Brain Pathology 8: 759-769(1998)

SYMPOSIUM: Transgenic Models of Neurodegeneration

Transgenic Mice in the Study of ALS: The Role of Neurofilaments

Jean-Pierre Julien, Sébastien Couillard-Després and Jurgen Meier

Centre for Research in Neuroscience, McGill University, The Montreal General Hospital Research Institute, Montreal H3G 1A4

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurological disorder of multiple etiologies that affects primarily motor neurons in the brain and spinal cord. Abnormal accumulations of neurofilaments (NFs) in motor neurons and a down-regulation of mRNA for the NF light subunit (NF-L) are associated with ALS, but it remains unclear to what extent these NF perturbations contribute to human disease. Transgenic mouse studies demonstrated that overexpression of normal and mutant NF proteins can sometimes provoke a motor neuronopathy characterized by the presence of abnormal NF accumulations resembling those found in ALS. Remarkably, the motor neuronopathy in transgenic mice overexpressing human NF heavy (NF-H) subunits was rescued by the co- expression of a human NF-L transgene at levels that restored a correct stoichiometry of NF-L to NF-H subunits. Transgenic approaches have also been used to investigate the role of NFs in disease caused by Cu/Zn superoxide dismutase (SOD1) mutations, which is responsible for ~2% cases of ALS. Studies with transgenic mice expressing low levels of a fusion NF-H/lacZ protein, in which NFs are withheld from the axonal compartment, suggested that axonal NFs are not toxic intermediates required for SOD1-mediated disease. On the contrary, overexpression of human NF-H proteins was found to confer an effective protection against mutant SOD1 toxicity in transgenic mice, a phenomenon that may be due to the ability of NF proteins to chelate calcium. In conclusion, transgenic studies showed that disorganized NFs can sometimes have noxious effects resulting in neuronopathy. However, in the context of motor neuron disease caused by mutant SOD1, there is emerging evidence that NF proteins rather play a protective role.

Corresponding author:
Jean-Pierre Julien, The Montreal General Hospital Research Institute, 1650 Cedar avenue, Montréal, Québec, Canada H3G 1A4; Tel.: 514 937 6011 ext.2361; Fax: 514 934 8265
E-mail: mdju@musica.mcgill.ca

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult-onset and heterogeneous neurological disorder that affects primarily large motor neurons in the brain and spinal cord. The degeneration of motor neurons leads to denervation atrophy of skeletal muscles and, ultimately, to paralysis and death. Current evidence suggests that multiple genetic and environmental factors may be implicated in ALS pathogenesis, including excitatory amino acid neurotoxicity (49, 65), increased protein kinase C activity (41, 43), impaired calcium homeostasis (40), neurofilament (NF) abnormalities (9, 31, 68, 24), oxidative injury (19, 66, 77) and autoimmune mediated injury (73). Approximately 10% of the ALS cases are familial with the disease inherited in an autosomal dominant manner with varying degree of penetrance. Mutations in the gene coding for the Cu/Zn superoxide dismutase (SOD1) located on chromosome 21 have been found in ~20% of familial cases (64, 6) but for the vast majority of sporadic ALS and familial cases the etiology remains unknown.

Mutations in the SOD1 gene

SOD1 is a ubiquitous cytosolic enzyme involved in the conversion of superoxide anion to hydrogen peroxide. To date, more than 50 different SOD1 mutations have been identified in familial ALS cases (64, 6, 77). These mutations are mostly inherited in an autosomal dominant fashion. The exception is the substitution of aspartic acid to alanine at position 90 of SOD1 ($SOD1^{D90A}$) that is autosomal recessive (1). The majority of SOD1 mutations are missense point mutations, and

Disease	Neurofilament Abnormalities	References
ALS (70% of cases)	Neurofilament accumulations in motor neurons Decline of 60% in NF-L mRNA	(9) (3)
Parkinson's disease (100% of cases)	Lewy bodies in substantia nigra and locus coreuleus	(70)
	Declines of 30% NF-L mRNA and 70% NF-H mRNA	(30)
Alzheimer's disease (20% of cases)	Cortical Lewy bodies Decline of 70% in NF-L mRNA	(72) (16)
Lewy body Dementia	Cortical Lewy bodies	(69)
Guam-Parkinsonism (100% of cases)	Neurofilament deposits in motor neurons	(62)
Peripheral Neuropathies	Neurofilament accumulations in peripheral axons that can be induced by various toxic agents, such as IDPN, hexanedione and acrylamide.	(5)
Giant Axonal Neuropathy	Neurofilament accumulations in peripheral axons	(39)

Table 1. Human diseases with abnormal neurofilament deposits.

Transgene	Neuronal populations affected	Motor Neuron Disease	References
Human NF-L	Thalamic neurons and cortical neurons	No	(50, 41)
Human NF-M	Cortical neurons and forebrain neurons	No	(82, 42)
Human NF-H	Spinal motor neurons and DRG neurons	Yes	(14, 15)
MSV/mouse NF-L	Spinal motor neurons and DRG neurons	Yes	(86)
MSV/mouse NF-M	Spinal motor neurons and DRG neurons	No	(84)
Mouse NF-H	Spinal motor neurons and DRG neurons	No	(51)
Mouse NF-H/lacZ	Perikaryal swellings and Purkinje cell degeneration	No	(22, 78)
MSV/mutant NF-L	Spinal motor neurons and DRG neurons	Yes	(44)

Table 2. Transgenic mice with abnormal neurofilament accumulations.

only two deletions have been reported (19, 58). Initially, the SOD1 mutations were believed to result in compromised enzymatic activity leading to accumulation of superoxide anions. However, many of the SOD1 mutants still maintain significant enzymatic activity (4, 76) and transgenic studies (80, 85, 29, 61) discussed below suggest that SOD1 mutations cause ALS through mechanisms involving gain of deleterious activities. Several hypotheses have been proposed concerning the noxious effects of SOD1 mutants. One of them is that mutations would render the copper in the SOD1 active site more accessible to peroxynitrite, allowing the formation of reactive nitronium-like intermediates resulting in nitration of proteins on tyrosine residues (2). Another proposal is that the mutations increase the peroxidase activity of SOD1, leading to the formation of more hydroxyl radicals from hydrogen peroxide (83). These aberrant activities of mutant SOD1 could contribute to damage proteins, mitochondria and other organelles, leading to cellular dysfunction and death. However, the pathogenic pathway of SOD1 toxicity underlying the selective degeneration of motor neurons has not been resolved.

Transgene	Pathology	References
SOD1 wild-type	No motor neuron disease but NF-rich spheroids and vacuoles in motor neurons	(18, 80, 85, 61)
SOD1 mutant hG93A	Prominent vacuoles in motor neurons and spheroids in proximal motor axons	(28, 79)
SOD1 mutant hG37R	Vacuoles and few neurofilament inclusions	(85)
SOD1 mutant mG86R	Argyrophilic perikarya and swollen processes in ventral horn gray matter	(61)
SOD1 mutant hG85R	glial inclusions that stain intensely for SOD1 and ubiquitin	(8)
Knock-out SOD1	No motor neuron disease	(59)

Table 3. Mutant and wild-type SOD1 transgenic mice.

