

Neurofilaments and Orthograde Transport Are Reduced in Ventral Root Axons of Transgenic Mice that Express Human SOD1 with a G93A Mutation

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Abstract. Mice engineered to express a transgene encoding a human Cu/Zn superoxide dismutase (*SOD1*) with a Gly⁹³ → Ala (G93A) mutation found in patients who succumb to familial amyotrophic lateral sclerosis (FALS) develop a rapidly progressive and fatal motor neuron disease (MND) similar to amyotrophic lateral sclerosis (ALS). Hallmark ALS lesions such as fragmentation of the Golgi apparatus and neurofilament (NF)-rich inclusions in surviving spinal cord motor neurons as well as the selective degeneration of this population of neurons were also observed in these animals. Since the mechanism whereby mutations in *SOD1* lead to MND remains enigmatic, we asked whether NF inclusions in motor neurons compromise axonal transport during the onset and progression of MND in a line of mice that contained ~30% fewer copies of the transgene than the original G93A (Gurney et al., 1994). The onset of MND was delayed in these mice compared to the original G93A mice, but they developed the same neuropathologic abnormalities seen in the original

G93A mice, albeit at a later time point with fewer vacuoles and more NF inclusions. Quantitative Western blot analyses showed a progressive decrease in the level of NF proteins in the L5 ventral roots of G93A mice and a concomitant reduction in axon caliber with the onset of motor weakness. By ~200 d, both fast and slow axonal transports were impaired in the ventral roots of these mice coincidental with the appearance of NF inclusions and vacuoles in the axons and perikarya of vulnerable motor neurons. This is the first demonstration of impaired axonal transport in a mouse model of ALS, and we infer that similar impairments occur in authentic ALS. Based on the temporal correlation of these impairments with the onset of motor weakness and the appearance of NF inclusions and vacuoles in vulnerable motor neurons, the latter lesions may be the proximal cause of motor neuron dysfunction and degeneration in the G93A mice and in FALS patients with *SOD1* mutations.

NEUROFILAMENTS (NFs)¹ comprise the major class of neuron-specific intermediate filaments and are the most abundant cytoskeletal components found in large myelinated axons (for reviews see Nixon, 1993; Fuchs and Weber, 1994). NFs are heteropolymers formed by three subunits known as the high (NFH; 110 kD), middle (NFM; 95 kD), and low (NFL; 62 kD) molecular weight NF proteins, all of which are synthesized in neuronal perikarya and transported in the SCa phase (i.e., slow component a) of axonal transport at ~0.2 to 1.2 mm/d (Lasek and Hoffman, 1976). Other cytoskeletal components (e.g., actin, tu-

bulin) are transported three to four times faster in the SCb of slow axonal transport (Hoffman and Lasek, 1980).

The carboxy termini or tail domains of NFH and NFM harbor tandem repeats of lysine-serine-proline (KSP) motifs, and the serine in this motif may be phosphorylated under both physiological and pathological conditions (Jones and Williams, 1982; Julien and Mushynski, 1982; Black and Lee, 1988; Lee et al., 1988*a,b*; Clark and Lee, 1991; Giasson and Mushynski, 1996). Normally, the serines in these motifs become highly phosphorylated only after NFH and NFM have been transported into axons where this phosphorylation regulates the caliber of axons (de Waegh et al., 1992; Cole et al., 1994; Tu et al., 1995). In a variety of human neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD), as well as in neurotoxin-induced neuropathies, such as those that result from exposure to aluminum and acrylamide (Troncoso et al., 1985; Yase, 1988; Johnson and Jope,

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1. *Abbreviations used in this paper:* ALS, amyotrophic lateral sclerosis; FALS, familial ALS; MND, motor neuron disease; NF, neurofilament; PD, Parkinson's disease.

1988; Johnson et al., 1990; Strong, 1994), NF inclusions typically form in the perikarya and processes of neurons, and these inclusions contain highly phosphorylated NFH and NFM (Tu et al., 1997a,b). The major functions of NFs are to provide mechanical support, especially in large myelinated axons, and to regulate axonal caliber. Thus, disruption of the NF network has been hypothesized to play a mechanistic role in the degeneration of selectively vulnerable neurons that accumulate inclusions in a subset of human neurodegenerative disease (Lee et al., 1994; Julien, 1995). Significantly, this hypothesis has been supported by several recent studies of a number of different lines of transgenic mice that develop an ALS-like phenotype, including prominent NF inclusions in motor neurons that subsequently degenerate (Côté et al., 1993; Xu et al., 1993; Eyer and Peterson, 1994; Lee et al., 1994; Tu et al., 1997a). Since only variations in the number of KSP motifs in NFH have been observed in some ALS patients (Figlewicz et al., 1994), other factors may lead to disruption of the NF network in neurodegenerative disorders. For example, mutations in the Cu/Zn superoxide dismutase gene (*SOD1*), which occurs in ~20% of familial ALS (FALS) kindreds, lead to perturbations of the NF network (for recent review see Tu et al., 1997b). FALS (as well as sporadic ALS) is a motor neuron disease (MND) characterized by progressive motor weakness due to the selective degeneration of motor neurons, many of which accumulate NF inclusions before their demise (Schmidt et al., 1987; Hill et al., 1991; Hirano, 1991; Tu et al., 1997b). Transgenic mice that express one of four different mutant forms of the human *SOD1* gene recapitulate many of the hallmarks of FALS including a fatal, progressive motor neuron weakness, the selective loss of motor neurons (Gurney et al., 1994; Ripps et al., 1995; Wong et al., 1995; Bruijn et al., 1997), fragmentation of the Golgi apparatus (Mourelatos et al., 1996), and the accumulation of NF inclusions in motor neurons that are vulnerable to degenerate (Dal Canto and Gurney, 1994, 1995, 1997; Tu et al., 1996). The precise mechanisms that lead to the selective degeneration of neurons in authentic ALS as well as in transgenic mouse models of this disorder remain enigmatic, but there is evidence to suggest that NF inclusions may impede axonal transport and thereby contribute to the degeneration of affected neurons (Collard et al., 1995). Alternatively, other studies suggest that NF inclusions may compromise the viability of affected neurons by sequestering vital organelles (Tu et al., 1997a). Thus, the present study exploited a classic experimental paradigm to determine if axonal transport in the ventral roots was impaired in transgenic mice that express human *SOD1* with a Gly⁹³→Ala mutation (G93A) and develop an ALS-like phenotype.

