

Chronic activation in presymptomatic amyotrophic lateral sclerosis (ALS) mice of a feedback loop involving Fas, Daxx, and FasL

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The reasons for the cellular specificity and slow progression of motoneuron diseases such as ALS are still poorly understood. We previously described a motoneuron-specific cell death pathway downstream of the Fas death receptor, in which synthesis of nitric oxide (NO) is an obligate step. Motoneurons from ALS model mice expressing mutant SOD1 showed increased susceptibility to exogenous NO as compared with controls. Here, we report a signaling mechanism whereby NO leads to death of mutant, but not control, motoneurons. Unexpectedly, exogenous NO triggers expression of Fas ligand (FasL) in cultured motoneurons. In mutant SOD1^{G93A} and SOD1^{G85R}, but not in control motoneurons, this up-regulation results in activation of Fas, leading through Daxx to phosphorylation of p38 and further NO synthesis. This Fas/NO feedback amplification loop is required for motoneuron death *in vitro*. *In vivo*, mutant SOD1^{G93A} and SOD1^{G85R} mice show increased numbers of positive motoneurons and Daxx nuclear bodies weeks before disease onset. Moreover, FasL up-regulation is reduced in the presence of transgenic dominant-negative Daxx. We propose that chronic low-level activation of the Fas/NO feedback loop may underlie the motoneuron loss that characterizes familial ALS and may help to explain its slowly progressive nature.

cell death | motoneuron disease | NO | p38 kinase | neurodegeneration

Amyotrophic lateral sclerosis (ALS) is the most frequent adult-onset motoneuron disease in humans. ALS is characterized by the selective degeneration of motoneurons in spinal cord, brainstem, and cerebral cortex leading to muscle atrophy and paralysis and ultimately to death. About 1 to 2% of all human ALS forms are caused by dominantly inherited mutations in the Cu/Zn superoxide dismutase (SOD1) gene. Mice transgenic for the ALS-linked SOD1 mutations G37R (1), H46R/H48Q (2), G85R (3), and G93A (4) develop an adult-onset motoneuron disorder that remarkably resembles human ALS.

Despite much intensive study, many questions remain concerning the mechanism(s) by which mutant SOD1 triggers specific motoneuron death. One unresolved issue is the cellular site of action of the gain-of-function mutations. Clement *et al.* (5) generated chimeric mice carrying a mixture of WT and mutant SOD1-expressing cells in the spinal cord. In these mice, WT motoneurons eventually showed stigmata of degeneration, whereas some mutant SOD1 motoneurons were protected from degeneration when surrounded by WT nonneuronal cells. These results suggest that mutant SOD1 in both motoneurons and surrounding cells may play a role in the disease process.

Our earlier studies using cultures of purified embryonic motoneurons reached similar conclusions. We found that motoneurons from SOD1^{G93A}, SOD1^{G37R}, and SOD1^{G85R} mice survived normally in the presence of optimal trophic support or when challenged by excitotoxic agonists. In marked contrast, compared with controls, they displayed a 10- to 100-fold increase in sensitivity to extracellular agonists of the Fas receptor or to exogenous nitric

oxide (6). Thus, motoneurons expressing mutant SOD1 have an intrinsic susceptibility that is only revealed when challenged with specific extrinsic agents. Only motoneurons, and no other cell type from mutant SOD1 mice, showed enhanced susceptibility. In motoneurons, we showed that the Fas receptor signals through two synergistic pathways involving Fadd/Caspase-8 and Daxx/Ask1/p38/nNOS, respectively (6).

Another open question in ALS relates to the kinetics of the neurodegenerative process. Mutant SOD1 mice display multiple features of programmed cell death at presymptomatic stages. Examples are proteolytic activation of caspases-1, -3, -7, -8, and -9 in the spinal cord (7–9), mitochondrial release of cytochrome *c* (10), and translocation of Bax from the cytosol to mitochondria (11). These modifications are manifest weeks to months before there is significant loss of motoneuron cell bodies or axons. One aspect of Fas-triggered motoneuron death seemed relevant to these slow kinetics. Whereas Fas activation *in vitro* kills lymphocytes within hours, motoneuron death in the same conditions takes days (12). The first aim of this study was therefore to identify the molecular mechanisms that underlie this exceptionally protracted cell death.