Neurofilament abnormalities in ALS

NFs are the major intermediate filaments of mature neurons and are particularly abundant in large myelinated axons. NFs are assembled by the copolymerization of three proteins, NF-L (61 kDa), NF-M (90 kDa) and NF-H (110 kDa) (35). The three NFs subunits share with other members of the intermediate filament family a central domain of approximately 310-amino acids, which is involved in the formation of coiled-coil dimers. Two coiled-coil dimers then line up in a staggered fashion to form an antiparallel tetramer (74, 33, 13). The subsequent chronology of linear and lateral associations between tetramers is difficult to discern although protofilaments consisting of tetramers linked end to end, and protofibrils consisting of two laterally associated protofilaments have been proposed. NFs are obligate heteropolymers requiring NF-L together with either NF-M or NF-H for polymer formation (10, 45). Following their synthesis in the perikaryon, the three NF proteins are transported down the axon with the slow axonal transport component. There is growing evidence that NFs are very dynamic structures (55, 56, 32) and for the view that NF proteins can move down the axon in an unpolymerized form along axonal microtubules (75).

In addition to conferring a mechanical support to the cell, NFs play a role in mediating the caliber of large myelinated axons. This is an important function because

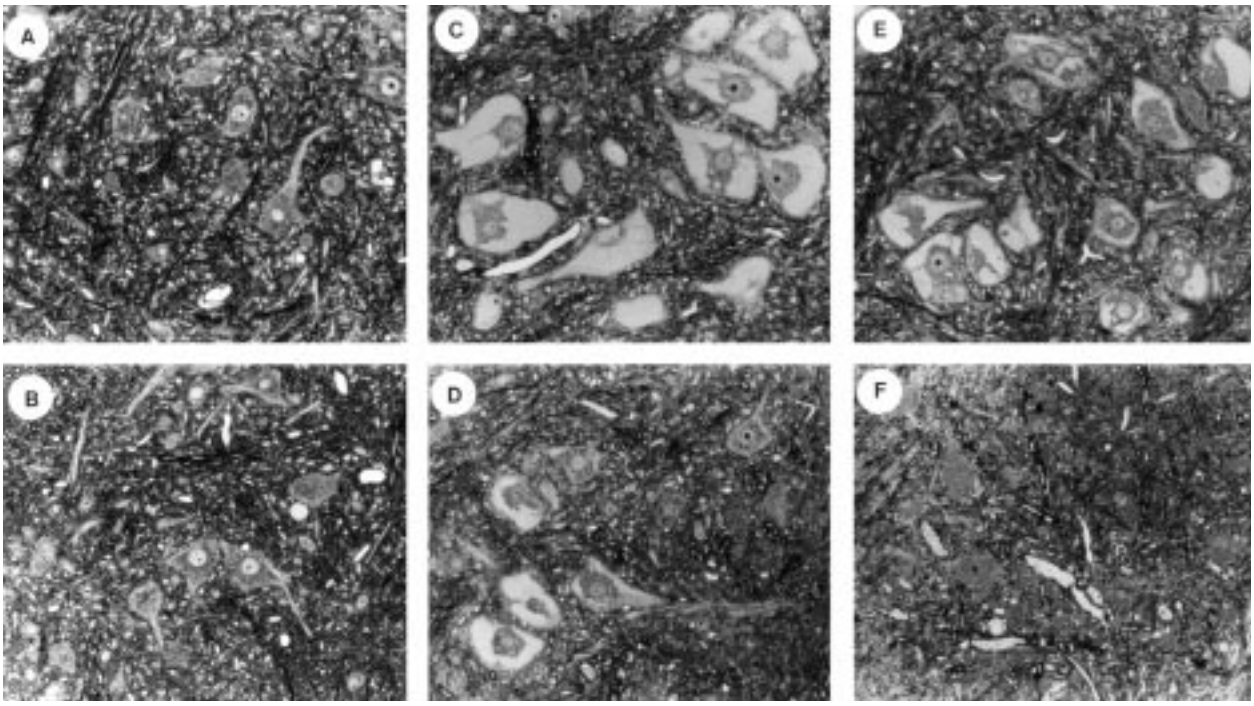

Figure 1. *Reduction of perikaryal swellings by restoration of NF-L to NF-H stoichiometry.* Light microscopy of spinal cord sections from mice homozygous (**C**) and heterozygous (**E**) for a human NF-H transgene showed large accumulations of NFs in the perikarya of motor neurons. The hNF-H transgenic mice were mated to transgenic mice overexpressing human NF-L (**B**) to derive doubly transgenic mice homozygous hNF-H+/+;hNF-L +/+ (**D**) and heterozygous hNF-H+/-;hNF-L +/- (**F**) for the two transgenes. Note the dramatic reduction of perikaryal swellings in doubly transgenic mice. In (**A**) is shown the spinal cord from a normal mouse. Tissues were embedded in Epon and 1 μm sections were stained with toluidine blue. Magnification 500X.

the axonal caliber is a major determinant of conduction velocity. The unequivocal proof of NF involvement in the control of axonal caliber was recently provided from the analysis of animals lacking axonal NF structures including a quivering quail mutant deficient in NF-L protein (87), a transgenic mouse expressing a NF-H/lacZ fusion construct (22) and a NF-L knock-out mice (88). Although a modest decline of ~10% in the number of ventral root axons was observed in NF-L -/- as compared to wild type (88), the absence of pathology in NF-L -/- mice demonstrates that the lack of intact NF structures is not by itself sufficient to grossly alter nervous system development or function in mice.

Perturbations in the normal metabolism of NFs are often associated with neurodegenerative diseases. Table 1 shows a lists pathologies where abnormal NF accumulations, often called spheroids or Lewy bodies, are frequently observed. Paradoxically, the NF deposits observed in neurodegenerative diseases are often associated with decreases in the levels of NF mRNAs. Bergeron et al. (3) reported a 60% reduction in the levels of NF-L mRNAs in motor neurons of ALS patients. In Alzheimer's disease, a decline of up to 70% in NF-L mRNA was reported as compared to age-matched controls (16) whereas in Parkinson's disease, the levels of both NF-L and NF-H mRNAs were found to be reduced in substantia nigra neurons as compared to age-matched controls (30). It has been hypothesized that the reduced NF protein synthesis occurring in affected neurons might lead to a reduction of axon caliber and synaptic dysfunction, possibly causing a loss of nigrostriatal projections (30). In fact, aging itself is a factor that may contribute to axonal atrophy. There is a normal decline in NF mRNA expression of 50-60% during aging (57, 42). The extent to which disorganized NFs or reduction of NF mRNA levels contribute to neurodegeneration in human diseases remains unknown.

Further evidence for NF involvement in motor neuron disease was provided by the finding of codon deletions in the Lys-Ser-Pro (KSP) phosphorylation domain of NF-H from some sporadic cases of ALS (24, 36). The combined results from various laboratories (24, 36, 63, 81) suggest that deletion mutations in the phosphorylation domain of the NF-H gene may be responsible for only a small percentage of ALS cases (~1.3%).