Materials and Methods

Experimental Animals

Transgenic mice engineered to overexpress mutant (G93A) and wild-type (N1029) human *SOD1* (Gurney et al., 1994) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained as hemizygotes for this study. Mice were screened by PCR with primers described by Gurney et al. (1994). All of these animals were bred and reared in a pathogen-free environment, and the experimental procedures used here were approved by the University of Pennsylvania.

Immunohistochemistry

16 G93A mutant *SOD1* transgenic and age-matched control mice (150, 180, 200, and 230 d, $n = 2$ /age group) were perfused with 4% paraformaldehyde in PBS after being lethally anesthetized by an intraperitoneal injection of ketamine hydrochloride (1 mg/10 g) and xylazine (0.1 mg/10 g). The spinal cords of these mice were harvested after a postperfusion interval of 2 h, immersed in the same fixative overnight, rinsed in PBS three times for 10 h, and embedded in paraffin as described (Tu et al., 1997a). Blocks of spinal cord were cut into 6- μ m-thick sections, mounted on poly-L-lysine-coated slides, and heated overnight at 40°C. Every other section was then examined by immunohistochemical methods using a panel of antibodies to NF and other proteins (Schmidt et al., 1987; Tu et al., 1996, 1997a).

Electron Microscopy

16 G93A *SOD1* transgenic and age-matched control mice of four different ages (150, 180, 200, and 230 d, $n = 2$ /age group) were deeply anesthetized as described above and sacrificed by intracardiac perfusion with 10 ml of 0.1 M cacodylate buffer (pH 7.4) and 50 ml of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. The L5 segments of the spinal cords of these animals and their corresponding ventral roots were removed, and processed for EM analysis. The ventral horns of the spinal cords were trimmed to blocks of ~1 mm³ and along with the nerve roots were postfixed in 1% osmium tetroxide for 20 min. After dehydration with graded ethanols, the tissue was embedded in Epon-Araldite resin at 60°C for 72 h. Ultrathin sections of these blocks were then cut and mounted on 100 mesh grids and stained with 1% uranyl acetate and 0.5% lead citrate. The EM grids were examined using an electron microscope (100CX; Jeol, Ltd., Tokyo, Japan) at 80 kV.

Analysis of Ventral Roots by Quantitative Western Blotting

The ventral roots of 24 G93A *SOD1* and N1029 transgenic mice as well as age-matched control mice of four different ages (150, 180, 200, and 230 d, $n = 3$ /age group) were harvested after being lethally anesthetized as described above. The L5 segment of the spinal cord was identified after laminectomy of the T13 to L6 vertebral bodies. It was then harvested and the attached ventral root was cut into five consecutive 2-mm-long segments for Western blot analysis (Fig. 1). Each segment was individually homogenized in 50 μ l of BUST buffer (0.5% SDS, 8 M urea, 2% β -mercaptoethanol, 0.01% proteinase inhibitor cocktail, and 0.1 M Tris HCl, pH 6.8) as described earlier (Cole et al., 1994; Tu et al., 1995). The solubilized samples were centrifuged in an ultracentrifuge (TL-100; Beckman Instruments, Inc., Fullerton, CA) for 30 min at 25°C, and 8 μ l of supernatant from each sample (containing ~5 μ g of protein) was loaded into individual lanes of a 6 or 7.5% polyacrylamide gel and separated by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes for quantitative Western blot analysis as previously described (Cole et al., 1994; Tu et al., 1995).

The following antibodies were used in this study: (a) polyclonal rabbit anti-NFH antiserum specific for the carboxy-terminus of NFH (1:500); (b) RMO24, a mouse mAb to a highly phosphorylated epitope of NFH (1:1,000); (c) RMO189, a mouse mAb to the rod domain of NFM (1:1,000); (d) a polyclonal rabbit antiserum specific for the carboxy terminus of NFL (1:1,000); and (e) a mouse mAb to β -tubulin (1:200; Sigma Chemical Co., St. Louis, MO). The specificity of these antibodies has been well documented (Tu et al., 1996, 1997a, and references therein) as have the procedures for quantitative Western blotting and statistical analysis of the Western blot data (Cole et al., 1994; Tu et al., 1995).

Axonal Transport Studies

24 G93A transgenic and N1029 transgenic mice as well as age-matched control mice of two different ages (150 and 200 d, $n = 3$ /age group) underwent laminectomy from segments T13 to L1 of the spinal cord under deep anesthesia. 200 μ Ci of ³⁵S-labeled methionine/0.6 μ l of saline was microinjected into two sites of the L5 ventral horn using a stereotaxic apparatus over a period of 2 min (Fig. 1) as previously described (Mitsumoto and Gambetti, 1986; Mitsumoto et al., 1993). Groups of animals were sacrificed 3 h and 7 d after microinjection for the analysis of fast and slow axonal transport, respectively. The L5 ventral roots were removed, pro-

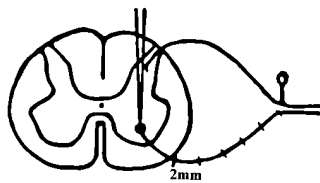


Figure 1. Schematic diagram showing the microdissection of L5 ventral roots and the microinjection of [³⁵S]methionine into the ventral horn of the mouse spinal cord in the experimental paradigm used here to analyze axonal transport.

cessed for SDS-PAGE, and subjected to quantitative Western blotting as described above.

Neuronal Counting

Quantification of ventral horn neurons was performed on spinal cord samples from the animals perfused for immunohistochemistry and EM as outlined above. To do this, 6- μ m-thick sections of spinal cord from the L5 segment of three sets of the G93A, N1029, and control mice of four different ages (150, 180, 200, and 230 d, $n = 3$ /group) were stained with the Nissl method. Images at 100 \times magnification were captured to the computer from slides using the NIH Image software (version 5.4). Only ventral horn neurons with clear nucleoli were counted in 10 different sections from each spinal cord. These sections were separated from each other by 30 μ m to avoid repetitive counting. Data were analyzed statistically using a Student's t test.

