Expression of the human neurofilament heavy gene (NF-H) in transgenic mice

Francine Côté

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Department of Neurclogical Sciences
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Francine Côté
Ph.D. 1994
Department of Neurological Science
McGill University

Abstract

Mammalian neurofilaments (NFs), the intermediate filaments (IFs) of nerve cells, are composed of three different polypeptides, commonly referred to as the low (NF-L), middle (NF-M) and heavy (NF-H) subunits. As members of the IF family and major components of large myelinated fibers, NFs have been assigned a putative role in maintaining the structural integrity of the neurons and in determining axonal caliber.

To study the expression and function of the human NF-H gene, a genomic clone encompassing the human NF-H gene was isolated (Cos4NF-H) from a chromosome 22 enriched library and transgenic mice were generated. Four transgenic lines with multiple copies of the intact human NF-H gene were obtained. Northern blot analysis revealed that the human transgene was expressed specifically in neurons at level of approximately two-fold the endogenous mouse level. In addition, the human NF-H gene was expressed at the correct stage during the mouse nervous system development. Furthermore, expression of the human NF-H gene appeared to be proportional to gene copy-number and independent of the position of integration into the mouse genome. This is the first example of such a pattern of transgene expression in nerve cells. These results suggest that the regulatory signals necessary to direct the correct expression of the human NF-H gene lie within the injected clone and are recognized between the two mammalians species. Once identified, the control elements may constitute a valuable tool to direct the expression of foreign genes specifically to neurons.

The human NF-H transgenic mice appear normal at birth but progressively develop tremors and pathological features reminescent of the ones observed in patients with amyotrophic lateral sclerosis (ALS). For instance, one sees selective enlargements of motor neuron perikarya, dorsal root ganglion (DRG) neurons and proximal axons in addition to distal axonal atrophy and muscle atrophy. Electron microscopy studies showed that the swellings consisted of intracytoplasmic accumulation of NFs. Axonal

transport experiments were undertaken to determine if cytoskeletal elements were abnormally transported as has been suggested for a number of neurodegenerative diseases such as ALS. The proteins synthesized in motor/sensory neuron cell bodies were labeled and their subsequent transport to distal axonal segments was analyzed by SDS gels and fluorography. The results showed an impairment of axonal transport of NF proteins, actin and tubulin. There was also retention of some rapidly transported organelles. A possible mechanism to explain these results is that overexpression of the human NF-H gene led to neurofilamentous swelling formation that disrupted axonal flow and eventually resulted in motor neuron degeneration. The human NF-H transgenic mice are therefore a valuable model of human motor neuron disorders and can be used to test therapeutic strategies aimed at reducing the neurofilamentous swellings.

Expression du gène de neurofilament humain NF-H chez les souris transgéniques

Francine Côté
Ph.D. 1994
Faculté des études supérieures
Département des Sciences Neurologiques
Université McGill

Résumé

Les neurofilaments (NF), les filaments intermédiaires (FI) spécifiques aux neurones sont constitués de trois sous-unités; légère (NF-L), moyenne (NF-M) et lourde (NF-H). En tant que membres de la famille des FI et constituants majeurs des grosses fibres nerveuses myélinisées, les NFs auraient pour rôle l'intégration mécanique de l'espace intracellulaire. De plus, les NF seraient impliqués dans la détermination et/ou le maintien du calibre des axones (Hoffman et al., 1984, 1987).

Afin de préciser les niveaux d'expression et la fonction du gène humain de la sous-unité lourde des NF (NF-H), un fragment génomique contenant le gène humain NF-H (Cos4NFH) fut isolé d'une banque de cosmides enrichie en séquences du chromosome 22 et inséré dans des souris. Quatre ligneés murines contenant des nombres différents de copies du gène humain NF-H ont ainsi été obtenues. Des analyses par Northern ont démontré que le gène humain NF-H était spécifiquement exprimé dans les tissus nerveux de la souris et que les niveaux d'expression étaient supérieurs au niveau du gène endogène (environ deux fois). De plus, le gène humain NF-H est exprimé correctement au cours du dévelopment. Finalement, l'expression du gène humain NF-H semble être dépendante du nombre de copies intégrées mais indépendante du site d'intégration dans le génome de la souris. Ces résultats suggèrent la présence, dans le cosmide Cos4NF-H, de facteurs importants qui sont reconnus par le système transcriptionnel de la souris. Lorsqu'identifiés, ces facteurs pourront servir à diriger l'expression d'autres gènes et ce de façon spécifique.

Bien que les souris exprimant le gène humain NF-H soient normales à la naissance, elles développent progressivement des tremblements et des pathologies semblables à celles que l'on retrouve chez les patients atteints de la sclérose latérale amyotrophique (SLA). Par exemple, des gonflements au niveau des corprs cellulaires

des neurones moteurs et des ganglions de la racine dorsale ainsi qu'au niveau des axones proximaux. Il y a également atrophie des axones distales et des fibres musculaires. L'analyse des tissus en microscopie électronique a révélé que ces gonflements étaient constitués de NF. Des études de transport axonal furent alors entreprises afin de déterminer si certains éléments du cytosquelette étaient anormalement transportés tel qu'il fut suggéré pour un certain nombre de maladies neurodégénératives incluant la SLA. Les protéines synthétisées dans les neurones moteurs et sensoriels furent marquées et leur transport le long de l'axone fut étudié sur gels polyacrylamide et fluorographie. Les résultats obtenus montrent que le transport des protéines de NF ainsi que celui de tubuline et d'actine est retardé. Il y a également rétention de certaines protéines transportées rapidement. L'apparition de gonflements qui résultent de l'accumulation des NF au niveau des corps cellulaires et des axones proximaux interfèrent avec le transport normal le long de l'axone et peut éventuellement mener à la mort des neurones moteurs. Les souris transgéniques exprimant le gène humain NF-H constituent donc un modèle unique de maladies dégénératives humaines et sont un outil indispensable pour tester différentes stratégies visant à diminuer les accumulations de NF.

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Abbreviations

ALS	amyotrophic lateral sclerosis
ATP	adenosine triphosphate
cAMP	adenosine 3',5'-cyclic monophosphate
BDNF	brain derived neurotrophic factor
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DNA	deoxyribonucleic acid
DMHD	3,4-dimethyl-2,5-hexanedione
DRG	dorsal root ganglion
EBS	epidermolysis bullosa simplex
EH	epidermolytic hyperkeratosis
FALS	familial amyotrophic lateral sclerosis
GAN	giant axonal neuropathy
GAP	growth-associated protein
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HCSMA	hereditary canine spinal muscular atrophy
2,5-HD	2,5-hexanedione
HRP	horse radish peroxidase
HS	hypersensitive site
IDPN	β, β'-iminodipropionitrile
IF	intermediate filament
K	keratin
kb	kilobase
kDa	kilodalton
LCR	locus control region
LIF	leukemia inhibitory factor
NF	neurofilament
NF-L	neurofilament light subunit
NF-M	neurofilament middle subunit
NF-H	neurofilament heavy subunit
NGF	nerve growth factor
MAP	microtubule-associated protein
3-MHD	3-methyl-2,5-hexanedione
mm	millimeter
Mnd	motor neuron degeneration mouse
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PKA/C	protein kinase A/C
PNS	peripheral nervous system
mRNA	messenger ribonucleic acid
SCa/b	slow component a/b
SDS	sodium dodecyl sulfate
SOD1	superoxide dismutase 1

Chapter I

Introduction

Most eukaryotic cells have an intricate cytoplasmic network of protein filaments. This network has been referred to as the cytoskeleton. The cytoskeleton serves as an intracellular framework and is responsible for most aspects of cell shape and movement (Alberts et al., 1989).

1. The neuronal cytoskeleton

The fundamental task of nerve cells is to receive, process and transmit signals. To perform these functions, nerve cells are branched structures with a central body or soma, several dendrites and a single axon. Dendrites, which extend from the cell body like antennae, receive information from other neurons. The axon, which may extend from a few millimiters (mm) up to a meter, eventually ends in many branches and serves as a transmission line from the soma. Dendrites, soma and axon assume characteristic shapes. The structure responsible for the organization and maintenance of these different shape is the neuronal cytoskeleton. The neuronal cytoskeleton is also involved in intra-axonal movement and synaptic transmission. It can be divided into three types of fibrillar elements varying in thickness; microtubules, microfilaments and intermediate filaments (Figure 1) (Burgoyne, 1991; Hirokawa, 1991).

1.1 Microtubules

Microtubules are hollow tubes built from dimers of α and β tubulin subunits. In *in vitro* preparations, the subunits first assemble themselves in strands and then in circular tubules with an outside diameter of 25-28 nanometers (nm). *In vivo*, microtubules are apparently in equilibrium with soluble tubulin subunits and can be readily assembled and disassembled. In normal axons, microtubules appear in groups and tend to form small bundles interspersed with neurofilaments. Microtubules are also found in all neuronal dendrites (Goldman and Yen, 1986; Hirokawa, 1991).

Electron microscopic studies have revealed regularly spaced fine strands on microtubules which are formed by microtubule-associated proteins (MAPs). MAPs serve both to stabilize microtubules against disassembly and to interconnect them with other cell components (Hirokawa, 1991). MAP1A, 1B, 1C, MAP2, 2C and Tau proteins are the major species isolated from mammalian brain in association with microtubules. MAP2 and 2C are components of cross-bridges between microtubules in dendrites and in axons respectively (Chen et al., 1992).

Microtubules are believed to be tracks along which vesicles and other membrane-bounded organelles are transported during bidirectional axonal flow (Vale et al., 1985b).

1.2 Microfilaments

Microfilaments are 5-7 nm in diameter. They are polymers of globular actin monomers (β-γ species) wound into a two-stranded helix. In neurons, actin exists mostly as unpolymerized or soluble monomers in a dynamic equilibrium with polymerized actin. In dendrites, much of it is concentrated at spines. In the axon and at nerve terminals, actin microfilaments are numerous and closely packed in a lattice underneath the axolemma. (Goldman and Yen, 1986; Hirokawa, 1991; Nixon, 1991).

Actin filaments accumulate in the growth cone of developing nerve cells and are possibly involved in their motility (Hirokawa, 1991).

1.3 Intermediate filaments

Unlike actin and tubulin, which are both globular proteins, the subunits of intermediate filaments (IFs) are intracellular fibrous proteins. IF protein molecules associate side by side to form long fibers in the cytoplasm of most higher eukaryotic cells. They are typically between 8 and 10 nm in diameter (Steinert and Roop, 1988)

Figure 1. Axoplasm in a rat sciatic nerve. Microtubules appear in groups interspersed with neurofilaments. Frequent cross-bridges are found between microtubules (arrows) and neurofilaments (curved arrow). (Reproduced from The Journal of Cell Biology (1988) 107, 1449-1461, by copyright permission of The Rockefeller University Press).

1.3.1 Family of Intermediate filaments

Intermediate filaments are encoded by a large multigene family displaying cell and tissue-specific expression patterns throughout development (Table I). Several different classes of IF have been recognized based upon sequence homology within the relatively conserved central rod domain and placement of introns within the genome (Steinert and Roop, 1988; Bloemendal and Rieper, 1989; Klymkowsky et al., 1989; Stewart, 1990).

Type I and II IF proteins, acidic keratins (40-52 kDa) and neutral or basic keratins (53-70 kDa) respectively, are found primarily in epithelial cells. The keratin are by far the most complex class of IF proteins (Fuchs and Hanukoglu, 1983; Steinert et al., 1984; Albers and Fuchs, 1987; Steinert and Roop, 1988).

Type III IF proteins include 1) vimentin (53 kDa), 2) desmin (52 kDa), 3) glial fibrillary acidic protein (GFAP) (45-50 kDa), 4) peripherin (57 kDa) and 5) plasticin (52 kDa). Vimentin is widely distributed in cells of mesenchymal origin, is often expressed by cells in culture and transiently by developing cells (Steinert and Roop, 1988). Desmin is found in both striated and smooth muscle cells. GFAP forms glial filaments in astroglia (Steinert and Roop, 1988). Peripherin is abundant in nerve cells of the peripheral nervous system (PNS) (Parysek et al., 1988; Greene, 1989). Plasticin was recently identified in regenerating fish optic nerve (Glasgow et al., 1992).

Type IV IF proteins include 1) α-internexin (66 kDa) (Fliegner, 1990), found mostly in embryonic neurons of the central nervous system (CNS) and 2) the neurofilament (NF) triplet proteins NF-L, NF-M and NF-H (about 68, 145 and 200 kDa respectively) present in most nerve cells of the PNS and CNS (Steinert and Roop, 1988).

Type V IF proteins are the nuclear lamins A, B and C (65-75 kDa). They form a lattice of filaments on the inner side of the nuclear membrane (Steinert and Roop, 1988). They are the only nuclear IF proteins, all others are cytoplasmic.

Type VI IF proteins include nestin (200 kDa) specifically expressed in neuroepithelial stem cells (Lendahl et al., 1990).

Table I

Classes of intermediate filament proteins

Class	Protein Names	Distribution
I	Acidic keratins	Epithelia
II	Basic/neutral keratins	Epithelia
III	Desmin	Muscle
	Vimentin	Mesenchyme
	Glial fibrillary acidic protein (GFAP)	Glia and astrocytes
	Peripherin	PNS neurons
	Plasticin	Regenerating retinal ganglion cells
IV	Neurofilaments: NF-L, NF-M, NF-H	CNS - PNS neurons
	Alpha-internexin	Embryonic CNS neurons
V	Lamins: A, B, C	Nuclear envelopes
VI	Nestin	Neuropithelial stem cells

1.3.2 Structural features and assembly of intermediate filaments

Despite the large differences in their size, sequence data revealed that each IF polypeptide chain has a three-domain structure made of a homologous central region of similar size (about 310 residues for cytoplasmic IFs; 356 residues for lamins) flanked by amino- and carboxy-terminal domains varying greatly in sequence and in length (Figure 2). In fact, the head and tail domains are unique to each protein and presumably confer specialized properties (Steinert and Roop, 1988; Klymkowsky et al., 1989).

The central region of all IF proteins forms an extended α-helical rod domain (typically referred to as coil 1A, 1B, 2A, 2B) with three short non α-helical breaks (referred to as L1, L2, L12). The helical domain has a distinctive sequence motif of a polypeptide that forms a coiled-coil. The sequence is a heptad repeat " a, b, c, d, e, f, g " where "a" and "d" are frequently apolar residues and amino acids in other positions are hydrophilic or charged residues. Residues "a" and "d" lie close together forming a stripe running around the α-helix. Consequently the coiled-coil is formed when two helices wrap around each other with their respective hydrophobic side chains facing each other (Steinert and Roop, 1988). Pruss et al. (1981), have described an antibody which is known to bind an epitope localized at the carboxy-terminal end of coil 2. As all IF proteins share this identical conserve sequence motif, reactivity with this antibody is one of the criteria for identification of new IF proteins.

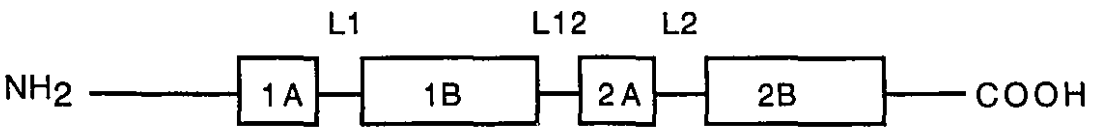
The conserved α-helical regions of all IF subunits form the basis of their structural uniformity. In particular, the amino- and carboxy-terminal sequences of the rod domain are highly conserved and play a critical role in IF assembly (Albers and Fuchs, 1987; Wong and Cleveland, 1990; Hatzfeld and Weber, 1992). For example, deletions including small portions of the helical rod domain of keratin K14 resulted in a mutant protein which could no longer be incorporated into the IF network (Albers and Fuchs, 1987; Stewart, 1990). Moreover, domain swapping experiments revealed that the carboxy-end of helix 1B was essential for subunit recognition and proper co-assembly of vimentin and keratin filaments in transfected mammalian cells (McCormick et al., 1991). In contrast, most sequences within the non-helical head and tail domains of IF proteins seem to be dispensable for IF assembly (for review see Fuchs and Weber, 1994 and reference therein). Nevertheless, the IF head domain possibly plays a role in both end to end and lateral association at the protofilament and/or protofibrillar level

while the tail domain seems to be involved in the control of lateral interaction between subunits (Fuchs, 1994; Fuchs and Weber, 1994).

The mechanism leading to polymerization of IF proteins is complex and still not completely understood. A current model of IF assembly involves 1) formation of a coiled-coil dimer from two monomers via the conserved α-helical regions aligned in parallel, 2) aggregation of two antiparallel dimers to form a tetramer or protofilaments and 3) association of the protofilaments into higher ordered structures. The final 10 nm filament is composed of eight protofilaments joined end on end to neighbors by staggered overlaps, where the non-helical end domains protrude from the filament backbone (Bloemendal and Pieper, 1989; Klymkowsky et al., 1989).

Most IF proteins, except the keratins and perhaps NF-M and NF-H will assemble spontaneously *in vitro* to form homopolymers. Epithelial keratins are obligatory heteropolymers. Evidence suggests that keratin filaments are formed from an equal number of acidic and basic or neutral subunits (Steinert et al., 1985; Klymkowsky et al., 1989; Stewart, 1990). Although NF-M and NF-H, by themselves, may not be competent for 10 nm filament formation, homodimers of NF-M and NF-H have been obtained under specific experimental conditions (Hirokawa, 1991).

Co-assembly between different IF proteins is possible and has been reported. Transfection studies of fibroblast cells demonstrated that all three NF subunits readily incorporate in IF-like arrays with endogenous vimentin (Monteiro and Cleveland, 1989; Chin and Liem, 1990, 1991). Alpha-internexin is also capable of co-assembly with vimentin to form a filamentous network in cell lines lacking an endogenous cytoplasmic IF network (Ching and Liem, 1993).



1A, 1B, 2A, 2B : Alpha-helical domains

L1, L12, L2 : Non alpha-helical domains

Figure 2. Structure of an intermediate filament protein.

1.3.3 Neurofilaments

Neurofilaments are stained with silver nitrate and correspond to the classic neurofibrils observed under light microscopy (Shaw, 1991). Early studies of axoplasmic flow led to the identification ot the three fibrous proteins as the neurofilament subunits: NF-L (light), NF-M (medium) and NF-H (heavy) (Hoffman and Lasek, 1975). The study also revealed the relative proportion of NF-L, NF-M and NF-H in the slow component triplet to be 10:5:3 or 6:2:1 (NF-L: NF-M: NF-H) (Hoffman and Lasek, 1975; Nixon and Lewis, 1986). In mammalian neurons, the three subunits co-polymerize and are distributed differentially. Axonal regions contain more NFs than dendritic and cell body regions. Electron microscopic studies revealed that NFs are found in bundles and run longitudinally and parallel with each other in the axon. They are the predominant intracellular structures of large myelinated fibers. Thin processes extending from NF cores were observed forming bridges between adjacent filaments and microtubules (Figure 1) (Hirokawa, 1982, 1986, 1991; Hisagana and Hirokawa, 1988).

There appear to be other IF proteins capable of incorporating into the IF network of cells of neuronal lineage: vimentin, nestin, peripherin, α-internexin and plasticin. However, for the purpose of this thesis and because the triplet NF-L, NF-M and NF-H are the major IF components of mature neurons, the terminology "neurofilament" will refer to assemblies of the triplet protein.

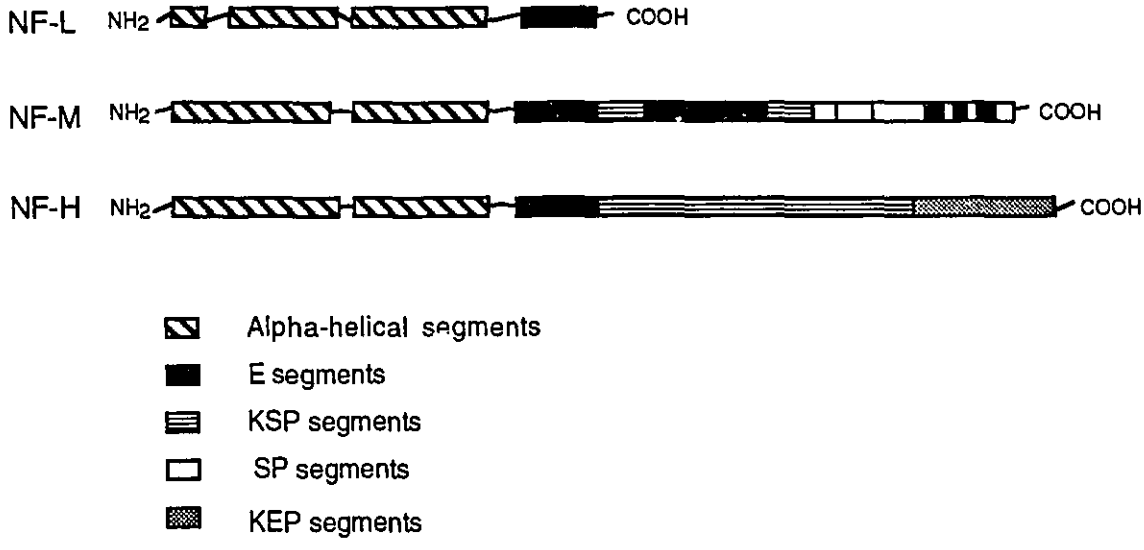


Figure 3. Structure of mammalian neurofilament proteins.

1.3.3.1 Neurofilament genes

The genes encoding non-neurofilament IF proteins share identical or near identical structures with respect to the placement of their introns. In contrast, placement of introns in NF genes occurs at entirely non-homologous locations to other IF genes (Julien and Grosveld, 1991). Human and mouse NF proteins are encoded by three different genes. There are similarities of intron positioning between the three NF genes. Human and mouse NF-L and NF-H genes have three introns and NF-M has two.

The differences in the position and number of introns between NF and other IF genes suggested that the primordial NF gene evolved from an RNA-mediated transposition event with the subsequent gain/loss of introns (Lewis and Cowan, 1986; Julien and Grosveld, 1991). It is clear, however, from their unique exon/intron organization, that genes encoding NF-L, NF-M and NF-H are derived from a common ancestor (Levy et al., 1987; Julien et al., 1988; Julien and Grosveld, 1991).

The genes encoding NF-L and NF-M are located some 30 kb apart on chromosome 14 in the mouse and on chromosome 8 in humans (Julien et al., 1986, 1987a). The NF-H gene is on mouse chromosome 11 and human chromosome 22 (Mattei et al., 1988; Lieberburg et al., 1989).

1.3.3.2 Chemical and immunological characterization of neurofilament proteins

The apparent molecular weights of NF-L, NF-M and NF-H as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are 68, 145 and 200 kDa respectively (Hoffman and Lasek, 1975; Liem et al., 1978; Julien and Mushynski, 1982). Complete sequence data predicted that mouse NF-L is actually 62 kDA, NF-M; 96 kDa and NF-H; 116 kDa, while human NF-L, NF-M and NF-H are 62, 102 and 110 kDa respectively (Lewis and Cowan, 1986; Julien et al., 1986, 1987a, 1988; Levy et al., 1987; Lees et al., 1988). The discrepancy is due to the unusual and unique structure of their carboxy-terminals (Shaw, 1991). Despite this difference, the three NF subunits share the highly conserved α-helical central domain common to all IF proteins.

The NF tail domain can be divided into specific regions (Figure 3). First, a short domain (tail A) is found immediately carboxy-terminal to the α-helical coil domain in the three subunits. This domain contains proline residues (one in NF-L, several in NF-M and NF-H) and is neutral or slightly basic (Geisler et al, 1983; Shaw, 1991). The next region has a glutamic acid rich segment and is referred as the E region. The length of the E segment is 50 and 60 amino acids in mammalian NF-L and NF-H subunit respectively and consists of two segments of 50 and 80 amino acids in NF-M, referred as E1 and E2 (Shaw, 1991). This region represents the entire carboxy-terminus of NF-L, whereas regions with repeated peptides containing the sequence Lysine-Serine-Proline (KSP) are found next to the E segment in NF-M and NF-H. There are 43-44 and 51 of these repeats in human and mouse NF-H respectively (Julien et al., 1988; Lees et al., 1988; Figlewicz et al., 1993). NF-M contains two different regions of KSP sequences separated by the E2 segment. Human and mouse have respectively 1 and 2 KSP sequences in the first region and 12 and 2 in the second region (Levy et al., 1987; Myers et al., 1987). A final stretch of lysine, glutamic acid and proline (KEP) immediately follows the last KSP sequence at the extreme carboxy-terminus of NF-H. Carboxy-terminal to the KSP2 region in NF-M are the SP and KE sequences (Harris et al., 1991; Shaw, 1991).

1.3.3.3 Neurofilament assembly

It is still not clear how the triplet of proteins is organized into NF. Quick-freeze, deep-etch technique coupled with rotary shadowing, assembly studies and antibody decoration experiments (against the three NF polypeptides) have provided some insights (Hirokawa, 1982, 1986, 1991). NF-L comprises most of the backbone of the filament. The rod domain of NF-M and NF-H co-assemble with NF-L into the backbone whereas their carboxy-terminal domains were observed as thin, flexible filaments projecting from the core (Hirokawa, 1982, 1986, 1991). These filaments are believed to be components of cross-bridges between microtubules and NFs (Hirokawa, 1982; Hisagana and Hirokawa, 1990).

Since all three NF proteins contain the conserved α-helical rod domain important for filament assembly, it was surprising to find that only NF-L could easily polymerize into 10 nm filaments *in vitro.* However no prominent projections were observed. *In vivo,* NF-L alone cannot form a filament, but aggregates into a punctate

structure (Lee et al., 1993). NF-M by itself can form a 10 nm filament under specific experimental conditions, while NF-H can only assemble into curly, non filamentous aggregates *in vitro* (Giesler and Weber, 1981; Gardner et al., 1984; Hirokawa, 1991). Nevertheless, *in vitro* experiments have demonstrated that NF-M and NF-H are readily incorporated into a filament of normal morphology when mixed with NF-L (Hirokawa, 1991). De novo assembly of NFs in the absence of any preexisting IF network requires the presence of NF-L and either NF-M or NF-H (Ching and Liem, 1993; Lee et al., 1993) Further *in vivo* evidence to support the requirement of NF-L in NF assembly comes from studies of the Japanese mutant quail. This quail has a nonsense mutation in the NF-L gene, produces NF-L mRNA level of only 5% of that in the wild-type quail and does not produce any NF-L protein but expresses both mRNA and protein for NF-M. However no filaments are seen in the axon (Ohara et al., 1993). This suggests that NF assembly process requires the NF-L subunit and involves NF-L/NF-M and/or NF-L/NF-H acting as the intermediate building blocks. This essential need for one subunit to be present is not without precedent in IF assembly as keratin filaments are formed from two types of keratin protein (Klymkowsky et al., 1989).

Assembly characteristics of NF subunits have also been studied by expressing the NF subunits in fibroblast cells and in mouse L-cells (Monteiro and Cleveland, 1989; Chin and Liem, 1990; Chin et al., 1991; Gill et al., 1990; Pleasure et al., 1990; Wong and Cleveland, 1990). The results showed that the rod and portion of the head and tail domains of the NF proteins are important for coassembly to form a filamentous network that co-localized with the endogenous IF network.

1.3.3.4 Sequential expression of intermediate filament proteins during nerve cell differentiation

The pattern of expression of each IF protein is of considerable importance and can be used to investigate gene regulation and expression during specific cell differentiation. In fact, during development of the nervous system, there are important transitions in IF gene expression which reflect major events in the pathway of an undifferentiated cell becoming a mature neuron.

Proliferating CNS stem cells initially co-express vimentin and nestin (Cochard and Paulin, 1984; Lendahl et al., 1990). Vimentin is not an exclusive neuronal marker since it is expressed in many cultured cells and in a variety of

developing and differentiated tissues. Nestin expression distinguishes the stem cell from the more differentiated cells in the neural tube. Expression of nestin is down regulated from a maximum on embryonic day 16 (E16) to none at all in adult cerebral cortex of the rat (Lendahl et al., 1990). The loss in nestin expression coincides with the appearance of α-internexin (Fliegner et al., 1990). As the neuronal precursor cell becomes post-mitotic, coincident with the end of migration and beginning of neuronal differentiation, abundant α-internexin mRNA is detected in the embryonic rat brain. Alpha-internexin expression is subsequently supressed in the adult, but it is found in certain cells of the CNS where the NF proteins are noticeably absent (Fliegner et al., 1990). Just prior to and as formation of axonal processes begins, cells start to express the NF proteins, while vimentin expression is rapidly lost. Developing neuronal cells do not synthesize the triplet NF proteins coordinately (Shaw and Weber, 1981). Expression of NF-L and NF-M mRNA starts around E8 to E13 depending on the species (Shaw and Weber, 1981; Julien et al., 1986; Lieberburg et al., 1989; Schlaepfer and Bruce, 1990; Kost et al., 1992). NF-H mRNA accumulates to a significant extent only in the early post-natal brain. Levels of expression of the three subunits are progressively upregulated after birth and remain constant in the adult. NF-L and NF-M expression were also shown to be coordinately up-regulated in PC12 cells when treated with nerve growth factor (NGF) while NF-H expression was delayed (Lindenbaum et al., 1988). NF protein expression is believed to be the terminal step in neuronal differentiation and thus make NF proteins excellent markers of mature nerve cells. NFs are also obvious components of large myelinated axons. In all peripheral post-mitotic neurons, peripherin is also expressed. Peripherin expression seems to be enriched in small-caliber fibers (Parysek et al., 1991). Peripherin and the NF proteins are colocalized in cultured PC12 cells and in some filaments isolated from the rat sciatic nerve (Parysek et al., 1991).

The large range of IF proteins utilized during neurogenesis, their orchestrated expression and their parallel distribution in the CNS and PNS is probably of significant importance for the elaboration and function of the nervous system but the mechanism regulating this expression remains to be defined (Kost et al., 1992) Similar patterns of differentially expressed IF proteins occur in epithelial cells. In the proliferative population, keratins K5 and K14 are expressed. Upon comitment to terminal differentiation, a new pair of keratins, K1 and K10 is expressed in the epidermis while K5 and K14 are repressed (Steinert and Liem, 1990).

1.3.3.5 Neurofilament phosphorylation

NFs are highly phosphorylated both *in vivo* and *in vitro*, as revealed by extensive shifts in their isoelectric points and apparent molecular weights following enzymatic digestion with phosphatases (Julien and Mushynski, 1982; Carden et al., 1985).