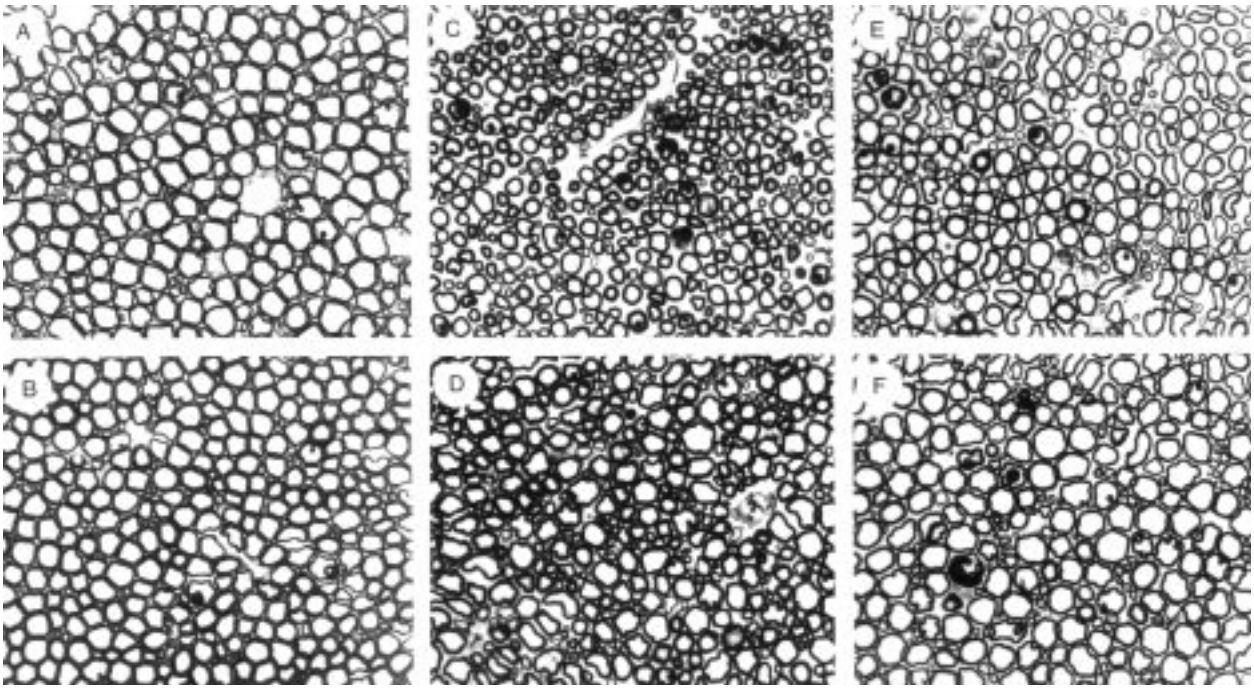

Figure 2. *Rescue of axonal atrophy by hNF-L overexpression in hNF-H transgenic mice.* Light micrographs of ventral root axons from normal mice (**A**), from mice homozygous for human NF-L transgene (**B**), from mice homozygous (**C**) and heterozygous (**E**) for human NF-H transgene, and from doubly transgenic mice hNF-L+/+; hNF-H +/+ (**D**) and hNF-L +/-; hNF-H +/- (**F**). Tissues were embedded in Epon and 1 μm sections were stained with toluidine blue. Magnification 550X.

Transgenic mice with abnormal NF accumulations

Mice expressing NF transgenes. As shown in Table 2, abnormal NF accumulations can be formed by overexpressing any one of the three NF genes. Several transgenic mouse lines expressing human NF-H proteins were generated by the microinjection of large genomic clones including human NF-H alleles (15). Two normal NF-H alleles exist in the human population, one bearing 43 KSP phosphorylation site repeats, and the other 44 KSP repeats (25). Both alleles were found to be capable of inducing motor neuron disease when overexpressed in transgenic mice, but an earlier onset of disease occurred with the allele having 43 KSP repeats (our unpublished observation). A modest two-to-three fold overexpression of this allele in mice provokes a late-onset motor neuron disease, characterized by the presence of aberrant NF accumulations in spinal motor neurons (15). These NF-H transgenic mice exhibit neurological abnormalities such as fine tremors and abnormal limb contraction reflexes during aging. The NF-H-induced pathology progresses with atrophy and slow degeneration of motor axons in old transgenic mice, resulting in secondary muscle degeneration. However, overexpression of human NF-H did not result into significant loss of spinal motor neurons and did not affect life-span. Our interpretation of this effect is that excessive levels of human NF-H protein alter the NF properties, resulting in retardation of axonal transport and aberrant aggregation of NFs in the cell body and proximal axon. Surprisingly, transgenic mice with a four-to-five fold increase in the level of wild type mouse NF-H protein did not develop a motor neuron disease despite the retardation of NF axonal transport, the presence of NF accumulations in motor neurons and the atrophy of myelinated axons (51). These results indicate that human NF-H is markedly more potent than murine NF-H in causing neuronopathy. The reason for this discrepancy could well be due to important differences in the phosphorylation consensus sequences of the KSP repeat domain (37, 46), a region that can affect filament interactions and perhaps axonal transport (52).

High-level overexpression of the wild-type mouse NF-L protein in transgenic mice achieved by use of the strong viral promoter from murine sarcoma virus (MSV), provoked an early-onset motor neuron disease accompanied by massive NF accumulations in spinal motor neurons and by muscle atrophy (86). A more robust phenotype was produced by expressing an assembly-disrupting NF-L mutant having a leucine to proline

substitution near the carboxy-terminal end of the rod domain (44). Although no such NF-L mutations have been reported in human ALS, similar mutations in keratins are the cause of severe forms of genetic skin diseases (26). Expression of this mutant NF-L protein at only 50% of the endogenous NF-L level was sufficient to induce within four weeks massive NF accumulations in spinal motor neurons and was accompanied by death of motor neurons, neuronophagia and severe denervation atrophy of the skeletal muscle.

These transgenic studies with the mouse NF-L and human NF-H genes provided the first demonstration that disorganized neurofilaments can provoke motor neuronopathy. The cellular selectivity of this disease was remarkable in that degeneration was restricted to spinal motor neurons even though the NF-L or NF-H transgenes were expressed throughout the nervous system. It is likely that neurons of small size and therefore having lower NF content were less vulnerable to develop noxious disorganized NFs. Nonetheless, additional unknown cellular factors may also contribute to the deleterious effects of NF inclusions since large NF accumulations were well tolerated within neurons of dorsal root ganglia (DRG) (15, 86, 44).

As shown in Table 2, some types of NF accumulations appear to be less toxic than others as not all transgenic mouse lines with NF abnormalities developed motor neuronopathy. For instance, no overt phenotypes occurred in transgenic mice expressing a mouse NF-H/lacZ fusion gene even though massive NF aggregates were detected in neuronal perikarya throughout the nervous system (22). Also, large NF swellings were found in DRG and motor neurons of transgenic mice overexpressing a NF-M transgene, but no axonal degeneration was observed (84). Moreover, transgenic mice with moderate overexpression of the human NF-L (38, 50) or NF-M genes (82) appeared normal, although a detailed analysis of the mice revealed the presence of abnormal perikaryal immunoreactivity in some populations of brain neurons.