Axonal Counting

The toluidine blue-stained semithin sections that were used to count axons in the L5 ventral roots came from three sets of the G93A, N1029, and control mice at 150, 180, 200, and 230 d of age that were prepared for the EM and immunocytochemical studies described above. To quantify the number of axons in these nerves, toluidine blue-stained transverse sections of L5 ventral roots were captured with Northern Exposure software (Empix Imaging Inc., Glen Mills, PA), and then printed at 400 \times magnification. An 8 \times 11 inch template transparency composed of identical squares was overlaid on the photograph to facilitate counting axons in each ventral root. The number of axons in each square was counted and pooled together to generate the total number of axons in a ventral root, and the data were analyzed statistically using a Student's t test.

Results

The Onset of MND Is Delayed in Rederived G93A Transgenic Mice with a Lower Copy Number of the Mutant SOD1 Gene

The G93A mutant and wild-type (N1029) *SOD1* transgenic mice used in this study were purchased from Jackson Laboratory and were rederived from the original G93A and the N1029 transgenic mice generated by Gurney et al. (1994). However, for unknown reasons, the rederived G93A mice contain 30% fewer copies of the *SOD1* transgene than the original G93A transgenic mice (this information is now posted on the Web page of Jackson Laboratory), and these mice develop an ALS-like phenotype later than the original line of mice. For example, muscle weakness and retraction of the hindlimbs with tail suspension were not observed in the rederived G93A mice until $\sim 200 \pm 7$ d of age, while the original mice become weak at ~ 120 d (Gurney et al., 1994). Further, the rederived G93A mice were completely paralyzed at ~ 250 d, rather than at 150 d, as in the original line. The rederived N1029 mice that over express wild-type *SOD1* transgene do not show evidence of MND for up to 2 yr just like the original line of these mice (Gurney et al., 1994; Tu et al., 1996).

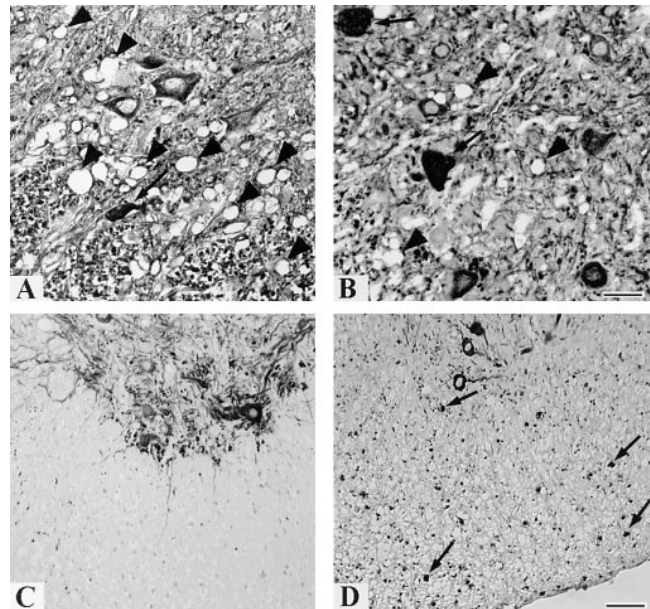


Figure 2. Photomicrographs showing NF-rich inclusions in the perikarya and proximal axons of the spinal cord of the G93A mice. The NF inclusions contain NFL epitopes revealed by a rabbit anti-NFL polyclonal antiserum (*A* and *B*). Notably, the vacuolar pathology (*arrowheads*) in the rederived G93A mice in the terminal stages of MND (230 d of age in *B*) is less prominent than in the original line of G93A mice at a similar terminal stage (140 d of age in *A*). However, the inclusions (*arrows*) in the rederived G93A mice in the terminal stages of MND (230 d of age in *B*) are more abundant than in the original line of G93A mice at a similar terminal stage (140 d of age in *A*). The large arrow in *B* indicates an inclusion with light staining. In *C* and *D*, the RMdO9 mAb to poorly phosphorylated NFH stains the white matter of the spinal cord of the G93A mouse (*D*) more intensely than in the control mouse (*C*), and the small arrows in *D* indicate RMdO9-stained axonal inclusions containing poorly phosphorylated NFH. Bars: (*B*) 20 μ m; (*D*) 40 μ m.

NF Inclusions Occur in Spinal Cord Motor Neurons of the Rederived G93A Transgenic Mice

We compared the extent of NF inclusions stained by hematoxylin and eosin (H&E) in spinal cord motor neurons of the rederived G93A transgenic mice with the original G93A mice and demonstrated Lewy body-like inclusions in the perikarya and proximal axons of spinal cord motoneurons by ~ 180 d of age (data not shown). Immunohistochemical studies performed with NF subunit-specific antibodies (e.g., RMO32 specific for highly phosphorylated NFM, RMO24 specific for highly phosphorylated NFH, and rabbit anti-NFL polyclonal antiserum) showed that these inclusions contained immunoreactivity for all three NF proteins (Fig. 2 and data not shown). The number of these NF-rich inclusions increased with the age, and vacuoles were seen in many of these neurons, although they were much less prominent than in the original G93A mice of the same stage of MND (Fig. 2, *A* and *B*, compare *arrowheads*). This is in agreement with a recent study, which demonstrated little or no vacuolar pathology in a line of G93A mice with very low transgene copy numbers and an attenuated (400 d) onset of an ALS-like phenotype (Dal Canto and Gurney, 1997). Interestingly, RMdO9 (a mAb