Following *in vivo* labeling of NFs with ^{32}P, NF-L, NF-M and NF-H were shown to contain multiple phosphorylation sites. Phospho-serine was present in all three subunits, while phospho-threonine was detected in NF-M only (Julien and Mushynski, 1982). Furthermore, it was shown that NF-M and to a larger extent NF-H were by far the most highly phosphorylated components and most of the phosphates were present within a confined region of the molecules (Julien and Mushynski, 1983). This region corresponds to the unique KSP sequence described previously and phosphorylation occurs predominantly on the serine residues (Carden et al., 1985; Lee et al., 1988). The mature NF-H protein appears to contains 20-100 phosphate groups/molecule of polypeptide compared to substantially less in NF-M (6 to 12) and NF-L (1 to 3) (Julien and Mushynski, 1982; Carden et al., 1985; Geisler et al., 1987; Levy et al., 1987; Myers, 1987; Lieberburg et al., 1989).

NFs display a different phosphorylation pattern during development as well as differential turnover of phosphate groups during their axoplasmic transport (Sternberger and Sternberger, 1983; Nixon and Lewis, 1986; Carden et al., 1987; Nixon et al., 1989; Lee et al., 1987; Nixon et al., 1989). The use of antibodies capable of distinguishing different phosphorylation states of NFs revealed a correlation between the location of NFs within neurons and their degree of phosphorylation (Sternberger and Sternberger, 1983; Lee et al., 1987; Lewis and Nixon, 1988). Studies using these antibodies showed that phosphorylated NF-M and NF-H epitopes were found predominantly in axons while non-phosphorylated epitopes were located in perikaryon. The phosphorylation process seems to occur as NFs move from the cell body to the axon.

Multiple kinases have been reported to phosphorylate both phosphorylated and dephosphorylated mammalian NF proteins (Sihag et al., 1988; Sihag and Nixon, 1989, 1990). These include cAMP-dependent protein kinase, Ca^{2+}/calmodulin dependent protein kinase II and casein kinases I and II. Phosphorylation sites near the amino-terminal region of NF-L and NF-M were shown to be substrates of PKA and PKC (Sihag and Nixon, 1991). On the other hand, the KSP segments in the carboxy-terminal

domains of NF-M and NF-H were shown not to be major *in vivo* phosphorylation substrates for any of the above kinases.

There have been many reports on the purification of a NF specific kinase. Xiao and Monteiro (1994) identified a NF associated kinase (NAK 115) from mouse brain extracts that is associated with NFs *in vivo*. There is a related kinase present in human brain extracts but the precise sites on NFs that are phosphorylated by that kinase have yet to be established. Shetty and co-workers (1993) characterized a $p34^{cdc2}$-like kinase isolated form rat spinal cords. The kinase was able to phosphorylate dephosphorylated NF-H and designed peptides with the KSP sequences. Hellmich et al., (1992) isolated a novel cyclin-dependent kinase from rat brain predominantly expressed in nervous system. Wible and colleagues (1989) have isolated a kinase from bovine spinal cord, that is tightly associated with NFs and which phosphorylated partially dephosphorylated NF-H. Hisanaga et al., (1991) reported a starfish oocyte CDC2 kinase specific for the dephosphorylated form of NF-H. Phosphorylation with this kinase shifted back to normal the electrophoretic mobility of the dephosphorylated form of NF-H on SDS-PAGE. Roder and Ingram (1991) identified two other NF specific kinases: PK36 and PK40, that induced mobility shifts of NF-H by phosphorylating KSP sites.

Even though all of the above could induce considerable shifts in the apparent molecular weight of NF-H, they only phosphorylated a small fraction of the possible sites, suggesting that the kinase specific to NFs has yet to be identified. Moreover, the fact that phosphorylation of NFs is usually delayed until after they have entered the axon and that there are several different phospho-isoforms of NF-H and NF-M throughout the axon may suggest that first, more than one kinase is active along the neuroaxis, second, that there is a mechanism that regulates the activation of specific kinase(s) at specific sites along the neuroaxis and third, that the kinase(s) may be transported together with the NFs (Pleasure et al., 1989).

Phosphorylation is one of the most important post-translational modifications that NF subunits undergo, nevertheless its functional significance is still unknown (Matus, 1988; Nixon and Sihag, 1991). Phosphorylation of vimentin, desmin and the nuclear lamins is important for the depolymerization of filaments, especially during mitosis (Chou et al., 1989; Steinert and Roop, 1988; Stewart, 1990), but nerve cells do not divide. However, the conservation of KSP motifs in the NF-H subunit from mammals and invertebrates (Way et al., 1992), the fact that these repetitive sequences

reside on part of the NF proteins that extend from the NF core as well as the presence of differentially phosphorylated NFs in functionally distinct regions of the nerve cell argue for an important role for phosphorylation at these sites in NF function and metabolism. Possible functions for NF phosphorylation are discussed below.

NF-L can be phosphorylated by a number of different kinases *in vitro* and *in vivo*. There are three potential phosphorylation sites in NF-L (Sihag and Nixon, 1989). Phosphorylation of the amino-head region of NF-L by PKA or PKC prevents *in vitro* assembly of NF-L into filaments and induces the disassembly of NF-L filaments (Sihag and Nixon, 1989, 1991; Hisagana et al., 1990). This is believed to permit exchange and integration of NF-M and NF-H subunits until appropriate stoichiometry of triplet proteins is achieved. There is a major PKA site on NF-L localized on the head domain at serine 55 (Sihag and Nixon, 1991). DNA transfection experiments have indicated that this site may be required for early steps in filament assembly (Gill et al., 1990). Such an event is also observed in type III IF proteins (vimentin, desmin and GFAP) which contain specific phosphorylation sites in their head domain which play a role in mitosis-mediated reorganization or dissassembly of these IF in some cell types (Chou et al., 1989).

On the other hand, phosphorylation of tail domains may regulate higher order interactions between NFs and other cellular components and may be involved in mediating the dynamic axon-specific function of NFs.

According to Price et al. (1987), phosphorylation of the repeat domain will introduce a large number of negative charges that could induce a repositioning or extension of NF-M or NF-H sidearms, therefore giving NFs a space occupying role. A report by Carden and colleagues (1987) further supports the idea that phosphorylation causes repulsion between filaments and may be a way of increasing axonal diameter during development.

Axonal NFs are not always evenly spread in the axon as a repulsive interaction among them would suggest (Hsieh et al., 1994) thus an opposite view proposed that the different states of phosphorylation may in fact modulate the surface properties of the filaments and in turn affect their functional interactions. In fact phosphorylation could connect NFs with each other or with other cytoplasmic structures (Nixon and Lewis, 1986). Indeed, Hisanaga et al., (1991) showed that phosphorylation of NF-H by the cdc2 kinase dissociated the binding of NF-H to microtubules. Axonal

transport studies also support this hypothesis (Willard and Simon, 1983). In the developing nerve cells as NFs enter and move down the axon, continued phosphorylation occurs, especially on the tail domain of NF-H. Consequently, cross-bridges are formed between adjacent NFs. These interactions tend to retard the movement of proteins down the axon. The end result is a decrease in NF transport velocity from 8mm/day to 1mm/day during post-natal development (Willard and Simon, 1983).

A recent report by Hisagana and Hirokawa (1993) revealed no structural differences in filaments formed by phosphorylated and dephosphorylated NFs. Non phosphorylated NF-H was still able to form cross-bridges between filaments. But these were *in vitro* experiments and it is known that enzymatic dephosphorylation does not remove all phosphate groups from NFs suggesting that the few phosphates remaining play a critical role in NF structure.

1.3.3.6 Neurofilament function

The presence of NFs throughout evolution argues for a fundamental role in nerve cells, but their precise function is still the subject of speculation. One hypothesis suggests that NFs form a cytoskeletal lattice via interaction of their carboxy-terminal domains and may be one means by which NFs control axonal caliber. Much direct and indirect evidence supports this idea. First, Hoffman et al., (1985b, 1987) demonstrated that a tight correlation between the number of NFs in axonal cross-section and axonal caliber persisted during axonal degeneration and subsequent regeneration. NF synthesis was shown to be down regulated when axons were severed accompanied by a subsequent decrease in axonal NF density and a shrinkage in axonal caliber. Second, developing axons are small in caliber and contained relatively few NFs compared to large myelinated mature axons where NFs are the predominant intracellular structures (Hoffman et al., 1985a, 1987; Hirokawa, 1991). Third, Yamasaki et al., (1991) identified a neurofilament-deficient mutant of Japanese quail. CNS and PNS axons do not contain any 10 nm filaments due to a nonsense mutation in the NF-L gene (Ohara et al., 1993). The quail showed no difference in survival and had well developed myelin sheaths. The most prominent change was that the size distribution of the axonal calibers significantly shifted to small size classes. Recently reported data by Eyer and Peterson (1994) provide additional evidence for a relation between axonal caliber and presence or absence of NF proteins. They produced transgenic mice expressing a NF-H/β

galactosidase fusion protein. The mice showed accumulation of NFs in perikarya, NF deficient axons and a correlated decrease in axonal diameter. Preliminary experiments revealed a reduction in conduction velocity concomitant with the decrease in caliber in myelinated fibers of both species (Yamasaki et al., 1991; Sakaguchi et al., 1993; Eyer and Peterson, 1994). The conduction of an action potential along an axon is one of the essential factors regulating information processing in the nervous system. As conduction velocity varies in parallel with fiber diameter, NFs possibly play an important role in regulating conduction velocity. From these data, it was speculated that the biological significance of NFs might be related to their role in maintaining the caliber of axons in a large size class that have a high conduction velocity (Yamasaki et al., 1991; Sakaguchi et al., 1993).

Price et al. (1988), have demonstrated that neighboring myelinated axons of the same size contain variable densities of NFs. Therefore, instead of being responsible for controlling axonal diameter, the phosphate groups on NFs do not promote interactions but impart a high charge to the proteins that results in mutual electrostatic repulsion. Consequently, repulsion between charged tails on neighbouring NFs might keep the projections distant from each other and give NFs a very effective space-occupying structural role in large caliber axons. Indeed, one important function of NFs is believed to be their contribution to the stability of the cytoplasmic matrix and as members of the IF family, they function as mechanical integrators of cytoplasmic space (Klymkowsky, 1991).

To define NFs function, cell culture studies were performed. Expression of the NF proteins in fibroblast cells and mouse L-cells resulted in copolymerization with the endogenous vimentin cytoskeleton (Monteiro and Cleveland, 1989; Chin and Liem, 1990; Pleasure et al., 1990; Chin et al., 1991). Overexpression of NF-L, NF-M and NF-H in transfected fibroblasts did not affect the cell function (Monteiro and Cleveland, 1989; Chin and Liem, 1990; Pleasure, 1990; Chin et al., 1991;Ching and Liem, 1993). These transient transfection studies have however pointed to particular regions of the NF proteins as important in their interactions with vimentin. Truncation of the α-helical rod domain of NF-L and NF-M led to dominant disruptions of the entire IF cytoskeleton (Gill et al., 1990; Wong and Cleveland, 1990; Chin et al., 1991). Part of the amino-terminal domain of NF-L seems to be required for early steps in filament assembly (Gill et al., 1990). But, NF-L and NF-M proteins lacking portions of the tail or head domains resulted in mutant NF proteins still capable of co-assembly with the endogenous vimentin network (Monteiro and Cleveland, 1989; Gill et al., 1990; Chin et

al., 1991). Finally, de novo assembly of NFs in the absence of any preexisting IF network requires the presence of NF-L and either NF-M or NF-H (Ching and Liem, 1993; Lee et al., 1993)

Tissue culture systems have been of limited use and no definitive function could be imputed to NF proteins from these studies. Cleveland and co-workers (1990) have generated transgenic mice overexpressing the mouse NF-L protein. Although they observed a four-fold increased expression of NF-L in peripheral axons, it did not affect axonal caliber (Monteiro et al., 1990). A simple explanation would be that an increase in levels of NF-L alone may not result in the formation of filaments with the same properties as those composed of the normal ratio of the three subunits. Furthermore, the NF-M and NF-H subunits may be involved in determining axonal caliber to a greater extent than NF-L. Even though no changes were observed in axonal diameter, the increased expression of the wild-type NF-L subunit generated an abnormal increase in NF density and a disorganization of the NFs network in axons (Monteiro et al., 1990). Finally, since these transgenic mice expressed high levels of the NF-L protein ectopically, they developed cataracts.

Whatever the function of NFs, it appears that they may not be essential for all axons. However, the functional change associated with neurons of NF deficient quail and mice suggest that their presence might be directly connected to the importance of axons in large size classes with a high conduction velocity (Yamasaki et al., 1991). In fact, the development of large myelinated nerve fibers enabling attainment of faster conduction velocities has played an important role in the evolution of larger species of animals (Yamasaki et al., 1991).

Absence of IF proteins has also been noticed in certain cell lines. Paulin-Levasseur and colleagues (Cochard and Paulin, 1988) have identified a number of mammalian cell lines lacking cytoplasmic IFs, suggesting that they were not critical to viability of eukaryotic cells. For instance, in epithelial cells, disruption of cytokeratin filaments by the injection of antibodies had little obvious effect on cell division, cellular morphology, cell-cell interactions or cell behavior (Klymkowsky et al., 1989). Nevertheless NF proteins, like other IF proteins, perform important function perhaps not within the isolated cell but at the level of more complex systems as we shall see in the next section.

2. Intermediate filament proteins in disease

As components of the cell cytoskeleton, IF networks provide structural stability to the cell. A recent series of reports on human skin and nervous system diseases provides the first real evidence of a direct involvement of IFs in determining and maintaining cell shape. In fact, the disorganization of neuronal or epithelial cytoskeleton had profound consequences on both cell structure and function.

2.1 Keratin proteins

Type I and type II keratins are characteristic of skin, hair and nails. Different laboratories have recently discovered that truncated forms of the human K1, K5, K10 and K14 keratin-type proteins disrupted endogenous keratin-filament organization. In doing so they have identified the underlying cause of two human inherited disorders of the skin: epidermolysis bullosa simplex (EBS) and epidermolytic hyperkeratosis (EH).

EBS is an autosomal dominant human skin disease in which the epidermis blisters following rupture of epidermal basal cells (for review see Fuchs and Coulombe, 1992). EH, also an autosomal dominant disease, is typified by blistering due to cytolysis this time manifested in the suprabasal layers of the epidermis. In addition, hyperproliferation in basal cells occurs in response to the overlying cellular degeneration (Cheng et al., 1992; Chipvez et al., 1992). The K5-K14 genes encode keratin proteins expressed in the basal cell layer of the mammalian epidermis. A different pair of keratins, K1-K10 is expressed in suprabasal cells. As epidermal cells differentiate, K1 and K10 protein levels increase while K5 and K14 expression is switched off (Steinert and Liem, 1990).

Point mutations in the beginning, middle and end of the rod domain of either K5 or K14 proteins have been identified in EBS patients (Coulombe et al., 1991; Fuchs and Coulombe, 1992). Coulombe and co-workers (1991) have engineered the precise EBS point mutation, transfected keratinocytes with the mutant and have perturbed the keratin network. Furthermore, transgenic mice expressing mutant human K14 gene exhibited profound defects in the epidermis similar to those found in patients with EBS (Vassar et al., 1991). A mutation in the helix termination peptide of K5 is also seen in EBS patients (Lane et al., 1992).

A correlation between EH phenotype and a truncated K1 gene has been identified through linkage studies (Chipev et al., 1992). A point mutation in the H1 subdomain of the keratin 1 was demonstrated to be the cause of EH. The H1 domain is a highly conserved sequence immediately next to the rod domain in type II keratin chain. Moreover, Cheng et al., (1992) have identified a point mutation in K10 of affected EH family members. Transgenic mice expressing a truncated human keratin 10 gene have skin with the morphological and biochemical characteristics of EH (Fuchs et al., 1992).

It is believed that in both EBS and EH, the mutant keratin can create differences in the stability of the IF network and interfere with the cytoskeletal organization of the epidermal cells. As a result, the keratin filament cytoskeleton may be structurally and functionally impaired and can not be properly assembled, giving rise to blistering human skin diseases (Chipez et al., 1992). This finding implies that keratin filaments may play a vital role in providing mechanical integrity to a keratinocyte (Fuchs and Coulombe, 1992).

2.2 Neurofilament proteins

Alterations in cytoskeletal protein organization are prominent in a number of human neurodegenerative disorders (Goldman and Yen, 1986). For example; 1) there is massive accumulation of NFs in perikarya, dendrites and proximal axons of motor neurons following experimental aluminum intoxication (Troncoso et al., 1986), 2) people in the polymer industry exposed to acrylamide have classic features of distal axonal degeneration (proximal axonal enlargements, sensory loss, weakness of the muscle of the feet and hands) (Gold et al., 1985), 3) giant axonal neuropathy is a rare inherited disease in children that exhibits massive segmental accumulation of packed NFs that distend axons to giant proportion (Klymkowsky and Plummer, 1985) and 4) accumulation of ultrastructurally normal NFs within anterior horn cells of amyotrophic lateral sclerosis (ALS) patients (Carpenter, 1968; Hirano, 1982, 1984; Munsat, 1992).

Giant axonal neuropathy (GAN), is a rare, slowly progressive neurological disorder, seen in children and in dogs (Klymkowsky and Plummer, 1985; Donaghy et al., 1988). It results from an autosomal recessive mutation (King et al., 1993). In children, the symptoms first appear between age 3 to 5 years old. GAN is characterized by dramatic focal axonal swellings containing abnormally oriented NFs in the CNS and

PNS. The swellings appear along the axon typically near nodes of Ranvier with paranodal myelin thinning. GAN is believed to affect cytoskeletal organization. NF-microtubule interactions are affected in the swellings (Donaghy et al., 1988). Microtubules are segregated into discrete bundles rather than being dispersed throughout the axon. NFs that accumulate in GAN are normal but there is abnormality in their spacing. They are more closely spaced and thicker due to probable flattening of their side-arms (Donaghy et al., 1988; King et al., 1993). This disorder can also be mimicked in mice/rats following exposure to toxins such as 2,5-hexanedione (2,5-HD) and β, β'-iminodipropionitrile (IDPN) (Griffin and Watson, 1988).

ALS syndrome is the most common form of motor neuron disease in humans. It is defined as a progressive disease of the voluntary motor system and includes signs of both upper and lower motor neuron abnormalities (Hirano et al., 1984; Munsat, 1992). ALS patients show loss of large motor neurons in their cortex, brainstem and anterior horn. Concomitant with that, there is axonal loss and secondary denervation and atrophy of the muscle fibers (Manetto et al., 1988; Glasberg and Wiley, 1991). In most cases, death usually results from respiratory failure. In ALS patients motor neurons of the anterior horn have swollen cell bodies (spheroids). The swellings are due to accumulation of 10 nm neurofilaments showing parallel or random orientation (Hirano et al., 1984). Whether or not the NFs are abnormally phosphorylated is still controversial. According to Schmidt et al., (1987) and Leigh et al., (1989) there is no difference in the phosphorylation of NFs between normal and ALS patients while Itoh et al., (1992) noticed increased phosphorylation of NFs in spinal ventral horn motor neurons but not in dorsal root ganglion (DRG) and sympathetic ganglion neurons of ALS patients. In addition, Manetto et al., (1988) using antibodies against NFs showed that a significantly higher number of perikarya and proximal axons of lower motor neurons reacted with the phosphorylated antibodies in ALS patients compared to aged-matched controls. Finally, Munoz et al., (1988) also noticed focal collection of phosphorylated NFs in soma of anterior horn motor neurons in ALS pateints.

Two major types of ALS have been identified; the classic or sporadic form (90-95 %) and the familial form (FALS) (5-10 %). Clinical presentation does not appear to differ between the two. Linkage studies have revealed a tight correlation between FALS and a mutation in the gene encoding the enzyme superoxide dismutase 1 (SOD1) (Deng et al., 1993; Rosen et al., 1993). In addition, transgenic mice expressing a mutated form of SOD1 developed pathological features of motor neuron disorder (Gurney et al., 1994). The etiology of the sporadic form is still unknown.

However, a recent report by Figlewicz et al., (1994) demonstrated the existence of mutations in the carboxy-terminal region of the NF-H gene in 5 patients with the sporadic form of the disease. The mutations consisted of either a 102 bp deletion eliminating 34 amino acids in the repeated tail region or a 3 nucleotides deletion in the same region. The link between these mutant alleles of the NF-H gene and the appearance of the disease is yet to be established but suggests a definite role of NFs in motor neuron disorders.

2.3 Models of neurodegenerative diseases

The cause of the neurofilamentous accumulation in the previously described disorders is still unclear. Fortunately, animal models showing similar features are available. These models give the possibility of studying the pathological processes underlying the disease. In many cases, NF accumulation appears to be related to a defect in the axonal transport mechanism.

2.3.1 Toxic substance models

Neurofilamentous accumulations within neuronal perikarya and most proximal axons are prominent features seen in animals following intoxication with β,β'-iminodipropionitrile (IDPN), 3,4-dimethyl-2,5-hexanedione (DMHD), aluminum and acrylamide. Distal and intermediate accumulation of NFs is seen after injection of 2,5-hexanedione (HD) and 3-methyl-2,5-hexanedione (3-MHD) respectively (Goldman and Yen, 1986).

Injection of IDPN into young rats produces axonal enlargements in the most proximal regions of large caliber fibers within the spinal cord and ventral roots. There is no significant accumulation in the cell body. The swellings are filled with densely packed phosphorylated NFs distending whole internodes. Spheroids similar to the ones in ALS patients are also seen. Axonal atrophy occurs below the neurofilamentous swellings. Ultrastructure studies have demonstrated segregation of organelles within axons where chaotically arranged NFs are distributed to the subaxolemmal region surrounding microtubules, mitochondria and smooth endoplasmic reticulum found in the center of the axon (Papasozomenos et al., 1982; Griffin et al., 1983; Goldman et al., 1986). With

time, the more distal regions of the axon undergo a decrease in caliber. When continuous administration is maintained, the swellings remain in the proximal axon. When stopped, the swellings are seen in the more distal regions. This suggests proximal to distal migration of the neurofilamentous enlargements and reversibility of the phenomenon. The mechanism underlying NF swellings is not clear, but IDPN was shown to increase the gelation of NFs *in vitro* due to increased phosphorylation of the NF subunits (Eyer et al., 1989). Little if any motor neuron loss occurs even after prolonged intoxication with IDPN.

The toxic effects seen after administration of aluminum into rabbit hypoglossal system include: accumulation of morphologically normal NFs (spheroid-like but abnormally phosphorylated) in the perikarya and proximal axons of many neuronal populations including anterior horn motor neurons, as well as motor neuron degeneration, muscle weakness, neurogenic muscle atrophy and distal axonal atrophy (Bizzi et al., 1984; Troncoso et al., 1985, 1990, 1992). The atrophic axon is surrounded by a thick myelin sheath, contains less NFs and a normal number of microtubules (Bizzi et al., 1984). The more distal axonal cytoskeleton and size are normal. Aluminum, like all multivalent cations, is believed to cause lateral aggregation of native NFs *in vitro*, as they possibly act as bridges between charged residues on adjacent filaments (Troconso et al., 1992). In addition, aluminum possibly increases NFs phosphorylation through activation of an unknown kinase (Manetto et al., 1988)

Experimental studies of acrylamide toxicity on rats showed the predominant degeneration of large sensory/motor fibers in their most distal regions as NFs accumulate in proximal regions (Griffin and Watson, 1988). Acrylamide apparently increases the phosphorylation and segregation of NF-M and NF-H at early stages of intoxication (Gold and Austin, 1991).

2,5-hexanedione (2,5-HD) is a neurotoxic metabolite of n-hexane and n-butyl ketone. 2,5-HD intoxication is a model of GAN, characterized by segmental swellings containing NFs at prenodal sites in the most distal region of peripheral axons (Monaco et al., 1990). In addition, it produces a decrease in distal axonal caliber followed by atrophy of the most proximal axons. The inter NF spacing proximal to the enlargement is increased (Monaco et al., 1989, 1990). Again NFs are dissociated from microtubules. NFs are located toward the center of the axon and microtubules predominate at the periphery. 3,4-dimethyl-2,5-hexanedione (3,4-DMHD), a more potent derivative of 2,5-HD produces mostly proximal axonal accumulation of NFs

whereas 3-methyl-2,5-hexanedione (3-MHD), a structural average between 3,4-DMHD and 2,5-HD induces NF accumulation in the distal two-thirds of rat sciatic nerve (Monaco et al., 1990). According to Graham et al., (1984), the three toxins act through the formation of pyrrol adducts on lysine residues of NF subunits so that progressive accumulation of NFs occurs due to intermolecular covalent crosslinking of NF proteins. In addition, the pyrrolation of NF proteins may disrupt the interactions between NFs and microtubules (Graham et al., 1984).

2.3.2 Naturally occuring animal models

Hereditary canine spinal muscular atrophy (HCSMA) is a motor neuron disorder of Brittany spaniels, characterized clinically by weakness and muscle atrophy and pathologically by progressive dysfunction and loss of motor neurons and abnormal accumulation of NFs in the anterior horn of the spinal cord (Cork et al., 1982; Messer, 1992). Early in the disease, the axonal caliber of affected motor neurons is smaller in distal regions and as the disorder progresses the proximal regions of these axons become distended with accumulations of NFs. The canine model exhibits three phenotypes distinguishable on the basis of age of onset and rate of progression: accelerated, intermediate and chronic (Messer, 1992). The disease is autosomal dominant, with homozygotes showing the accelerated form as early as sixteen weeks of age and heterozygotes showing the intermediate or chronic form at two to seven years of age.

Mice with the autosomal recessive gene "wasted" develop extensive vacuolar degeneration of motor neurons within the anterior horn of the spinal cord and motor nuclei of the brainstem, due to a spontaneous mutation in inbred mouse HRS/j (Lutsep and Rodriguez, 1989; Messer, 1992). There is also prominent accumulation of phosphorylated NF-H proteins within the vacuolated neurons. Occasional neurons will show axonal swelling. The disease is early in onset starting by 14-18 days and most affected mice die by 28-31 days of age. Clinical features include tremors and weakness of the forelimbs and paralysis affecting mainly the hindlimbs (Lutsep and Rodriguez, 1989; Messer, 1992).

Motor neuron degeneration mice (Mnd mouse) have a neurological disorder which is semi-dominant with variable penetrance in the C57BL/6.KB2 strain due to a double recombination in the mouse histocompatibility region. It is characterized by a redistribution of NF proteins to the margins of the cell bodies of lumbar spinal cord

motor neurons, leaving a large area in the cytoplasm devoid of NFs. No neurofilamentous swellings are observed. There is also a decrease in the number of lumbar anterior horn neurons and numerous inclusion bodies in the affected soma. The disease is late in onset, identified by 6-7 months of age with weakness of the hindlimbs which progresses over a course of about 3 months to total paralysis and premature death (Callahan et al., 1991; Messer, 1992). Upper and lower motor neurons are affected in the severe stage.

The wobbler mouse shows clinical symptoms of lower motor neuron disorder where forelimbs not hindlimbs are affected (Andrew, 1975; Mitsumoto and Gambetti, 1986; Monaco, 1990). It is an early-onset (3 weeks) autosomal recessive mouse mutant. Pathology has revealed vacuolarization of the soma of cervical motor neurons and a decrease in the number of axons from loss of anterior horn neurons. The number of NFs is reduced in the affected cells and axonal swellings and perikaryal NF accumulations are rare (Messer, 1992).

The progressive motor neuronopathy mouse (pmn) is an autosomal recessive mutant with early onset (around 3 weeks old). Morphological manifestation includes caudo-cranial motor neuron degeneration. The disease progresses rapidly with death occuring around 6-7 weeks of age due to respiratory failure (Hirano and Iwata, 1979).

These animals models have been useful in studying motor neurons diseases and as we shall see in the next section, the pathogenesis of these disorders appears to result from changes in the kinetics of axonal transport.

3. Axonal transport

The cell body which contains the protein-synthetic machinery of neurons, is responsible for maintaining the whole neuronal mass. It does so by transporting materials, synthesized in the perikaryon, down the axon via a continuous stream, called anterograde movement. Associated with this motion, is a movement in the opposite direction; retrograde transport. In this case, endogenous material is returned to the cell body, either for destruction or redeployment. Exogenous material taken up at the nerve terminal also travels this way to the soma (Grafstein and Forman, 1980; Brady and Lasek, 1982; Lasek et al., 1984; Griffin and Watson, 1988; Vallee and Bloom, 1991).

Six groups of axonal transport have been defined based on 1) average velocity of material transported and 2) composition and localization of materials within nerve cells (Table 2) (Willard et al., 1974; Levine and Willard, 1980; Tytell et al., 1981; Brady and Lasek, 1982; Vallee and Bloom, 1991). Groups I and II consist mostly of rapidly transported membrane bound constituents. Groups III contains polypeptides migrating at an intermediate rate. Groups IV and V, also referred to as slow component b and a (SCb, SCa), are composed mainly of slowly transported cytoplasmic proteins. Finally, retrogradely transported organelles form a distinct class, moving at a rapid rate (Group VI).

Table II

Rate components of axonal transport and cytological structures

Rate component			Velocity (mm/day)	Type of materials
Anterograde				
Fast		I-II	200-400	Synaptic vesicles, glycoproteins, glycolipids, neuropeptides, neurotransmitters and enzymes related to neurotransmission, tubulovesicular structures, neurosecretory granules
Intermediate		III	50-100	Mitochondrial proteins, associated enzymes and lipids
Slow	SCb	IV	2-8	Actin, clathrin, tubulin, calmodulin, nerve specific enolase, creatinine phosphokinase
	SCa	V	0.25-2	Cytoskeletal proteins: NFs and tubulin
Retrograde				
Fast		VI	100-200	Growth factors, lysosomal enzymes, preslysosomal, lamellar and multivesicular bodies.

3.1 Approaches to study axonal transport

Initial recognition of multiple forms of axonal transport was obtained through studies in many different nerve pathways and animal species using different technical approaches (Grafstein and Forman, 1980; Brady and Lasek, 1982; Vallee and Bloom, 1991).

3.1.1 Studies of axonal transport from accumulation at a block

The classic experiments on nerve compression performed by Weiss and Hiscoe (1948) clearly demonstrated the existence of slow axonal transport. They observed that the fibers proximal to a point of compression swelled because they could not pass the constricted region. Once the compression was relieved, the accumulated material moved down the axon at a rate of 1-2 mm/day. Furthermore, the observation that accumulation of material occured on both sides of the constriction provided evidence for retrograde transport.