Rescue of motor neuronopathy by restoration of subunit stoichiometry. It has been proposed that the piling-up of NFs can have a noxious effect by reducing the delivery of components required for axonal maintenance (14, 12). However, it remains unclear whether the transport defect results from a physical block by NF deposits or from the indirect effect of disorganized NFs on other cellular components required for axonal transport. Interestingly, we discovered that the motor neuronopathy in transgenic mice overexpressing human NF-H proteins can be rescued by overexpressing human NF-L. Doubly hNF-H;hNF-L transgenic mice were derived by mating mice bearing a human NF-H transgene from line 200 (15) with mice bearing a human NF-L transgene from line 29 (38). Overexpression of human NF-L together with human NF-H in doubly transgenic mice virtually eliminated the massive perikaryal swellings (Fig. 1) and the axonopathy (Fig. 2) observed in mice expressing the human NF-H transgene alone. Moreover, the doubly NF-H;NF-L transgenic mice did not develop overt neurological defects even after one year of age. The levels of human NF-H proteins were slightly more elevated in the doubly transgenic mice (Meier J., Couillard-Després S., Gravel C. and Julien J.-P., paper in preparation). Therefore, the noxious effect of human NF-H overexpression is not due to a toxic property of human NF-H in itself as suggested before (51) but rather related to a cytoskeletal disorganization resulting from an aberrant subunit stoichiometry.

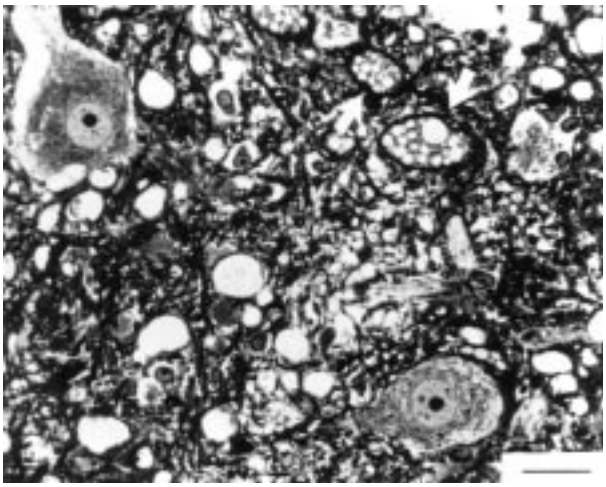

Figure 3. *Vacuolar degeneration of motor spinal neurons in SOD1^{G37R} transgenic mice at end-stage of disease.* Arrows show vacuoles in degenerating neurons. Tissues were embedded in Epon and 1 μm sections were stained with toluidine blue. Calibration bar equals 25 μm.

Transgenic mice expressing SOD1 mutants

Several lines of transgenic mice expressing various SOD1 mutants found in human ALS have been generated in different laboratories. Table 3 summarizes the neuropathological changes occurring in SOD1 transgenic mice that have been reported to date. Transgenic mice expressing three different SOD1 mutants developed motor neuron disease even though the SOD activities in mice were not reduced (28, 85, 80, 61). Mice expressing wild-type human SOD1 to high levels (28, 85, 80) or

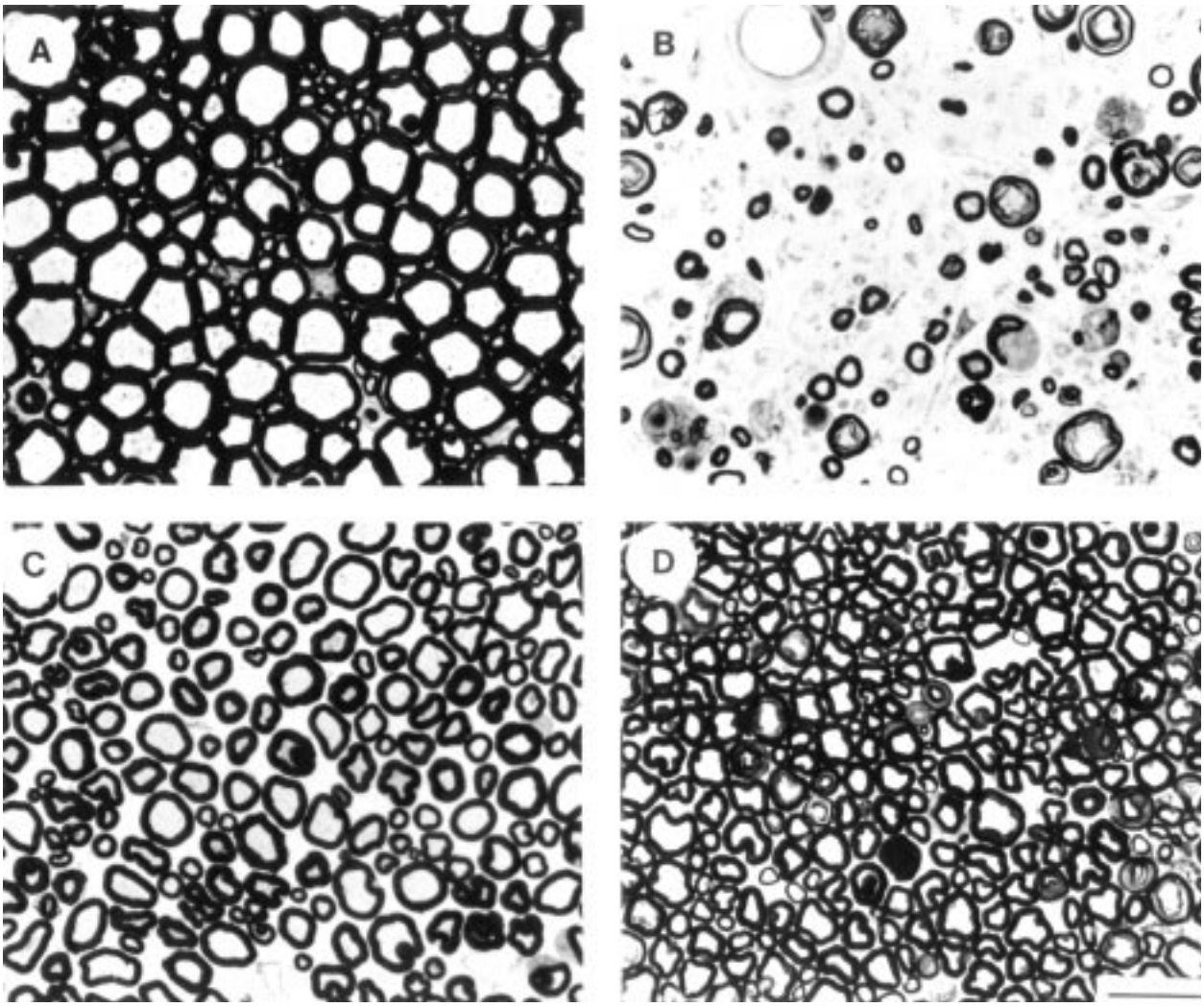

Figure 4. *Less neurodegeneration in SOD1^{G37R} transgenic mice co-expressing human NF-H proteins.* Light micrographs show the lumbar (L5) ventral root from a normal mouse (**A**), from a singly SOD1^{G37R} transgenic mouse (**B**), from a transgenic mouse overexpressing human NF-H (**C**), and from doubly SOD1^{G37R};NF-H transgenic mouse (**D**). All mice were one-year old. Tissues were embedded in Epon and 1 μm sections were stained with toluidine blue. The bar equals 10 μm.

mice homozygous for the targeted disruption of the SOD1 gene (59) did not develop motor neuron disease. This demonstrated that SOD1 mutations cause ALS through a mechanism involving a gain of adverse function rather than a loss of superoxide dismutase activity.