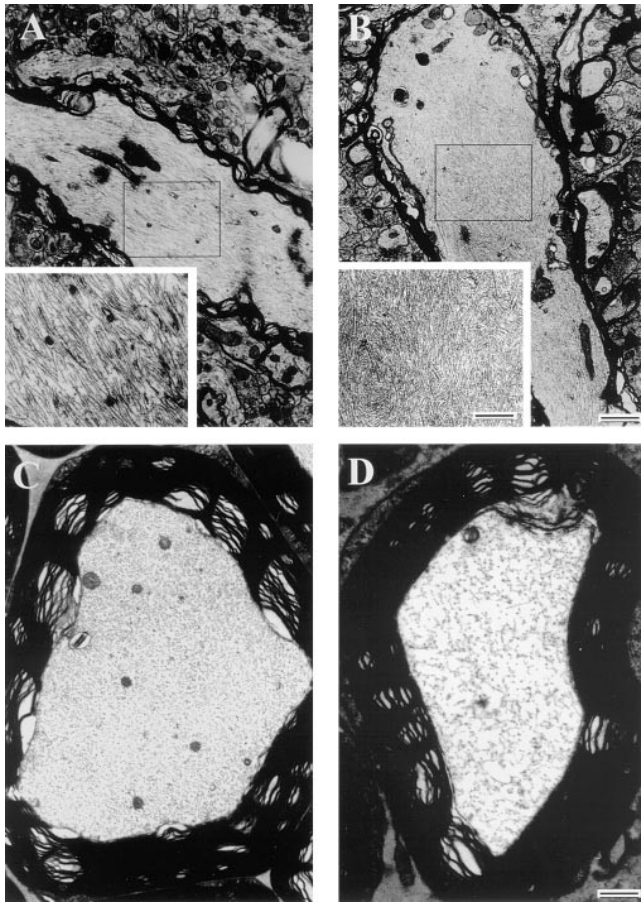


Figure 3. Electron micrographs showing NF accumulations in a proximal axon of a spinal cord motor neuron and reduced NFs in an L5 ventral root of a 200-d-old G93A mouse. NFs are aligned in parallel in the normal axon (A). The NF inclusion is a mass of tightly packed disorganized NFs (B). This inclusion fills the axon and displaces cellular organelles in the proximal ventral root axons of the G93A mice. Normal NFs are shown in a cross-section of a ventral root axon from a control mouse (C), while there is a decreased density of NFs in the L5 ventral root axon of G93A mice (D). Bars: (B) 1 μ m; (B, inset, and D) 0.5 μ m.

specific for a non- or poorly phosphorylated NFH epitope), intensively stained axons in the white matter of the spinal cords of the G93A mice (Fig. 2 D), but this mAb stained the same region of control mice very weakly (Fig. 2 C), suggesting that the phosphorylation state of NFH in white matter axons of the G93A mice may be markedly reduced.

Ultrastructural Changes in the Rederived G93A Transgenic Mice

Since NF inclusions were detected in neuronal perikarya and axons of G93A mice, we compared the ultrastructural changes of NF morphology in proximal axons and ventral roots of G93A and control mice. NFs were aligned in parallel in the normal proximal axons (Fig. 3 A), but masses of tightly packed, disoriented filaments similar to NFs were detected in proximal axons of the G93A mice (Fig. 3 B). These inclusions resembled the spheroids seen in human ALS (Gurney et al., 1994; Tu et al., 1995) as well as in transgenic mice that over express NF proteins and develop

MND (Côté et al., 1993; Xu et al., 1993; Lee et al., 1994; Wong et al., 1995; Tu et al., 1997a). Aggregates of dilated mitochondria and other organelles also were seen proximal to these inclusions in some axons (data not shown). Notably, the number of normal NFs in the L5 ventral root axons of the rederived G93A mice was dramatically reduced (Fig. 3 D) at 200 d compared to age-matched control mice (Fig. 3 C), but the thickness of the myelin sheath around large axons appeared similar in the ventral roots of the G93A and age-matched control mice.

NF Triplet Proteins and Tubulin Are Reduced in the Ventral Root Axons of the Rederived G93A Mice

Since NF inclusions are found in proximal axons of the G93A mice and the number of NFs in the ventral roots of these mice are reduced, we asked whether or not the amount of NF subunit proteins was reduced in the G93A mice. To do this, we performed quantitative Western blot analysis on 2-mm segments along the ventral roots of the G93A and N1029 transgenic mice as well as control mice to compare the relative levels of the NF triplet proteins in the ventral root axons (see Fig. 1 for orientation). At 150 d, the levels of NF proteins in the L5 ventral root axons of the G93A mice were similar to N1029 and control mice (Fig. 4, A and E), but there were no NF inclusions or motor neuron degeneration at this time in the G93A mice (data not shown). However, by 180 d, a substantial decrease in NF proteins was detected in the G93A mice compared to age-matched N1029 and the control mice (Fig. 4, B and E). Specifically, a 50% decrease in NFL was first detected in 180-d-old G93A mice (Fig. 4, B and E), and the levels of NFL decreased progressively by 70 and 90% when these mice were 200 (Fig. 5, C and E) and 230 d of age (Fig. 4, D and E), respectively. An obvious decrease in the NFM immunoreactivity also was observed in the ventral root axons of the G93A mice, although it occurred slightly later and to a lesser extent than the decrease in NFL. For example, the levels of NFM were reduced by 50 and 70% when the G93A mice were 200 and 230 d of age, respectively. Similarly, the levels of NFH in the ventral root axons of the G93A mice decreased at 200 and 230 d of age by 90 and 95%, respectively (Fig. 4 E). Since the decrease in the levels of NF proteins was similar in all five consecutive segments of the ventral roots of the G93A mice, this suggests that orthograde transport of NF proteins may be retarded at one or more sites proximal to the ventral root axons studied here. Our analysis also showed that the amount of NF proteins in the ventral root axons of the N1029 transgenic mice was comparable to that seen in the ventral root axons of the control mice at all four ages examined here (data not shown). Thus, both the N1029 transgenic and the wild-type littermates of G93A mice were used as controls and are referred to as such together here.

To determine if the levels of NF proteins were reduced selectively in ventral root axons of the G93A mice, β -tubulin levels were analyzed in the same ventral root samples using the methods outlined above. As shown in Fig. 4, the levels of β -tubulin in the ventral root axons of the N1029 transgenic mice were similar to those in the control mice at 150 d (Fig. 4 A). However, like NF proteins, the β -tubulin levels in the ventral root axons of the G93A mice de-