It is now possible to reversibly (for example by lowering the temperature) disrupt the continuity of a nerve fiber and study the material accumulating on either side of the lesion. The technique can be very useful in determining the axonal transport characteristics of any neuronal constituents provided they can be identified either through biochemical, histochemical or electron microscopic assays (Grafstein and Forman, 1980).

3.1.2 Studies of axonal transport involving radioactive labeling

Radioactive amino acid precursors such as [^{35}S] methionine, are widely used to study axonal transport. The technique is non damaging to the nerve cell and yields important information on the rate, amount and composition of materials transported in the nerve in different situations (Grafstein and Forman, 1980).

Radioactive amino acids, applied in the region of the cell bodies of choice, are incorporated into proteins and transported down the nerve fibers. A few examples of sites of injection include: 1) vitreous humour of the eye to study axonal transport in the

optic system, 2) floor of the fourth ventricle to label transported material in the hypoglossal and vagus nerve, 3) the region of motor neurons in the spinal cord or sensory neurons which supply the sciatic nerve. Retrograde transport can also be studied by applying radiolabeled [^{125}I] coupled to a trophic factor, to nerve endings and examining subsequent labeling in the cell bodies.

The motor neurons, DRG systems and their associated sciatic nerve provide the opportunity to examine transport in long time course experiments. The L4, L5 spinal cord segments or L4, L5 DRG of the animal are exposed by laminectomy. The isotope is then injected using a glass micropipette, to label motor or sensory axons. At various intervals thereafter (hours, days, weeks) the labeled sciatic nerve is removed and cut in consecutive segments. Using SDS-PAGE, the specific transport and velocity of labeled proteins are detected autoradiographically (Figure 4).

The labeling technique coupled to the constriction technique is also useful to study the different classes of transport (Ochs, 1972).

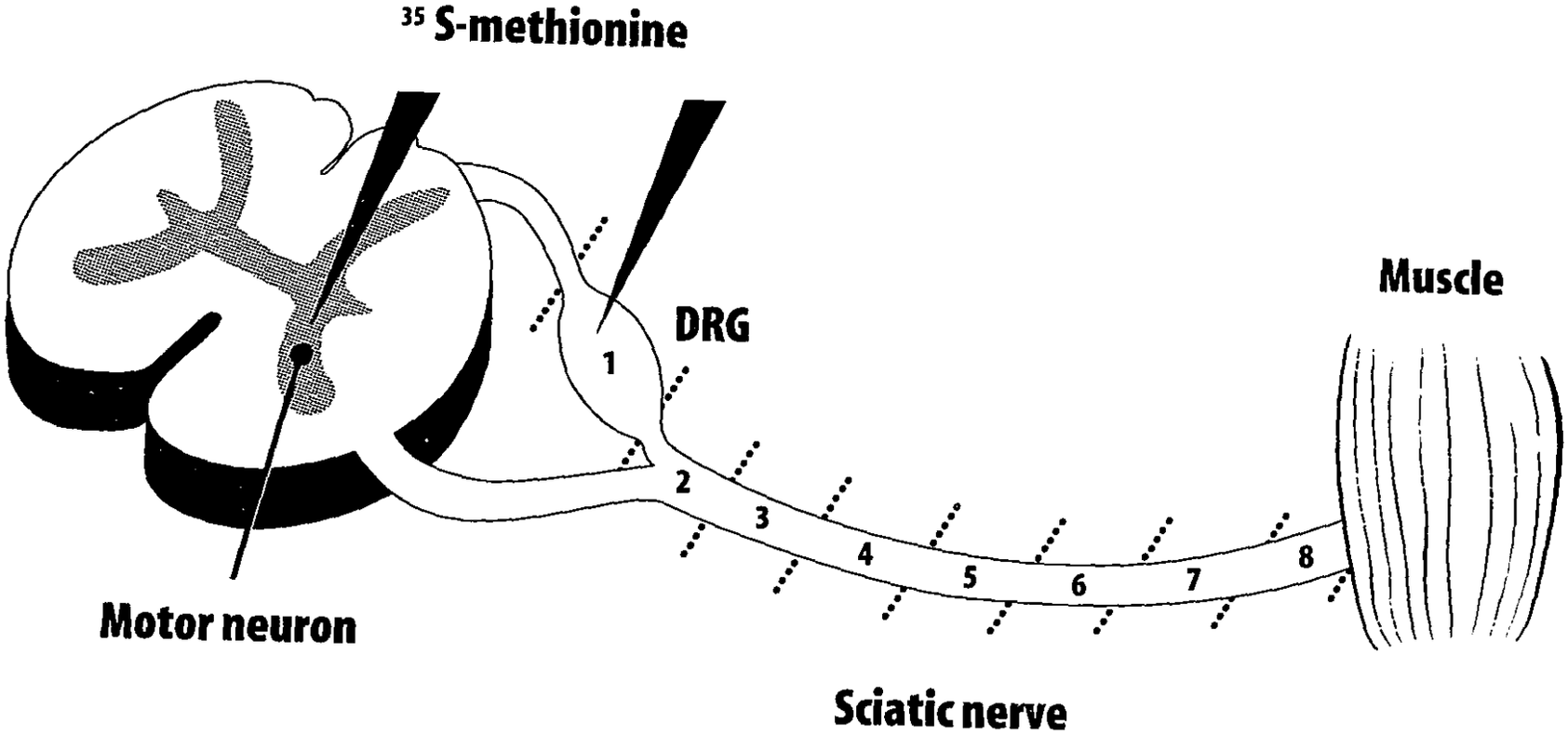
3.1.3 Studies of axonal transport by direct visualization of microscopic organelles

Visualization of intra-axonal movement can be done using the extruded axoplasm of the giant axon from the squid *Loligo pealeii* (Brady, 1991). Such a preparation lacks a surrounding plasma membrane and retains many of the structural features of the intact axon for hours. Axonal transport continues and using video-enhanced light microscopy, vigorous bidirectional movement of membranous organelles can be observed. Because the isolated axoplasm lacks a surrounding membrane, the axon interior becomes readily accessible to externally applied buffer solutions and many properties of rapid axonal transport have been defined using this preparation (Breuer et al., 1991; Brady et al., 1982).

An important limitation of this technique is that only the transport of larger structures such as mitochondria and multivesicular bodies can be studied.

Figure 4. Illustration of experimental paradigm to study axonal transport.

The lumbar spinal cord and DRG (L4-L5) are exposed by laminectomy and newly synthesized proteins in motor/sensory axons in the sciatic nerve are labeled by the injection of [^{35}S]mehtionine. Mice are sacrificed various time thereafter and the sciatic nerve is removed and cut in consecutive 5mm segment.


^{35}S-methionine
DRG
Muscle
1
2
3
4
5
6
7
8
Motor neuron
Sciatic nerve

3.2 Fast anterograde axonal transport

Fast anterograde axonal transport is involved in delivery of organelles and plasma membrane and targeting of materials to specific functional domains, for example components important for neurotransmission, synaptogenesis and axonal maintenance. The typical rate of fast transport in mammalian species is 50-400 mm/day as defined by labeling, stop-flow and accumulation studies (Table II) (Grafstein and Forman, 1980; Lasek et al., 1984).

3.2.1 Nature of rapidly transported material

Classes I and II carry a large variety of membranous organelles such as smooth endoplasmic reticulum, plasma membrane constituents, vesicles of various kinds and size, glycoproteins, lipids, nucleosides and sugar (Grafstein and Forman, 1980; Lasek et al., 1984). The velocity rate varies from to 200-400 mm/day. Group III consists of proteins of intermediate velocity (50-100 mm/day) and includes mitochondria.

No major differences were noted between the profiles of the rapidly transported proteins in various neuronal systems and species (Ochs, 1972; Stone and Wilson, 1972; Bisby, 1977). Only slight differences in the relative abundance of some peripherally and centrally transported proteins were observed (Stone and Wilson, 1972). This suggests that the most abundant proteins of the different types of nerve cells subserve common neuronal function (Stone and Wilson, 1972).

3.2.2 Dynamic properties of fast anterograde transport

To achieve the maximum velocity rate of 400 mm/day, fast transport requires the coordinated action of several components such as a transport track, molecular motor(s) and regulatory factor(s).

Microtubules were shown to serve as the rails for newly formed organelles destined for anterograde movement. In axons, microtubules are oriented with their plus

end toward the axon terminal and their minus end in the opposite direction (Vale et al., 1985a).

The protein kinesin is the motor generating the movement. (Vale et al., 1985a). Using electron microscopy and immunocytochemistry, kinesin was found to accumulate on the proximal side of a peripheral nerve ligation at a rate correlated with fast axonal transport and also to partition with anterogradely moving organelles (Hirokawa et al., 1991). Kinesin is a microtubule activated ATPase and fast axoplasmic transport depends on continued supply of ATP to the transport mechanism (Vale et al., 1985a). Kinesin can translocate endogenous axoplasmic vesicles along microtubules. It does so by forming a complex with microtubules and the vesicles. The mechanism responsible for fast transport presumably involves cycles of attachment, motion and detachment of kinesin protein to microtubules to which the hydrolysis of ATP is coupled.

3.3 Slow anterograde axonal transport

The slowly transported material observed by Weiss and Hiscoe (1948) was further characterized by Hoffman and Lasek (1975) and involves the movement of components of the cytoskeleton and associated proteins including cytoplasmic enzymes of intermediary metabolism. Slow transport has been resolved into two subcomponents: SCa or group V proteins and SCb or group IV. SCa and SCb are differentiated on the basis of their protein composition and velocity (Hoffman and Lasek, 1975, 1980). The fastest of the two components, SCb migrates at rates ranging from 2.0 to 8.0 mm/day while SCa proteins move at 0.25 to 2.0 mm/day. SCa is referred as the microtubule-neurofilament network, while SCb is known as the cytoplasmic matrix or microfilament network (Hoffman and Lasek, 1980). SCa is believed to play an important role in supporting neuronal shape. SCb is thought to be more active and plays a number of roles in neurons including an involvement in growth cone mobility and moving the cytoplasmic complex through the axon in both normal and regenerating conditions.

3.3.1 Nature of slowly transported material

The constituents of SCa are quite simple and include the major structural elements of the axon: neurofilaments and microtubules. The composition of SCb is more

complex and is characterized by the presence of up to 100 different proteins. Among these are tubulin, actin, clathrin, brain spectrin, calmodulin and cytoplasmic enzymes such as nerve specific enolase and creatinine phosphokinase (Table II) (Lasek et al., 1984).

A number of studies suggest differences in the composition of SCa and SCb in specific nerves (Grafstein and Forman, 1980; Hoffman and Lasek, 1980; Lasek et al., 1984; McQuarrie et al., 1986; Oblinger et al., 1987). In general, the composition of SCb and SCa in optic axons differed from that in DRG and motor neurons. In mammalian species, the relative proportion of NF proteins to tubulin in SCa is higher in DRG and ventral motor neurons than in optic axons. In fact, NF proteins outnumber microtubules by a factor of 10 to 1 in large caliber axons (Hoffman et al., 1985b). However in rat optic nerve, tubulin is more abundant than the NF proteins (70 % versus 13 %) and is present in SCa only (Oblinger et al., 1987). In the motor and sensory neurons of the rat sciatic nerve, tubulin is found in SCa but is the major protein of SCb. Several neurospecific isoforms of tubulin have been characterized in the nervous system (Denoulet et al., 1989). Tashiro and Komiya (1984) also found two subpopulations of transported tubulin which could be differentiated by their transport rate and isoform composition in both the sensory and motor axons of the sciatic nerve. For example, a specific β-tubulin subcomponent termed β' is enriched in SCb (Denoulet et al., 1989). Actin is more abundant in SCb in DRG and ventral motor neurons. Actin is also found as part of SCa in spinal motor neurons (McQuarrie et al., 1986). Actin and tubulin found in SCa or SCb reflect the state in which they are in, i.e. stably polymerized (SCa) or dynamic (SCb) and in turn probably reflect their function in different situations as we shall see in sections 3.5.1 and 3.5.2.

3.3.2 Dynamic properties of slow anterograde transport

In addition to differences in SCa and SCb content in optic, sensory and motor axons, there are also variations in their velocity rates (Grafstein and Forman, 1980; McQuarrie et al., 1986; Oblinger et al., 1987; Nixon, 1991). In general peripheral axons move group IV and V proteins at faster rates than central axons. For example, the velocity for SCa is 0.1-0.75 mm/day in optic axons and 0.5-1.0 mm/day in central sensory ganglion and 1.0-1.5 mm/day in peripheral sensory ganglion. Rates for SCb

are between 2-3 mm/day in optic axons and 3-4 mm/day in spinal motor axons and 4-5 mm/day in DRG neurons.

Although the composition of SCa and SCb is well documented, little is known about the form in which the proteins are transported and the mechanism responsible for slow transport. Unlike actin and tubulin, NFs are found exclusively in SCa and the newly synthesized subunits of NF are believed to be rapidly converted to stable polymerized structures. Initial experiments on slow axonal transport (Hoffman and Lasek, 1975) identified the NF proteins moving as a coherent mass without material trailing behind. This led to the structural or polymer hypothesis (Tytel et al., 1980; Lasek et al., 1984) which states " that proteins within a class are organized into linear structures actively transported in the axon as an interconnected matrix that ultimately undergoes proteolysis at the terminal ". Transport of the three NF subunits provided evidence to support this hypothesis as they were shown to be assembled in the cell body and translocated in the axon with identical transport kinetics in a stoichiometric ratio of 10:5:3 or 6:2:1 (NF-L:NF-M:NF-H) and not as individual subunits (Hoffman and Lasek, 1975; Nixon and Lewis, 1986; Nixon, 1991).

The polymer sliding hypothesis suggested interactions between SCa and SCb matrix as responsible for their movement (Nixon, 1991). During the sliding movement the NF side-arms interact with proteins in SCb. Through these interaction NF proteins may be pulled by the faster moving components of group IV which interact with the motor molecule (Lasek, 1986). It is clear however that the possible interactions between SCa and SCb are only transient as the structures in each component move separately and form a separate transport wave (Hoffman and Lasek, 1980; Lasek, 1986; Vallee and Bloom, 1991).

An alternative model by Nixon and Logvinenko (1986) proposed that NFs are not moving as polymer but are mostly stationary. While studying rat optic nerve, they observed that the newly synthesized NF proteins advanced along the axon and were incorporated into a stationary cytoskeleton in the axon. The stationary NFs were replenished by individual migrating NF or small bundles of NF proteins. The study also suggested that the extensively phosphorylated NF-H isoform was associated with stationary NFs. The axonal cytoskeleton has therefore a moving unpolymerized NF network that maintains a stationary NF network where the movement of NF proteins is powered by a kinesin- or dynein-like motor which is linked to a carrier protein (for example, tubulin) (Nixon, 1991).

Using 2-D gels, Lasek et al (1992), contradicted this view and demonstrated that slow axonal transport move NFs continuously along the axon at a rate between 0.05-3.0 mm/day and if there were any stationary NFs in the axons, their number would be relatively small, that is, the NF structure and not individual subunits are the units of transport. This finding is in agreement with the polymer sliding model.

Hirokawa's group (Okabe et al., 1993) provided evidence for a non-polymer transport of NFs. They produced fluorescent NF-L proteins and introduced them in primary cultures of mouse sensory neurons. Following photobleaching of a small region of the axon, fluorescence recovery was observed. This study contradicted the polymer transport model and suggested that freely diffusible NF-L subunits were translocated across the bleach spot and incorporated into pre-existing filaments.

Tubulin is also actively transported down the axon and again whether in the form of assembled microtubules, as free subunits or in some as yet unidentified form is uncertain. Okabe and Hirokawa, (1990) used fluorescent tubulin injected into mouse or chick DRG neurons to demonstrate that a large fraction of tubulin is transported as free tubulin dimers and does not exist strictly as a stable polymer. Most of the insoluble form of tubulin moves with the NF proteins while SCb is enriched with soluble tubulin. On the contrary, Reinsch et al., (1991) using a photoactivation technique in Xenopus neurons demonstrated polymer movement of tubulin without any stationary phase.

The form in which actin is transported is also not known (Nixon, 1991). In sciatic motor axon, actin advances at group IV velocity, which is believed to be in a monomeric form. Actin moving in group V is recovered in a polymeric enriched fraction (Nixon, 1991). Again photobleaching experiments on actin filaments revealed a continuous flow of actin molecules (Okabe and Hirokawa, 1990). Therefore, axonal actin and tubulin do not exist strictly as stable polymers. Actin and tubulin transported in SCb are mostly in their unpolymerized or soluble forms readily available for transport and exist in equilibrium with their respective polymers in SCa.

3.4 Retrograde fast axonal transport

Endogenous and exogenous substances are carried from the most distal part of the axon to the cell body by means of retrograde transport. A large proportion of constituents carried by fast anterograde transport is returned to the cell body after

undergoing endocytosis to be degraded or recycled (Brady and Lasek, 1982). Exogenous substances carrying information from the periphery accumulate in the perikaryon as well. They transmit important signals and influence the metabolic activity of the cell body to respond to events in the terminal regions.

3.4.1 Nature of retrogradely transported material

Material transported retrogradely includes; multivesicular bodies, lysosomal structures, glycoproteins, phospholipids, enzymes, trophic factors for example, NGF, CNTF, certain types of viruses (herpes simplex, polio) and the widely used exogenous protein tracer horseradish peroxidase (HRP) (Grafstein and Forman, 1980; Griffin and Watson, 1988).

3.4.2 Dynamic properties of retrograde transport

Studies with exogenous radioactive proteins indicate a retrograde transport velocity of about 200-300 mm/day (Grafstein and Forman, 1980).

Cytoplasmic dynein is the molecular motor responsible for the retrograde motion. Dynein has a microtubule activated ATPase activity. Once a particle is attached to it, dynein moves it towards the minus end of microtubules, thus from the distal part of the axon towards the soma (Paschal and Vallee, 1987; Vallee, 1992; Vallee and Bloom, 1991).

3.5 Involvement of axonal transport in morphological and functional integrity of the nerve cell

The axon is dependent on the continuous flow and replacement of the axoplasmic proteins by axonal transport to maintain its integrity (Hoffman and Lasek, 1980). Arrest of axonal transport leads to changes in the axon trunk, axon terminals and post-synaptic cells and reflects the importance of an adequate transport system. This section will discuss the rate and composition of the different classes of transport as they change following axonal injury and under certain pathological conditions.

3.5.1 Axonal transport in normal, axotomized and regenerating axons

Intra-neuronal transport is a crucial event during initial elongation processes and subsequent normal conditions of growth (Brady and Lasek, 1982). Normal growth of an axon towards its target requires 1) fast transport to provide the membrane essential for elaboration of the new axon and to supply trophic factors and 2) after synthesis of cytoskeletal proteins, delivery of these proteins via slow transport to the distal axon and to the growth cone and organization of the axonal cytoskeleton at the entry of the growth cone to permit its active motility.

Microtubules and actin filaments are major structural elements of the advancing growth cone (Sabry et al., 1991). In neonatal rabbits, their transport velocity is increased by two-fold as compared to adult (Willard and Simon, 1983). NF proteins also move at faster speeds in newborn animals. During maturation of the nerve cell and radial axonal growth, their speed is reduced and the cytoskeleton is laid down. The number of NFs in the axon increases rapidly and soon outnumber the microtubules in large caliber axons (Hoffman and Lasek, 1980; Hoffman et al., 1985a). The increased ratio of NFs to microtubules favors stabilization of neuronal morphology in the adult neuron that is associated with a decrease in neuronal plasticity. In aged animals, transport rates slow significantly (McQuarrie et al., 1986).

When a peripheral axon is injured, the nerve fibers disconnected from the cell body degenerate and are replaced by new outgrowths from the proximal stump (Oblinger and Lasek, 1988). These events are correlated with changes in mRNA and protein metabolism. Following axotomy, there is an increase in mRNA for tubulin and actin while the expression of the NF genes is reduced and proportional reductions in the amount (not in the transport velocity) of NF proteins undergoing axonal transport are observed (Hoffman et al., 1987). The decrease in axonal NF content is also associated with reduced axonal caliber of the proximal stump. The atrophic segment further extends distally. This phenomenon is called somatofugal axonal atrophy and proceeds anterogradely along the nerve fibers and is correlated with kinetics of NF transport (Griffin and Watson, 1988). On the other hand, during outgrowth and regeneration, actin and tubulin transported in SCb, move at a velocity comparable to the the rate of elongation. This led to the hypothesis that they are directly involved in the regenerative process (Hoffman and Lasek, 1980; Oblinger and Lasek, 1984). In regenerating axons,

there is no change in the velocity but rather a change in the form and the relative amount of materials transported in the two subcomponents. Within a few days after axotomy of peripheral sensory neurons, there is elevated actin and classes II-III tubulin transported mainly in SCb and a decreased amount of tubulin and NFs in SCa (Hoffman and Cleveland, 1988; Muma et al., 1990; Jacob and McQuarrie, 1991; Tashiro and Komiya, 1991; McKerracher and Hirscheimer, 1992; McKerracher et al., 1993). The successful regeneration and reconnection that follows is characterized by return of cytoskeletal protein delivery to the normal level and resumption of radial growth of the axon. The sequential and coordinated changes in tubulin, actin and NFs synthesis represent an interchange between a regenerating plastic axon capable of sprouting and a stable established axon (Hoffman and Lasek, 1980).

Following axotomy, there is a latent period (1 to 4 days) during which time SCb proteins are prepared for initial neurite elongation (Hoffman and Lasek, 1980). During that period, fast transport carries new axolemmal constituents to the growing tip. The content and rate of rapidly transported proteins remains mostly the same after axotomy, except for the induction of a subset of proteins known as growth-associated proteins (GAPs) (Skene and Willard, 1981a, 1981b).

Finally, retrograde transport provides chemical sampling of the milieu of the growing tip. Transport of neurotrophic factors plays an essential role in the regenerating axons: for example, atrophy and decrease in NF content in the proximal axon can be prevented by exogenous application of NGF (Gold et al., 1991). Injured motor neurons in experimental animals have been shown to retrogradely transport BDNF and NT-3 and enhanced CNTF and LIF retrograde transport was also seen following peripheral nerve injury (DiStefano et al., 1992; Curtis et al., 1993, 1994).

3.5.2 Axonal transport in pathological conditions

As mentioned previously, constriction of an axon results in the formation of swellings on both proximal and distal sides. The origin of these accumulations are due to interruption of bulk axoplasmic flow. This suggests that damming of transport is causing axonal enlargement. Therefore the prominent focal enlargement of an axon or soma seen in a number of human, animal and experimental conditions can be related to a selective defect in axonal transport (Grafstein and Forman, 1980; Gajdusek, 1985).

In ALS patients, Gadjusek (1985) hypothesized that there was interference with normal transport of NFs without interruption of their synthesis. Manetto and co-workers (1988) proposed that premature phosphorylation of NF within perikarya of ALS patients may alter/reduce the transport of NFs in lower motor neurons. In ALS patients, there is an increased mean anterograde velocity for fast transport and a reduction in the speed and the amount of material transport back to the cell body (Mitsumoto and Gambetti, 1986; Breuer et al., 1987; Breuer et al., 1991).

In the wobbler mouse, fast and slow axonal transport are impaired. There is a reduction in the amount and speed of retrogradely transported material as well as a decrease in NFs transported (Mitsumoto and Gambetti, 1986).

In HCSMA, a dog model of motor neuron disorder, the transport of NF proteins, actin and tubulin is lower (Griffin et al., 1982)

Administration of IDPN to rats disrupted the NF-microtubule meshwork and selectively blocked NF transport in the more distal region of the axon. The decreased delivery of NF occurs below the proximal neurofilamentous swellings. It is possible that the observed increase in NFs autophosphorylation may be related to the retardation of NF transport (Gold and Austin, 1991). In addition, it is likely that the observed reorganization of the cytoskeleton reflects loss of interactions of NFs with microtubules and other axonal organelles. It therefore underlies the failure of normal translocation of NFs. Tubulin, actin, fast anterograde and retrograde transports appear relatively normal (Griffin et al., 1978; Papasozomenos et al., 1982; Griffin et al., 1983). Rapid transport occurs exclusively in the central microtubule domain.

The toxin 2,5-HD causes NFs to be disconnected from microtubules. The transport distribution of the NF triplet in rats chronically intoxicated with 2,5-HD and 3-MHD is significantly faster in sciatic nerve central and peripheral axons in the region proximal to the enlargements (Monaco et al., 1989, 1990; Watson et al., 1991). The increased rate of NF transport in proximal somatic motor axons causes the distal swellings because it provokes a decrease in the residence time of NFs in proximal axons and an increase in the pool of NFs reaching the distal end (Watson et al., 1991). There is also retention of rapidly transported components in 2,5-HD neuropathy (Griffin et al., 1984). By contrast, the potent analogue of 2,5-HD, DMHD, significantly reduced the rate of transport of NF proteins at the level of the proximal axons in rats, while tubulin and actin proteins were only slightly retarded (Griffin et al., 1984; Monaco et

al., 1989). In this situation, the changes are very similar to the ones observed following IDPN intoxication where there is progressive slow down and arrest of NF transport.

Intoxication with aluminum retards transport of all three NF proteins in the proximal sciatic nerve (Bizzi et al., 1984; Troncoso et al., 1985). NF transport in the distal region, tubulin, actin and fast transport are normal.

In acrylamide intoxication, the transport defect is predominantly distal and impairs bidirectional fast transport (Griffin and Watson, 1988). There is only a modest retardation of NFs and tubulin transport in the proximal axon.

4. Control of gene expression in the nervous system

The nervous system is a complex organ. The mammalian nervous system contains approximately 10^{12} nerve cells (McKay, 1989). The generation of a nerve cell during development involves the coordinate activation of a battery of genes. As mentioned in section 1.3.3.4, several IF genes are expressed throughout neural differentiation. For example, undifferentiated precursor cells contain the type III IF protein vimentin, which is expressed in the developing embryo, while following the formation of definitive neuronal cells, the NF proteins appear (Shaw and Weber, 1981; Cochard and Paulin, 1983) . Therefore the activation of NF gene expression is a key point in the development of a neuronal cell type and the understanding of the mechanism that regulates the switch in neuronal gene expression is essential to the understanding of neuronal development. However, the molecular mechanisms controlling the activation and/or repression of the NF genes and other neuron-specific genes at specific times remain to be determined.

Transcriptional regulation of NF gene expression was investigated *in vitro*. After transfection into non-neuronal cells such as fibroblasts, the mouse and human NF-L genes were transcribed correctly even though the corresponding endogenous genes were not expressed (Julien et al., 1987b, Monteiro and Cleveland, 1989). Tissue culture systems have therefore been of limited use in finding cis-acting elements within the NF genes responsible for their expression (Monteiro and Cleveland, 1987; Pleasure et al., 1989). By comparison, DNA fragments containing either the complete human or mouse NF-L genes were correctly regulated in transgenic mice (Julien et al., 1987b; Monteiro et al., 1990). In addition, deletion mutant analysis of the human NF-L gene in transgenic mice indicated the presence of intragenic elements involved in the regulation of neuronal specificity (Beaudet et al., 1992). The use of transgenic mice technology therefore can help in defining the molecular signals that regulate the cell-type specific activation of genes during nerve cell differentiation.

4.1 Transgenic mouse technology to study gene regulation

The transgenic technology offers the opportunity to determine regulatory elements involved in the region-specific expression, developmental expression and level of expression of the transgene. Once the gene of interest is microinjected into a

fertilized mouse egg, it usually becomes integrated, in head to tail tandem arrays, into a chromosome at a randomly selected site (Jaenisch, 1988). In most cases, the site of integration of the gene does not affect the cell-type specificity. For example, expression of a 21.5 kb fragment containing the human NF-L gene in transgenic mice was neuro-specific (Julien et al., 1987b). By contrast, no correlation between the number of gene copies integrated into the mouse genome and transgene activity is usually observed (Palminter and Brinster, 1986). For example, transgenic mice with more than 200 integrated copies of the human NF-L gene showed levels of expression similar to those with just a few copies (Julien et al., 1987b). In addition, the human NF-L gene failed to demonstrate an increase in protein level in transgenic mice even though mRNA levels were increased from three to five fold (Beaudet et al., 1993). This effect is called " a chromosomal position effect " and is interpreted as insufficient sequence information in the injected DNA to be independent of the influence of the surrounding chromatin in the integration locus (Palminter and Brinster, 1986).

4.2 The mechanism of integration-position independent, copy-number dependent and proper developmental gene expression

A number of studies including experiments in cell culture and transgenic mice have been done to define the regions containing regulatory elements responsible for copy-number dependent, integration site independent and developmentally correct expression of the inserted gene. The most studied example of such control regions is the human β-globin gene (Grosveld et al., 1987; Blom van Assendelft et al., 1989; Ryan et al., 1989; Talbot et al., 1989).

The human β-like globin genes are a cluster of five active genes occupying approximately 70 kb of DNA on chromosome 11. The different genes are expressed in a precise developmental-stage, copy-dependent and tissue-specific manner. This is achieved through stable interactions between basal transcriptional complexes at their promoters and regulatory sequences associated with erythroid-specific deoxyribonuclease I (DNaseI) hypersensitive (HS) sites lying 6-40 kb upstream of the structural genes. There are 6 HS sites in the β-globin gene which are referred to as the "Locus control region" (LCR) (Stamatoyannopoulos, 1991) and carry out two separated albeit related functions. They organize the entire β-globin locus for expression specifically in erythroid tissues and act as powerful enhancers to direct high level

expression. They do that by first organizing the β-globin locus into an open and active chromatin domain regardless of the site of integration. Once the open domain is established, local regulatory promoter and enhancer sequences are then accessible to trans-acting factors that stimulate the average level of β-globin gene expression. In these studies, the human ε-globin gene was first expressed in the embryonic yolk sac then the two γ-globin genes were expressed in the fetal liver and finally the β-globin gene was activated near the time of birth. The location of the HS sites defines the active β-like globin domain in cells of the erythroid lineage. Experiments using various constructs in cell cultures and in transgenic mice showed that the LCR may contain regions of functional redundancy, but the results also suggested that all six HS sites, as well as all the five genes in their normal chromosomal arrangement, are necessary for the proper regulation of the entire human β-globin locus or to direct the expression of other genes (Collis et al., 1990; Dillon et al., 1991). For example, the HS sequences were shown to disrupt normal temporal-specific expression when inserted immediately upstream of either the human γ or β-globin gene alone (Behringer et al., 1990).

Although not as well studied, other examples suggesting the presence of LCR have been reported in a number of mammalian genes including S100 β and CD2.