Our results linking Fas signaling to mutant SOD1 were obtained by using purified motoneurons *in vitro*. A second important aim of this study was therefore to investigate the significance of this signaling pathway in mutant SOD1 mice *in vivo*. Since our earlier publication (6), other groups studying mutant SOD1 mice have reported activation of certain intermediates in the Fas pathway we described. In the spinal cord of SOD1^{G93A} mice, Tortarolo *et al.* (13), Hu *et al.* (14), and Ackerley *et al.* (15) observed increased p38 activation, whereas Wengenack *et al.* (16) reported increased levels of Ask1. However, these results concern intermediates that are common to several signaling mechanisms. We therefore focused our attention on Fas and Daxx, which are specific to this pathway.

We report here that nitric oxide (NO), an end-product of the Fas pathway in motoneurons, unexpectedly induces expression of the endogenous Fas ligand (FasL), which in mutant SOD1 motoneurons then triggers further chronic activation of the pathway downstream of Fas. This feedback loop is necessary for motoneuron death induced by NO or FasL *in vitro*. Furthermore, because all elements of this feedback loop show perturbed expression in mutant SOD1 mice at presymptomatic stages, it is possible that this

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Abbreviations: DIV, day(s) *in vitro*; NB, nuclear bodies.

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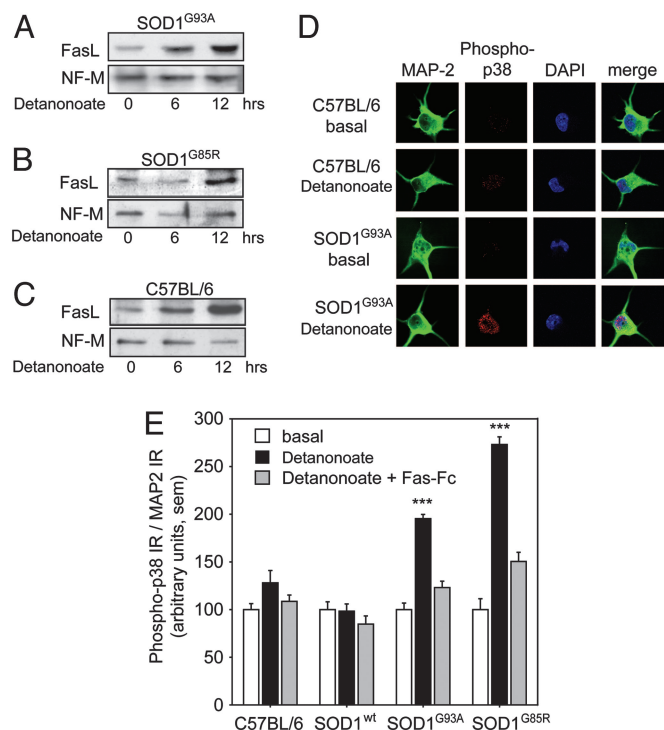


Fig. 1. Regulation of FasL and p38 kinase by NO in cultured motoneurons. Motoneurons were cultured for 16 h and then treated for 6 or 12 h with the NO donor Detanonoate (20 μ M). (A and B) Western blots of SOD1^{G85R} and SOD1^{G93A} motoneuron protein extracts demonstrated that the 38 kDa form of FasL protein was strongly up-regulated by NO within 6–12 h. (C) Similar up-regulation of FasL protein was seen in C57BL/6 motoneuron extracts after Detanonoate addition. Control protein extracts were prepared immediately before Detanonoate addition (0). Blots were reprobed with an antibody against neurofilament medium chain (NF-M). (D) Immunocytochemistry revealed a strong increase in phosphorylated p38 kinase in SOD1^{G93A} motoneurons exposed for 6 h to Detanonoate as compared with SOD1^{G93A} motoneurons cultured under basal conditions or motoneurons from C57BL/6 mice. Motoneurons are colabeled with anti MAP-2 antibodies and DAPI. (E) Histograms showing that Detanonoate increased the phospho-p38 immunoreactivity (IR) in mutant SOD1^{G93A} and SOD1^{G85R} motoneurons by 2- and 2.8-fold, respectively, although having no significant effect on C57BL/6 or SOD1^{WT} motoneurons. Detanonoate-induced phosphorylation of p38 kinase in mutant motoneurons was blocked by preincubation with Fas-Fc. Values for phospho-p38 kinase IR were normalized to MAP-2 IR; error bars represent SEM.

pathway is linked to the slowly progressive nature of motoneuron loss *in vivo*.