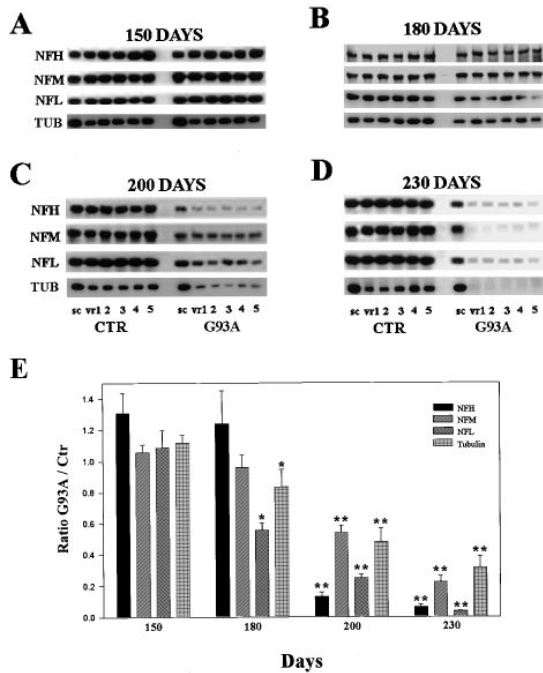


Figure 4. Western blots showing a progressive decrease in the level of NF proteins in the L5 ventral roots of the G93A transgenic mice. The ventral roots of 24 G93A *SOD1* and N1029 transgenic mice as well as age-matched control mice of four different ages (150, 180, 200, and 230 d, $n = 3/\text{age group}$) were harvested after lethal anesthesia as described. The relative levels of NFH are revealed by a rabbit anti-NFH polyclonal antiserum; those of NFM, by the mAb RMO189, and those of NFL, by the rabbit anti-NFL polyclonal antiserum. The levels of NF proteins and tubulin in the 150-d-old G93A mouse are comparable to that of the age-matched control mouse (A, CTR). However, there is a progressive decrease in NF proteins and tubulin in the ventral roots of the G93A mice at 180, 200, and 230 d of age compared with the age-matched CTR mice (B–D). Quantification of these data (E) shows that NFL and tubulin start to decrease in the G93A mice as early as 180 d, while a significant reduction in the levels of NFM, NFH, and tubulin occurs when the G93A mice reach 200 d of age. The ventral roots of the G93A mice lose 80–90% of NF proteins when they are at the terminal stages of MND (230 d). *vr1-5*, Ventral root segments from 1 to 5; * $P < 0.05$; ** $P < 0.01$.

creased progressively with age. For example, a 20% decrease in the levels of β -tubulin was first detected at 180 d in the ventral root axons of the G93A mice (Fig. 5, B and E). Further, this decrease progressed to 50 and 70% of the control values by 200 or 230 d, respectively, in the G93A mice (Figs. 5 C and 4, D and E). Since the decrease in β -tubulin levels in the ventral root axons of G93A mice also was observed in all of the ventral segments examined here, these findings support the hypothesis that axonal transport of β -tubulin also is impeded proximal to the ventral root. Taken together, our data suggest that the translocation of multiple proteins is affected in axons of spinal cord motor neurons of the G93A mice.

The Decreased Level of NF Proteins Is Not Due to the Loss of Ventral Root Axons in Rederived G93A Mice

To determine if the decrease in NF proteins in the ventral

Table I. Number of Ventral Motorneurons and Ventral Root Axons

Age groups	<i>n</i>	Number of neurons	Number of axons
150 Ctr	3	92.10 \pm 1.13	869.00 \pm 61.16
G93A	3	86.65 \pm 3.75	860.67 \pm 60.41
180 Ctr	3	87.60 \pm 2.15	890.33 \pm 56.88
G93A	3	86.13 \pm 2.40	881.00 \pm 52.25
200 Ctr	3	92.62 \pm 3.18	871.33 \pm 17.37
G93A	3	85.73 \pm 1.53	755.33 \pm 29.63
230 Ctr	3	95.52 \pm 5.76	873.33 \pm 51.54
G93A	3	72.48 \pm 5.71**	651.00 \pm 49.22**

Table I indicates the number of ventral motor neurons and L5 ventral root axons in G93A versus control (Ctr) at 150 to 230 d of age.

\pm , SE.

** $P < 0.01$.

root axons of the G93A transgenic mice resulted exclusively from the loss of axons, we determined the number of axons in the L5 ventral roots as well as the number of neurons in the L5 ventral horn from three pairs of control and G93A transgenic mice at 150, 180, 200, and 230 d of age. At 150 and 180 d of age, the number of neurons in the L5 ventral horns of the G93A mice was comparable to that of age-matched control mice (Table I). Similarly, the number of axons in the L5 ventral roots of the G93A mice was not significantly different from that of the age-matched control mice (Table I and Fig. 5, A and B). However, there was a \sim 15% decrease in the number of L5 ventral root axons as well as in the number of the ventral horn neurons in the G93A mice at 200 d, and by 230 d of age, the numbers of these axons and neurons were reduced by \sim 25% (Table I and Fig. 5, C–F). Furthermore, the L5 ventral roots of the G93A mice at 200 d appeared shrunken and disorientated suggesting that the content of the ventral root axons may be reduced (compare Fig. 5, C and E with D and F). Since the magnitude of the reduction in the levels of NF proteins exceeds the extent of axon loss by two to three times in the ventral roots of the G93A transgenic mice, we infer that the progressive diminution in the levels of NF proteins in L5 ventral root axons is caused by mechanisms other than axon loss in the G93A mice.

Orthograde Slow Axonal Transport of Cytoskeletal Proteins Is Retarded in the Ventral Root Axons of the G93A Mice

The accumulation of NFs in proximal axons has been implicated in the retardation of axonal transport in various experimental paradigms (Griffin et al., 1982; Troncoso et al., 1985; Collard et al., 1995), and it is speculated that this may lead to the degeneration of affected axons and their corresponding neurons (Collard et al., 1995). Since NF-rich spheroids in the proximal axons of motor neurons and the reduction of NF proteins in the ventral roots of G93A transgenic mice suggest that impaired axonal transport could lead to the MND phenotype, we monitored the transport of cytoskeletal proteins in the slow component at 7 d after microinjection of ^{35}S -labeled methionine into the L5 ventral horns of G93A, N1029, and control mice. Radiolabeled cytoskeletal proteins traveling in slow axonal transport components were monitored in 2-mm segments along the entire length of the ventral roots by SDS-PAGE (see Fig. 1 for orientation), and the identity of several cy-