The S100 β is a calcium binding protein present primarily in brain astrocytes. It is believed to be involved in the development of the mammalian brain by stimulating the proliferation of glial cells and differentiation of nerve cells (Allore et al., 1990). Potential cis-acting regulatory elements have been identified in the promoter region of the human S100 gene and at location similar to the one found in the promoter region of the human β-globin gene (Friend et al., 1992).

CD2 is a glycoprotein present on most thymocytes and probably on all peripheral T cells. It is involved in adhesion to CFA-3 molecules and participates in the T cell activation pathway. Using different constructs, DNase HS sites in the CD2 3'-flanking sequence were identified. Sequences like the ones present in β-globin were also capable of directing the expression of heterologous promoters in a tissue-specific manner with levels proportional to gene copy number and independent of the integration site into the mouse genome (Greaves et al., 1989).

5. Objectives

The neurofilament triplet proteins (NF-L, NF-M and NF-H) are cytoskeletal proteins specifically expressed in nerve cells. While a great deal is known about the molecular biology of NFs, most of their precise functions are still the subject of speculation (Liem, 1990, 1993). The principal objective of my research thesis was to study the function of the human NF-H gene and its possible involvement in determining axonal caliber. A human genomic clone coding for the neurofilament largest subunit (NF-H) was isolated and expressed into mice. Transgenic mice expressing the human NF-H gene provided a means to directly address questions regarding its function and control of its expression.

5.1 Overexpression of the human NF-H gene in transgenic mice

Neurofilaments as members of the intermediate filament family and major components of large myelinated fibers, have been assigned a putative role in maintaining the structural integrity of the nerve cell and in determining axonal caliber. Previous work with the neurofilament light subunit (NF-L) fail to show any significant effect on axonal caliber following overexpression of that subunit in transgenic mice (Monteiro et al., 1990). No increase in axonal diameter was observed in transgenic mice over-expressing the mouse NF-L subunit by four-fold (Monteiro et al., 1990). The only change noted was the formation of cataracts. A possible explanation for these results is that although NF-L alone can form a filament, it does not have any projections at the periphery. On the other hand, the long carboxy-terminal domain of NF-H is associated with the projections observed on the filament periphery. These projections are believed to mediate interactions between NFs and therefore may have a particular influence on axonal caliber.

The approach used to study the function of NF-H and its possible involvement in axonal volume was to direct its expression in transgenic mice. A cosmid clone encompassing the complete human NF-H gene (Cos4NFH) was isolated from a chromosome 22 genomic library and microinjected into mice. Four transgenic lines with multiple copies of the intact human gene were obtained. In all four lines, the transgene expression was tissue-specific. Unexpectedly, mice expressing the human NF-H gene at approximately two-fold the endogenous level exhibited pathological

features resembling those found in human motor neuron diseases including ALS and an inherited giant axonal neuropathy observed in children (GAN). For instance, selective enlargements of motor neuron perikarya, DRG cell bodies and proximal axons were observed. Electron microscopy studies revealed that the enlargement consisted of intracytoplasmic accumulation of NFs. In addition distal axonal atrophy and muscle atrophy were observed. The human NF-H transgenic mice therefore provide a useful experimental model of human motor neuron disorder.

5.2 Axonal transport studies

As the protein synthetic machinery is excluded from the axon, proteins synthesized in the soma are transported via a sophisticated mechanism along the neuroaxis. Therefore, normal process growth of an axon is dependent on synthesis, assembly and axonal transport of cytoskeletal components. The neurodegenerative process that occurs following intoxication with IDPN, hexacarbons and aluminim described in section 2.3 were perceived as a secondary effect of an impairment in axonal transport (Gajdusek, 1985; Chou, 1992). To determine whether the perikaryal and axonal swellings in the NF-H transgenic mice were the cause or consequence of a defect in axonal transport, analysis of axonal transport of labeled proteins in normal and transgenic motor/sensory axons were performed. Comparison of the distribution of labeled proteins in transgenic and control sciatic nerve fibers demonstrated that there was alteration in the compositon, rate and amount of proteins transported. Impairment of axonal transport was not selective for NFs but also affected other polypeptide components. There was a decrease rate of transport of actin, tubulin and NF proteins in motor axons as well as retention of rapidly transported proteins. Furthermore, the stoichiometry of radiolabeled NF-L, NF-M and NF-H was significantly altered. These results suggest that the accumulations of NF within perikarya and proximal axons are not a by-product of a defective axonal transport but play a critical role in the evolution of the motor neuron disorder observed in the human NF-H transgenic mice.

5.3 Regulation of the human NF-H gene expressed in transgenic mice

As mentioned in sections 4.1 and 4.2 factors such as the site of chromosomal integration, the presence of introns and essential regulatory elements can affect the

expression of a transgene in mice. A locus control region has been identified in the human β-globin gene that circumvent these factors resulting in high-level, tissue-specific expression of the human β-globin in transgenic mice. To determine if our Cos4NFH cosmid contains such regulatory region, slot blots, immunoblots and Northern blots were performed. Results provided evidence for copy-dependent, integration-independent and correct developmental expression of the human NF-H gene in transgenic mice. This is the first example of a transgene being expressed in such a manner in nerve cells. This finding therefore suggests first, the presence of an LCR in the 39 kb injected human NF-H cosmid and second, that the regulatory sequences present in the LCR are recognized by the mouse transcription system.

Chapter II

Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: A mouse model of amyotrophic lateral sclerosis

Francine Côté, Jean-François Collard and Jean-Pierre Julien

Montreal General Hospital Research Institute

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Progressive Neuronopathy in Transgenic Mice Expressing the Human Neurofilament Heavy Gene: A Mouse Model of Amyotrophic Lateral Sclerosis

Francine Côté, Jean-François Collard, and Jean-Pierre Julien
Centre for Research in Neurosciences
McGill University
Montreal General Hospital Research Institute
Montreal H3G 1A4
Canada

Summary

We generated four transgenic mice with a 34 kb genomic fragment including the complete human neurofilament heavy (NF-H) gene. This human NF-H fragment contained all regulatory elements for tissue-specific expression, and in two transgenic lines, human NF-H proteins were produced at levels up to 2-fold the levels of endogenous mouse NF-H protein. By 3–4 months of age, these NF-H transgenics progressively develop neurological defects and abnormal neurofilamentous swellings that are highly reminiscent of those found in amyotrophic lateral sclerosis (ALS). We propose that a modest up-regulation of NF-H cross-linkers can result in an impairment of neurofilament transport, causing neuronal swellings with ensuing axonopathy and muscle atrophy, a mechanism of pathogenesis pertinent to the possible etiology of ALS.

Introduction

Neurons contain three major classes of cytoskeletal organelles: microtubules, neurofilaments, and microfilaments. Neurofilaments are found predominantly in axons, where they run longitudinally and parallel to each other. In mature neurons, neurofilaments are composed of three protein subunits, light (NF-L) (68 kd), medium (NF-M) (145 kd), and heavy (NF-H) (200 kd) (Hoffman and Lasek, 1975; Liem et al., 1978). During neurogenesis, there is a differential expression of the three subunits. The NF-L and NF-M proteins are coexpressed during early embryonic development, while the activation of NF-H expression is delayed to the postnatal period (Shaw and Weber, 1982; Julien et al., 1986; Carden et al., 1987). The three subunits share with each other and with all intermediate filament proteins a conserved central domain of approximately 310 amino acids that is involved in the formation of coiled-coil structures (Geisler et al., 1984; Franke et al., 1987; Steinert and Roop, 1988). In vitro reassembly experiments showed that the NF-L subunit is able to assemble into homopolymeric 10 nm filaments (Geisler and Weber, 1981; Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1990). In contrast, the NF-M and NF-H self-assemble in vitro into short filament structures and require coassembly with NF-L for formation of intermediate filaments (Gardner et al., 1984; Hisanaga and Hirokawa, 1990; Hirokawa, 1991).

A role of neurofilaments in the control of axon caliber was first suggested by the close correlation between the number of neurofilaments and the diameters of large myelinated axons (Hoffman et al., 1984, 1985, 1987; Griffin and Watson, 1988). Nonetheless, a subsequent study in transgenic mice revealed that overexpression of NF-L protein alone was not sufficient to affect axonal caliber (Monteiro et al., 1990). This finding raised the possibility that normal properties of neurofilaments required the correct ratios of the two other subunits, the NF-M and NF-H proteins. A striking feature of the NF-M and NF-H proteins is their long carboxy-terminal tail regions that form side arms unique to neurofilaments (Julien and Mushynski, 1982, 1983; Hirokawa et al., 1984; Hisanaga and Hirokawa, 1990). At the electron microscopic level, the side arms appear to cross-link neurofilaments and other neuronal structures. A number of observations suggested that the side arms can modulate the spacing between neurofilaments, thereby regulating the caliber of axons (Carden et al., 1987; Lee et al., 1988; Julien and Grosveld, 1991; de Waegh et al., 1992). In addition, there is evidence that the side arms can affect the rate of neurofilament transport down the axon (Willard and Simon, 1983; de Waegh et al., 1992). This notion came first from the observation that the postnatal appearance of NF-H protein coincides with the decreased velocity of cytoskeletal transport (Willard and Simon, 1983).

The NF-H tail domain is particularly intriguing, with multiple repeats of KSP (Julien et al., 1988; Lees et al., 1988) that account for the unusual high content of phosphoserine residues in this protein (Julien and Mushynski, 1982, 1983; Carden et al., 1985; Zimmerman and Schlaepfer, 1986; Lee et al., 1988). The presence of charged amino acids in a domain of NF-H that form sidearm projections led to the suggestion that local changes in phosphorylation of NF-H could regulate the spacing between neurofilaments (Carden et al., 1987; Lee et al., 1988; Julien et al., 1988). Indeed, a recent analysis of trembler mice provided direct evidence that changes in NF-H phosphorylation mice can alter neurofilament densities, and thereby the axonal caliber, and slow axonal transport (de Waegh et al., 1992).

The abnormal accumulation of neurofilaments in the perikarya and proximal axons is a characteristic pathological finding in amyotrophic lateral sclerosis (ALS) (Carpenter, 1968; Hirano et al., 1984; Schmidt et al., 1987; Sasaki et al., 1989; Chou, 1992), the most common form of human motor neuron disease. Although the cause of ALS is unknown (Armon et al., 1992; Figlewicz and Rouleau, 1992), an impairment of axonal transport has been proposed as a plausible mechanism of pathogenesis (Gajdusek, 1985; Goldman and Yen, 1986; Griffin and Watson, 1988; Breuer et al., 1992; Chou, 1992). Evidence for this hypothesis is supported by the selective alterations of axonal transport by chemical agents in experimental models that result in neurofilamentous swellings. Thus, abnormal accumulations of neurofilaments in distinct regions of the neuron

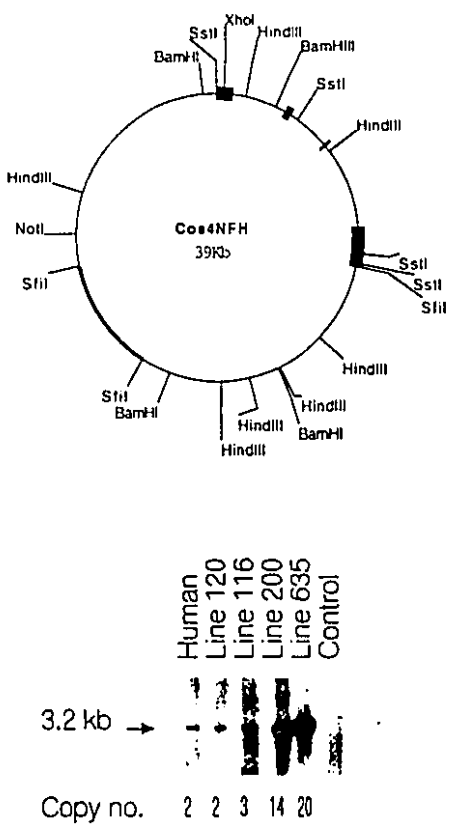


Figure 1. Restriction Cleavage Map of the Injected Human NF-H Fragment and Southern Blot Analysis of Mouse Tail DNA

(A) The 39 kb cosmid clone that contains the complete human NF-H gene was linearized with NotI before microinjection. The wide, filled area corresponds to NF-H exons and the narrow area to vector sequences. Not all XhoI sites are indicated.

(B) Four transgenic founders were identified by Southern blotting using 10 μg of tail DNA. Genomic mouse and human DNA were digested with BamHI, electrophoresed, and hybridized to a 361 bp SstI–XhoI fragment of the human NF-H gene. The human NF-H gene yields a signal at 3.2 kb. The four transgenic lines have a different number of integrated copies. The control lane contained DNA of a normal mouse.

occur in a variety of other disorders, including an inherited giant axonal neuropathy in children (Carpenter et al., 1974) and toxic neuropathies induced by β,β′-iminodipropionitrile (IDPN) (Griffin et al., 1978), 2,5-hexanedione (Graham et al., 1984), acrylamide (Asbury and Johnson, 1978), and aluminium (for review see Troncoso et al., 1992). The pathology in the case of IDPN (Griffin et al., 1978), 2,5-hexanedione (Graham et al., 1984), and aluminium (Bizzi et al., 1984; Troncoso et al., 1985) intoxications has been shown to result directly from abnormalities in intracellular transport of neurofilaments.

We generated transgenic mice using a large genomic fragment including the human NF-H gene. We report here that a modest overexpression of human NF-H proteins in transgenic mice causes a progressive neuronopathy with pathological features that resemble those found in ALS.

Results

Generation of Transgenic Mice

Lees et al. (1988) previously reported the isolation of two genomic clones containing partial sequences for the human NF-H gene. To obtain a genomic fragment including the complete NF-H gene, we screened a human genomic library enriched in chromosome 22 using a mouse NF-H cDNA probe (Julien et al., 1988). We isolated one cosmid clone that contained the complete NF-H transcriptional unit flanked with 9.6 kb of 5′ sequences and 13.4 kb of 3′ sequences (Figure 1A). Its identity was confirmed by mapping with various restriction enzymes and determination of partial sequences. The cosmid was linearized with NotI before its microinjection into fertilized mouse eggs.

Four transgenic founders were obtained out of 94 offspring. All four founders transmitted the transgene to further generations in a Mendelian fashion. The number of copies in each transgenic line was estimated by Southern blot analysis of genomic tail DNA digested with BamHI and hybridized with a ^{32}P-labeled human NF-H probe of 361 bp (SstI–XhoI). Based on densitometric analysis, we estimate that hemizygous mice from lines 120, 116, 200, and 635 carry, respectively, 2, 3, 14, and 20 copies of the human NF-H gene (Figure 1B).

Neuron-Specific Expression of the Human NF-H Transgene

To examine whether the human NF-H transgene was correctly expressed in mice, Northern blot analysis was carried out on 20 μg of total RNA obtained from different tissues of F1 offspring (2 months old) from two transgenic lines, lines 200 and 635. The Northern blot in Figure 2 (upper panel) shows the result of an hybridization at high stringency using the SstI–XhoI human NF-H probe described above for Southern analysis. The results with a nontransgenic littermate reveal that at high stringency, this probe hybridizes very poorly to mouse NF-H messenger RNA (mRNA). The human NF-H mRNA of 3.9 kb in size was detected only in the brain, cerebellum, and spinal cord of transgenic mice. Human NF-H transcripts were not detected in liver, kidney, lung, spleen, muscle, or heart. Thus, expression of the human NF-H transgene was strictly limited to nervous tissue.

To assess the level of overexpression of NF-H mRNAs in transgenic mice, the same blot was stripped and rehybridized at reduced stringency with a 414 bp fragment derived from the first exon of the mouse NF-H gene (Figure 2, middle panel). This probe, which spans a region with 87% homology between mouse and human NF-H, cross-hybridizes with human NF-H mRNA under the hybridization conditions used here. Note that the mouse and human NF-H transcripts, which are of similar size (Julien et al., 1988; Lees et al., 1988), are not distinguishable on the Northern blot shown in Figure 2. As expected, in all mice examined, the mouse NF-H probe mRNA detected transcripts in RNA samples derived from the nervous system. However, stronger NF-H signals are observed in transgenic samples. Densitometric analysis on blots that were

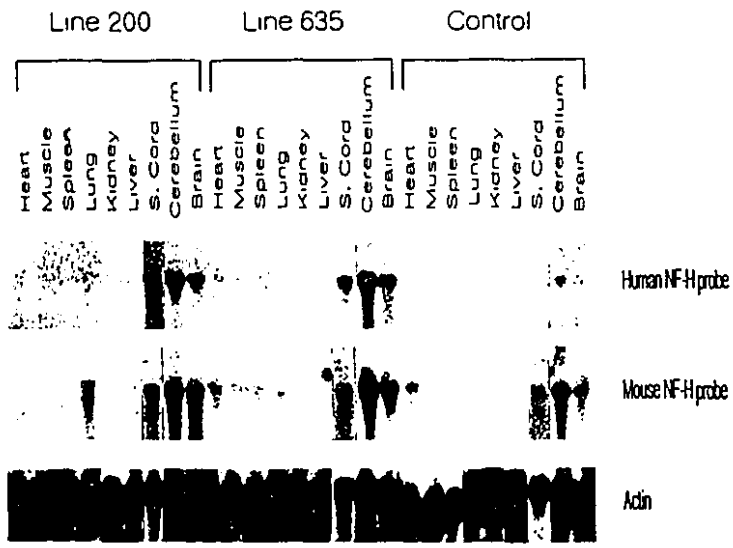


Figure 2. Tissue-Specific Expression of Human NF-H mRNA in Transgenic Mice

Northern blot analysis was carried out using total RNA (20 μg) isolated from various tissues of transgenics from lines 200 and 635 and of a normal mouse. The membrane was hybridized with a human NF-H probe at high stringency (upper panel), with a mouse NF-H probe at reduced stringency (middle panel), and with actin (lower panel).

less exposed revealed in mouse line 200 increases of total NF-H transcripts of 1.5-, 1.8-, and 3.0-fold in brain, cerebellum, and spinal cord, respectively, and in mouse line 635 increases of 1.7-, 2.0-, and 4.0-fold in brain, cerebellum, and spinal cord, respectively. Thus, similar levels of overexpression of NF-H mRNA occur in the two transgenic lines, 200 and 635, that contain the highest number of copies of the transgene. Milder increases of 20%–50% in total NF-H mRNAs were detected in various central nervous system regions of transgenic lines 120 and 116 that contain fewer transgene copies (data not shown). To examine expression of human NF-H protein in transgenic mice from line 200, SDS–gel electophoresis and immunoblotting were performed on various tissue homogenates from both the central and peripheral nervous systems. As shown on the Coomassie blue–stained gel in Figure 3A, the human NF-H protein with its higher electrophoretic mobility is easily distinguishable from the endogenous mouse NF-H protein. Expression of human NF-H proteins was further confirmed by immunoblotting of a duplicate gel using a monoclonal antibody, designated OC95 (provided by V. M.-Y. Lee, University of Pennsylvania), which

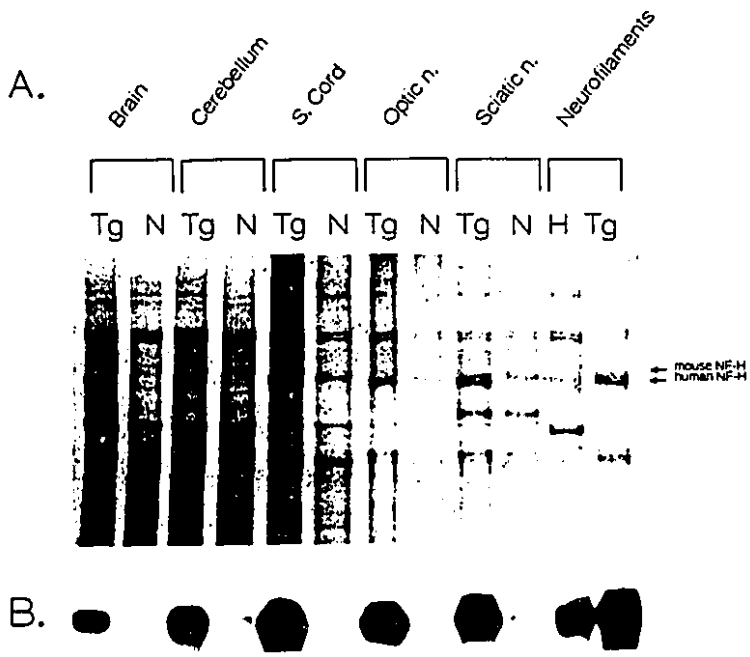


Figure 3. Detection of Human NF-H Protein in Nervous Tissue

(A) Tissue homogenates from the central and peripheral nervous systems were electrophoresed on a 6.5% SDS gel. The gel was stained with Coomassie blue. Having a higher electrophoretic mobility, the human NF-H protein is easily distinguishable in total homogenates of cerebellum, spinal cord, and optic and sciatic nerves.

(B) The presence of human NF-H proteins was further confirmed by immunoblotting of a duplicate gel using a specific anti-human NF-H antibody (OC95; provided by V. M.-Y. Lee).

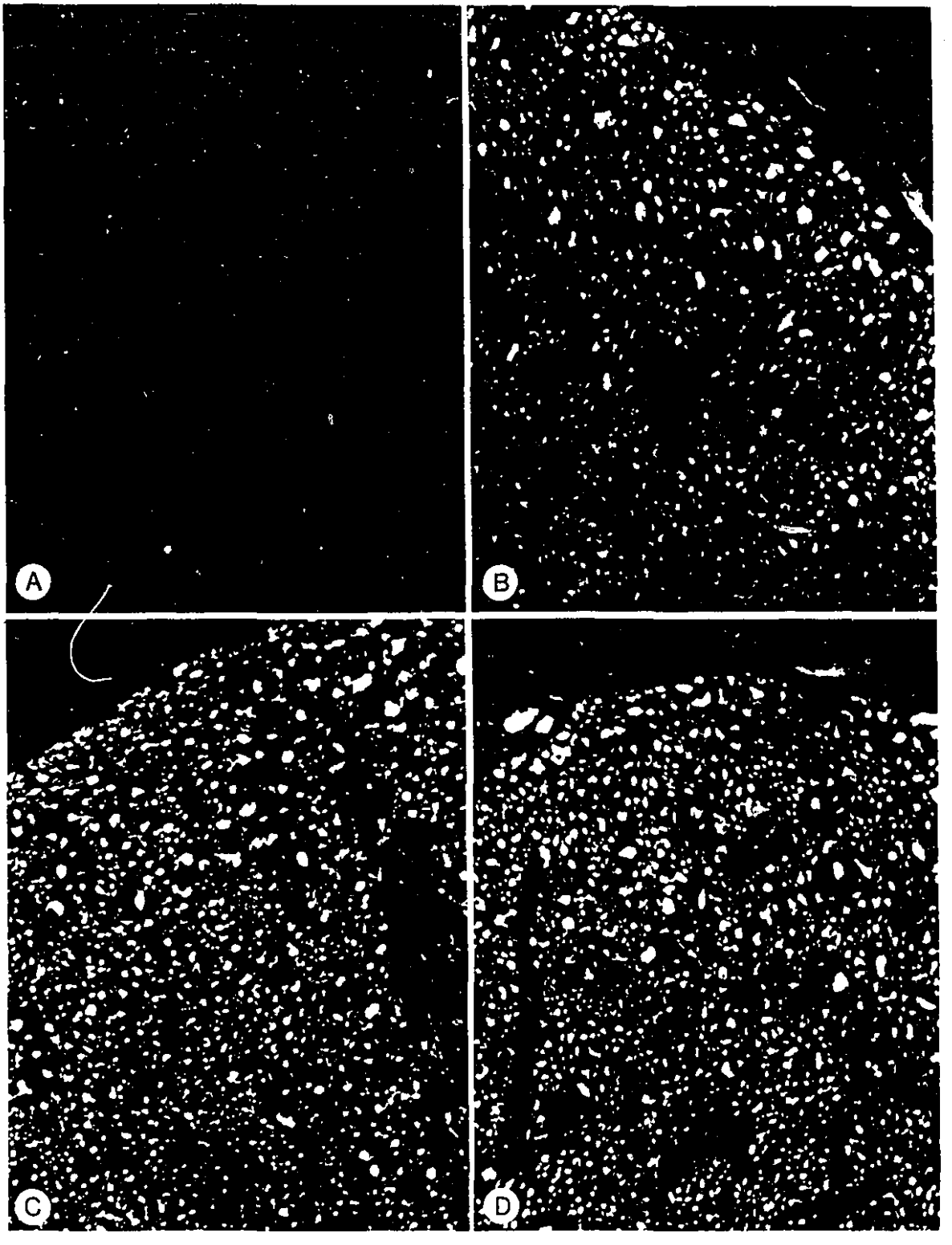
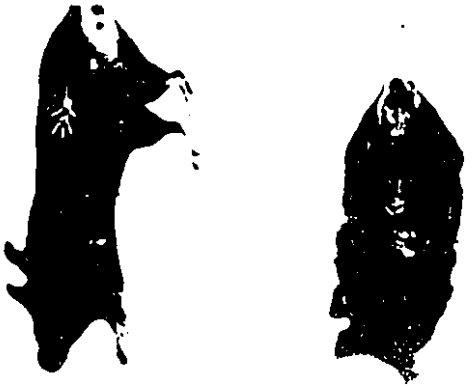


Figure 4. Immunofluorescence Detection of Human and Mouse NF-H Proteins in Axons of Transgenic Spinal Cord

The anti-human NF-H monoclonal antibody (OC95) does not immunolabel spinal cord of a normal mouse (A), but it strongly labels axons of a spinal cord from a transgenic of line 200 (3 months old) (B). The staining pattern resembles the one obtained with the anti-mouse NF-H antibody (OC59) that detects axons of both normal (C) and transgenic (D) spinal cord sections. These two antibodies (provided by V. M.-Y. Lee, University of Pennsylvania) recognize phosphorylated epitopes on NF-H proteins present in axons but not in neuronal perikarya. Magnification, 350 ×.



A B

Figure 5. Abnormal Limb Flexions in NF-H Transgenic Mice

When lifted by the tail, normal mice extend their legs (A), whereas homozygous NF-H transgenic mice of line 200 (3 months old) progressively lose this reflex (B).

recognizes specifically the phosphorylated form of human NF-H protein (Figure 3B).

Based on densitometric analysis of Coomassie blue–stained bands from the spinal cord, optic nerve, and sciatic nerve of a hemizygous transgenic (2 months old), we concluded that human NF-H protein was expressed in both the central and peripheral nervous systems at levels that exceed by approximately 2-fold those of the endogenous mouse NF-H (Figure 3A). Similar results were obtained with the analysis of transgenics from line 635 (data not shown). Thus, in contrast with the situation previously observed with NF-L transgenes (Monteiro et al., 1990), overexpression of NF-H in transgenic mice was not impeded by the existence of posttranscriptional controls.

The increased levels of NF-H proteins in transgenic mice had no effect on the levels of NF-M and NF-L proteins. We obtained biochemical data indicating that the extra NF-H proteins are incorporated into neurofilament structures. Neurofilament preparations made by homogenization of spinal cords of transgenic mice in 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM sodium phosphate (pH 6.5) were centrifuged at 15,000 × g for 20 min. The pellets and supernatants were analyzed by SDS–polyacrylamide gel electrophoresis. The NF-H proteins are unable to self-assemble in 10 nm filaments (Hirokawa, 1991), and therefore one would expect them to appear in the supernatant if unassembled into neurofilaments. Instead, the human and mouse NF-H proteins, together with NF-L and NF-M proteins, were entirely recovered into the insoluble fraction, suggesting their incorporation into neurofilaments. The NF-H proteins were not simply trapped in the pellet since unassembled NF-H subunits following in vivo disassembly of neurofilaments by okadaic acid treatment can be recovered in the soluble fraction under similar experimental conditions (Sacher et al., 1992).

As expected for detection of subunits assembled into neurofilaments, the anti-human NF-H antibody (OC95) yielded a strong immunofluorescence staining of transgenic axons throughout the nervous system. Figure 4 shows the specific immunodetection of both human and mouse NF-H proteins, respectively, in axons of a transgenic spinal cord from line 200. As revealed in Figure 4A, the anti-human NF-H antibody does not immunolabel endogenous mouse neurofilament proteins. The intensity and distribution of the staining with the anti-human NF-H monoclonal are comparable with those obtained with the anti-mouse NF-H monoclonal (OC59).

Clinical Symptoms

Transgenic mice derived from the founder lines 200 and 635 appear normal during the first few weeks of postnatal development. Then, progressively, the mice manifest signs of neurological abnormality. They develop fine tremors. When lifted by their tails, they reflexively contract their forelimbs and hindlimbs while normal mice extend their legs (Figure 5). The disease progresses with signs of weakness. Severely affected mice are unable to support their weight when grasping a pencil, and they have difficulty in ambulating. During the moderate stage, the mice breed well. Some of our NF-H transgenic mice are more than 18 months of age. However, two NF-H transgenic mice from line 635, one homozygous female and one hemizygous male of more than 1 year old, were recently sacrificed after they started to demonstrate breathing problems.

The founders from lines 200 and 635 as well as their F1 transgenic offsprings began to show overt phenotypes by 3–4 months of age. However, we observed that homozygous F2 offsprings of line 200 were more severely affected than their hemizygous littermates, implying a correlation between the neurological symptoms and the levels of NF-H expression. We also noted an earlier onset on symptoms after successive inbreedings of this mouse line. In the F6 generation, disease symptoms became evident as early as 4 weeks of age. This variability in timing is likely the result of the different genetic backgrounds of mice that evolved from a transgenic founder with a hybrid C57BL6/C3H background.