The transgenic mice expressing SOD1 mutants exhibit many aspects of the pathology observed in human ALS. The neuropathology in the SOD1^{G93A} and SOD1^{G37R} mice appeared mainly in the motor neurons in spinal cord and brain stem (28, 85, 80). The pathology in these transgenic mice is characterized by the presence of prominent vacuoles representing dilated mitochondria and endoplasmic reticulum in motor neurons (Fig. 3). Fragmentation of Golgi apparatus was also identified in the SOD1^{G93A} mice, which is similar of human ALS (54). NF-rich inclusions were also detected in the proximal axons and perikarya in the SOD1^{G93A} (80) and mSOD1^{G86R} mice (61), but to a lower extent in the SOD1^{G37R} mice (85, 85). It is unclear why the SOD1^{G37R} mice developed less NF-rich inclusions. This could reflect differences in the expression levels of SOD1 transgenes or differences in the targets of oxidative damage due to the site of mutation. The pathological characteristics of the SOD1^{G85R} mice are even more intriguing. These mice exhibit glial inclusions immunoreactive for SOD1 and ubiquitin (8) that are similar to those

reported in some human ALS cases (71). However, such inclusions were not observed in mice expressing the $SOD1^{G37R}$, $SOD1^{G93A}$ and even $mSOD1^{G86R}$, which is the mouse SOD1 mutation equivalent to the human $SOD1^{G85R}$.

The role of NFs in SOD1-mediated disease

The mechanism underlying the selective degeneration of motor neurons by expression of SOD1 mutants is not fully understood. The presence of abnormal NF accumulations in motor neurons of some familial ALS cases caused by mutations in SOD1 (67) and the finding of similar NF inclusions in transgenic mice expressing SOD1 mutants (80, 61, 85) suggested the possibility that NF proteins could be primary targets in SOD1-induced disease. In support of this view was a report of immunoreactivity to nitrotyrosine residues in NF-L found in spheroids of motor neurons from familial ALS cases (11). It has been speculated that tyrosine nitration of NF-L may impede its assembly and lead to formation of disorganized filaments (17). However, recent lines of evidence do not support the view that NFs are a toxic intermediate in SOD1-mediated disease. First, nitrotyrosine modification of NF proteins was not detectable in $SOD1^{G85R}$ transgenic mice (7). Second, Eyer et al. (21) observed no changes of disease progression due to $SOD1^{G37R}$ in a context where NFs are withheld from the axonal compartment by expression of a NF-H/lacZ fusion construct. Although the authors concluded that axonal NFs are not required for SOD1- mediated disease, their results must be interpreted with caution because neurons in the NF- H/lacZ mice are not devoid of NFs. In fact, there are enormous NF swellings in neuronal perikarya of NF-H/lacZ mice that can be viewed as a relocalization of endogenous NFs. Thirdly, we recently discovered that NF proteins may have a protective rather than noxious effect in the context of SOD1-mediated disease.

To investigate the role of NFs in SOD1-mediated disease, we mated transgenic mice expressing $SOD1^{G37R}$ (85) with transgenic mice overexpressing human NF-H by 1.5 fold. Transgenic mice expressing $SOD1^{G37R}$ alone in a wild-type NF background had a mean life expectancy of 9.5 months and most of them (75%) died of paralysis before 1 year of age. In contrast, 100% of $SOD1^{G37R}$;NF-H transgenic mice were still alive after 1 year for an average life span of almost 16 months. Thus, overexpression of the human NF-H transgene extended the mean longevity of $SOD1^{G37R}$ mice by ~6 months which represent an increase of 65% of their life-span. It should be noted that while the onset of paralysis and death due to $SOD1^{G37R}$ toxicity were dramatically delayed in $SOD1^{G37R}$;NF-H transgenic mice, these doubly transgenics exhibited the neurological phenotypes characteristic of the parental NF-H transgenic line. Microscopic examination corroborated the protective effect of NF-H protein against SOD1 toxicity (Fig. 4). Whereas massive neurodegeneration occurred in 1-year old mice expressing $SOD1^{G37R}$ alone (Fig. 4 B), spinal root axons and motor neurons were remarkably spared in doubly $SOD1^{G37R}$;NF-H transgenic littermates (Fig. 4 D).

What mechanism can account for the apparent paradoxical effects of NF-H overexpression? To date, we have found no evidence that the extra NF-H proteins would act as a sink for toxic oxygen radical species thereby reducing damage to other essential cellular components. Experiments carried out with mice at different ages revealed no detectable changes in the pattern or amount of protein-bound nitrotyrosine or carbonyl modifications in the doubly $SOD1^{G37R}$; NF-H transgenic mice when compared to normal, $SOD1^{G37R}$ and NF-H transgenic mice (data not shown). Other groups recently published studies supporting these observations. Following infection of differentiated PC12 cells with adenovirus coding for mutant SOD1 genes, the resulting cell death occurred without increases in the content of protein-bound nitrotyrosines, nor was the cell death nitric oxide-dependent (27). The level of protein-bound nitrotyrosine was also reported to remain unchanged in a different mutant SOD1 transgenic mouse line (7). In another study with ALS cases, the level of carbonyls was reported not to be significantly increased in brain samples of FALS patients bearing SOD1 mutations (23). Therefore, we conclude that buffering of oxidative modifications by the NF-H protein is unlikely to be responsible for the protection against SOD1-induced damage.

One attractive explanation for the protective effect of NF-H in the context of SOD1-mediated pathogenesis is based on previous reports that NF proteins have multiple calcium binding sites, including high affinity sites, suggesting NF involvement in calcium homeostasis (47, 48). From these studies, it is therefore conceivable that increasing the NF-H protein levels might confer protection against rises in intracellular calcium resulting from oxidative stress and particularly from mitochondrial damage reported in $SOD1^{G37R}$ transgenic mice (85). There is emerging evidence of calcium involvement in cell death caused by SOD1 mutations. For instance, the calcium-binding protein, calbindin D_{28K}, was recently found to confer protection against mutant SOD1-mediated death of PC12 cells in culture (27) and in primary

motor neuron expressing $SOD1^{G93A}$ in culture (personal communication from Heather Durham, Montreal Neurological Institute). A calcium involvement in ALS is also strongly supported by the selective vulnerability of motor neurons lacking typical calcium-binding proteins, parvalbumin and calbindin as shown in studies on ALS patients and monkeys (60, 20, 34), as well as in a line of SOD1 transgenic mice (53). If NF proteins act as calcium chelators, the dramatic declines in neurofilament mRNA levels occurring during aging (57, 42) and to a greater extent in motor neurons of ALS patients (3) may contribute to increase the vulnerability to calcium-mediated cell death.