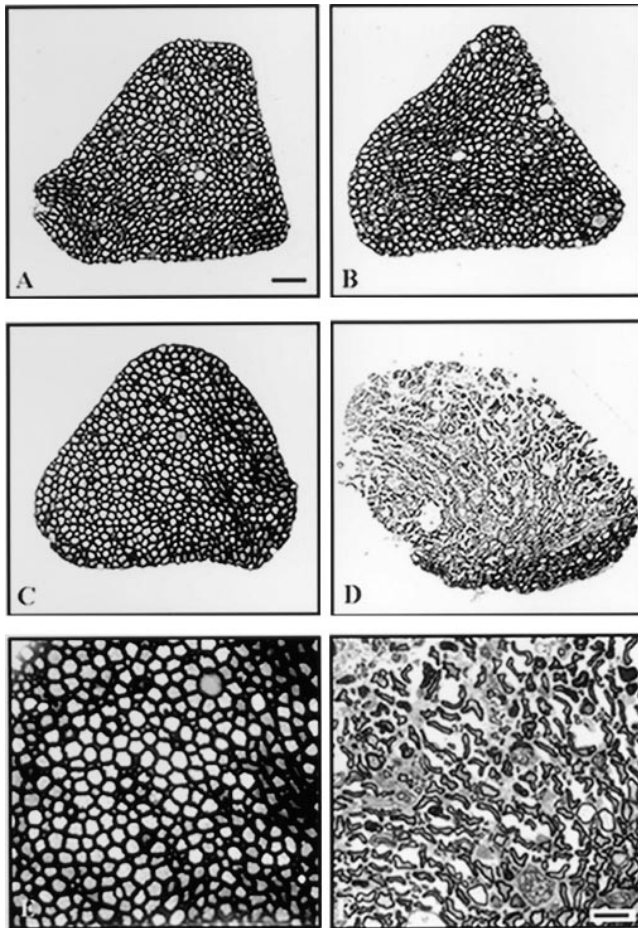


Figure 5. EM photomicrographs show a drastic reduction in the axonal caliber of the L5 ventral roots of the G93A transgenic mice by 200 d of age. The ventral roots of the control mouse (A, C, and E) contain many large myelinated axons that are tightly packed and evenly distributed in the nerve. Note the relative abundance of small- and intermediate-sized axons (A, C, and E). There is no obvious difference in the ventral roots of the G93A transgenic mice (B) compared to control mice (A) at 150 d. In contrast, the ventral roots of the G93A mouse are mainly composed of axons of smaller caliber (D and F) compared to age-matched control mice (C and E). The abundant interaxonal space and the tangential or longitudinal orientation of axons probably reflects the collapse of these axons due to impaired transport. Bars: (A) 20 μ m; (F) 10 μ m.

cytoskeletal proteins was determined by Western blots (Fig. 6, A and B). This showed there was no reduction in the intensity of any of the radiolabeled proteins traveling along the ventral roots by slow axonal transport in 150-d-old G93A transgenic compared to control mice (Fig. 6 A). For example, the ratio of the radioactivity of each of the NF subunit proteins, tubulin subunits, and actin between 150-d-old G93A and control mice was close to one, suggesting that there was no difference in the slow axonal transport of these mice (Fig. 6, A and C-E). By contrast, the movement of these cytoskeletal proteins was considerably reduced in the 200-d-old G93A mice (Fig. 6 B). Quantitative analysis showed that the transport of these proteins was retarded mainly in the most proximal segment of the ventral roots (Fig. 6, D and E). The transport of NFH and

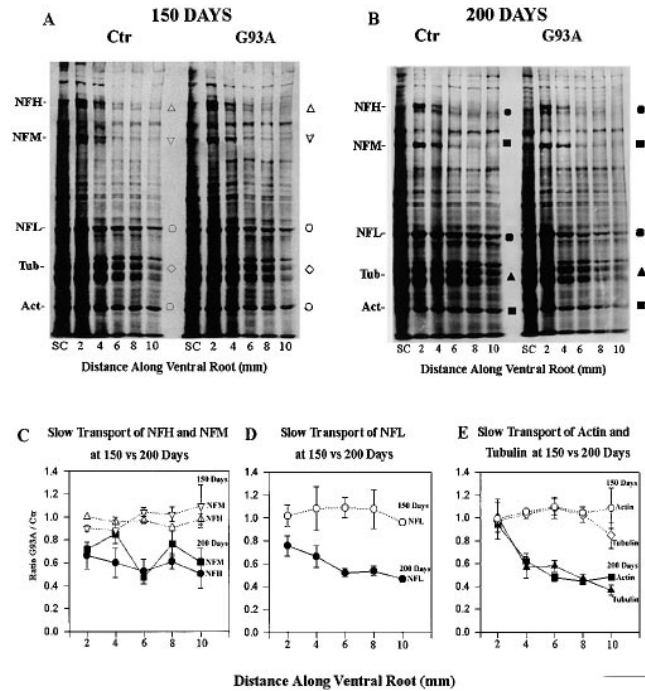


Figure 6. SDS-PAGE shows a progressive retardation in slow transport in the L5 ventral root of the spinal cord in the G93A transgenic mice compared to the control (CTR) mice. 12 G93A and N1029 transgenic mice as well as age-matched control mice of two different ages (150 and 200 d, $n = 3$ /age group) were killed 7 d after microinjection. Fluorographs show a decrease in the transport of a variety of cytoskeletal proteins such as NFH, NFM, NFL, tubulin, and actin in the 200-d-old G93A mice (B) but not in the 150-d-old G93A mice (A). The graphs in C and D illustrate quantitative measurements of individual proteins conveyed by slow axonal transport in pairs of age-matched G93A and CTR mice. The 150-d-old G93A mice fail to show any significant retardation of slow transport (C). However, the slow transport of several proteins is retarded in the 200-d-old G93A mice (D). The symbols to the right of each fluorograph in A and B are aligned with the respective proteins identified on the left, and they are used in C through E. *Tub*, Tubulin; *Act*, actin.

NFH proteins in the distal ventral root of 200-d-old G93A mice decreased by 30%, and the transport of NFL was reduced by ~50% in these mice compared to controls (Fig. 6, C and D). Significantly, the impairment of axonal transport in motor neurons of G93A transgenic mice was not limited to NF proteins. As shown in Fig. 6, B and E, 200-d-old G93A mice exhibited a 50% reduction in tubulin and actin transport, i.e., two major cytoskeletal proteins of slow axonal transport. Thus, our data suggest that there are defects in the slow axonal transport of NF proteins as well as of other proteins including tubulin and actin in the G93A mice.