Swellings of Perikarya and Proximal Axons

Light microscopic examination of spinal cord sections from homozygous NF-H transgenic mice of line 200 (3 months old) revealed striking abnormalities in motor neurons of the anterior horn (Figures 6A and 6B). Many neurons with eccentrically localized nuclei show prominent swellings of their perikarya and proximal axons. Examination with electron microscopy demonstrated that the swellings consist of densely packed 10 nm neurofilaments (Figures 6C and 6D). Other organelles, including mitochondria and vesicles, are displaced into a submembranal disposition (Figure 6D) or squeezed through the masses of neurofilaments (Figure 6C). Evidence that the filaments in the perikaryal swellings are composed by heteropolymerization of multiple neurofilament subunits was provided by their immunofluorescence staining with either an anti-

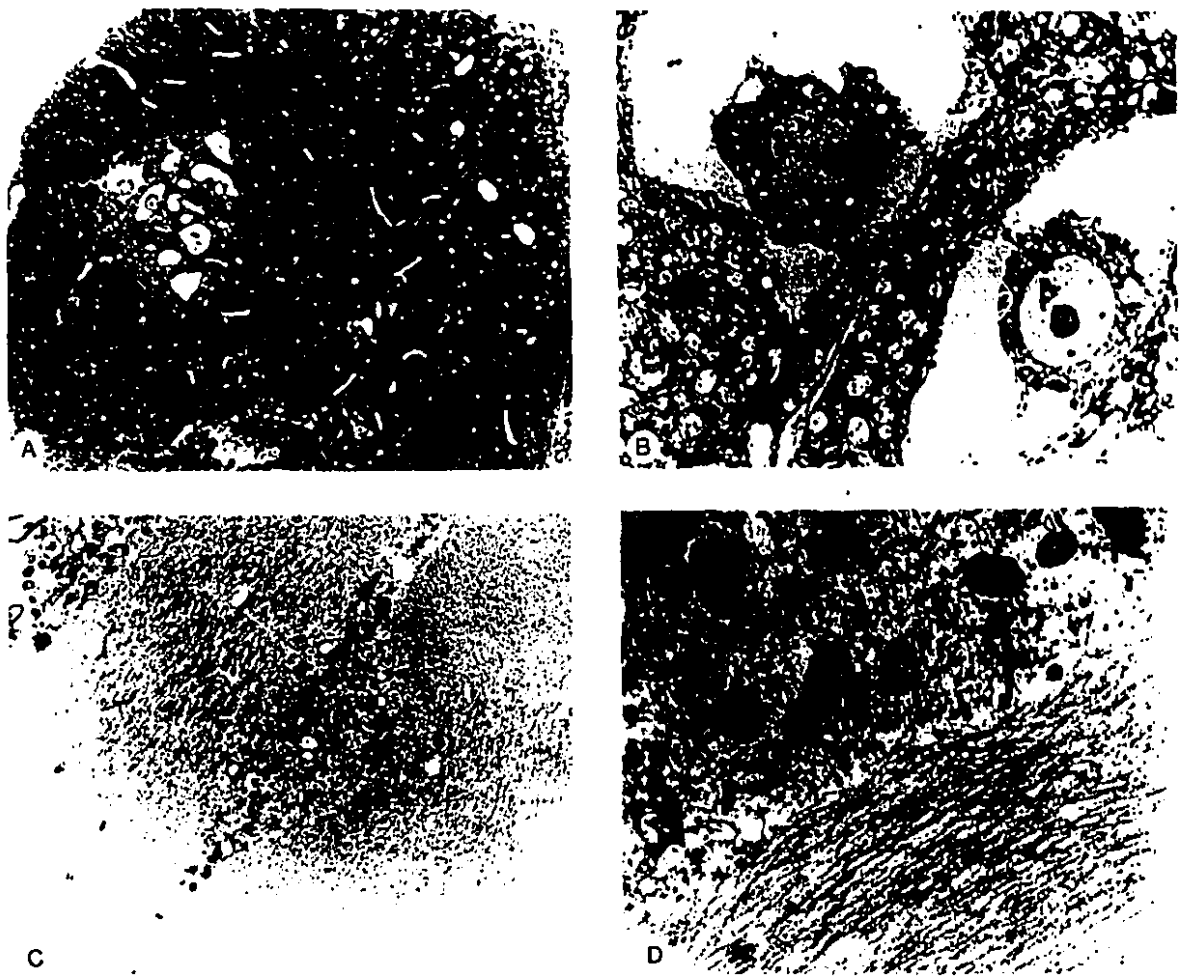


Figure 6. Swellings of the Perikarya and Proximal Axons of Anterior Horn Motoneurons

Light microscopy of a spinal cord section from a transgenic mouse from line 200 (3 months old) (magnification, 70×) showing prominent swellings in soma and proximal axons of motoneurons (A). The arrow points to a motoneuron magnified by 10× more in (B). In electron microscopy, the intracytoplasmic material consisted of accumulations of densely packed neurofilaments with displacement of other organelles. Magnification in (C), 3,170×; magnification in (D), 17,200×.

NF-L antibody or the SMI-32 monoclonal antibody (Sternberger and Sternberger, 1983) that recognizes the dephosphorylated NF-H protein (data not shown).

Such dramatic swellings also occur in many neurons of the dorsal root ganglia (DRG) in these NF-H transgenics. The light micrographs in Figures 7A and 7C show the organization and morphology of cells and axons in a DRG section from a normal mouse. The appearance of DRGs from transgenic animals is very different (Figures 7B and 7D). Many cells have eccentric nuclei and their perikarya are swollen. Numerous proximal axons are also enlarged with thin myelin sheaths (see arrows in Figure 7D). Electron microscopy confirmed that perikaryal and axonal enlargements in DRG are caused by the aberrant accumulations of neurofilaments (data not shown).

Atrophy of Distal Axons and Muscle Fibers

Electron microscopic analysis of the ventral spinal roots and of the sciatic nerve in homozygous NF-H transgenics revealed abnormalities indicative of axonal atrophy. As shown in Figure 8, distal axons of the sciatic nerve have shrunken in caliber, as indicated by their relatively thick myelin sheaths. Myelin ovoids are abundant. In some instances, axons contain only microtubules, mitochondria, and vesicles and are devoid of neurofilaments (Figure 9). The progressive axonopathy in the NF-H transgenics is accompanied by secondary atrophy of skeletal muscle fibers (Figure 10).

Discussion

Impairment of Neurofilament Transport

We report in this paper that a modest overexpression of human NF-H proteins in transgenic mice provokes a progressive neuronopathy with features that resemble those observed in ALS. It is unlikely that the pathology is the consequence of genetic material other than the NF-H gene contained within the Cos4NF-H clone. Based on Northern

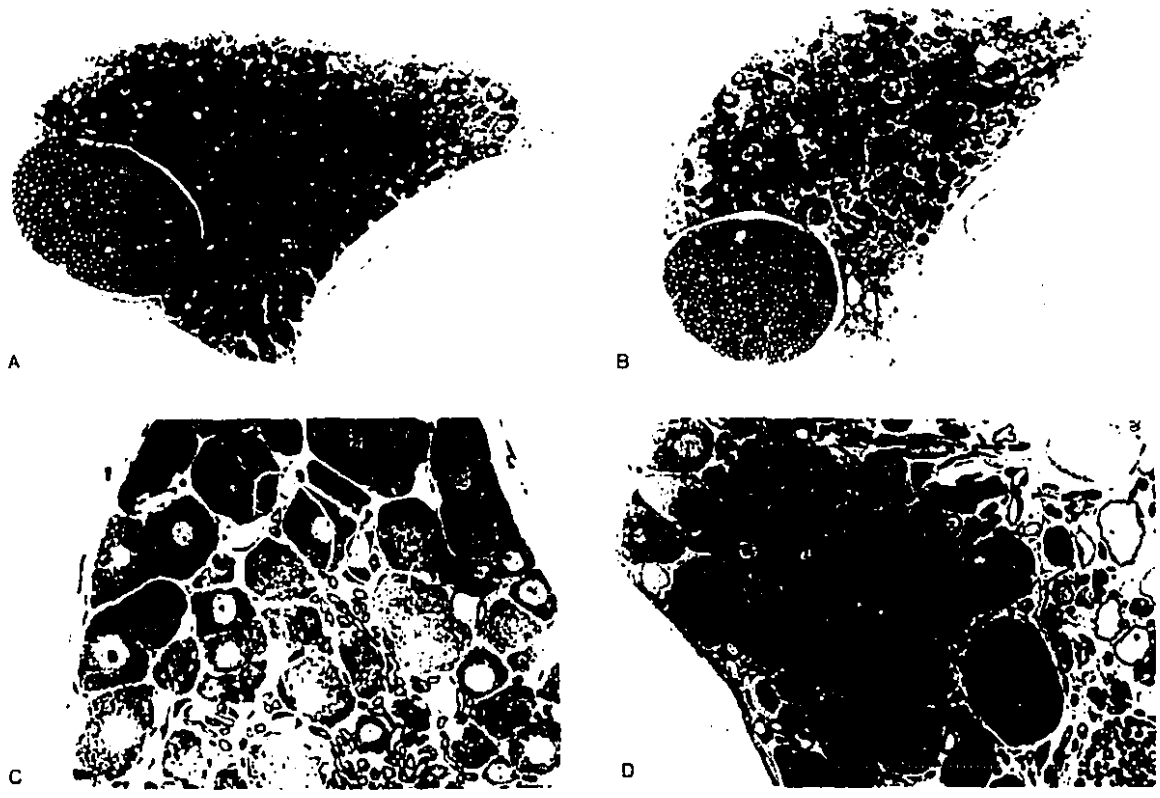


Figure 7. Perikaryal Swellings and Giant Axons in DRG Sensory Neurons

(A and C) Light microscopy of normal mouse DRG. (B and D) Abnormal swellings in large DRG sensory neurons from a transgenic of line 200 (3 months old). The arrowhead points to prominent neurofilament accumulations in a perikaryon. The arrows point to giant axons. Note the population of sensory neurons that do not exhibit abnormal swellings. Magnification of (A) and (B), 63×; magnification of (C) and (D), 250×.

blot analyses, there is no indication for the presence of another gene in this clone. RNA samples from transgenic lines 200 and 635 were hybridized with DNA probes spanning the different regions of the clone. The NF-H mRNA was the only detectable RNA species on Northern blots (data not shown).

The pathology in NF-H transgenics is characterized by abnormal accumulations of neurofilaments in the perikarya and proximal axons of anterior horn motoneurons and of DRG neurons. Similar neurofilamentous swellings of proximal axons can be induced experimentally by IDPN (Griffin et al., 1978) or aluminium (Bizzi et al., 1984; Troncoso et al., 1985) intoxications. The accumulations of neurofilaments by IDPN administration result from a selective impairment of the slow axonal transport (Griffin et al., 1978). On the other hand, it was proposed that aluminium neurotoxicity is caused by abnormal cross-linking of neurofilaments leading to impairment in transport of perikaryal neurofilaments to the initial axon (Bizzi et al., 1984; Troncoso et al., 1985).

In the NF-H transgenics, exceeding levels of incorporated NF-H might affect intracellular transport of neurofilaments either by imposing additional drag due to extra NF-H cross-linkages or by impeding normal interactions of neurofilaments with a slow transport motor. Indeed, a defect in neurofilament transport would be consistent with previous studies demonstrating a close correlation between decreased velocity of the slow axonal transport and the appearance of NF-H during postnatal development (Willard and Simon, 1983). Additional evidence for a role of NF-H in mediating neurofilament transport was recently provided by the report showing that decreased levels of NF-H phosphorylation in trembler mice produce a closer packing of neurofilaments and thereby a reduction in the rate of the slow axonal transport (de Waegh et al., 1992).

An impairment of neurofilament transport by extra NF-H cross-linkers is likely responsible for the gradual piling up of newly synthesized neurofilaments in the perikarya and proximal axons with ensuing distal axonopathy and muscle atrophy. This "dying back" model has been hypothesized as a plausible mechanism underlying ALS (Gajdusek, 1985; Griffin and Watson, 1988; Chou, 1992).

It is remarkable that a relatively modest increase in NF-H expression was sufficient to generate such severe neurological defects. Even in the transgenic founders of lines 116 and 120, having approximately 50% increase in total NF-H mRNA expression (data not shown), we started to notice mild neurological symptoms at the age of 6 months. It is possible that the human NF-H protein holds a certain susceptibility to induce aberrant neurofilament accumulations, perhaps through the formation of shorter and stronger neurofilament cross-linkages. Sequence data on

Figure 8. Axonal Atrophy in the Distal Region of a Sciatic Nerve

Electron microscopic examination of a cross section of a sciatic nerve distal to axonal swellings from homozygous NF-H transgenics of line 200 (3 months old). Signs of axonal atrophy are evident. Myelin ovoids are abundant within axoplasm. Magnification, 1760×.

NF-H tail regions reveal considerable variations from one species to another in the length of the carboxyl terminals, the number of NF-H phosphorylation repeats, and the exact sequences between the repeats. Nonetheless, it is striking that the phosphorylation domain in human NF-H (Lees et al., 1988) is highly homologous to the corresponding region of NF-H protein in rabbit (Mack et al., 1988), a species especially susceptible to accumulations of neurofilaments by aluminium intoxication (Troncoso et al., 1990).

A Unique Mouse Model of ALS

There are other hereditary mouse models with motor neuron diseases (Messer, 1992). However, in these models the genetic defects have yet to be identified, and several aspects of their pathologies differ from those described in ALS. The wobbler (Mitsumoto and Bradley, 1982; Kaupmann et al., 1992), wasted (Lutsep and Rodriguez, 1989), and pmn (Sendtner et al., 1992) are recessive mutant mice that develop early manifestations of motor neuron degeneration in contrast with ALS, which is basically a disease of older individuals. In both wasted and pmn, the disease progresses very rapidly, with death at about 4 and 7 weeks, respectively. In the wobbler mouse, the pathology affects predominantly cervical motor neurons (Mitsumoto and Bradley, 1982), and neurofilamentous swellings are infrequent (Andrews, 1975). The motor neuron degeneration (mnd) mouse exhibits an adult onset motor neuron disease, with signs of neurological disorder identifiable by 6–7 months of age, followed by a progression to total paralysis with premature death (Callahan et al., 1991). Although many clinical symptoms in the mnd mice resemble those described in ALS, the pathology does not produce neurofilamentous swellings, a hallmark of ALS, but rather a marked redistribution of neurofilaments within the cytoplasm of anterior horn neurons (Callahan et al., 1991).

The NF-H transgenic mice described here present pathological features with striking similarities to those

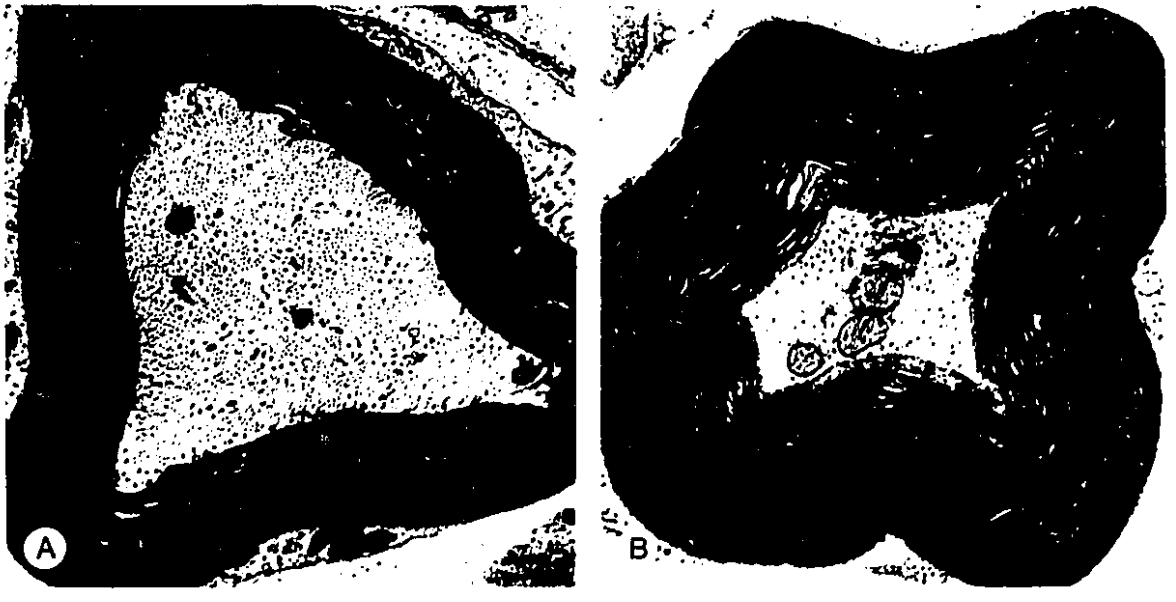


Figure 9. High Magnification of Normal and Transgenic Axons of a Sciatic Nerve

(A) The normal axon contains numerous neurofilaments, and it has a ratio of myelin:axoplasm of 1:3. (B) The axon from the NF-H transgenic has shrunken from a previously larger caliber, as indicated by the excessive thickness of the myelin sheath (ratio of myelin:axoplasm, 1:1). Note that the axon is devoid of neurofilaments. Magnification, 16,100×.

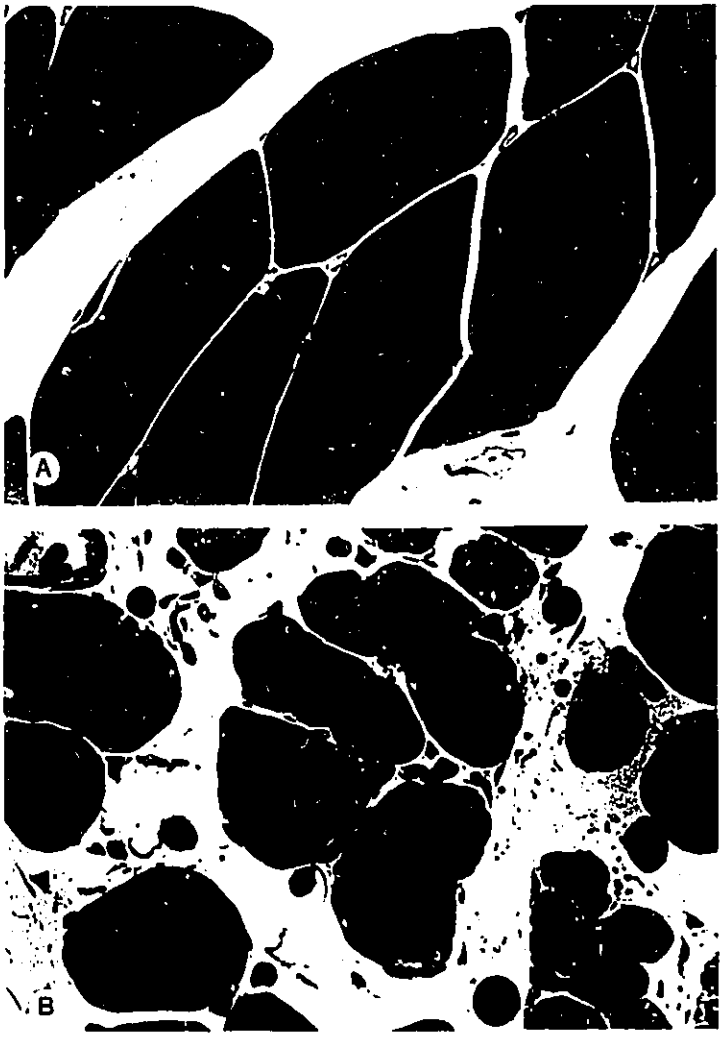


Figure 10. Light Microscopy of Normal and Degenerating Skeletal Muscle Fibers

Light microscopy of normal (A) and degenerating (B) skeletal muscle fibers of homozygous NF-H transgenics of line 200 (7 months old). Magnification, 530×.

found in ALS. The progressive neuronopathy in these mice is characterized by neuronal swellings that resemble those found in motor neurons of ALS patients (Carpenter, 1968; Hirano et al., 1984; Schmidt et al., 1987; Sasaki et al., 1989; Hirano and Kato, 1992; Chou, 1992). As discussed above, the "dying back" axonopathy in NF-H transgenics is likely the result of a defect in neurofilament transport, a model mechanism pertinent to the prevailing hypothesis of an axonal transport defect underlying ALS (Gajdusek, 1985; Goldman and Yen, 1986; Griffin and Watson, 1988; Chou, 1992; Breuer et al., 1992). The neurofilamentous accumulations occur in large motor and sensory neurons, which are high producers of neurofilaments. This shared property can explain, in part, the cellular selectivity of the pathology. Although ALS is clinically a motor neuron disorder, there is an increasing recognition of degeneration outside the motor system (for review see Norris, 1992). Proximal axonal swellings have been observed in sensory neurons of central pathways in ALS (Averback and Crocker, 1982). Swellings have not yet been reported in peripheral sensory neurons of ALS patients, but a selective decrease in the number of large myelinated fibers in the dorsal root was described in ALS patients (Kawamura et al., 1981), and Radtke et al. (1986) found abnormal sensory-evoked potentials in 47% of their ALS patients.

The cumulative poisoning produced by extra NF-H can explain the delayed onset and progressiveness of the disease. An involvement of gene dosage is also illustrated by an earlier onset and more severe symptoms in homozygous as compared with hemizygous transgenic mice of line 200. In addition, our successive inbreedings of this mouse line suggest an influence of the genetic background on the phenotypes.

The NF-H transgenic model offers an interesting perspective not only for testing therapeutic strategies but also for investigating in a systematic way the various genetic and environmental factors controlling the onset and progression of the disease. The present study reveals a neurotoxicity produced by the human NF-H protein. Accordingly, the elucidation of cis- and trans-acting factors regulating NF-H expression, now in progress, might yield new insights on the etiology of ALS.

Experimental Procedures

Production of Transgenic Mice with a Human NF-H Clone

A cosmid clone bearing the human NF-H gene was isolated by screening a cosmid library of a human lymphoblastoid cell line enriched in chromosome 22 using a cDNA probe for mouse NF-H (Julien et al., 1988). The library was constructed by partial MboI cumulative digestion in the vector *Lawrist V* (Aubry et al., 1992). Restriction mapping and partial sequencing of the clone confirmed the presence of the complete human NF-H gene. This cosmid, designated Cos4NF-H, was linearized with NotI that cuts at 9.6 kb upstream of the transcription start site. Microinjection of the linearized 39 kb fragment was performed as described by Brinster et al. (1981). Integration of human transgene into the mouse genome was assessed by Southern blot analysis of genomic DNA isolated from the mouse tail. Human and mouse DNA was digested with BamHI, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane (Gene Screen Plus, New England Nuclear–Dupont). A 361 bp SstI–XhoI fragment from the first exon of the human gene was subcloned into the Bluescript SK(+) vector and used as probe for both Southern and Northern hybridizations. The DNA probe was labeled with [α-^{32}P]dATP (3000 Ci/mmol) (Amersham) by random priming using deoxynucleotides and random hexanucleotides (Pharmacia). The membrane was prehybridized in 5× SSC (1× SSC is 150 mM NaCl and 15 mM Na_3 citrate [pH 7.0]), 1% SDS, 20 mM Tris (pH 7.5), 5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinyl pyrolidone), 10% dextran sulfate, and 100 μg/ml heat-denatured salmon sperm DNA at 65°C for at least 2 hr. Radiolabeled DNA probe was added to the filter. After overnight hybridization at 65°C, membranes were washed 15 min at room temperature in 5× SSC, 1% SDS; 30 min at 65°C in 1× SSC, 1% SDS; and 30 min at 65°C in 0.5× SSC, 1% SDS. The filters were exposed to Kodak X-Omat AR film with an intensifying screen. Transgene copy number was estimated by densitometric analysis using SciScan 5000 (U. S. Biochemicals). Sequencing of the human first exon was performed by the dideoxy chain termination method (Sanger et al., 1980).

RNA Analysis

Mice were sacrified by cervical dislocation. Following dissection, tissues were immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was isolated by homogenization in guanidinium thiocyanate and ultracentrifugation through a CsCl cushion (Chirgwin et al., 1979). Each RNA sample (20 μg) was fractionated on a 1% agarose–formaldehyde gel (Sambrook et al., 1989) prior to blotting. The filter was prehybridized, hybridized, and washed as previously described for Southern analysis, with the following modification. An additional wash was done for 30 min at 65°C in 0.05× SSC, 1% SDS for the Northern membrane hybridized with the human probe. The same membrane was also hybridized with a mouse probe, using a 414 bp fragment (XhoI–NruI) from the first exon of the mouse gene. The hybridization solution was the same, but the temperature was lowered to 60°C. The filter was finally probed with actin.

SDS Gels and Immunoblotting

Total protein extracts from brain, cerebellum, spinal cord, optic nerve, and sciatic nerve were prepared by direct homogenization of the tissues into 1× SDS sample buffer (1× SDS buffer is 15% glycerol, 2% SDS, 80 mM Tris–HCl [pH 6.8], 5% β-mercaptoethanol, and 0.01% bromophenol blue). Each sample (5 μg) was loaded on a 6.5% acrylamide gel and run according to Laemmli (1970) using the Bio-Rad miniprotean system. Neurofilament-enriched preparations from mouse spinal cord and human brain were prepared as described previously (Julien et al., 1987), and 1 μg of protein was loaded on the gel. The gels were stained with Coomassie blue.

Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). After blocking the membrane with 3% powdered milk in a phosphate-buffered saline solution, the human NF-H protein was detected by incubation with an anti-human NF-H antibody that recognizes the phosphorylated carboxy-terminal (OC95; provided by V. M.-Y. Lee, University of Pennsylvania), followed by incubation with a peroxidase-linked anti-rat immunoglobulin G (Organo Teknica) using the Amersham ECL detection kit.

Microscopic Analysis

Mice were anesthetized with nembutal and perfused via 0.9% NaCl and 2.5% glutaraldehyde, 0.5% paraformaldehyde in sodium phosphate buffer (pH 7.4). Tissue samples were immersed in fixative for 2 hr, rinsed in phosphate buffer, and postfixed in 1% osmium tetroxide. After three washes with phosphate buffer, each sample was dehydrated in a graded series of ethanol and embedded in Epon. The thin sections were stained with toluidine blue and examined under a Nikon Labophot microscope. Ultrathin sections were stained for 8 min in lead citrate and examined with a Philips CM10 electron microscope.

Immunofluorescence Staining

Anesthetized mice were perfused via 0.9% NaCl and 4% paraformaldehyde in sodium phosphate buffer (pH 7.4). Tissue samples were rinsed in phosphate buffer and immersed in 15% sucrose and phosphate buffer. Cryostat sections of 10 μm were first incubated with rat anti-human NF-H antibody (OC95) or anti-mouse NF-H antibody (OC59) and then incubated with fluorescein-labeled goat anti-rat immunoglobulin G (Jackson Laboratories).

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Chapter III

Motor neuron degeneration by disruption of axonal transport in transgenic mice with ALS-like neurofilament accumulations.

Jean-François Collard*, Francine Côté* and Jean-Pierre Julien

* J.-F. C. and F.C. made equal contributions to this work

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Abstract

The abnormal accumulation of neurofiaments (NFs) in the perikarya and proximal axons of motor neurons is a characteristic pathological finding in amyotrophic lateral sclerosis (ALS) (Carpenter, 1968; Hirano et al., 1984; Schmidt et al., 1987). A causative role of NFs in the development of ALS was recently suggested by studies in which forced overexpression of NF proteins in transgenic mice provoked motor neuron disease (Xu et al., 1993 and Chapter II, this thesis) and by the finding of variant alleles of the human NF heavy gene (NEFH) gene in some ALS patients (Figlewicz et al., 1994). Moreover, NF swellings have been detected in degenerating motor neurons of transgenic mice expressing a mutant form of the human superoxide dismutase 1 (SOD1) found in familial ALS (Gurney et al., 1994). To investigate the mechanism by which disorganized NFs may result in neurodegeneration, we examined via pulse-labeling with [^{35}S]methionine if axonal transport of newly-synthesized proteins was impaired in spinal motor neurons of NEFH transgenics, a mouse model with ALS-like features (Chapter II, this thesis). Our data reveal dramatic decreases in the rates of intracellular protein transport into NEFH motor axons not only for NF proteins but also for other components including tubulin, actin and some rapidly transported proteins. Microscopic examination confirmed a paucity of organelles including cytoskeletal elements, smooth endoplasmic reticulum (SER) and especially mitochondria in degenerating axons of NEFH transgenic mice. From these results, we propose that NF accumulations, similar to those found in ALS, can play a central role in motor neuron degeneration by impeding the intracellular transport of components required for axonal maintenance.

Results and discussion

We reported previously that transgenic mice with a modest overexpression of the human NEFH gene (line 200) developed a progressive ALS-like pathology characterized by the presence of abnormal NF accumulations in the perikarya and proximal axons of spinal motor neurons (Chapter II, this thesis). We proposed that excess levels of NEFH protein, which forms NF cross-linkages, might reduce intracellular transport of newly synthesized NFs resulting in the gradual piling up of these structures in large motor neurons. The motor dysfunction in NEFH transgenics progresses during aging by the atrophy and subsequent degeneration of axons distal to the NF swellings. Light microscopy examination of the L5 ventral roots from a NEFH transgenic of two years old revealed a massive degeneration of large axons derived from spinal motor neurons (figure 1).

To clarify the mechanism of neurodegeneration in NEFH transgenics, we examined whether intracellular transport of axonal proteins was impaired. [^{35}S]methionine was injected into the spinal cord and following various intervals, the profiles of ^{35}S-labeled proteins in 5 mm consecutive segments of the sciatic nerve were analysed by fluorography of SDS polyacrylamide gels as described before (Hoffman and Lasek, 1975). Figure 2 shows fluorographic patterns of radiolabeled proteins transported in the sciatic nerve at 7 and 28 days after injection of [^{35}S]methionine into spinal cord of normal mice and NEFH transgenic mice. In normal mice (figure 2A and 2B), the NF-L, NF-M and NF-H proteins move into motor axons as a triplet with a velocity of approximately 0.5mm/d, in agreement with previous reports, (Hoffman and Lasek, 1975; Nixon, 1991). In motor axons of NEFH transgenics, there is a remarkable reduction in the rates of transport of NF proteins. In 3 month old transgenics, all three NF subunits moved into motor axons at a decreased rate (figure 2C and 2D). Note the anomalous stoichiometry of transported NF proteins and the exceedingly low levels of NF-M protein (figure 2D). During aging, the impairment in NF protein transport into motor axons becomes even more apparent. In 18 month old NEFH transgenics, sacrificed 28 days post-injection of [^{35}S]methionine, the bands corresponding to NF-L and phosphorylated NF-H are barely visible in the sciatic nerve while NF-M is not detectable (figure 2F).

The impairment of axonal transport in motor neurons of NEFH transgenic mice is not limited to NF proteins. As shown in figure 2, there is a reduction in the rate

of transport of tubulin and actin proteins, two cytoskeletal proteins of the slow moving components. In normal mice, 28 days following [^{35}S]methionine injection into spinal cord, the peak of incorporated radioactivity for tubulin and actin proteins occurred in the 10 and 15 mm segments of the sciatic nerve, respectively (figure 2B). In contrast, for NEFH transgenics of either 3 or 18 month old, the peaks of tubulin and actin remained in the proximal 5 mm segment of the sciatic nerve.