Results

Identification of a Fas/NO Feedback Loop in Mutant SOD1 Motoneurons. Because purified mutant SOD1 motoneurons show increased susceptibility to activation of the Fas death pathway, we asked whether expression of key signaling molecules differed between mutant SOD1 and control mice. Levels of Fas receptor and Fas ligand (FasL) were estimated by Western blotting. Levels of Fas were similar between C57BL/6 and mutant SOD1 motoneurons and unchanged by the presence of NO donors such as Detanonoate {Z-1-[2-(2-aminoethyl)-N-(2-aminonioethyl)amino]diazene-1-ium-1,2-diolate} (data not shown). The levels of the 38-kDa form of FasL (17), however, were increased 10- to 15-fold within 6–12 h of addition of Detanonoate (20 μ M) to cultures of SOD1^{G85R} and SOD1^{G93A} motoneurons (Fig. 1A and B). FasL was up-regulated to a similar degree in motoneurons from C57BL/6 mice (Fig. 1C), indicating that this event was independent of the presence of mutant SOD1. FasL up-regulation was concentration-dependent in the

range from 0 to 20 μ M Detanonoate and motoneuron-specific (Figs. 6A–D and 7A, which are published as supporting information on the PNAS web site). FasL up-regulation was also observed after addition of the NO donor sodium nitroprusside (Fig. 6E). FasL can exist in both membrane-bound and soluble forms (18). However, by using a sensitive ELISA assay (detection limit 3.6 pg/ml), no soluble FasL could be detected in media conditioned by Detanonoate-treated motoneurons. This result suggests that the FasL expressed after NO exposure is mostly membrane-bound, and therefore more likely to exert autocrine than paracrine effects under these experimental conditions.

We next asked whether the up-regulated FasL was capable of activating the Fas receptor expressed on the same motoneurons. As a reporter for Fas activation, we quantified phosphorylation of p38 kinase, a key event in the Fas pathway in cultured motoneurons (6), by immunolabeling of phospho-p38 (Tyr-180/Tyr-182) followed by quantitative confocal microscopy. Phospho-p38 was only weakly detected in untreated motoneurons, irrespective of the genotype (mutant SOD1, SOD1^{WT}, or nontransgenic). However, in mutant SOD1 motoneurons treated with 20 μ M Detanonoate, phospho-p38 kinase became clearly apparent in the nucleus and cytoplasm (Fig. 1D). Quantitative image analysis demonstrated that, after treatment with Detanonoate, immunoreactivity for phospho-p38 kinase increased by 2- and 2.8-fold in SOD1^{G93A} and SOD1^{G85R} motoneurons, respectively ($P < 0.0005$; Fig. 1E). In contrast, identical treatment of control motoneurons from C57BL/6 or SOD1^{WT} mice gave no significant increase in p38 activation (Fig. 1E).

To confirm that p38 kinase activation was a result of FasL up-regulation, we preincubated motoneurons for 2 h with Fas-Fc (19), an extracellular decoy that competes with interactions between Fas and FasL, before exposing them to NO donors. Fas-Fc inhibited the NO-induced phosphorylation of p38 kinase in both SOD1^{G93A} and SOD1^{G85R} motoneurons by >70% (Fig. 1E). These findings indicate that in both control and mutant SOD1 motoneurons, exogenous NO leads to up-regulation of FasL. However, only in the presence of G93A or G85R mutant forms of SOD1 does this mechanism lead to a Fas-dependent increase in phosphorylation of p38 kinase.

Functional Evidence for a Role of the NO/Fas Feedback Loop in Mutant Motoneuron Death. To confirm that FasL-Fas interactions and p38 activation were functionally involved in NO-triggered death of mutant SOD1 motoneurons, we used dominant-negative constructs and pharmacological inhibitors. We previously reported that 20 μ M Detanonoate triggers death of $\approx$ 45% of mutant SOD1^{G85R} and SOD1^{G93A} motoneurons, whereas it does not affect survival of motoneurons from C57BL/6 mice (6) or SOD1^{WT} mice (Fig. 2E). To detect potential involvement of Daxx in the NO/Fas feedback loop, we electroporated purified SOD1 motoneurons with vectors encoding either a dominant-negative form of Daxx (Daxx-DN), WT Daxx, or a control vector. An EGFP vector was coelectroporated to monitor survival of transduced motoneurons (Fig. 2A and B). After administration of 20 μ M Detanonoate, the survival of SOD1^{G85R} and SOD1^{G93A} motoneurons transduced with WT Daxx or control vector was reduced by 42% and 55%, respectively (Fig. 2C and D). These figures are close to those for nonelectroporated cells, demonstrating that the transduced neurons are representative of the whole population. In contrast, expression of Daxx-DN almost completely protected mutant SOD1^{G85R} and mutant SOD1^{G93A} motoneurons against NO-induced cell death (Fig. 2C and D). Mutant motoneuron death induced by exogenous NO was also strongly inhibited by Fas-Fc, SB203588, an inhibitor of p38 kinase, and L-VNIO, an inhibitor of nNOS (Fig. 3A and B). Thus, in agreement with the expression data, FasL-Fas interactions, p38 kinase, and nNOS are all required for NO-triggered death.