Components of Orthograde Fast Axonal Transport Are Selectively Retarded in the Ventral Root Axons of the G93A Mice

Since all major cytoskeletal components of slow axonal transport were reduced in G93A mice that developed MND, we asked whether proteins transported in the fast components also are impaired. To do this, we analyzed radiolabeled proteins at 3 h after microinjection of 35 S-labeled

methionine into the L5 ventral horn of G93A, N1029, and control mice, and we showed that there was no reduction in the intensity of any of the radiolabeled proteins traveling along the ventral roots by fast axonal transport in 150-d-old G93A and control mice (Fig. 7 A). For example, we compared the ratio of three different radiolabeled proteins with molecular mass 100, 43, and 20 kD from G93A mice versus control mice and demonstrated that their movement along all five segments of the ventral roots did not differ significantly (Fig. 7, A and C). In contrast, the fast axonal transport of radiolabeled proteins in 200-d-old G93A mice appeared to be variable when compared to control mice of the same age (Fig. 7 B). For example, the migration of a radiolabeled protein with molecular mass 100 kD (Fig. 7 B) was similar to that of the control, and the transport of a second 43-kD protein was only slightly affected (Fig. 7 B) while the movement of a third 20-kD protein (Fig. 7 B) was reduced by 60% in the ventral root of G93A transgenic mice compared to controls, indicating a selective retardation of specific proteins (Fig. 7, B and D).

Discussion

Several lines of transgenic mice carrying human or mouse *SOD1* transgenes with mutations identified in FALS patients have been shown to develop an MND phenotype similar to FALS, and these mice serve as useful animal models for research into the pathobiology of FALS and sporadic ALS (for recent review see Tu et al., 1997b). For example, the G93A transgenic mice used in this study, which were rederived from the original line generated by Gurney et al. (1994), develop the cardinal features of human ALS. However, the transgene copy number in the rederived G93A mice is 30% lower than in the original G93A line, and this significantly affects several key aspects of the MND phenotype in these mice. For example, the onset of MND was delayed by 80 d, and the duration of MND was prolonged by 20 d in the rederived mice compared to the original G93A line. Further, the initial appearance of vacuoles and NF-rich inclusions also occurred later in the life span of the rederived G93A mice. Additionally, the number of vacuoles was decreased, while the NF-rich inclusions were more abundant in both perikarya and proximal axons of spinal cord motoneurons. These observations are in agreement with a recent report demonstrating that another line of G93A transgenic mice with a low transgene copy number did not develop MND until 400 d of age (Dal Canto and Gurney, 1997). Significantly, these mice showed almost no vacuoles, but they had many more NF-rich inclusions in their spinal cord motoneurons. Taken together, these data clearly demonstrate a direct correlation between the expression levels of the mutant transgene, the age at which MND first emerges, and the severity or rate of progression of MND. Thus, increasing levels of the mutated gene correlates with an earlier onset of MND, more abundant vacuoles, and less prominent NF-rich inclusions (Wong et al., 1995; Tu et al., 1996; Dal Canto and Gurney, 1997).

Since mice carrying the murine equivalent of the human G85R *SOD1* mutation or low copy numbers of the G93A mutant human *SOD1* transgene develop MND, but far fewer mitochondrial vacuoles, it is likely that NF-rich in-

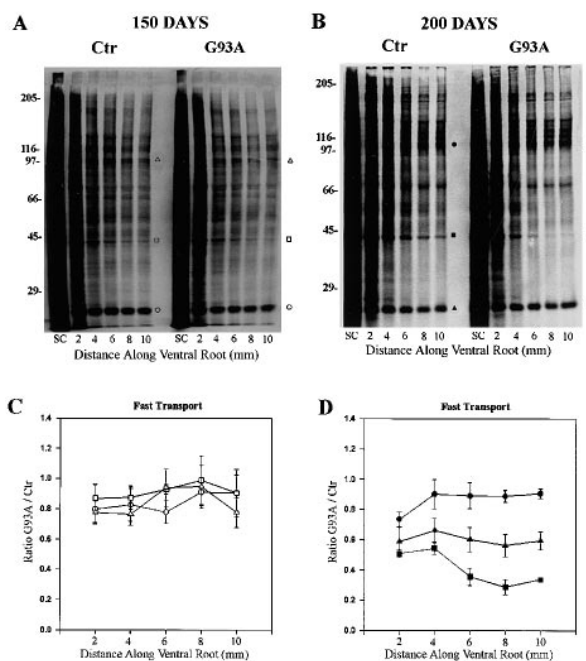


Figure 7. SDS-PAGE shows a progressive retardation in fast transport in the L5 ventral roots of the spinal cord in the G93A transgenic mice compared to the control (CTR) mice. 12 G93A and N1029 transgenic mice as well as age-matched control mice of two different ages (150 and 200 d, $n = 3$ /age group) were killed 3 h after microinjection. Fluorographs show no change in the fast transport of several proteins (closed triangle, closed square, and closed circle) in the 150-d-old G93A mice (A and C), but the fast transport of some proteins is retarded in the 200-d-old G93A mice (B and D). The graphs in C and D illustrate quantitative measurements of individual proteins conveyed by fast axonal transport in pairs of age-matched G93A and CTR mice. The 150-d-old G93A mice fail to show any significant slowing of fast transport (C), but the fast transport of several proteins is retarded in the 200-d-old G93A mice (D). The symbols in A and B correspond to proteins analyzed in the graphs in C and D.

clusions are the proximal cause of MND. This view is compatible with reports that similar vacuoles have not been detected in FALS patients with a G93A mutation in the *SOD1* gene, and that patients with FALS develop prominent NF-rich inclusions in their surviving spinal motor neurons with very little, if any, vacuolar pathology (Kato et al., 1996). Thus, we conclude that the mice with low copy numbers of a G93A *SOD1* transgene are much more authentic models for FALS than mice with high mutant transgene copy numbers, and that NF-rich inclusions may play a causal role in the onset and progression of FALS. Accordingly, we sought to identify any potentially detrimental consequences induced by these NF-rich inclusions. Here, we report significant decreases in both the levels and transport rates of NF and other proteins in the L5 ventral roots of aged G93A transgenic mice, and we speculate that this impaired axonal transport is a consequence of the accumulation of NF-rich inclusions. Thus, the accumulation of these inclusions may be the proximal cause of the demise of spinal cord motor neurons in these mice as well as in ALS.