To determine if the axonal supply of rapidly transported proteins associated with membrane-bound structures and mitochondria was also impaired, we anayzed the radiolabeled proteins in the sciatic nerve 15h after injection of [^{35}S]methionine into the spinal cord of 3 month old NEFH transgenics. Remarkably, the results in figure 3 indicate that some major radiolabeled proteins of the fast transport component seen in normal mice (figure 3A) were not transported into motor axons of NEFH transgenics (figure 3B).

Electron microscopy confirmed a general depletion of crucial organelles in atrophied axons of the sciatic nerve from NEFH transgenic mice of 3 month old. As shown in the longitudinal sections in figure 4, when compared to normal axons of similar myelin thickness (A), shrunken transgenic axons (B) are clearly deficient in filamentous structures, mitochondria and SER. The lack of mitochondria in motor axons of NEFH transgenics was further confirmed by the very weak immunofluorescence staining of longitudinal sections of L5 ventral roots (figure 5B) with an antibody specific to inner membrane mitochondria (monoclonal MOM/H6/C12 from Serotec Inc.). This anti-mitochondria antibody yielded an intense staining of L5 ventral root axons from normal mice (figure 5A). The immunofluorescence staining of spinal cord sections from NEFH transgenic mice revealed the trapping of mitochondria within perikaryal NF accumulations of motor neurons (data not shown).

The abnormal NF accumulations in ALS have been widely viewed as a consequence of neuronal dysfunction, perhaps reflecting defects in axonal transport (Griffin et al., 1978; Gadjusek, 1985; Chou, 1992). However, the results presented here indicate that NF accumulations themselves can play a central role in motor neurons degeneration by disrupting the intracellular supply of components required for axonal integrity. Of particular interest is our finding of a deficient transport of mitochondria into motor axons distal to the NF swellings. A shortage in mitochondria might be expected to cause a severe disturbance on energy metabolism resulting in a neuropathy. Such " dying back " model of nerve degeneration has been proposed as a plausible

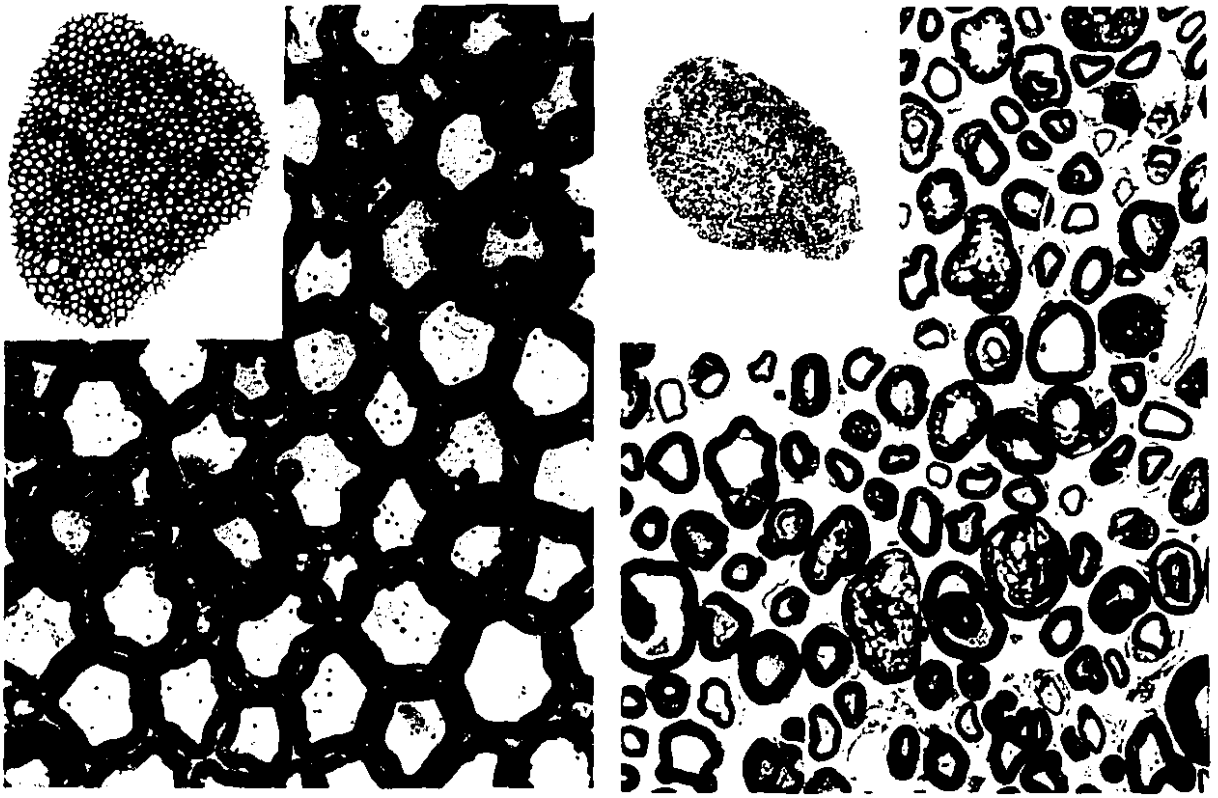
mechanism underlying ALS (Griffin et al., 1978; Griffin and Watson, 1988; Chou, 1992). The formation of NF aggregates in proximal axons are likely to be more damaging than those occuring in the cell bodies. Thus, large NF aggregated sequestered in perikarya of spinal motor neurons of transgenic mice expressing a mouse NF-H/lacZ fusion gene were well tolerated (Eyer and Peterson, 1994). The milder phenotype in the NF-H/lacZ transgenic mice, as compared to those seen in NEFH transgenics, is probably due to the failure of NFs to invade proximal axons and thereby to block transport.

A disruption of axonal transport by NF disorganization is a pathological mechanism that can account for the sporadic (90% of cases) and the hereditary (10% of cases) forms of ALS, as both forms present similar symptoms and characteristic NF accumulations in spinal motor neurons (Hirano et al., 1984). This mechanism is consistent with several aspects of ALS. First, it provides an explanation for the cellular selectivity of the disease. Large motor neurons represent a class of neurons to be particularly vulnerable to NF abnormalities because of their high synthesis of NF proteins (Oblinger et al., 1987). Second, there is a retardation in the slow axonal transport of cytoskeletal elements during aging (McQuarrie et al., 1989), a factor that can predispose to the disease. Third, a NF involvement in ALS pathogenesis is compatible with the recent report of aberrant NF swellings in degenerating motor neurons of transgenic mice expressing a mutant form of human SOD1 found in a subset of familial ALS (Gurney et al., 1994). Although the link between SOD1 and NFs has yet to be elucidated, it has been suggested that SOD1 mutations in ALS may increase nitration by peroxynitrite of tyrosine residues in cellular proteins, perhaps resulting in alteration of NF phosphorylation and assembly. A pathway involving NF phosphorylation is indeed supported by the recent report of codon deletions in the NEFH phosphorylation domain of some ALS patients (Figlewicz et al., 1994).

Figure 1. Motor axon degeneration in L5 ventral root of NEFH transgenic mice. A, light microscopic examination of cross-sections of L5 ventral roots from normal (left) and NEFH transgenic mice (right) at two years of age. The insets, at the same magnification, show sections of the entire roots. Magnification 1440X, insets 140X. B, selective loss of large motor axons in L5 ventral root.

Methods. Microscopic analysis. Mice were anesthetized with nembutal and perfused via 0.9% NAcl, and 2.5% glutaraldehyde, 0.5% paraformaldehyde in sodium phosphate buffer and postfixed 1.5h in 1% osmium tetroxide. After three washes with phosphate buffer, each sample was dehydrated in a graded series of ethanol and embedded in Epon. The thin sections were stained with toluidine blue and examined under a Nikon Labophot microscope.

A.



Control Transgenic

B.

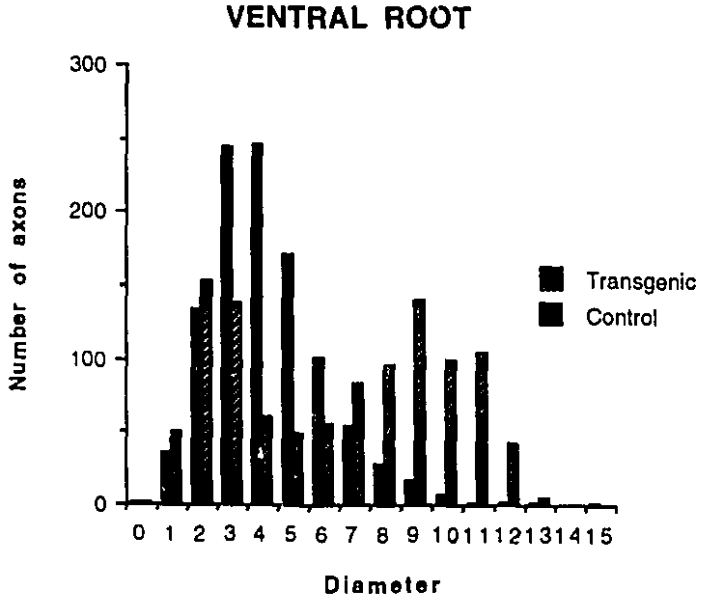


Figure 2. Fluorographs of slow transport profiles in motor axons of the sciatic nerve from normal and NEFH transgenic mice. Panels A and B represent the profiles of normal mice 7 and 28 days following intraspinal injection of [^{35}S]methionine. Panels C and D show the corresponding results from NEFH transgenics 3 month old while E and F show the results from transgenic mice 18 month old. For each panel, lane 1 contains proteins extracted from the site of injection (L4-L5 spinal cord) and each successive lane represent a 5mm nerve segment extending distally to the right. Note the reduced rate of transport for all three NF subunits in transgenic mice (D and F). Axonal transport of actin and tubulin is also retarded in transgenic mice. In normal mice after 28 days of pulse-labeling, the peaks for tubulin and actin proteins are found at 10 and 15mm away from the spinal cord whereas the peaks for these two proteins in transgenic mice are detected in the 5mm segment.

Methods. 3 month and 18 month old homozygous C57Bl/6j transgenic mice from line 200 and aged-matched control mice were used. Mice were anesthetized with nembutal. The lumbar spinal cord was exposed by laminectomy and newly synthesized proteins in motor axons in the sciatic nerve were labeled by the injection of a total of 3 µl of [^{35}S]methionine (Amersham, specific activity > 1000Ci/mmol) through a glass micropipette connected to a Hamilton syringe filled with mineral oil, into each of 3 sites of the right anterior horn of the lower lumbar enlargement (L4-L5). Cells in that region give rise to motor axons of the sciatic nerve. The isotope was dried down and resuspended in distilled water to a final concentration of 150µCi/µl prior to injection. The animals were sacrificed 7 and 28 days later. The sciatic nerves were removed in continuity, cut into 5mm segments and homogenized in 115µl of SUB buffer (0.5% SDS, 8M Urea, 2% β-mercaptoethanol). Homogenates were centrifuged at 10,000 rpm in a table top centrifuge. A 10 µl aliquot was analyzed by 8% SDS-PAGE. Following electrophoresis, gels were stained with Coomassie blue, destained in methanol/acetic acid, treated with Amplify (Amersham), dried and processed for fluorography. The labeled proteins on the gels were visualized by exposing dried gels to Kodak XAR films at -80°C for 2 to 14 days.

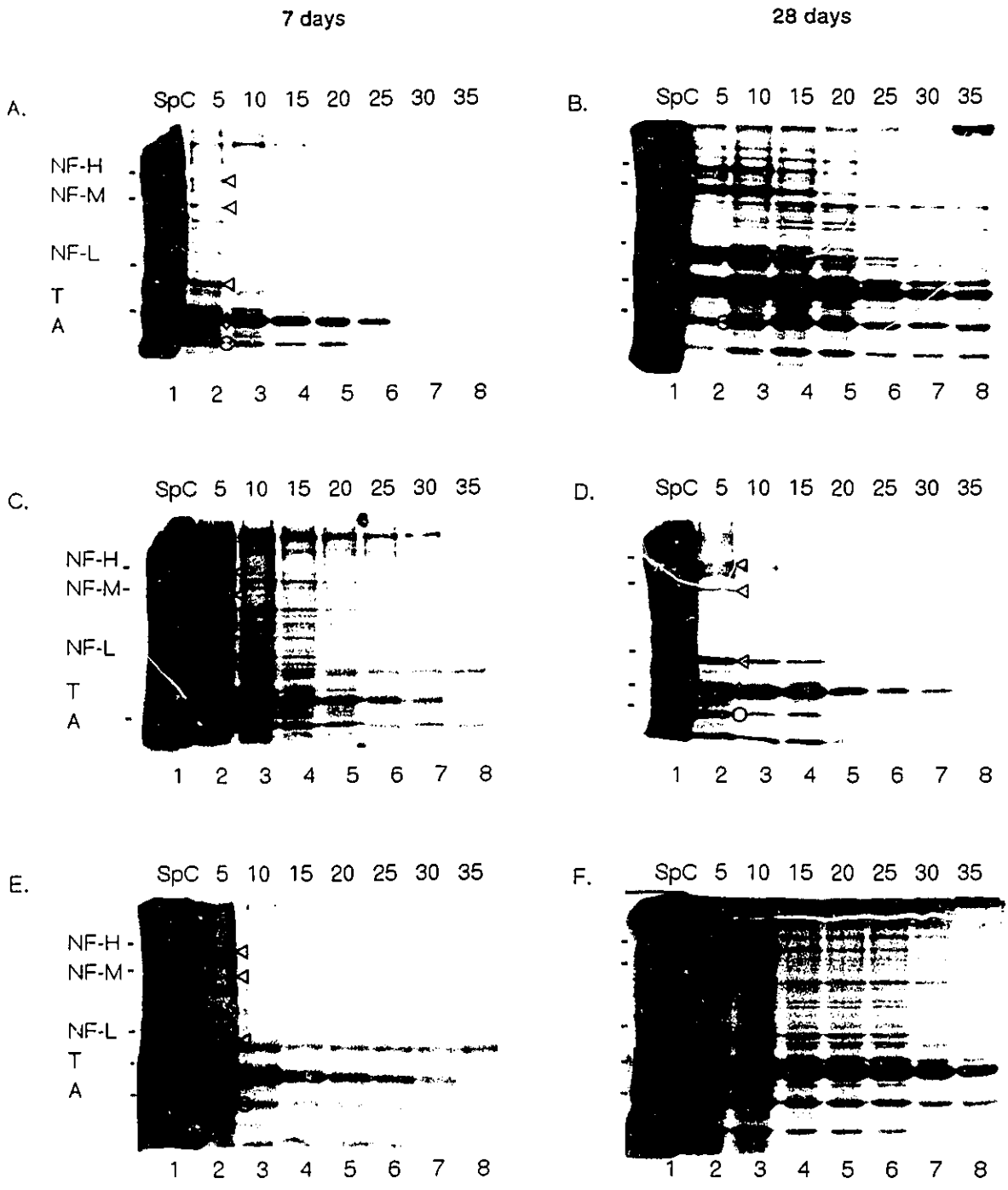
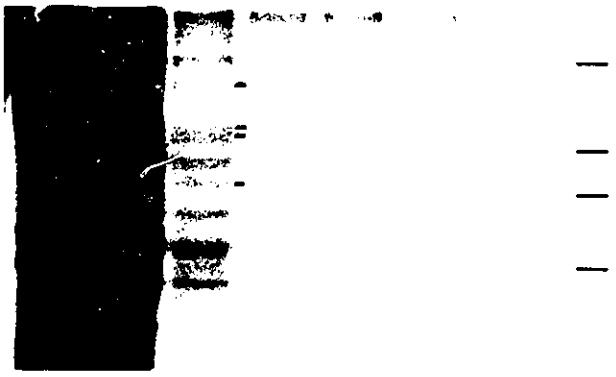

7 days
28 days
A.
B.
C.
D.
E.
F.
SpC 5 10 15 20 25 30 35
NF-H
NF-M
NF-L
T
A
1 2 3 4 5 6 7 8

Figure 3. Fluorographs of fast transport profiles in motor axons. Segments of sciatic nerve from normal (A) and transgenic (B) 3 month old were analysed 15h following [^{35}S]methionine as described in figure 2. Note the poor detection of some proteins (-) of the fast transport component in NEFH transgenics motor axons. Bars on the right indicate the postion of molecular weight standards (200, 116, 97, 66 kDa, reading top to bottom).

Methods. The methods are described in figure 2.

A.



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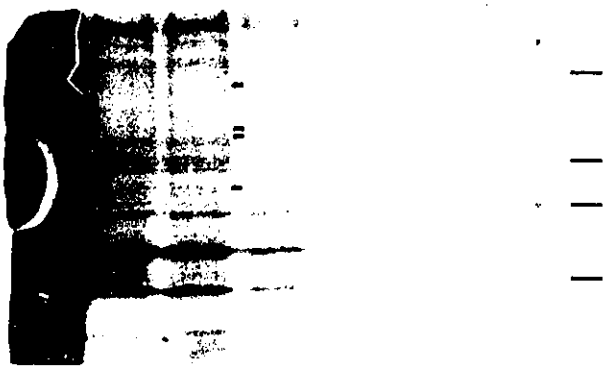


Figure 4. Electron microscopy of longitudinal sections of myelinated axons from the sciatic nerve of normal and NEFH transgenic mice. Note the depletion of filaments, mitochondria and smooth endoplasmic reticulum in the shrunken myelinated axon of NEFH transgenic (B) as compared with a normal axon of similar myelin thickness (A). Magnification 12,500X.

Methods. Electron microscopic analysis. The samples were prepared as described in figure 1. Ultrathin sections of the sciatic nerve were stained for 8 minutes in lead citrate and examined with a Philips CM10 electron microscope.

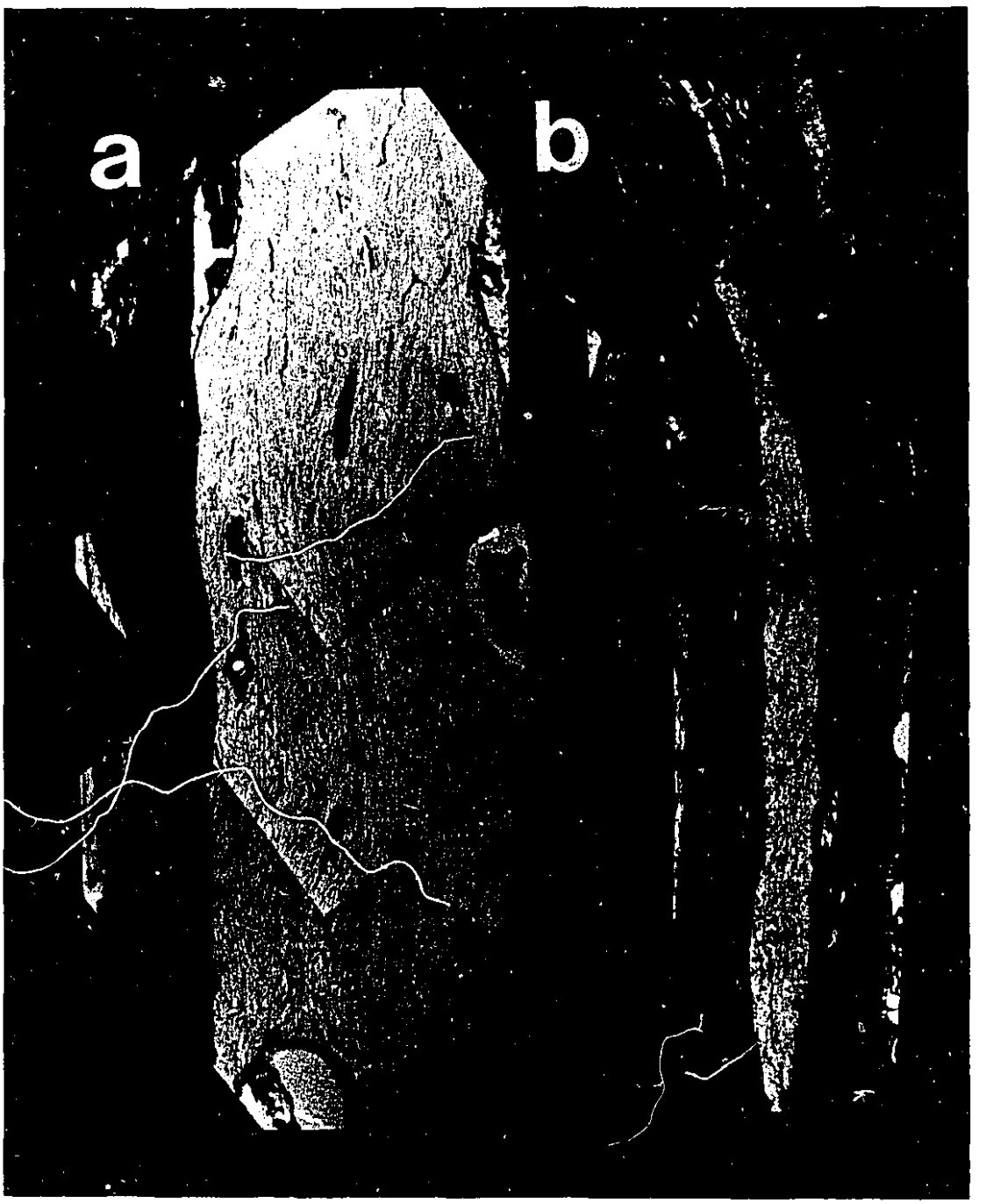
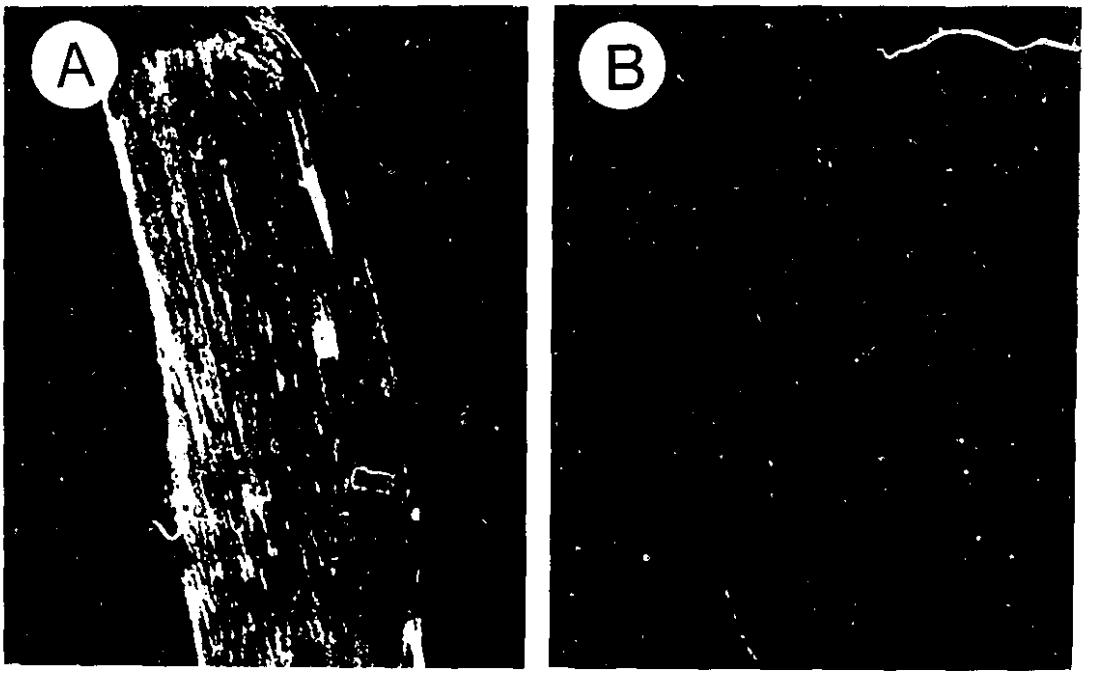

a
b

Figure 5. Immunofluorescence detection of mitochondria in ventral roots of normal (A) and NEFH transgenic mice (B). Magnification 285X.

Methods. Immunofluorescence microscopic analysis. Anesthetized mice were perfused with 0.9% NAcl and 4% paraformaldehyde in sodium phosphate buffer, pH 7.4 Sciatic nerves were rinsed in phosphate buffer and immersed in 15% sucrose and phosphate buffer. Cryostat sections of 10 μ were first incubated with anti-inner membrane mitochondria antibody (Serotec MOM/H6/C12) and then incubated with anti-mouse biotin (Jackson Laboratories) and fluorescein-labeled streptavidin. Sections were examined under a Nikon Labophot microscope.


A
B

Chapter IV

Copy-dependent and correct developmental expression of the human neurofilament heavy gene in transgenic mice

Francine Côté, Jean-François Collard, Daniel Houle and Jean-Pierre Julien

Montreal General Hospital Research Institute

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Research Report

Copy-dependent and correct developmental expression of the human neurofilament heavy gene in transgenic mice

Francine Côté, Jean-François Collard, Daniel Houle, Jean-Pierre Julien *

Centre for Research in Neurosciences, McGill University, The Montreal General Hospital Research Institute, Montreal, Que., Canada H3G 1A4

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Abstract

We recently produced four lines of transgenic mice bearing a 34 kb DNA fragment that includes the human gene coding for the neurofilament heavy (NF-H) chain. Analysis of the NF-H transgenics revealed an increase in human NF-H mRNA and protein that parallels the increase in gene copy number, providing the first example of a transgene with copy-dependent expression in neurons. In addition, expression of the human NF-H transgene is induced post-natally following a developmental pattern similar to the endogenous mouse NF-H gene.

Keywords: Neurofilament; Neuronal expression; Gene regulation; Transgenic mouse; Intermediate filament

1. Introduction

Neurofilaments (NFs) are the major intermediate filaments (IFs) of nerve cells. They are formed by the copolymerization of three subunits; light (NF-L), medium (NF-M) and heavy (NF-H) [15,22]. The three neurofilament subunits are encoded by different genes and several reports have shown that the genes are under separate developmental control [3,7,18,20,21,26]. Neurofilament expression occurs after nerve cells have undergone their last mitotic division. Significant expression of NF-L and NF-M is first seen in the embryonic brain, while for most neurons, NF-H expression is delayed relative to the other subunits and occurs in the post-natal period. There is clear evidence for a role of neurofilaments in the control of axonal calibers [16,29]. Abnormal expression of neurofilaments in distinct regions of the neuron occur in a variety of disorders including amyotrophic lateral sclerosis (ALS) [4,6], an inherited giant axonal neuropathy [4] and toxic neuropathies [1,11,13,28].

We have recently derived four lines of transgenic mice carrying multiple copies of the human NF-H gene [8]. The NF-H transgenic mice progressively develop physiological and pathological features reminiscent of amyotrophic lateral sclerosis, which make them a unique mouse model of ALS. We now report that neuronal expression of human NF-H mRNA in transgenic mice increases with the number of gene copies integrated and it parallels expression of the mouse NF-H gene throughout development.

2. Materials and methods

2.1. Generation of transgenic mice

The four lines of transgenic mice were obtained by microinjection of a *Not*1 linearized cosmid containing the human NF-H gene into male pronuclei of fertilized mouse eggs. The 39 kb fragment was purified and prepared for injection as previously described [8]. Transgenic mice were identified by Southern blot analysis of tail DNA using a 361 bp *Sst*1–*Xho*1 fragment from the first exon of the human gene. The number of human NF-H gene copies in mice from each of the transgenic lines was determined by densitometric analysis and comparison with a human diploid DNA standard, using the SciScan™ 5000 (U.S.B.)

2.2. RNA analysis

For studies of copy-dependent expression, RNA was prepared from total cerebral cortex, cerebellum and spinal cord by the guanidinium thiocyanate procedure and applied to slot blot apparatus

Abbreviated title: Copy-dependent and developmental expression of a human neurofilament transgene.

* Corresponding author. Fax: (1) (514) 934-8265.

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according to Sambrook et al. [25]. Five μg of RNA were used in each slot. For the developmental studies, homozygous males from lines 200 and 635 were mated to normal females. Cortex from heterozygous progeny was removed at different developmental stages and RNA was extracted as described above. For both Northern and slot blot analysis, RNA was transferred to Hybond+ membrane (Amersham). The membranes were prehybridized in 5×SSC (1×is 150 mM NaCl and 15 mM Na_3 citrate (pH 7.0), 1% SDS, 20 mM Tris (pH 7.5), 5×Denhardt's solution (1×is 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinyl pyrolidone), 10% dextran sulphate and 100 μg/ml heat denatured salmon sperm DNA at 65°C for at least 2 h. A 1.2 kb PCR fragment from the 3' end of the human NF-H gene was used as a probe. A 416 bp *Nru*I–*Xho*I fragment of the 3' region of the mouse NF-H gene was used to identify mouse NF-H mRNA. The mouse NF-L probe is a 300 bp *Sst*I fragment and finally, in order to control for equivalent loading of RNA, Northern blots were probed with actin. All DNA probes were radiolabelled by random priming, added to the filters and left overnight at 65°C. Membranes were then washed 15 min at room temperature in 5×SSC, 0.5% SDS; 30 min at 65°C in 1×SSC, 0.5% SDS and 30 min at 65°C in 0.1×SSC, 0.5% SDS. Filters were exposed to Kodak X-Omat AR film in the presence of an intensifying screen at −70°C.

2.3. Protein analysis

For studies of copy-dependent expression, total protein extracts from the cortex of adult transgenic mice of each line and of normal mice were prepared by homogenization of the tissue in 0.5% SDS, 8 M urea and 2% β-mercaptoethanol. Ten μg of each sample was loaded and electrophoresed on a 7.5% SDS-polyacrylamide gel. For developmental studies, normal and homozygous males from transgenic line 200 were mated to normal C57BL females. The cortex from heterozygous transgenics and normal progeny was removed at different developmental stages and proteins were extracted and fractionated on SDS-PAGE as described above. For both studies, two identical gels were run simultaneously. One gel was stained with Coomassie blue to confirm equivalent loading and the other one was used for immunoblotting. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell inc.). After blocking the membrane with 3% powdered milk in phosphate-buffered saline solution, the human NF-H and mouse NF-H proteins were detected by incubation with anti-human NF-H monoclonal (OC95) and anti-mouse NF-H monoclonal (OC59), respectively (kindly provided by V.M.-Y. Lee, University of Pennsylvania), followed by incubation with a peroxidase-conjugated AffiniPure goat anti-rat IgG (Jackson ImmunoResearch) using the Amersham ECL detection kit.

2.4. Immunofluorescence staining

Anesthetized mice were perfused via 0.9% NaCl and 4% paraformaldehyde in phosphate buffer, pH 7.4. Tissue samples were rinsed in phosphate buffer and immersed in 15% sucrose. Cryostat sections of 10 μm were first incubated with rat anti-human NF-H antibody (OC95) or anti-mouse NF-H (OC59) and then incubated with fluorescein-labeled goat anti-rat IgG (Jackson ImmunoResearch).