Conclusion

Studies with mice overexpressing NF transgenes demonstrated that disorganized NFs can sometimes have noxious effects and provoke neuronopathies presumably by interfering with axonal transport (15, 14, 86, 44). Factors and signalling pathways that can potentially induce noxious NF inclusions are still poorly understood although alterations in the stoichiometry of NF-L to NF-H subunits were sufficient to either provoke or alleviate neuronopathy in transgenic mice. Thus, there is a need to study the factors that can affect the metabolism, transport, phosphorylation and other posttranslational modifications of NF proteins. Moreover, it remains unclear why certain types of NF accumulations are more toxic than others in specific cell types.

Although transgenic studies demonstrated that disorganized NFs can have noxious effects on neuronal function, recent data indicated that NF proteins are not toxic intermediates required for disease caused by mutant SOD1 (21), which is responsible for ~2% of ALS cases. On the contrary, NF-H overexpression was found to confer a remarkable protection against mutant SOD1 toxicity. This rescue of SOD1-induced degeneration may be due to the ability of NF-H protein to chelate calcium (47, 48), a property of NF proteins that have been neglected so far. Thus, it is possible that the decline in NF mRNA levels occurring during aging (57, 42) and to a greater extent in ALS (3) could be a factor that contribute to increase the susceptibility of motor neurons to oxidative stress and calcium-mediated death. In this regard, the breeding of knock- out mice for NF genes (88) with transgenic mice expressing SOD1 mutants should provide a powerful approach to resolve this issue.

Acknowledgments

This work was supported by the ALS association (USA), the American Health Assistance Foundation and the Medical Research Council of Canada (MRC). S. C.-D. has a MRC studentship, J. M. was supported by a fellowship from the German Government and J.-P. J. has a MRC Senior Scholarship.

Transgenic mouse studies recently published by Couillard-Després et al. (Proc Natl Acad Sci USA 95: 9626-9630) and by Williamson et al. (Proc Natl Acad Sci USA 95: 9631-9636) provide compelling evidence that levels of NF proteins can affect the progression of disease caused by SOD1 mutations.

References

1. Anderson PM, Nilsson P, Ala-Hurula V, Keranen ML, Tarvainen I, Haltia T, Nilsson L, Binzer M, Forsgren L, Marklund SL (1995) Amyotrophic lateral sclerosis associated with homozygosity for an Asp90Ala mutation in Cu/Zn superoxide dismutase. *Nature Genet* 10: 61-66
2. Beckman JS, Carson M, Smith CD, Koppenol, WH (1993) ALS, SOD and peroxynitrite. *Nature* 364: 584
3. Bergeron C, Beric-Maskarel K, Muntasser S, Weyer L, Somerville MJ, Percy ME (1994) Neurofilament Light and Polyadenylated mRNA Levels Are Decreased in Amyotrophic Lateral Sclerosis Motor Neurons. *J Neuropathol Exp Neurol* 53: 221-230
4. Borchelt DR, Lee MK, Slunt HS, Guarnieri M, Xu ZS, Wong PC, Brown RH Price DL, Sisodia SS, Cleveland DW (1994) Superoxyde dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc Nat. Acad Sci USA* 91: 8292-8296
5. Brimijioin S (1984) The role of axonal transport in nerve disease. In Dyk, P.J., Thomas P.K., Lambert E.H., Bunge R. eds. *Peripheral Neuropathy*, Vol. 1, Philadelphia: W.B. Sunders Company., 477-493
6. Brown RH (1995) Amyotrophic lateral sclerosis: Recent insights from genetics and transgenic mice. *Cell* 80: 687-692
7. Bruijn LI, Beal MF, Becher MW, Schulz JB, Wong PC, Price, DL, Cleveland DW (1997) Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked superoxide dismutase 1 mutant. *Proc Natl Acad Sci USA* 94: 7606-7611
8. Bruijn LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW (1997) ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18: 327-338
9. Carpenter S (1968) Proximal enlargement in motor neuron diseases. *Neurology* 18: 841- 851

10. Ching G, Liem R (1993) Assembly of type IV neuronal intermediate filaments in nonneuronal cells in the absence of preexisting cytoplasmic intermediate filaments. *Cell Biol* 122: 1323-1335
11. Chou SM, Wang HS, Koma IK (1996) Colocalization of NOS and SOD1 in neurofilament accumulation within motor neurons of amyotrophic lateral sclerosis: an immunohistochemical study. *J Chem Neuroanat* 10: 249-258
12. Cleveland DW (1996) Neuronal growth and death: order and disorder in the axoplasm. *Cell* 84: 663-666
13. Cohlberg JA, Hajarian H, Tran T, Alipourjeddi P, Noveen A (1995) Neurofilament protein heterotetramers as assembly intermediates. *J Bio Chem* 270: 9334-9339
14. Collard JF, Côté F, Julien JP (1995) Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* 375: 61-64
15. Côté F, Collard JF, Julien JP (1993) Progressive neuronopathy in transgenic mice expressing the human neurofilament heavy gene: A mouse model of amyotrophic lateral sclerosis. *Cell* 73: 35-46
16. Crapper McLachlan DR, Lukiw WJ, Wong L, Bergeron C, Bech-Hansen NT (1988) SelectivemessengerRNA reduction in Alzheimer's disease. *Molec Brain Res* 3: 255-262
17. Crow JP, Ye YZ, Strong M, Kirk M, Barnes S, Beckman JS (1997) Superoxide dismutase catalyzes nitration of tyrosines by peroxynitrite in the rod and head domains of neurofilament-L. *J Neurochem* 69: 1945-1953
18. Dal Canto MC, Gurney ME (1994) The development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. *Am J Pathol* 145: 1271-1279
19. de Belleroche J, Orrell, RW, Virgo L (1996) Amyotrophic lateral sclerosis:recent advances in understanding disease mechanisms. *J Neuropathol Exp Neurol* 55: 747-757
20. Elliott JL, Snider WD (1995) Parvalbumin is a marker of ALS-resistant motor neurons. *Neuroreport* 6: 442-452
21. Eyer J, Cleveland DW, Wong PC, Peterson AC (1998) Pathogenesis of two axonopathies does not require axonal neurofilaments. *Nature* 391: 584-587
22. Eyer J, Peterson A (1994) Neurofilament-deficient axons and perikaryal aggregates in viable transgenic mice expressing a neurofilament—galactosidase fusion protein. *Neuron* 12: 389-405
23. Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, Macgarvey U, Kowall NW, Brown RH, Beal MF (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J Neurochem* 69: 2064-2074
24. Figlewicz DA, Krizus A, Martinoli MG, Meininger V, Dib M, Rouleau GA, Julien JP (1994) Variants of the heavy neurofilament subunit are associated with the development of amyotrophic lateral sclerosis. *Hum Mol Genet* 3: 1757-1761
25. Figlewicz DA, Rouleau GA, Krizus A, Julien JP (1993) Polymorphism in the multi- phosphorylation domain of the human neurofilament heavy-subunit-encoding gene. *Gene* 132: 297-300
26. Fuchs E (1994) Intermediate filaments and disease: mutations that cripple cell strength. *J Cell Biol* 125: 511-516
27. Ghadge GD, Lee JP, Bindokas VP, Jordan J, Ma L, Miller RJ, Roos RP (1997) Mutant superoxide dismutase-1-linked familial amyotrophic lateral sclerosis: molecular mechanisms of neuronal death and protection. *J Neuroscience* 15: 8756-8766
28. Gurney ME, Pu H, Chiu AY, Canto MCD, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, Chen W, Zhai P, Sufit RL, Siddique T (1994) Motor neuron degeneration in mice expressing a human Cu Zn superoxide dismutase mutation. *Science* 264: 1772-1775
29. Gurney ME, Cutting FB, Zhai P, Andrus PK, Hall ED (1996) Pathogenic mechanisms in familial amyotrophic lateral sclerosis due to mutation of Cu, Zn superoxide dismutase. *Pathologie Biologie* 44: 51-56
30. Hill WD, Arai M, Cohen JA, Trojanowski, JQ (1993) Neurofilament mRNA is Reduced in Parkinson's Disease Substantia Nigra Pars Compacta Neurons. *J Comp Neurol* 329: 328-336
31. Hirano A, Donnenfeld H, Sasaki S, Nakano I (1984) Fine structural observations of neurofilamentous changes in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 43: 461-470
32. Hirokawa N (1991) Molecular architecture and dynamics of the neuronal cytoskeleton. in R. D. Burgoyne, Ed., *The Neuronal Cytoskeleton*. Wiley-Liss Incorporated, New York, 5- 74.
33. Hisanaga S, Hirokawa N (1990) Molecular architecture of neurofilament: in vitro reassembly process of neurofilament-L protein. *J Mol Biol* 211: 871-882
34. Ho BK, Alexianu ME, Colom LV, Mohamed AH, Serrano F, Appel SH (1996) Expression of calbindin-D28K in motoneuron hybrid cells after retroviral infection with calbindin-D28K cDNA prevents amyotrophic lateral sclerosis IgG-mediated cytotoxicity. *Proc Natl Acad Sci USA* 93: 6796-6801
35. Hoffman PN, Lasek RJ (1975) The slow component of axonal transport: identification of the major structural polypeptides of the axon and their generality among mammalian neurons. *J Cell Biol* 66: 351-366
36. Julien JP (1997) Neurofilaments and motor neuron disease. *Trends Cell Biol* 7: 243-249
37. Julien JP, Cote F, Beaudet L, Sidky M, Flavell D, Grosveld F, Mushynski WE (1988) Sequence and structure of the mouse gene coding for the largest neurofilament subunit. *Gene* 68: 307-314
38. Julien, JP, Tretjakoff I, Beaudet L, Peterson A (1987) Expression and assembly of a human neurofilament protein in transgenic mice provide a novel neuronal marking system. *Genes Dev* 1: 1085-1095
39. Klymkowsky MW, Plummer DJ (1985) Giant axonal neuropathy: A conditional Mutation Affecting Cytoskeletal Organization. *J Cell Biol* 100: 245-250
40. Krieger C, Jones K, Kim SU, Eisen AA (1994) The role of intracellular free calcium in motor neuron disease. *J Neurol Sciences* 124: 27-32