Although the G93A transgenic mice develop a clinical and pathologic phenotype similar to FALS and sporadic ALS (Gurney et al., 1994), the mechanism whereby *SOD1*

mutations cause the formation of NF-rich spheroids and Lewy body-like inclusions in spinal cord motor neurons remains unknown (Tu et al., 1996). *SOD1* is a member of the *SOD* gene family, and it is found in the cytosol and peroxisomes of most cells including neurons (Bannister et al., 1987; Beyer et al., 1991). The function of *SOD1* is to convert the toxic superoxide anion radical O_2^- to H_2O_2 (Coyle and Puttfarcken, 1993; Olanow, 1993) which is further reduced to H_2O by glutathione peroxidase and/or catalase. Since normal levels of *SOD1* in neurons are necessary for antioxidant defense mechanisms, previous studies have shown that an imbalance of *SOD1* activity can induce the accumulation of excess amounts of O_2^- or other free radicals resulting in oxidative damage to neurons (Coyle and Puttfarcken, 1993; Olanow, 1993). This oxidative stress may directly or indirectly lead to a retardation in axonal transport resulting in the formation of NF aggregates in the proximal axons of motor neurons. Alternatively, since a gain of adverse function has been hypothesized for the mutant *SOD1* gene (Gurney et al., 1994), it is possible that the NF abnormalities are a direct consequence of this yet to be identified adverse function.

A slight reduction in the NF levels was detected in the G93A mice at the onset of MND at 180 d of age, and the level of NF proteins progressively dropped to only 10 to 20% of the levels seen in age-matched control mice by the time the G93A mice were completely paralyzed at 230 d of age. At the same time, a prominent increase in the staining intensity of RMdO9 was noted in the white matter of the spinal cords of the G93A mice when compared with age-matched controls. Since this mAb is specific for poorly phosphorylated NFH epitopes, this observation indicates that the phosphorylation state of axonal NFH also is reduced in the G93A mice.

NFs are the most important determinants of axonal caliber, but previous studies have shown that Schwann cells also regulate axonal caliber by modulating the number of NFs and the phosphorylation state of NFH and NFM (de Waegh et al., 1992; Cole et al., 1994; Tu et al., 1995). Thus, a reduction in the level of NF proteins and the phosphorylation state of NFH and NFM results in a contraction in the diameter of affected axons. Notably, the decrease in NF proteins correlates closely in time with shrinkage of the axons in the ventral roots and the appearance of clinical MND in the G93A mice. Since axons that become devoid of cytoskeletal structures are prone to degenerate (Côté et al., 1993; Xu et al., 1993; Eyer and Peterson, 1994; Lee et al., 1994), and axonal degeneration may play a vital role in the emergence of disease (Sagot et al., 1996), our data suggest that the loss of NF proteins could lead to the shrinkage and degeneration of the ventral roots, which may in turn contribute to the onset of MND in the G93A transgenic mice.

There are three probable mechanisms that may explain the depletion of NFs in the ventral roots of the G93A mice. The first mechanism is the formation of NF-rich inclusions which can retain NF subunits in the perikarya and proximal axons, thereby reducing the amount of NFs transported into the distal axons. Interestingly, NFL decreased by 50% as early as 180 d of age, whereas NFH and NFM decreased to the same extent later than NFL. This differential decrease may be related to the different roles of these NF triplet proteins. Since NFL forms the back-

bone of NFs (for review see Nixon, 1993), the earlier decrease in NFL may be caused by the preferential incorporation of NFL into inclusions followed by NFM and NFH. Although the mechanism responsible for the transport of NF subunit proteins is not clear, specific motor protein(s) may be involved. It is likely that mitochondrial damage due to the G93A mutation in these mice may lead to an energy shortage for these putative NF motor proteins resulting in the failure to transport NF proteins into axons, thereby causing deposition of assembled NF proteins in abnormal loci such as perikarya and proximal axons. Also, FALS mutations in the *SOD1* gene have been shown to convert the protein product of this gene from a free radical scavenger to an oxidative stressor. This gain of adverse function by the mutant *SOD* protein may alter the behavior of NF proteins and lead to the formation of inclusions (for detailed review see Tu et al., 1997b). However, the level of tubulin in the ventral roots also decreases, suggesting that factors other than NF inclusions also contribute to the reduction of proteins in the ventral roots.

A second mechanism to consider is a physical blockage of axonal transport by these NF inclusions. EM studies showed that the proximal axons of the G93A mice frequently were dilated by aggregated vacuoles and filamentous inclusions with cellular organelles such as mitochondria clustered at one side of the aggregates. These data suggest that the axonal transport of organelles could be blocked by these inclusions. The observation that NF as well as other proteins (e.g., tubulin) are diminished in the ventral roots is also consistent with a nonselective mechanism due to a physical blockage of axonal transport. Indeed, our transport studies showed that the movement of three NF proteins and other cytoskeletal components such as tubulin and actin in the ventral roots of the 200-d-old G93A mice all were retarded. Furthermore, transport of some of the radiolabeled proteins in the fast phase also was retarded in the same G93A mice. Currently it is unclear why some but not all proteins transported by fast axonal transport are retarded. It is possible that proteins in larger organelles, such as those found within mitochondria, are selectively blocked. Since the transport of these proteins in the fast and slow components involves a complex motor machinery, the most reasonable explanation for these observations is a physical blockage of axonal transport by NF-rich inclusions and aggregated vacuoles at the level of proximal axons. Finally, a third mechanism involved here may be reduced NF protein synthesis in the affected motor neurons, resulting in the diminished export of NF proteins into their axons. However, additional studies are needed to address the validity of these hypothetical mechanisms.

In conclusion, our data demonstrate that mutations in the *SOD1* gene lead to an alteration in the NF network, which may impede both fast and slow axonal transport in affected spinal cord motor neurons. Thus, our data support a causal role of NF inclusions in the demise of spinal cord motor neurons. In view of the striking similarity in the MND phenotype of these mice and human FALS patients, this mechanism may be highly relevant to the death of the same neuronal populations in human FALS.

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