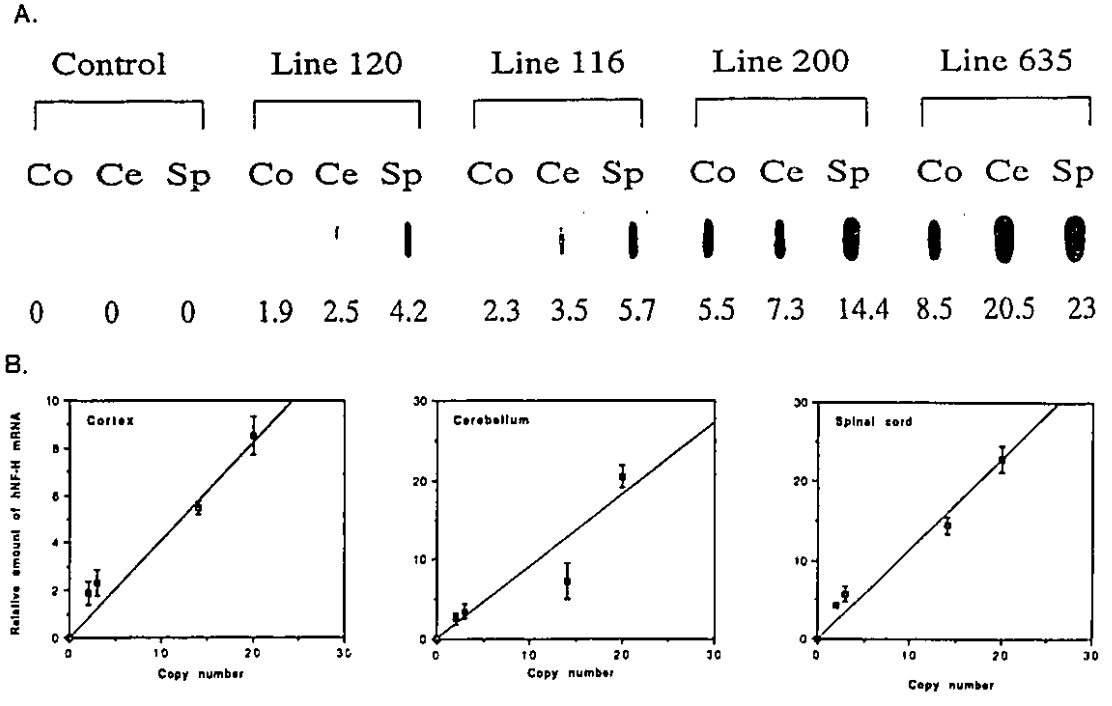

Fig. 1. Copy-dependent expression of the human NF-H gene in transgenic mice. A: 5 μg of total RNA from cortex, cerebellum and spinal cord of the four lines were applied to slot blot apparatus and hybridized with a 3' specific human NF-H probe. Densitometric analysis was done and values below each spots are relative amount of human NF-H mRNA normalized to the background value, set at 0. B: relative amounts of human NF-H mRNA in cortex, cerebellum and spinal cord were plotted against the human NF-H transgene copy number. There is a strict correlation between the two. Vertical bars represent the ± S.E.M.

3. Results

3.1. Copy-dependent expression of the human NF-H transgene

A 39 kb genomic fragment (Cos4NFH) containing the four exons of the human NF-H gene flanked by 9.6 kb of upstream and 13.4 kb of downstream sequences was introduced into the genome of mice. Four independent lines were established, line 120, 116, 200 and 635 containing respectively 2, 3, 14 and 20 copies of integrated transgene [8]. We have previously shown that Cos4NFH contains the control elements required for specific neuronal-expression of the human NF-H gene in transgenic mice [8]. To study the correlation between the number of gene copies and the level of expression of human NF-H mRNA, we performed slot blot hybridization of RNA. The membranes were hybridized with a 3' specific human NF-H probe that does not recognize mouse NF-H mRNA. Fig. 1A shows that the signal obtained for the human NF-H mRNA increases in parallel with the number of gene copies. Slot blot analysis allows the results to be quantitated by scanning densitometry. Numbers below the spots, in Fig. 1A, are relative amount of human NF-H mRNA normalized to the background value which was set at 0, using the SciScan™ 5000 scanning system. The values were derived from scanning of autoradiographs exposed for different period of time, from three independent experiments. The linear correlation between the relative level of expression of cortex, cerebellum and spinal cord human NF-H mRNA and copy number in the four lines is illustrated in the graphs of Fig. 1B.

3.2. Correct developmental expression of the human NF-H transgene

We examined the expression of the human NF-H gene during development in the cortex of mice from transgenic lines 200 and 635. Both lines yielded identical results. In Fig. 2 is shown the Northern blot analysis of total RNA from cortex of heterozygous transgenic mice of line 2000 hybridized with (A) human NF-H specific probe, (B) mouse NF-H specific probe, (C) mouse NF-L probe and (D) actin. Using the human NF-H specific probe, we detected a single mRNA species of 3.9 kb. No expression was detectable in the embryonic cortex and there is only a very weak signal at birth. Significant expression of the human transgene does occur at post-natal day 5 (P5). The signal for human NF-H mRNA then increases gradually between P5 and P10 and more rapidly thereafter (Fig. 2A). The pattern of expression of the human NF-H transgene follows that of the endogenous mouse NF-H gene (Fig. 2B). The same blot was then rehybridized with a mouse NF-L probe to determine if NF-H overexpression had

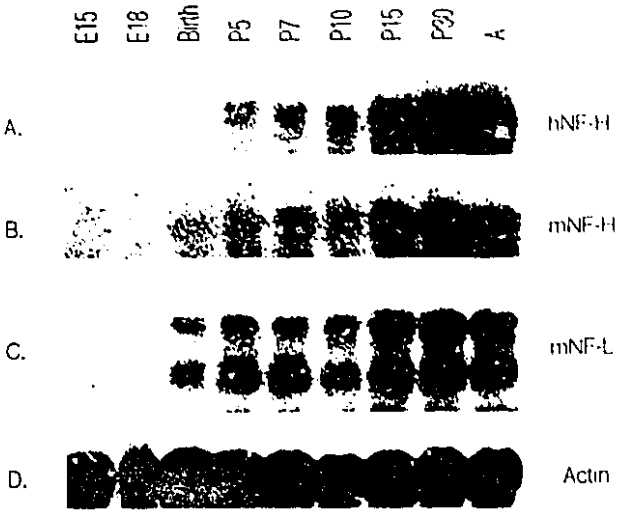

Fig. 2. Developmental pattern of expression of the human NF-H transgene. Ten μg of total RNA from cortex of heterozygous mice of increasing age, were fractionated on a 1% agarose-formaldehyde gel prior to blotting. Membranes were hybridized with probes (A) specific to human NF-H, (B) specific to mouse NF-H, (C) specific to mouse NF-L and (D) actin.

any effect on normal developmental pattern of expression of the mouse NF-L gene. Figure 2C shows the two species of mouse NF-L mRNA (2.2 kb and 3.5 kb) present at embryonic day 15 (E15) and whose expression progressively increase with time. This pattern of expression corresponds to the previously published pattern of NF-L expression in normal mice [18].

3.3. The levels of NF-H protein expression comply with mRNA levels

To determine if the increase in NF-H mRNA levels was translated into protein levels, total protein extracts of brain cortex from a normal mouse and from transgenic mouse lines were analyzed by immunoblotting following electrophoresed on SDS-PAGE. The anti-human NF-H (OC95) and anti-mouse NF-H (OC59) monoclonals were used to assess the levels of human and mouse NF-H proteins, respectively. The results shown in Fig. 3A reveal a correlation between the levels of human NF-H proteins and mRNA levels detected in each transgenic line. Note that the level of the endogenous mouse NF-H proteins was not altered by the high-level expression of human NF-H proteins in transgenic lines 200 and 635.

The monoclonals OC95 and OC59 were also utilized by immunoblotting to monitor the human and mouse NF-H protein expression during development of the cortex from line 200. As shown in Fig. 3B, the developmental expression of human NF-H proteins in transgenic line 200 corresponds to the one observed for the mouse NF-H protein. The results of the developmental

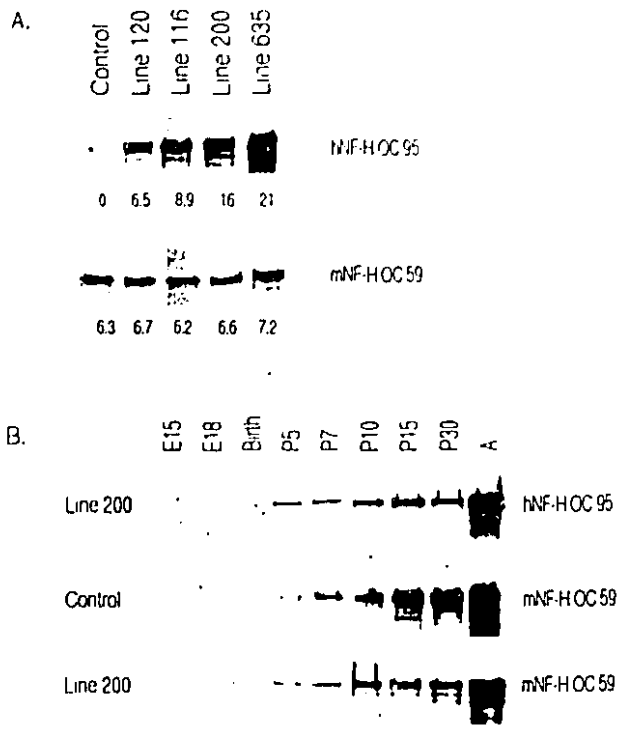

Fig. 3. Copy-dependent and correct developmental expression of human NF-H proteins in transgenic lines. A: immunodetection of human NF-H and mouse NF-H proteins in total protein extracts of brain cortex from a normal mouse and from transgenic mouse lines following electrophoresed on SDS-PAGE. B: immunodetection of human and mouse NF-H proteins in total protein extract of the cortex from mice of transgenic line 200 and from normal mice at different developmental stages.

expression of NF-H proteins (Fig. 3B) are in agreement with the developmental pattern of expression of NF-H mRNAs shown in Fig. 2.

3.4. Immunofluorescence detection of human NF-H proteins

The distribution of human NF-H proteins in the CNS of transgenic lines 200 and 635 was examined by immunofluorescence using the OC95 monoclonal antibody. As shown in Fig. 4, the distribution of immunostaining with this anti-human NF-H antibody in the spinal cord and brain of line 200 (B,F) and 635 (C,G) is similar to the one obtained with the anti-mouse NF-H (OC59) in corresponding regions of a normal mouse (D,H).

4. Discussion

The 39 kb cosmid clone bearing the human NF-H gene represents the first example of a transgene being expressed in a copy-dependent manner in neurons. Tissue-specific genes are often expressed appropriately in transgenic mice generated by the microinjected method but there is generally no correlation between the number of copies of inserted genes and the levels of transgene expression [17]. Only a few examples of copy-dependent expression of transgenes have been reported. They include the β-globin gene in the erythroid lineage [14], the CD2 gene in T cells [12] and the gene for the S100 β-subunit in astrocytes [10]. The DNA region conferring copy-dependent expression of the human β-globin gene has been the best characterized [9,14,24,27]. It consists of a sequence containing six nuclease hypersensitive sites that form what is termed the Locus Control Region (LCR). The LCR may act in two ways; first it modulates changes in chromatin structure and second it enhances transcription activity of the transgene [9]. Thus, the globin LCR is able to confer position-independent expression in erythroid cells of each integrated gene copy, usually arranged in a head to tail tandem array at the integration site. In contrast, the copy-dependent expression in glial cells described for the S100β gene did not reflect such strict position-independent profile. Thus, approximately 10 copies of integrated S100β genes were required to achieve a level of transcription activity equivalent to one endogenous S100β allele [10]. The DNA regions of the S100β gene responsible for copy-dependent expression has yet to be identified [10]. The copy-dependent activity of the human NF-H fragment resembles the latter situation. Thus, the levels of human NF-H mRNA increase with the gene copy number but the activity of each copy is not equivalent to one endogenous NF-H mouse allele. For instance, in transgenic mice of line 200 bearing 14 copies of the human NF-H gene, the levels of human NF-H mRNA in nervous tissue correspond to approximately 2-fold those of the endogenous mouse NF-H mRNA.

Our developmental study in two NF-H transgenic lines indicate that expression of the human NF-H gene was correctly regulated following the developmental pattern of the endogenous mouse NF-H gene. This is in contrast with a previous transgenic mouse study with a 21.5 kb fragment bearing the human NF-L gene [19].

Fig. 4. Immunofluorescence detection of human and mouse NF-H proteins in the CNS of transgenic mice. The anti-human monoclonal (OC95) does not react with the spinal cord (A) or brain (E) of normal mice but it recognized axons from the spinal cord and brain of mice from two different transgenic lines overexpressing human NF-H proteins. B and C show immunostaining with OC95 of spinal cord sections from lines 200 and 635, respectively. F and G show OC95 immunofluorescence staining of brain sections (the region of dorsal lateral geniculate nucleus) of lines 200 and 635, respectively. Note the weaker immunofluorescence signal of line 635 as compared with line 200 for OC95 in this region of the brain. The expression patterns for human NF-H protein is similar to the one observed for the mouse NF-H protein in corresponding spinal cord and brain regions (D and H, respectively). Magnification, 950×.

The human NF-L transgene was expressed specifically in neurons but it failed to confer copy-dependent expression and to comply with the developmental profile of the endogenous mouse NF-L gene.

Our results revealed levels of human NF-H protein expression that corresponded to mRNA levels for the four different transgenic lines. This is in contrast to the situation reported previously for NF-L gene in which overexpression in transgenic mice of the NF-L gene did not result in corresponding increase in the levels of proteins due to the existence of posttranscriptional controls specific for NF-L regulation [2,23].

In conclusion the 34 kb human NF-H fragment described here contains regulatory sequences which are recognized by mouse transcription factors to confer correct temporal activation and copy-dependent expression in neurons. Work is now in progress using the transgenic approach to identify the regulatory elements of the human NF-H gene responsible for copy-dependent and post-natal activation in neurons. These NF-H regulatory elements may constitute a valuable tool in the development of future vectors for gene therapy in the nervous system.

Acknowledgments

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Chapter V

Discussion

Expression pattern of the human NF-H gene in transgenic mice

A 39 kb genomic fragment containing the four exons of the human NF-H gene as well as 13.4 kb and 9.6 kb upstream and downstream sequences (Cos4NFH) was microinjected into fertilized mouse eggs to produce transgenic mice (Chapter II, figure 1). Four independent lines were established with 2 to 20 copies of the transgene. Specific probes from the human NF-H gene were used to investigate its expression in transgenic mice. Northern blot analysis revealed that the introduction of the human NF-H gene into the mouse genome resulted in an expression pattern that is tissue-specific with a level of expression approximately two-fold that of the endogenous mouse level (Chapter II, figure 2). In addition, the temporal pattern of expression of the human NF-H gene paralleled that of the endogenous mouse NF-H gene (Chapter IV, figure 2). Despite the increase in human NF-H mRNA, no changes occurred in the developmental pattern of expression of either the endogenous mouse NF-L or NF-H mRNA and proteins (Chapter IV figures 2 and 3). Thus, the presence of the human NF-H transgene did not appear to affect the normal developmental pattern of expression of the mouse NF-L or NF-H genes. Finally, slot blot analysis indicated copy number dependent and position-independent expression of the human NF-H gene in transgenic mice (Chapter IV, figure 1). This is the first example of a transgene being expressed in such a manner in neurons. These results suggest that the signals necessary to direct the expression of the human NF-H gene lie within the injected fragment and are recognized by murine transcription factors.

The pattern of expression of the human NF-H gene contrasts with that of the human NF-L gene. Expression of a fragment containing 14 kb of 5' and 3.2 kb of 3' sequences of the NF-L gene was restricted to nerve cells in transgenic mice but its developmental profile did not follow the endogenous one (Julien et al., 1987). In addition, the levels of transgene NF-L mRNA were not proportional to copy number. Moreover, transgenic mice expressing increasing levels of NF-L mRNAs did not show a corresponding increase in the protein level (Beaudet et al., 1993). These data provided

evidence for the existence of different posttranscriptional controls for human light and heavy NF subunits (Beaudet et al., 1993).

In contrast to the expression of the human NF-L gene, transgenic mice expressing human β-globin, S100 β and CD2 genes show copy-dependent and position-independent expression. The sequences responsible for their correct expression have been delineated and are referred to as the " locus control region " (LCR) (Grosveld et al., 1987). The LCR organizes the exogenous gene into an active chromatin configuration not influenced by the chromosomal site of insertion. This renders the chromatin accessible to trans-acting factors which bind to regulatory promoter and enhancer sequences in the gene and stimulate the level of transgene expression. Such a LCR must be present in the injected human NF-H clone, but some regulatory elements must be missing since the level of expression of one transgene copy is not comparable to that of the endogenous mouse level as with the β–globin and CD2 genes (Grosveld et al., 1987; Greaves et al., 1989). Instead, mRNA expression in the 14 copy human NF-H transgenic (line 200) is approximately equivalent to twice that of the endogenous mouse gene.

Transcripts for the human NF-H gene were first detectable post-natally. This delayed expression of the NF large subunit has also been noticed in other species and its significance is related to its putative neuronal function (see below). As several IF genes are sequentially expressed during development of nerve cells, the maturation of a neuron is reflected by a particular sequence of IF gene expression. For instance, vimentin/nestin IF proteins are expressed in dividing neuroepithelial cells and upon commitment to neuronal differentiation, nestin is down regulated (Lendahl et al., 1990). Upon terminal differentiation, CNS cells express α-internexin which most likely plays a role in stabilizing axons in early growing neurites (Fliegner et al., 1990; Lendahl et al., 1990; Ching and Liem, 1993). Alpha-internexin expression persists through development but decreases as NF-L and NF-M are expressed (Fliegner et al., 1990). The initial expression of NF-L and NF-M subunits following the last mitotic division is concomittant with the initiation of axon extension and maintenance of neuritic outgrowth (Shaw and Weber 1981; Cochard and Paulin, 1984). The expression of NF-H lags somewhat behind the expression of NF-L and NF-M as the neuron mature (Glasgow et al., 1992). Most adult cell types of the CNS and PNS express NFs. The post-mitotic expression of the NF proteins and especially the late expression of the large subunit mean that NFs are not required at early stages of development (Shaw and Weber, 1981). Instead, NFs fufill structural requirements of the cytoskeleton later in neuronal development. Indeed, Carden et al., (1987) demonstrated that the delayed appearance of

NF-H coincided in time with maturation and stabilization of neuronal circuitries which may be important in modulating axonal events including the slowing of cytoplasmic transport and the growth of axonal caliber (Simon and Willard, 1983; Hoffman et al., 1985a). However, the unified expression of NF-L and NF-M in the embryo and their close proximity on chromosome 11 in mouse and on human chromosome 8 possibly indicates a common regulatory mechanism for these two which is different from the NF-H subunit. However, the signals that trigger or shut down the differential expression of the three subunits still remain to be determined. Some axonal or extrinsic factors might be important in the repression/induction of the NF genes. For example, there is a decrease in NF mRNA and protein following nerve transection (Hoffman et al., 1987). The signal from the periphery that initiates this sequence is unknown but the same signal or a different one could help to coordinate the expression of the NF proteins (Schlaepfer and Bruce, 1990). Several reports (Lindembaum et al., 1988; Verge et al.,1990; Gold et al., 1991) also demonstrated that the trophic substance NGF is capable of up-regulating NF gene expression. Finally, recently reported data by White et al., (1994) identified intracellular pathways that control NF expression. The authors treated RN46A cells (a neuronal cell line) with forskolin and dibutyryl cAMP. Results showed that both substances up-regulated NF expression.

As mentioned above it is likely that the injected human NF-H cosmid contains a control region simiar to the β-globin gene which in addition to being involved in copy-dependent, position-independent expression is also responsible for regulating its precise developmental expression. For that purpose, the control of the human NF-H gene expression may involve the presence of binding sites within its putative LCR for developmental stage specific transcriptional activators and/or repressors.

In fact, Lazzarini's group using a combination of DNaseI footprinting, methylation interference and gel shift analyses have mapped multiple binding sites for nuclear proteins including some novel ones for trans-acting factors within the 5' flanking sequence of the human NF-H gene (Elder et al., 1992). Analyses of the human NF-H promoter also revealed functional elements for basal promoter activity located within the first 205 bp upstream of the transcription initiation site but no tissue-specific signals were found.

Zimmerman et al., (1994) using sequences from the rat nestin gene fused to LacZ gene in transgenic mice identified enhancer elements in the second intron. The sequences gave a specific pattern of expression throughout the neuroepithelia and

position-independent expression. The location of this intron is also shared by the three NF genes and may be important for their stage specific development.

Identification and characterization of the regulatory elements of the human NF-H gene could lead to design of elements for directing expression of other genes in a cell-type specific, position independent, copy number dependent fashion in transgenic mice. Furthermore, the NF-H gene is of particular interest because it is activated predominantly in the post-natal period and can represent an alternative promoter to circumvent an embryonic lethality when directing foreign and possibly harmful gene products. Finally, understanding the regulation of the human NF-H gene offers the possibility to dissect the transcriptional mechanisms that control changes in gene expression during the differentiation of neurons.

NF dynamics

Compared with microtubules and microfilaments, NFs and IFs in general have been considered static components of the cell cytoskeleton. The data presented in Chapter III bring interesting new facts concerning NF dynamics and transport in the axon. Most NFs in the mature neuron are presumed to be in the triplet subunit form (Hoffman and Lasek, 1975). The irreversible polymerization of most NFs, at least in mature axons is suggested by the low percentage of free NF subunits found in the soluble state in axon preparations. It has been shown that although newly synthesized NF proteins are triton-soluble, more than 99% of this pool is translocated down the axon in a triton-insoluble form and in the same subunit stoichiometry as that found in isolated axonal NF (Nixon et al., 1989). It is believed that NFs can not enter the axon unless they are in the polymerized form. This forms the basis of the structural hypothesis of axonal transport, where proteins are transported in the axon as component parts of intact cytological structure (Lasek et al., 1984). Recent evidence however, supports the idea that there is NF subunit exchange between soluble and filamentous pools once the triplet is assembled. Isolated NF-L subunit assembled into homopolymers *in vitro* and were shown to undergo exchange with soluble NF-L subunits (Angelides et al., 1989). The exchange was preceded by dissociation of NF-L subunits from NF-L filaments. In their discussion, Angelides and colleagues (1989) also suggested that phosphorylation was an important regulatory process for assembly/dissassembly of NFs. Furthermore, Okabe et al., (1993) used photobleaching of fluorescently labeled NF-L protein to visualize NF

dynamics in living neurons. Again, incorporation of fluorescent NF-L subunits occurred at discrete sites along the entire surface of the filament, which argues against NFs moving as polymers. Furthermore, photobleaching experiments with vimentin, another IF protein, by Vikstrom et al., (1989) also demonstrated that newly synthesized vimentin subunits incorporated progressively into endogenous vimentin network along the entire length of the existing IF network. These data collectively indicate that a continuous exchange/incorporation of soluble IF subunits along the entire length of the polymerized IF network does occur and is possibly required for the maintenance of the filament system (Visktrom et al., 1989; Okabe et al., 1993).

In the axonal transport studies presented in Chapter III, NFs in control animals, were thought to be transported mainly as assembled polymers as defined by the coherence of the labeled wave. (Chapter III, figure 2A and 2B). However, in transgenic motor/sensory axons NF-L proteins were present up to 15/25mm away fom the site of injection while NF-M and NF-H proteins were not or barely translocated down the axon (Chapter III, figure 2D and 2F; Appendix A, figure 1D and 1F). This data provides evidence for non-polymer transport of NFs and support Hirokawa and co-workers's hypothesis (Okabe et al., 1993) that there is NF subunit exchange between soluble and filamentous pools once the triplet is assembled and that NF subunits assembly can proceed by incorporation of those soluble subunits into pre-existing filaments.

The human NF-H transgenic mice as a model of human motor neuron disease

The production of transgenic mice expressing the human NF-H protein at a level approximately two-fold the endogenous level (line 200) led to a progressive neuropathy. The transgenic mice were born normal but subsequently developed tremors and weakness or their hindlimbs and forelimbs. In addition, when compared to aged-matched controls, they did not reflexively extend their legs to form a 120° angle with their bodies (Chapter II, figure 5). Electron microscopy studies revealed massive accumulation of NFs into spinal motor neurons, DRG neurons and their proximal axons (Chapter II, figure 6 and 7). Distal axonal atrophy accompanied by atrophy of muscle fibers was also observed (Chapter II, figure 8 and 10). The similarities between the abnormalities seen in the human NF-H transgenic mouse and those encountered in ALS patients are striking and thereby make the NF-H transgenics a very good model of this

disorder. But how can the changes seen in the human NF-H transgenic mice neuronal cytoskeleton be sufficient to trigger a neurodegenerative process ?

As proteins are almost exclusively synthesized in the soma, all the material necessary for the axon and its synaptic terminal are transported there by cytoplasmic axonal flow (Grafstein and Forman, 1980; Lasek, 1981). The concept that defective axonal transport may be associated with or be the cause of pathogenesis leading to disruption of the neuronal cytoskeleton and eventually to neuron disorder is not new (Gajdusek, 1985). To address this issue directly, axonal transport studies were performed in two types of neurons: DRG and ventral motor neurons. Following various intervals after motor/sensory neurons were labeled by injecting [^{35}S]methionine, mice were sacrificed and the labeled proteins in consecutive 5 mm long segments of the sciatic nerve were analyzed by SDS gels and fluorography. Comparison of the distribution of labeled proteins in transgenic and control motor/sensory fibers demonstrated that NF proteins extended farther distally in control than in transgenic fibers (Chapter III, figure 2A and 2B). The anterograde movement of the SCa proteins in control motor neurons at a rate of 0.35mm/d was illustrated by the presence of a labeled peak of NF proteins 10mm from the spinal cord at 28 days (Chapter III, figure 2B). The triplet proteins have coincidental distribution within the control axon at all post-labeling times. A number of striking differences were noted in the transport kinetics of cytoskeletal proteins of transgenic mice (Chapter III, figure 2C through F). First, there was a net slowing of axonal transport of labeled NF proteins, actin and tubulin. Second, the stoichiometry of labeled NF-L, NF-M and NF-H was not constant. Similar results were seen in studies of slow axonal transport following labeling of sensory neurons, i.e. slower transport of NF proteins and altered subunit ratio (Appendix A, figure I). However, the transport of actin and tubulin seemed to be unaffected in that situation. The significance of this finding will be discussed later. Third, in addition to a defect in slow transport, the rapid component of transport was affected in human NF-H transgenics. Representative fluorographic patterns of labeled proteins rapidly transported in motor axons of control and transgenic mice can be seen in figures 3A and 3B of Chapter III. Overall comparison of transported proteins revealed impaired transport of important cytological structures such as mitochondria. The reduced number of mitochondria from the transgenic axons was further confirmed by electron microscopy and immunofluorescent studies (Chapter III, figure 4 and 5). The impaired transport of mitochondria in human NF-H transgenic mice may be relevant to the neuropathy by selectively hindering the transport of energy products. Based on these

data we can suggest that the defect in the transport of materials resulted from NF abnormalities while distal axonal atrophy and degeneration are secondary events to failed delivery of the transported material.

The preceding discussion has summarized the pathological features occuring in the human NF-H transgenic mice. Other hereditary canine and mouse models of motor neuron disorders including wobbler, wasted, pmn, Mnd and HCSMA have been reported (Chapter I, section 2.3.1). Unfortunately the genetic defects have yet to be identified and certain aspects of their pathologies differ from the ones described in patients with ALS. For instance, early onset of motor neuron degeneration occurs in the wobbler mouse, only cervical motor neurons, no upper motor neurons are affected and no NF swellings are observed as in ALS (Mitsumoto and Bradley, 1982; Kaupmann et al., 1992). The wasted and pmn models also develop early manifestations of motor neuron disease compared to ALS, which is a disease of older individuals (Lutsep and Rodriguez, 1989; Sendtner et al., 1992). In both wasted and pmn mice the disease progresses quite rapidly resulting in death around 4 and 7 weeks respectively. Wasted mice will show only occasional neurons with prominent axonal swelling. The Mnd model represents an adult onset disease starting around 6-7 months (Callahan et al., 1991). In the Mnd mouse, a rearrangement of NF cytoskeleton is seen, there are no NF accumulations but a redistribution of NFs within the cytoplasm of anterior horn neurons occurred leaving a large area of cytoplasm without NFs. In the dog model (HCSMA), the changes are restricted to ventral horn motor neurons where numerous NF-containing enlarged axons are observed but there is no change in motor cortex or corticospinal tract as in ALS patients.

Experimental animals models in which NF accumulations similar to the one observed in human motor neuron diseases (including ALS and GAN) have also been reported (Chapter I, section 2.3.2). They include intoxication with substances such as aluminum, acrylamide, IDPN and hexacarbons (2,5-HD, 3-MHD and 3,4-DMHD) (Griffin et al., 1983, 1984). However, the precise mechanism of action of these toxins is only partially understood and they probably have multiple effects on the neuron.

The structural changes observed in the spontaneous and induced models of motor neuron diseases are understood in terms of underlying transport abnormalities (Griffin and Watson, 1988). However, it is not clear if NF accumulations occuring in these models are a cause or a consequence of a defective transport (Griffin and Watson, 1988). Furthermore in the wobbler mouse model, the impairment of NF transport is

likely related to a reduction in NF proteins synthesis and not to a block of NF transport (Mitsumoto and Gambetti, 1986). In contrast, the aberrant accumulation of filaments in cytoplasm and proximal axons of human NF-H transgenic mice is not merely a by-product of a defect in transport but a major participant in axonal transport dysfunction that results ultimately in motor neuron death. It is this finding that makes the human NF-H transgenic mice a unique model of motor neuron disease and illustrates a new mechanism of neuronal death.

The next section discusses the possible events leading to the cytoskeletal alterations in the human NF-H transgenics in comparison with previously published models of motor neuron diseases. A mechanism resulting in neurofilamentous accumulations could be increased cross-linking between the filaments. A second one could be a post-translational modification of NFs (for example, a change in the phosphorylation state of NF proteins). Finally, a change in filament structure and organization resulting either from an alteration in the relative proportion of the NF proteins composition or from a mutation (mutation in the α-helical rod domain or deletion in the amino-terminal domain), could also lead to neurofilamentous accumulation.