These results provided strong functional evidence for a feedback loop triggered by NO in mutant SOD1 motoneurons. However, it

Disease course varies between different lines of mutant SOD1 mice: SOD1^{G93A} mice expressing a catalytically active form of human SOD1 display an early disease onset ≈ 100 days of age (4), whereas SOD1^{G85R} mice express an inactive form of SOD1 leading to late onset around day 200 (3). We therefore chose to analyze these mouse lines at comparable presymptomatic stages when the total number of motoneurons is still normal: day 75 for SOD1^{G93A} mice and day 120 for SOD1^{G85R} mice (7, 20) (*Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). We found that not only the Fas receptor, as shown in ref. 21, but also its endogenous ligand FasL and its signaling intermediate Daxx were expressed in control spinal cord (Fig. 4A). We therefore quantified the expression of different elements of the pathway in presymptomatic mutant mice.

In nontransgenic mice, $31 \pm 2\%$ of all choline acetyl transferase (ChAT)-positive motoneurons in the lumbar spinal cord segment L4 expressed significant levels of FasL at day 75 ($n = 3$ mice) and $29 \pm 4\%$ were stained at day 120 ($n = 2$). In SOD1^{WT} mice, the number of FasL-positive motoneurons ($26.5 \pm 3.2\%$, $n = 3$) was close to that in nontransgenic mice. Strikingly, the proportion of FasL-positive motoneurons was at least two-fold higher in SOD1^{G93A} mice at 75 days ($70 \pm 1.9\%$, $n = 4$) and in SOD1^{G85R} mice at 120 days ($60.2 \pm 6.6\%$, $n = 4$; Fig. 4B; $P < 0.001$, Student's *t* test). We therefore asked whether FasL might be able to engage Fas receptor signaling in motoneurons *in vivo*. Double immunofluorescence labeling of presymptomatic SOD1^{G93A} spinal cord showed that all FasL-positive motoneurons coexpressed significant levels of Fas (Fig. 4C).

Daxx Accumulates in Nuclear Speckles in Motoneurons of Mutant SOD1 Mice. Immunostaining for Daxx in control and mutant SOD1 lumbar spinal cords revealed a diffuse cytoplasmic and nuclear localization in a broad range of neurons in the ventral and dorsal horn (Figs. 4A and 5A). Interestingly, in motoneurons, Daxx accumulated in discrete subnuclear domains (Fig. 5B) that stained positive for sc35, a general splicing factor and marker of nuclear speckles. Only a subpopulation of nuclear speckles were stained for Daxx. Colocalization of Daxx and sc35 was confirmed by Z-scan confocal analysis (data not shown). These nuclear domains (Daxx-NBs) are reminiscent of promyelocytic leukemia nuclear bodies (PML-NB) which, in other cell types, have been shown to contain sc35 (22–24). We quantified Daxx-NBs in control and mutant SOD1 motoneurons (Fig. 5C). Lumbar motoneurons contained 2.2 ± 0.9 Daxx-NBs and 1.9 ± 1.1 Daxx-NBs per section in C57BL/6 mice aged 75 and 120 days, respectively, and 1.8 ± 1.2 Daxx-NBs per section in SOD1^{WT} mice aged 75 days. In contrast, the number of Daxx-NBs was increased to 5.5 ± 1.5 per section in SOD1^{G93A} mice and 3.4 ± 0.2 in SOD1^{G85R} mice (mean $\pm$ SD, $n = 3$, $P < 0.001$, Student's *t* test). In conclusion, therefore, two key intermediates of the Fas/NO signaling loop, FasL and Daxx, are activated in motoneurons of presymptomatic ALS mice.

