terada et al. (35) reported the successful injection of adenoviral vectors directly into DRG neurons, this approach yielded very low gene transfer efficiency in anterior horn motor neurons. We have also considered the direct injection of viruses into sciatic nerve but we discovered that nerve injury itself could cause a reduction of perikaryal swellings in the spinal cord. One week after crush axotomy of the sciatic nerve of hNF-H+/+ mice by the method described previously (Zhu et al. [5]), the number and size of perikaryal swellings were dramatically reduced on the crush side of the spinal cord (data not shown). A down-regulation of NF gene expression after nerve damage (36, 37) was likely responsible for the reduction of NF swellings.

As an alternative, we then established an in vivo gene transfer protocol to motor neurons by injecting Ad5-CMV-LacZ viruses into the right tibialis muscle of hNF-H+/+ mice. This approach, which is based on the retrograde axonal transport from nerve endings of virus particles to the nucleus of spinal motor neurons, offers many advantages. For gene therapy, it is less invasive and more reliable than direct virus injection into nervous tissue. No obvious cytotoxic effect due to viral infection could be detected and no inflammatory reaction leading to destruction of motor neurons was triggered by muscle injection. Moreover, as described below, long-term expression of foreign gene was also observed with the use of the hNF-L gene promoter. As shown in Figure 8, muscle injection of the Ad5-CMV-lacZ viruses resulted in a robust and specific expression of β-gal in motor neurons innervating the injected muscle. These experiments also revealed that the viral infection and the CMV-driven expression of lacZ did not reduce the neurofilamentous swellings in hNF-H+/+ mice (Fig. 8a, b). At 7 days post-injection, we noticed that normal mice showed prominent X-gal staining of motor neuron perikarya, whereas the hNF-H+/+ mice had limited number of positive cells with staining restricted to a spotty pattern. The poor β-gal staining in the hNF-H+/+ mice observed at a relatively short time interval after viral injection probably reflects an impairment of retrograde axonal transport, as a consequence of NF accumulations.

We injected the Ad5-hNFL viral vector into the right tibialis muscle of NF-H+/+ mice and examined the spinal cord of these animals 21 days postinjection. As shown in Figure 8, the immunodetection of hNF-L proteins using a specific anti-human NF-L antibody (DP5-112) occurred only in spinal motor neurons ipsilaterally to the injected side (Fig. 8d, e). Moreover, no perikaryal swellings occurred in the hNF-L-positive motor neurons. Thin sections of Epon-embedded spinal cord further demonstrated that the number of neurofilamentous swellings in the perikary of motor neurons were reduced in the spinal cord ipsilateral to the Ad5-hNFL injected side, as compared with the noninjected contralateral side (Fig. 8f, g). Moreover, we demonstrated that the Ad5-hNFL vector is suitable to direct a long-term expression of hNF-L protein. The insert in Figure 8d shows detection of hNF-L proteins in a motor neuron of hNF-H+/+ mice 9 months after muscular injection of the viruses.

DISCUSSION

The results reported here demonstrate that extra hNF-L proteins can suppress motor neuron disease caused by the overexpression of hNF-H proteins in transgenic mice. When overexpressed alone, the hNF-H transgene induces a neuropathy characterized by the perikaryal accumulation of NFs and impairment of axonal transport (24). However, the coexpression of the hNF-L transgene reduced in a dose dependent fashion these perikaryal accumulations caused by hNF-H overexpression. Since the hNF-H protein levels in doubly hNF-L-hNF-H transgenic mice were higher than those found in the parental hNF-H transgenic mice, we conclude that increasing the ratio of NF-L to NF-H subunits can alleviate the toxicity of hNF-H.

The scarcity of perikaryal swellings in doubly hNF-L-hNF-H transgenic mice (Fig. 3) indicates that a correct ratio of NF-L to NF-H subunits is required for efficient translocation of newly synthesized NF proteins into the axonal compartment. It also suggests that NF subunits are translocated from the perikarya to the axons as hetero-oligomeric structures. While the mechanism of NF transport is a controversial issue, many lines of evidence suggest that NFs are highly dynamic structures (38, 39). Whether NF-L and NF-H are translocated into axons as dimers, tetramers, or even larger oligomeric structures remains unclear. However, the NF-L and NF-H subunits have been reported to dimerize in vitro (32) and the existence of tetramers containing NF-L and NF-H have also been demonstrated (3). Moreover, recent studies on NF-M knockout mice revealed that NF-L and NF-H proteins are able to form NF structures in absence of NF-M (6, 40). This suggests that extra NF-L and NF-H proteins in the doubly hNF-L-hNF-H transgenic mice can assemble and form additional NF structures whose dynamics allow an efficient translocation of subunits into axons. Our axonal transport studies revealed that NF protein transport was essentially restored to normal values in doubly hNF-L-hNF-H mice, as compared with the parental hNF-H mice. Furthermore, other axonal components, such as tubulin, were also transported at greater rates in the doubly hNF-L-hNF-H transgenic mice than in the
hNF-H mice, supporting the view of an improved distal delivery of material required for maintenance of axonal integrity.

Our results also demonstrate that the coexpression of hNF-L together with hNF-H proteins in an adequate ratio can restore a normal NFs density and radial growth of axons (Fig. 4). An increase in axonal NF content would explain why some motor axons in doubly hNF-L:hNF-H transgenic mice exceeded by few micrometers the caliber typically found in normal mice. The axonal calibers increased similarly in transgenic mice coexpressing mouse NF-L with either mouse NF-M or NF-H subunits (41). It is noteworthy that a large excess of each subunit alone does not increase the caliber of axons (14, 15, 17). Overexpression of hNF-H alone caused axonal atrophy, while a modest up-regulation of NF-L alone increased the NF density with no effects on axonal calibers (Figs. 3, 5).

The data presented here emphasize the importance of subunit stoichiometry in NF functions and neuronal integrity. This may be of relevance to ALS since there is evidence of perturbations in the normal metabolism of NFs in this disease. The abnormal accumulation of NFs is frequently observed within degenerating neurons in ALS (for review see 42). Moreover, Bergeron et al (1994) reported a 60% decrease in the levels of NF-L mRNA in motor neurons of ALS patients. It is noteworthy that neurons with spheroids exhibited greater reduction (~87%) in NF-L mRNA levels. It would be of particular interest in future studies to determine whether changes in the ratio of NF proteins do occur in ALS. Our findings raise the possibility that a decreased ratio of NF-L to NF-H mRNAs might exacerbate disease by causing NF depositions, axonal transport defects, and axonopathy. In any case, the finding of mutations in the hNF-H gene of a small number of ALS patients provided compelling evidence for NF involvement in this disease. To date, the combined results from 3 different groups revealed the presence of deletion or insertion of codons in the hNF-H gene of 9 ALS cases, including a familial case (21–23). The results suggest that subtle changes in the NF-H tail structure can be a primary event in ALS. In this regard, it is noteworthy that the hNF-H, when expressed alone, is acting as a mutant protein in the murine cellular environment, as suggested previously (19). This likely reflects the sequence disparities between the tail domains of the human and mouse NF-H proteins. In any case, our results suggest that NF-L overexpression may provide a suitable approach to protect against disease due to NF-H abnormalities in ALS. A gene delivery approach based on muscle injection of recombinant adenoviruses would offer a means to up-regulate the NF-L levels in a sustained manner and hence slow down disease progression.

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