Increased cross-linking

The NF triplet protein contains a central α-helical rod domain responsible for the formation of the filament core while the tail domain of NF-M and NF-H protrude from the filament backbone and interact with other cell components (Hisagana and Hirokawa, 1988; Hirokawa, 1991). As mentioned in section 1.3.3.4, during neuronal development there is a reduction in the rate of NFs transport velocity (from 8mm/d to 1mm/d) concomittant with the appearance of NF-H (Willard and Simon, 1983). This result suggested that increased cytoskeletal interactions due to NF-H expression could affect the kinetics of transport. Although not shown, we obtained biochemical data suggesting that the human and mouse NF-H proteins were incorporated into NFs. Hence, a possible explanation for the neurofilamentous accumulation and impaired transport of NF proteins in human NF-H transgenic mice is that the excess level of human NF-H protein will incorporate into NFs and promote additional NF/NF interactions. These interactions will promote accumulation of NFs in cell bodies and proximal axons and retard even more the movement of NF proteins. These additional drags imposed upon the

cell body may form a lattice that could also prevent the transport of tubulin, actin and mitochondrial proteins as observed in the motor axons of human NF-H transgenic mice.

Change in subunit ratio

A second possibility for abnormal NF accumulation in cell body and proximal axon of human NF-H transgenics may be a disruption in the normal ratio of NF subunits. The molar ratio of NF-H, NF-M and NF-L in a filament corresponds to about 1:2:6 as established by [^{32}P]phosphate incorporation and initial transport studies (Hoffman and Lasek, 1975; Nixon and Lewis, 1986). The molecular ratio in each filament is precisely regulated to confer differential stability in development (Okabe, 1993). The ratio may be a gate of entry for NF into the transport system in which case only those filaments composed of an appropriate subunit ratio may be loaded into the axon (Muma et al., 1990). As mentioned previously, we obtained data suggesting that the mouse and human NF-H proteins were incorporated into NFs Therefore in the human NF-H transgenics the unusual presence of human NF-H subunits in the NF polymers may alter their ratio and in turn their assembly and eventually promote a gradual rearrangement of the entire cytoskeleton. Support for this hypothesis comes from microinjection studies which revealed that NFs form a dynamic network with subunit exchange, therefore the filament network can be susceptible to disruption upon incorporation of abnormal subunits (Okabe et al., 1993).

There are examples where a change in NF subunit ratio is related to motor neuron diseases. In HCSMA, there is a selective decrease in the levels of the NF-L gene expression (Muma and Cork, 1993). Such a disruption of the normal subunit ratio of NF is sufficient to lead to NF accumulation (Muma and Cork, 1993). In addition, slow axonal transport of the SCa cytoskeletal proteins was reported to be slower. Overexpression of the mouse NF-L subunit resulted in increased NF density and swollen motor neurons (Monteiro et al., 1992; Zu et al., 1993). Finally, transgenic mice expressing the human NF-M protein at a low level in their brain (25 % relative to the endogenous level of murine NF-M) developed neurofilamentous swellings that are age-dependent (Vickers et al., 1994). In these mice, there is co-localization and possibly co-assembly of human and mouse NF-M species (Lee et al., 1992). This highlights the possibility that extra human NF-H protein present only in small amounts in transgenic mice may effectively perturb a normal NF cytoskeleton. In accordance with that, the

slower progression of the disease in the human NF-H transgenic lines 116 and 120, which express about 50% relative to the endogenous mouse NF-H level, suggests a progressive accumulation of NF and a gradual rearrangement of the neuronal cytoskeleton.

More evidence that an alteration in the IF protein composition can affect the well being of the cell comes from transgenic mice overexpressing a wild-type hair keratin gene. These mice have fragile cortical cells leading to hair brittleness and breakage. The hypothesis is that the excess expression of a type II relative to a type I keratin gene possibly leads to protein aggregation that interferes with normal IF organization (Powel and Roger, 1990) Therefore an imbalance in the subunit ratio disrupted the normal ordered arrays of keratin proteins. A similar hypothesis can be made from the human NF-H transgenic mice where changes in filament structure and organization resulting from an altered ratio of NF-L to NF-M to NF-H due to the presence of human NF-H and this would lead to NF accumulation and a decreased efficiency of transport of these filaments.

Mutation in rod domain of IF proteins

Although the IF proteins differ significantly in amino- and carboxy-terminal sequences, they all have a common structure based on a conserved helical central rod domain (Steinert and Roop, 1988). This region was shown to be crucial for proper IF assembly. Recently, the first three genetic diseases of IF genes have been discovered (for review see Fuchs and Weber, 1994). They are blistering skin diseases involving mutations in the highly conserved α-helical rod domain. The mutations may have created instability in the primary structure of the keratin filament network which resulted in altered interaction between keratin molecules which in turn compromise the mechanical integrity of the epidermal cells (Stewart, 1993; Fuchs, 1994; Fuchs and Weber, 1994). In fact, there is a correlation between the site of the mutation in the rod domain, the degree to which filament assembly is perturbed and the severity of the human disease (Fuchs and Coulombe, 1992; Fuchs and Weber, 1994).

Transgenic mice with point mutation in the mouse NF-L rod domain similar to the one in keratin genes, have been produced to examine the consequence on NF assembly (Lee et al., 1994). The mice express only a modest level of the mutated NF-L protein (about 50% of the endogenous NF-L). All animals were phenotypically normal

at birth and around three weeks of age, they started to show weakness in both upper and lower limbs. The transgenic mice have abnormal NF accumulation and show massive degeneration of spinal motor neuron cell bodies and axons. In addition, there is severe denervation induced atrophy of the skeletal muscle. In contrast, sensory neurons remain mostly unaffected. The ultrastructure of the axonal swellings revealed segregation of microtubules, membrane organelles and mitochondria toward the central portion of the swellings surrounded by NFs. Together these results point to the hypothesis that an assembly-disrupting point mutation in NF-L resulted in complete reorganization of the neuronal cytoskeleton and cause neuronal death. Therefore, this demonstrated a direct link between NF mutations and motor neuron diseases.

Although not in the same critical rod domain, a mutation in the carboxy-terminal region of NF-H may also underly a motor neuron degeneration. Polymorphism in the KSP region of the human NF-H gene has been reported (Figlewicz et al., 1993). There are 43 and 44 KSP repeats present in the population. Codon deletions in this domain of NF-H have recently been identified in five patients with the sporadic form of ALS (Figlewicz et al., 1994). These mutations indicate that changes in filament phosphorylation may have dramatic effects on organization and assembly resulting in motor neuron diseases.

Aberrant phosphorylation of NFs

Most of the axonal matrix is filled with longitudinal oriented NFs cross-linked to each other by NF side-arms (Hirokawa, 1982, 1986, 1991). The NFs side-arms are made by the carboxy-terminal domain of NF-H which has an unusually high content of glutamic acid and phosphorylated serine. The functional significance of this region remains to be determined. One hypothesis is that phosphorylation serves as a mechanism for coordinating the interaction of NFs with each other and with other cytoskeletal element in events such as axoplasmic transport (Hirokawa, 1982; Nixon et al., 1987). Indeed, phosphorylation must be functionally related to axonal transport since continued phosphorylation and dephosphorylation events take place during NFs transit in the axon (Tashiro and Komiya, 1989). Moreover, de Waegh et al., (1992) using the Trembler mouse model showed that decrease in NF transport was correlated with reduced NF phosphorylation.

Within the lattice of NFs, microtubules appear in groups and actin-like filaments occupy the subaxolemmal space. Membrane organelles including mitochondria tend to localize in the microtubule domain in the subaxolemmal region (Hirokawa, 1991). MAPs are components of cross-bridges between microtubules/microfilaments microtubules/NFs and between microtubules and membrane organelles. (Chen et al., 1992). These cross-bridges can also be controlled dynamically by phosphorylation (Hirokawa, 1991).

Although the mechanism for slow axonal transport has not been defined yet, a model has proposed the movement of soluble subunits (NFs, tubulin and actin monomers for example) that replace the preexisting network by dynamic incorporation/exchange (Steinert and Liem, 1990; Okabe et al., 1993). In this situation, the propulsive mechanism for the migration is possibly present all along the axon (Bizzi et al., 1984).

The orderly movement of axonally transported proteins in discrete waves has led to an alternative proposal where the proteins are transported in the form of structure rather than as individual subunits. It is believed that NF polymers move passively through their attachment to motile microtubules (polymer transport) (Lasek, 1986; Joshi and Bass, 1993). The coherent transport of NF proteins and microtubules also indicate a possible structural association between the two (Tashiro et al., 1984). However, the cross-linker system between NFs and microtubules is not a rigid permanent strucuture and can be affected by phosphorylation (Hisagana and Hirokawa, 1990). Whether or not microtubules are the motor vehicle or they are linked to the motor transport which may be a component of SCb is not known (Nixon, 1991). However, McQuarrie et al., (1986) provided evidence that the motor for transport of the axonal cytoskeleton is contained in SCb.

A common feature of the accumulation of NFs in perikarya and/or proximal axons found in a variety of motor neuron disorders and in experimental intoxication is reactivity with antibody to phosphorylated NFs (Shaw, 1991). Labeling with the SM31 antibody, which recognizes phosphorylated epitopes on NFs, was present in cell bodies of motor and DRG neurons of the human NF-H transgenic mice. Why NFs are abnormally phosphorylated is unknown but this phosphorylation of NF tails in cell bodies could weaken the affinity of NF with microtubules or the transport motor thereby preventing their entry into axons and their decrease in transport velocity. In view of the fact that NFs are highly phosphorylated proteins, and given the interactions between different cytoskeletal systems described earlier, it seems an attractive hypothesis that the NF

accumulation followed by blockage of NF, actin and tubulin transport observed in the human NF-H transgenic mice is related to phosphorylation of NF proteins that leads to disrupted interactions of NFs, microtubules and actin proteins with the transport molecule as opposed to being the consequence of increasing drag due to formation of cross-bridges (Goldman and Yen, 1986; Watson et al., 1991). Indeed, Hisagana et al., (1991) demonstrated that phosphorylation of NF-H dissociated the binding of NFs to microtubules.

Some of the transport characteristics that differ between motor/sensory axons in human NF-H transgenic mice can be examined in light of the hypothesis that differences exist in SCa and SCb proteins content and velocity. NFs are exclusively transported in SCa in motor/sensory axons (Hoffman and Lasek, 1975; Oblinger et al., 1987) compared to tubulin and actin proteins that are mostly transported with SCa in motor neurons while a significantly greater amount of actin/tubulin moves with SCb in peripheral than in central axons (McQuarrie et al., 1986). In fact, several microtubule subpopulations differing in their velocity can be distinguished on the basis of their isotubulin composition in motor/sensory neurons. In SCa the isotubulin population is enriched in the most acidic β-isoforms (Denoulet et al., 1989) while type II β'-tubulin, Tα1 α-tubulin are found mostly in SCb (Tashiro and Komiya, 1991). These subsets of microtubules could also differ by their interaction with the NF network with the result that one population may be more tightly linked to NFs (Tashiro et al., 1984). Therefore one subset of microtubules could be transported independently at a faster rate and the other in association with NFs (Tashiro et al., 1984). NFs, tubulin and actin transport being altered in motor neurons therefore suggests a complete block of SCa movement. The cause of the defect in their transport may result from their physical separation from a common carrier structure. In sensory neurons, a block of SCa movement also occured in which NF, tubulin (same isoforms as in motor axons) and actin proteins are retained in cell bodies and proximal axons. However, since there is substantially less actin and tubulin in SCa of motor neurons, their defect is unnoticed. Actin and tubulin proteins present in SCb being different isoforms are still linked to the transport motor and are translocated down the axon. As a result there is normal transport of actin and tubulin in SCb in sensory axons of human NF-H transgenic mice (Appendix A, figure 1). Work by Tashiro et al., (1984) support this hypothesis, they have observed that following IDPN intoxication, NF transport is slowed down whereas that of tubulin remained unaffected, so that a complete separation of the main wave of tubulin from that of the retarded NF triplet was noticed. However, some polypeptides in

the tubulin region remained arrested together with the NF proteins, these tubulins were enriched in the most acidic subtype of β-tubulin. In that case, systemic administration of IDPN produced neurofilamentous swellings in the most proximal axons and resulted in slowing of NF transport coincident with increased phosphorylation on NF-H (Griffin et al., 1978; Griffin and Watson, 1988; Watson et al., 1989; Gold and Austin, 1991). Intoxication with IDPN increases NF autophosphorylation in such a way that they self aggregate. Therefore, the physical dissociation of axonal NFs and microtubules underlies the failure of the normal translocation of NFs (Griffin and Watson, 1988). The similarity in the transport defect seen in the human NF-H transgenic mice sensory axons and following IDPN intoxication support the physical dissociation of NFs from microtubules as a possible underlying cause for their accumulation and defective transport.

In view that phosphorylation of NFs may be related to the neurofilamentous swellings, other examples where NFs are abnormally phosphorylated have been reported in which similar swellings occurred and selective alterations of NF transport have been observed.

In rabbits intoxicated with aluminum, there is retention of newly synthesized NF proteins in the neuronal cell bodies and initial axons followed by decreased entry of NFs into the nerve fiber and distal axonal atrophy (Bizzi et al., 1984; Troncoso et al., 1992). Aluminum may act on a kinase since there is abnormal phosporylation of NFs which may in turn cause their aggregation in cell bodies and proximal axons. Indeed reports by Schmidt et al., (1987) and Durham (1990) showed that aluminum induced aggregates are labeled intensively with antibody to phosphorylated NFs.

The common observation in IDPN, aluminum intoxication and in the human NF-H transgenic mice is that NFs which accumulate in perikarya of motor neurons are inappropriately phosphorylated, as they contain phosphorylated epitopes that are normally present only in axonal domains. Thus, it is possible that this abnormal phosphorylation of NFs occurs first and that this process, in turn, leads to NF accumulation and impaired transport of NFs. However, the reverse sequence of events is also possible (Troncoso et al., 1992). As suggested by Troncoso et al., (1990, 1992) following aluminum administration, the neuronal concentration of aluminum reaches a critical level capable of causing aggregation of NFs, which are then phosphorylated and subsequently there is impairment of NF transport.

Following 2,5-HD intoxication, distal axonal swellings in which NFs are found in the center of the axon surrounded by microtubules are observed. In the enlargements, 2,5-HD is believed to cause inter-molecular covalent cross-linking of NFs whereas the non-covalent interaction between NFs and microtubules are disrupted possibly via interference in the phosphorylation/dephosphorylation events. Here NF transport is faster in the region proximal to the distal enlargements. In those regions, NF phosphorylation is reduced and NF inter-spacing is increased (Monaco et al., 1987). Perhaps, because NFs are dephosphorylated they are dissociated from one another and they are relatively free to distribute and to interact more readily with a rapid transport motor vehicle until they reach the swellings where axonal transport of NFs distal to the enlargements was reported to progressively slow down.

In early stages of ALS, clusters of morphologically normal NF also accumulate in the neuronal cell body (Hirano, 1982). Genetic studies linked one familial form of ALS to a mutation in the SOD1 enzyme (Rosen et al., 1993). In contrast, the pathogenesis of the motor neuronal degeneration in sporadic ALS is unclear and several possible etiological factors can be involved (Tandan and Bradley, 1985; Chou, 1992; Tyler and Shefner, 1991; Messer, 1992). These include: an age-related deficiency of a neurotrophic factor, metal intoxication, immunological dysfunction, environmental neurotoxins, viral infection, a primary impairment of axonal transport or axostasis with a dying back degeneration of both upper and lower motor nerve fibers. The intracytoplasmic NF accumulations observed in ALS, support the idea that transport of NF is possibly impaired and the type of impairment that we have uncovered in the human NF-H transgenic mice is consistent with several aspects of ALS (Chou, 1992) . First, in ALS pathology, the slowly evolving sequence of changes termed dying back implies initial involvement of long large axons with degeneration beginning in distal region and progression to proximal with time. The selective involvement and loss of large myelinated motor/sensory fibers are likely to result from the fact that NFs are the most abundant cytoskeletal structures in large diameter axon (Lee et al., 1988; Chou, 1992). In addition, these fibers put a large demand on axonal transport. Second, the decrease in axonal transport of cytoskeletal proteins observed in the human NF-H transgenic is progressive and age-dependent and ALS is a disease of older individuals. Finally, further evidence for an involvement of NFs in ALS comes from transgenic mice expressing a mutant form of human superoxide dismutase 1 (SOD1) which causes one hereditary form of ALS (Rosen et al., 1993). The SOD1 transgenic mice developed neurofilamentous swellings in spinal motor neurons (Gurney et al., 1994) and the

accumulated NFs were apparently phosphorylated (Gurney et al., 1994). A direct link between SOD1 and NFs has not been determined yet. However, a defect in SOD1 enzyme activity results in the formation of the powerful oxidant peroxynitrite and motor neurons possess targets susceptible to nitration by peroxynitrite such as tyrosine kinases which can in turn alter NF phosphorylation and assembly (Beckman et al., 1993).

Some rapidly transported organelles are retained in the human NF-H transgenics while fast axonal transport was reported to be increased in ALS patients (Breuer et al., 1987). Axonal transport in HD neuropathy also demonstrated defects in fast axonal transport similar to the ones observed in human NF-H transgenic mice, where excessive retention of rapidly transported material within giant axonal swellings were found (Griffin et al., 1984). The discrepancy between the results in ALS patients compared to the human NF-H transgenic mice may be due to the site where the studies were performed; distal to the enlargements in ALS and proximal in transgenic mice. Therefore, the axonal transport measurements made in ALS patients were in a region distal to the NF swellings. We can speculate that in the human NF-H transgenic mice, organelles such as mitochondria are retained in the swellings. In contrast, in regions distal to the enlargements, there may be less organelles (hence less interactions) which could move more rapidly as in ALS patients.

Breuer et al., (1987) observed a diminished number of organelles moving in the retrograde direction in ALS axons and interruption of a target derived trophic factor as a possible cause underlying ALS has also been suggested. The neurotrophin family of proteins supports the development and survival of vertebrate neurons. NGF is the best characterized factor without which sympathetic neurons die during development. The search for an equivalent neurotrophic factor for motor neurons has lead to the identification of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophins 3 and 4/5 (NT-3, NT4/5), acidic and basic fibroblast growth factor (FGF) and leukemia inhibitory growth factor (LIF) among others. Recently, Henderson et al., (1994) identified a glial cell line-derived neurotrophic factor (GDNF) as a powerful trophic factor to support the survival of purified embryonic rat motor neurons in culture. GDNF could also prevent death and atrophy of axotomized neonatal motor neurons.

Retrograde transport is significant in the pharmacological delivery of neurotrophic factors to the CNS and PNS and receptor mediated retrograde axonal

transport of BDNF, CNTF and LIF to the cells bodies of motor neurons was demonstrated (DiStefano et al., 1992; Curtis et al., 1993, 1994). CNTF supports the survival of sensory/motor neurons both *in vivo* and *in vitro* (Sendtner et al., 1992; Curtis et al., 1993, 1994). Infusion of CNTF in the pmn mice efficiently rescued motor neurons from degeneration, prolonged their survival and improved their motor function (Sendtner et al., 1992). Further additive interactions between BDNF and CNTF arrested the development of motor dysfunction in wobbler mice (Mitsumoto et al., 1994). BDNF expressed in skeletal muscles is transported retrogradely to spinal motor neurons and prevents the retrograde degeneration of axotomized neonatal motor neurons. Beneficial effects of CNTF were observed in pmn mice even though they were in an advanced stage of motor neuron degeneration (Sendtner et al., 1992). Even though in ALS patients, the initial symptoms of motor neuron deficiency usually occur late and by that time about 50% of the spinal motor neurons are lost, the beneficial effect of CNTF observed in pmn mice suggests the use of trophic substances to treat the disease.

In summary, transgenic mice with variable copy numbers of the human NF-H gene were generated. Expression of the human gene was tissue-specific, copy-dependent and independent of the site of integration into the mouse genome. A slight overexpression of the human NF-H gene in transgenic mice resulted in accumulation of NFs in perikarya of motor and sensory neurons and their proximal axons. Similar neurofilamentous accumulations are observed in human motor neuron disorders including ALS and GAN. We provided evidence that accumulation of NFs was a primary event leading to the transport defect of polypeptide components of both SCa and fast transport with the subsequent degeneration of distal axons. Such a defect in axonal transport is pertinent to the etiology of human motor neuron disease including ALS in which NFs transport is also suspected to be impaired. We can tentatively reconstruct the pathogenesis of human NF-H induced neuropathy as follows. The presence of the human NF-H protein initiates a disorganization of the neuronal cytoskeleton (due to aberrant phosphorylation of NFs, increased cross-linking of NFs, or another as yet unknown cause). The large myelinated motor, DRG neuron cell bodies and their proximal axons are filled with NFs. Eventually the swellings hinder the transport of cytological structures such as mitochondria and SCa cytoskeletal proteins through the affected axonal region. The distal part of the axon becomes cut off from its cell-body generated supply of essential elements and it degenerates producing the clinical neuropathy. The NF-H transgenic mice are therefore a very good model of human neurodegenerative disease and can be used to test therapeutic

strategies. The reported beneficial effect of neurotrophins on motor neuron rescue and survival in animals models of human motor neuron disorders introduce the possibility of using them for treating human degenerative diseases. It would be of interest in future work to test the effects of the different neurotrophins on motor neurons of the human NF-H transgenics.

Contribution to original knowledge.

Using transgenic mouse technology, we demonstrated that:

- Expression of the human NF-H gene (Cos4NFH) in mice was tissue-specific, copy-dependent, independent of the site of integration into the mouse genome and followed the same developmental pattern as the mouse endogenous NF-H gene. These results established that DNA elements within the Cos4NFH coding sequences or introns confer this pattern of expression and that these regulatory elements are recognized by the murine transcription system.

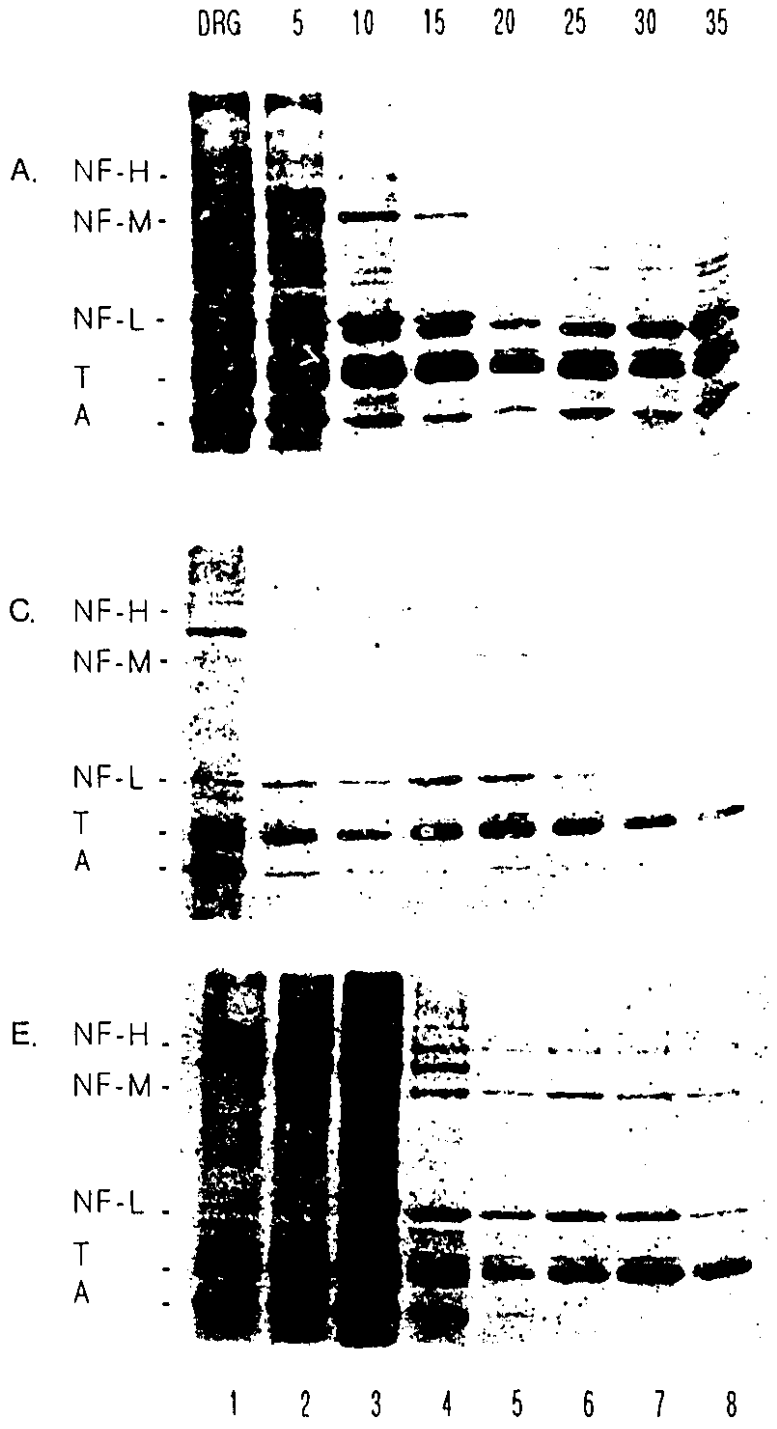
- Forcing neurons to overexpress the human NF-H protein was sufficient to yield morphological features of human motor neuron disorders. These include perikaryal and proximal axonal swellings filled with 10 nm NFs, distal axonal atrophy and muscle atrophy. Results suggested that accumulation of NF proteins and the associated disorganization of perikaryal and axonal cytoplasm could be an integral part of the pathogenic parhway leading to motor neuron death. We showed that the neurofilamentous accumulation provoked neuronal death by disrupting not only NF transport but also transport of other components essential for axonal maintenance.

In conclusion, the use of transgenic mice have provided a powerful experimental system in which to dissect complex issues of NF expression and their involvement in motor neuron diseases.

Appendix A

Figure 1. Fluorographs of slow transport profiles in sensory axons of the sciatic nerve from normal and N-FH transgenic mice. Panels A, C and E represent the profiles of normal mice 7, 14 and 28 days following injection of [^{35}S]methionine into L4-L5 DRG. Panels B, D and F show the corresponding results from NF-H transgenic mice. For each panel, lane 1 contains proteins extracted from the site of injection (l4-L5 DRG) and each successive lane represents a 5mm sciatic nerve segment extending distally to the right. Note the reduced rate of transport for all three NF subunits in transgenic mice, whereas actin and tubulin transport appear normal (B, D, F).

Methods. The methods are described in Chapter III, figure 2.


DRG 5 10 15 20 25 30 35
A.
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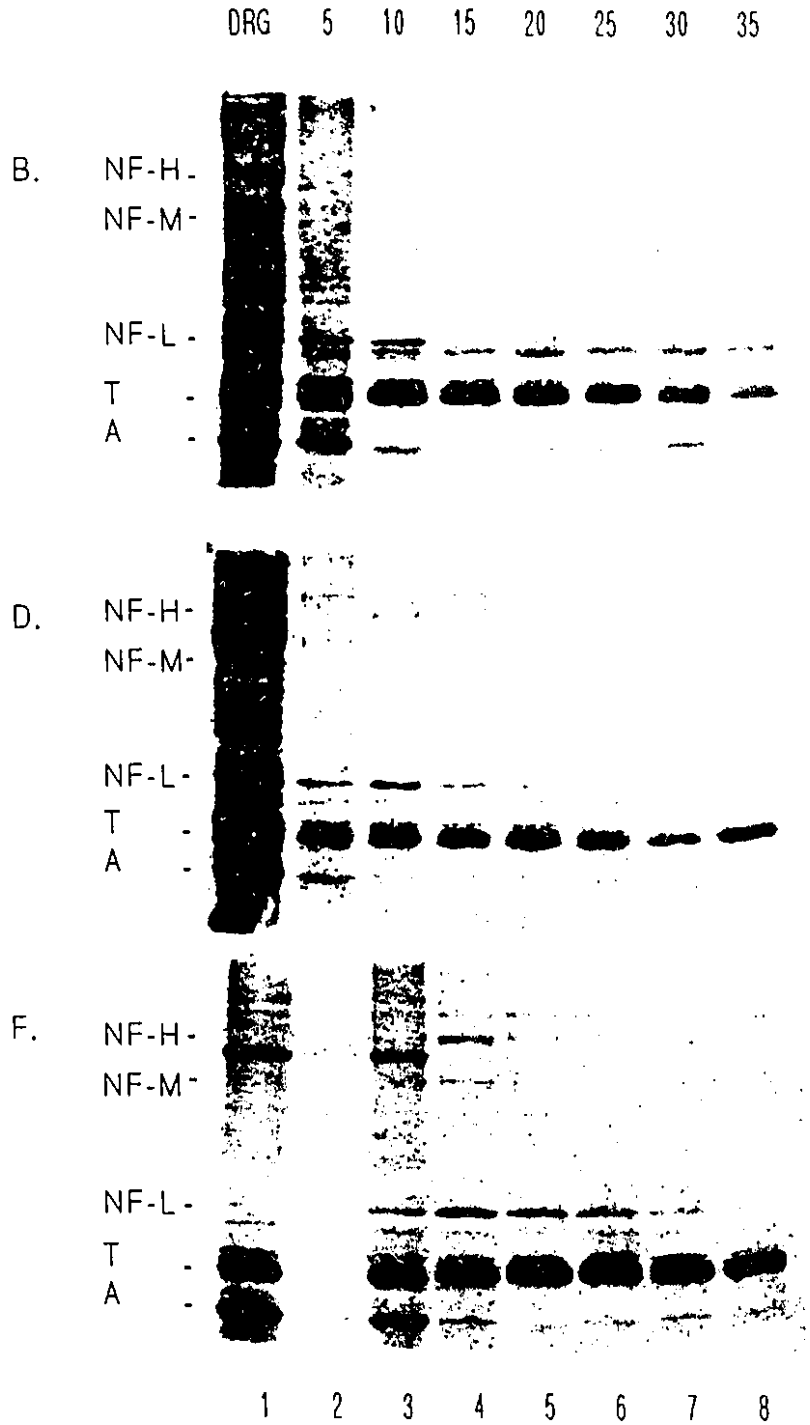
1 2 3 4 5 6 7 8


DRG 5 10 15 20 25 30 35
B.
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NF-H
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NF-L
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F.
NF-H
NF-M
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1 2 3 4 5 6 7 8

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