A Dominant-Negative Form of Daxx Inhibits FasL Up-Regulation *in Vivo*. To address the functional relevance of Fas-Daxx signaling in mutant SOD1-linked motoneuron disease, we crossbred SOD1^{G93A} mice with transgenic mice expressing a dominant negative form of Daxx. These Daxx-DN mice (25) show a weaker phenotype than the Daxx null mutants, which are embryonically lethal (26). Western blot analysis demonstrated that SOD1 and Daxx-DN transgenes were expressed in lumbar spinal cord of double transgenic SOD1^{G93A};Daxx-DN mice at levels similar to those in the parental strains (Fig. 5D). Endogenous Daxx expression was not influenced by Daxx-DN (Fig. 5D). Interestingly, in SOD1^{G93A};Daxx-DN mice, only $56.4 \pm 3.9\%$ of L4 motoneurons were FasL-positive, as compared with $70 \pm 1.9\%$ in SOD1^{G93A} and $32.6 \pm 7.9\%$ in Daxx-DN mice (mean $\pm$ SD, $n = 3$ each, $P < 0.001$; Fig. 5E). Thus, expression of Daxx-DN leads to a reduction of 36% in the mutant SOD1^{G93A}-induced increase in FasL-positive motoneurons. These

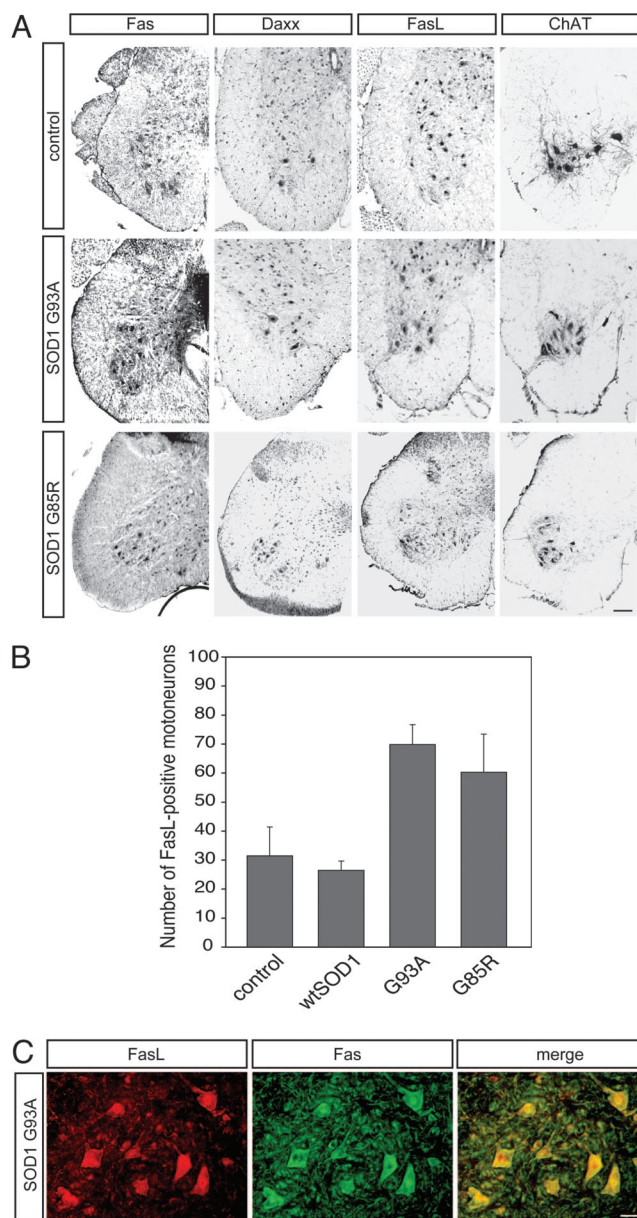


Fig. 4. Up-regulation of FasL in lumbar motoneurons of mutant SOD1 mice. (A) Transverse sections of L4 lumbar spinal cords of C57BL/6 (control), 75-day-old SOD1^{G93A}, and 120-day-old SOD1^{G85R} mice immunostained for Fas, Daxx, FasL, and choline acetyl transferase (ChAT). Fas and Daxx displayed a scattered expression pattern in various cell types of the gray and white matter. In control mice, FasL was mainly detected in dorsal and ventral horn neurons, including a few motoneurons. In SOD1^{G93A} and SOD1^{G85R} mice, a high proportion of motoneurons, as identified by ChAT staining on adjacent sections, was positive for FasL. (Scale bar: 100 μ m.) (B) Histograms showing the percentage of FasL-positive motoneurons in serial sections of L4 lumbar spinal cord. Note the increased percentage of FasL-positive motoneurons in SOD1^{G93A} and SOD1^{G85R} mice as compared with C57BL/6 and SOD1^{WT} mice. (C) Immunolabeling reveals coexpression of FasL and Fas in spinal cord motoneurons in a 75-day-old SOD1^{G93A} mouse. (Scale bar: 50 μ m.)

data are consistent with a model in which chronic cycling of the mutant SOD1-dependent Fas feedback loop is required to build up signaling intermediates to levels at which they can trigger neurodegeneration.