41. Krieger C, Lanius RA, Pelech SL, Shaw CA (1996) Amyotrophic lateral sclerosis: the involvement of intracellular calcium and protein kinase C. *Trends Pharmacol Sci* 17: 114-120

42. Kuchel GA, Poon T, Irshad K, Richard C, Julien JP, Cowen T (1997). Decreased neurofilament gene expression is an index of selective axonal hypotrophy in ageing. *Neuroreport* 8: 799-805

43. Lanius RA, Paddon HB, Mezei M, Wagey R, Krieger C, Pelech SL, Shaw CA (1995) A role for amplified protein kinase C activity in the pathogenesis of ALS. *J Neurochem* 65: 927-930

44. Lee MK, Marszlek JR, Cleveland DW (1994) A Mutant Neurofilament Subunit Causes Massive, Selective Motor Neuron Death: Implication for the Pathogenesis of Human Motor Neuron Disease. *Neuron* 13: 975-988

45. Lee MK, Xu Z, Wong PC, Cleveland DW (1993). Neurofilaments Are Obligate Heteropolymers In Vivo. *J Cell Biol* 122: 1337-1350

46. Lees JF, Shneidman PS, Skuntz SF, Carden MJ, Lazzarini RA (1988) The structure and organization of the human heavy neurofilament subunit (NF-H) and the gene encoding it. *EMBO J* 7: 1947-1955

47. Lefebvre S, Mushynski WE (1987) Calcium binding to untreated and dephosphorylated porcine neurofilaments. *Biochem Biophys Res Commun* 145: 1006-1011

48. Lefebvre S, Mushynski WE (1988) Characterization of the cation-binding properties of porcine neurofilaments. *Biochemistry* 27: 8503-8508

49. Leigh PN, Meldrum BS (1996) Excitotoxicity in ALS. *Neurology* 47: 221-227

50. Ma D, Descarries L, Julien JP, Doucet G (1995) Abnormal perikaryal accumulation of neurofilament light protein in the brain of mice transgenic for the human protein: sequence of postnatal development. *Neuroscience* 68: 135-149

51. Marszalek JR, Williamson TL, Lee MK, Xu Z, Hoffman PN, Crawford TO, Cleveland DW (1996) Neurofilament subunit NF-H modulates axonal diameter by selectively slowing neurofilament transport. J Cell Biol 135: 711-724

52. Miyasaka H, Okabe S, Ishiguro K, Uchida T, Hirokawa N (1993) Interaction of the tail domain of high molecular weight subunits of neurofilaments with the COOH-terminal region of tubulin and its regulation by tau protein kinase II. *J Biol Chem* 268: 22695- 22702

53. Morrison BM, Gordon JW, Ripps ME, Morrison JH (1996) Quantitative immunocytochemical analysis of the spinal cord in G86R superoxide dismutase transgenic mice: neurochemical correlates of selective vulnerability. *J Comp Neurol* 373: 619-631

54. Mourelatos Z, Gonatas NK, Stieber A, Gurney ME, Dal Canto MC (1996) The Golgi apparatus of spinal cord motor neurons in transgenic mice expressing mutant Cu,Zn superoxide dismutase becomes fragmented in early, preclinical stages of the disease. *Proc Natl Acad Sci USA* 93: 5472-5477

55. Nixon RA (1991) Axonal Transport of Cytoskeletal Proteins in R. D. Burgoyne, Ed., *The Neuronal Cytoskeleton*. Wiley-Liss, inc., 283-307

56. Nixon RA, Lewis SE, Mercken M, Sihag RK (1994) [32P]Orthophosphate and [35S]Methionine Label Separate Pools of Neurofilaments with Markedly Different Axonal Transport Kinetics in Mouse Retinal Ganglion Cells In Vivo. *Neurochem Res* 19: 1325- 1333

57. Parhad IM, Scott JN, Cellars LA, Bains JS, Krekoski CA, Clark AW (1995) Axonal atrophy in aging is associated with a decline in neurofilament gene expression. *J Neurosci Res* 41: 355-366

58. Pramatarova A, Goto J, Eiji N, Nakashima K, Takahashi K, Takagi A, Kanazawa I, Figlewicz DA, Rouleau GA (1994) A two basepair deletion in the SOD1 gene causes familail amyotrophic lateral sclerosis. *Hum Mol Genet* 3: 2061-2062

59. Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH, Scott RW, Snider WD (1996) Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nature Genet* 13: 43-47