Discussion

Amplification mechanisms play an important role in intracellular signaling pathways. The best studied examples are posttranslational

motoneurons induced by avulsion (35) or neurofilament gene mutations (36).

Feedback amplification loops involving other intermediates in the Fas signaling, such as caspase-8 and caspase-3 or Bid have been reported in acellular systems and nonneuronal cells (37, 38). In the context of ALS, the Fas/NO feedback loop is of particular interest because it involves an extracellular step and diffusible factors. The cell death trigger NO is known to be produced not only by motoneurons but also by microglia and activated astrocytes (34, 39) and Fas agonists have been detected in sera of patients with sporadic ALS (40). "Community effects" may thus allow for cellular neighbors to accelerate or to inhibit motoneuron death (5) and also underlie the clinical finding that ALS often progresses locally, between adjacent muscles or motor pools. Further studies are required to better understand the molecular and cellular basis of these phenomena.

We believe that chronic cycling of feedback loops of the type described here may provide a general approach to understanding the delayed onset and relatively slow progression of many neurodegenerative diseases. As has been proposed for nucleation of mutant proteins with polyglutamine expansions (41), the initial insult produced by the feedback loop may be subliminal and without phenotype. However, as levels of toxic intermediates and death signals build up, they may reach a threshold that can trigger the pathological process. If this model is correct, then therapeutic intervention at the level of "death receptors" and cell death pathways should be envisioned at much earlier stages in the disease process than is generally imagined.

Materials and Methods

See *Supporting Materials and Methods* for details.

Animals and Reagents. SOD1^{G85R} mice, line 148 (3), were maintained as homozygotes, SOD1^{G93A} mice (4) and SOD1^{WT} mice, line 76 (3) as hemizygotes. All mice were on a pure C57BL/6 background. The following reagents and antibodies were used: brain-derived neurotrophic factor and ciliary neurotrophic factor (R & D Systems), glial cell line-derived neurotrophic factor, sodium nitroprusside (Sigma), Detanonoate, Fas-Fc, L-VNIO (Alexis, San Diego), SB203580 (Calbiochem), anti-mouse Fas (JO2, Pharmin-

gen); anti Fas (M-20) and anti FasL (N-20 and N-20-G, Santa Cruz Biotechnology); anti-FLAG (M2, Sigma), anti-NF-M (Ab1987, Chemicon), anti-sc35 (S4045, Sigma), anti-phospho-p38 (Cell Signaling Technology, Beverly, MA), anti-MAP2 (Sternberger-Meyer, Jarrettsville, MD), and secondary antibodies (Jackson ImmunoResearch or Molecular Probes).

Immunohistochemistry of Spinal Cord. Spinal cord cryosections (16 μ m) were incubated with primary antibodies [1:100 for anti-FasL, N-20-G, and anti-Fas, M-20; 1:200 for anti-Daxx, M112; 1:500 for anti-sc35, S4045, and anti-choline acetyl transferase (ChAT)], revealed with the ABC staining kit (Vector Laboratories) or with fluorochrome-conjugated secondary antibodies and analyzed by confocal microscopy. FasL-positive motoneurons or fluorescently labeled Daxx nuclear bodies were counted on at least 20 different sections originating from three mice per genotype.

Motoneuron Culture and Analysis. Male transgenic SOD1 mice were mated with female C57BL/6 mice, embryos harvested at embryonic day 12.5, and genotyped by PCR (6). Motoneurons were purified from ventral spinal cords by using a metrizamide density gradient (42), cultured in the presence of neurotrophic factors and treated at 1 DIV with Detanonoate or anti-Fas antibodies. Electroporation was done as described (6). Cell survival was determined by fluorescence or phase-contrast microscopy. Immunohistochemistry, quantitative confocal microscopy, and Western blot analysis were performed as described in *Supporting Materials and Methods*. Soluble FasL in conditioned media was measured by ELISA (R & D Systems). All experiments were performed in triplicate or quadruplicate and repeated at least twice.

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