60. Reiner A, Medina L, Figueredo-Cardenas G, Anfinson S (1995) Brainstem motoneuron pools that are selectively resistant in amyotrophic lateral sclerosis are referentially enriched in parvalbumin: evidence from monkey brainstem for a calcium-mediated mechanism in sporadic ALS. *Exp Neurol* 131: 239-250

61. Ripps ME, Huntley GW, Hof PR, Morrison JH, Gordon JW (1995) Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 92: 689-693

62. Rodgers-Johnson P, Garruto RM, Yanagihara R, Chen KM, Gajdusek DC, Gibbs CJ (1986) Amyotrophic lateral sclerosis and parkinsonism-dementia on Guam: a 30-year evaluation of clinical and neuropathological trends. *Neurology* 36: 7-13

63. Rooke K, Figlewicz DA, Han FY, Rouleau GA (1995) Analysis of the KSP repeat of the neurofilament heavy subunit in familial amyotrophic lateral sclerosis. *Ann Neurol* 46: 789-790

64. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, Rachmani Z, Krizus A, McKenna-Yasek D, Cayabyab A, Gaston SM, Berger R, Tanzi RE, Halperin JJ, Herzfeldt B, Bergh RVD, Hung WY, Bird T, Deng G, Mulder DW, Smyth C, Laing NG, Soriano E, Pericak-Vance MA, Haines J, Rouleau GA, Gusella JS, Horvitz HR, Brown RH (1993) Mutation in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362: 59- 62

65. Rothstein JD (1996) Excitotoxicity hypothesis. *Neurology* 47: S19-S26

66. Rothstein JD (1996) Therapeutic horizons for amyotrophic lateral sclerosis. *Cur Opin Neurobiol* 6: 679-687

67. Rouleau GA, Clark AW, Rooke K, Pramatarova A, Krizus A, Suchowersky O, Julien JP, Figlewicz D (1996) SOD1 mutation is associated with accumulation of neurofilaments in amyotrophic lateral sclerosis. *Annals Neurol* 39: 128-131

68. Schmidt ML, Carden MJ, Lee VMY, Trojanowski JQ (1987) Phosphate dependent and independent neurofilament epitopes in the axonal swellings of patients with motor neuron disease and controls. *Lab Invest* 56: 282-294

69. Schmidt ML, Murray JM, Lee VMY, Hill WD, Wertkin A, Trojanowski JQ (1991) Epitope map of neurofilament protein domains on cortical and peripheral nervous system lewy bodies. *Am J Pathol* 139: 53-65

70. Schmidt ML, Murray JM, Lee VMY, Hill WD, Wertkin A, Trojanowski JQ (1991) Epitope map of neurofilament protein domains on cortical and peripheral nervous system Lewy bodies. *Am J Pathol* 139: 53-65

71. Shibata N, Hirano A, Kobayashi M, Sasak IS, Kato T, Matsumoto S, Shiozawa Z, Komori T, Kemoto A, Umahara T (1994) Cu/Zn superoxide dismutase-like immunoreactivity in Lewy body-like inclusions of sporadic amyotrophic lateral sclerosis. *Neurosci Letters* 179: 149-152

72. Smith MA, Rudnicka-Nawrot M, Richey PL, Paprotnick D, Mulvihill P, Miller CA, Sayre L, Perry G (1995) Carbonyl-related post-translational modification of neurofilament protein in the neurofibrillary pathology of Alzheimer`s disease. *J Neurochem* 64: 2660-2666

73. Smith RG, Siklos L, Alexianu ME, Engelhardt JI, Mosier DR, Colom L, Habib Mohamed A, Appel SH (1996) Autoimmunity and ALS. *Neurology* 47: S40-S45

74. Steinert PM, Roop DR (1988) Molecular and cellular biology of intermediate filaments. *Annu Rev Biochem* 57: 593-625

75. Terada S, Nakata T, Peterson AC, Hirokawa N (1996) Visualization of slow axonal transport in vivo. *Science* 273: 784-788

76. Tsuda T, Munthasser S, Fraser PE, Percy ME, Rainero I, Vaula G, Pinessi L, Bergamini L, Vignocchi G, McLachlan DRC, Tatton WG, George-Hyslop PS (1994) Analysis of the funtional effects of a mutation in SOD1 associated with familial amyotrophic lateral sclerosis. *Neuron* 13: 727-736

77. Tu PH, Gurney ME, Julien JP, Lee VM, Trojanowski JQ (1997) Oxidative stress, mutant SOD1, and neurofilament pathology in transgenic mouse models of human motor neuron disease. *Lab Investig* 76: 441-456

78. Tu PH, Robinson KA, de Snoo F, Eyer J, Peterson A, Lee VM, Trojanowski JQ (1997) Selective degeneration fo Purkinje cells with Lewy body-like inclusions in aged NFHLACZ transgenic mice. *J Neurosci* 17: 1064-1074

79. Tu PH, Elder G, Lazzarini RA, Nelson D, Trojanowski JQ, Lee VMY (1995) Overexpression of the Human NFM Subunit in Transgenic Mice Modifies the Level of Endogenous NFL and the Phosphorylation State of NFH Subunits. *J Cell Biol* 129: 1-13

80. Tu PH, Raju P, Robinson KA, Gurney ME, Trojanowski JQ, Lee VMY (1995) Transgenic Mice Carrying a Human Mutant Superoxide Dismutase Transgene Develop Neuronal Cytoskeletal Pathology Resembling Human Amyotrophic Lateral Sclerosis Lesions. *Proc Natl Acad Sci USA* 93:3155-3160

81. Vechio JD, Bruijn LI, Xu Z, Brown RHJ, Cleveland DW, Vechio JD, Bruijn L, Xu Z, Brown RH, Cleveland DW (1996) Sequence variants in human neurofilament proteins: absence of linkage to familial amyotrophic lateral sclerosis. *Ann Neurol* 40: 603-610

82. Vickers JC, Morrison JH, Friedrich VL, Elder GA, Perl DP, Katz RN, Lazzarini RA (1994) Age-associated and cell-type-specific neurofibrillary pathology in transgenic mice expressing the human midsized neurofilament subunit. *J Neuroscience* 14: 5603-5612

83. Wiedau-Pazos M, Goto JJ, Rabizadeh S, Gralla EB, Roe JA, Lee MK, Valentine JS, Bredesen DE (1996) Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. *Science* 271: 515-518

84. Wong PC, Marszalek J, Crawford TO, Xu Z, Hsieh ST, Griffin JW, Cleveland DW (1995) Increasing neurofilament subunit NF-M expression reduces axonal NF-H, inhibits radial growth, and results in neurofilamentous accumulation in motor neurons. *J Cell Biol* 130: 1413-1422

85. Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jemkins NA, Sisodia SS, Cleveland DW, Price DL (1995) An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 14: 1105-1116

86. Xu Z, Cork LC, Griffin JW, Cleveland DW (1993) Increased expression of neurofilament subunit NF-L produces morphological alterations that resemble the pathology of human motor neuron disease. *Cell* 73: 23-33

87. Yamasaki H, Itakura C, Mizutani M (1991) Hereditary hypotrophic axonopathy with neurofilament deficiency in a mutant strain of the Japanese quail. *Acta Neuropathol* 82: 427-434

88. Zhu Q, Couillard-Després S, Julien JP (1997) Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. *Exp Neurol* 148: 299-316