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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 SOD1G93A and SOD1G85R, 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 SOD1G93A and SOD1G85R 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. This paper was submitted directly (Track II) to the PNAS office. Abbreviations: DIV, day(s) in vitro; NB, nuclear bodies.

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indicating that this event was independent of the presence of mutant addition of Detanonoate (20 μM). FasL (17), however, were increased 10- to 15-fold within 6–12 h of 1,2-diolate} (data not shown). The levels of the 38-kDa form of SOD1G93A motoneuron protein extracts demonstrated that the 38 kDa form of SOD1G93A motoneurons (Fig. 1A) were similar between C57BL/6 mice (Fig. 1A) and unchanged by the presence of NO donors such as Detanonoate (20 μM) 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 SOD1G93A and SOD1G85R 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 decay 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 SOD1G93A and SOD1G85R 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 μM Detanonoate triggers death of ~45% of mutant SOD1G85R and SOD1G93A 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 μM Detanonoate, the survival of SOD1G85R and SOD1G93A 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 SOD1G85R and mutant SOD1G93A motoneurons against NO-induced cell death (Fig. 2 C 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
remained possible that NO from the exogenous donor was provided at levels in excess of those generated by the Fas/H20862 NO pathway, and thereby led to nonphysiological activation of Fas. We therefore looked for evidence of the feedback loop in neurons triggered to die by another element of the pathway, the Fas receptor itself. Mutant SOD1 motoneurons were exposed to agonistic anti-Fas antibodies in the absence or presence of Fas-Fc (Fig. 3). Fas-Fc does not functionally interact with the Fas antibodies used to trigger death (Fig. 7). Blockade of FasL/H20862 Fas interactions by Fas-Fc gave nearly complete protection against cell death (Fig. 3 and D). This result demonstrates that even when the pathway is triggered by the endogenous Fas receptor, further activation of Fas by FasL is required for cell death to occur (Fig. 3E).

Fig. 2. Role of Daxx in mutant SOD1 motoneuron death. (A and B) Plasmid electroporation was used to coexpress a dominant negative FLAG-tagged form of Daxx (Daxx-DN) and EGFP in cultured motoneurons. (C and D) SOD1<sup>G85R</sup> or SOD1<sup>G93A</sup> motoneurons were electroporated with the EGFP expression vector in combination with an empty control vector or vectors encoding either WT Daxx or Daxx-DN. At 1 day in vitro (DIV), motoneurons from each electroporation were either exposed to the NO donor Detanonoate or kept under basal conditions. Motoneuron survival at 3 DIV was expressed as the percentage of EGFP-positive cells in the presence versus absence of NO. Daxx-DN protected mutant SOD1<sup>G85R</sup> and SOD1<sup>G93A</sup> motoneurons from NO-triggered cell death. Expression of Daxx-WT had no effect on survival. The differences between the effects of Daxx-DN and Daxx-WT were statistically significant: *P < 0.005 for SOD1<sup>G85R</sup>; *P < 0.01 for SOD1<sup>G93A</sup>. Student's t test. (E) Survival of SOD1<sup>WT</sup> motoneurons transduced with control, Daxx-WT, or Daxx-DN vectors with or without NO challenge. Histograms represent means and SEM from two independent experiments.

Fig. 3. Role of FasL, Fas, p38 kinase, and nNOS in mutant SOD1 motoneuron death. (A–D) Mutant SOD1 motoneurons were treated (or not treated) at 1 DIV with Detanonoate (A and B) or agonistic anti-Fas antibodies (C and D). Cell survival was quantified at 3 DIV by phase-contrast microscopy and expressed relative to the number of motoneurons alive under basal conditions (0). Addition of the extracellular part of Fas receptor, Fas-Fc, the p38 kinase inhibitor SB203580 (5 μM), or the nNOS inhibitor L-VNIO (10 μM) significantly reduced the NO-triggered death of SOD1<sup>G85R</sup> (A) or SOD1<sup>G93A</sup> (B) motoneurons. Mutant SOD1 motoneuron death mediated by agonistic anti-Fas antibodies at 0.5 ng/ml or 100 ng/ml was prevented or significantly reduced by Fas-Fc. Error bars show SEM. *P < 0.05; **P < 0.01; ***P < 0.001, Student's t test. (E) Model illustrating the Fas/NO feedback loop in mutant SOD1 motoneurons.

**FasL Is Up-Regulated in Mutant SOD1 Spinal Cord.** Our in vitro results showed that, to kill a mutant SOD1 motoneuron, Fas or NO need to trigger a feedback loop. The involvement of such a loop, together with the requirement for transcriptional up-regulation of nNOS (6) may explain the relatively slow time course of motoneuron death when compared with other cellular models of Fas-triggered apoptosis. We reasoned that it might also be related to the late onset and prolonged time course of the neurodegenerative process in mutant SOD1 mice in vivo. We therefore asked whether the elements of the Fas/NO feedback loop were expressed in the spinal cord and whether they showed alterations during the period leading up to the onset of clinical symptoms.

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Disease course varies between different lines of mutant SOD1 mice: SOD1G93A mice expressing a catalytically active form of human SOD1 display an early disease onset ~100 days of age (4), whereas SOD1G85R 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 SOD1G93A mice and day 120 for SOD1G85R 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 ± 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 ± 4% were stained at day 120 (n = 2). In SOD1WT mice, the number of FasL positive motoneurons (26.5 ± 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 SOD1G93A mice at 75 days (70 ± 1.9%, n = 4) and in SOD1G85R mice at 120 days (60.2 ± 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 SOD1G93A 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 SOD1WT mice aged 75 days. In contrast, the number of Daxx-NBs was increased to 5.5 ± 1.5 per section in SOD1G93A mice and 3.4 ± 0.2 in SOD1G85R mice (mean ± 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 motoneuronal disease, we crossedbred SOD1G93A 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 SOD1G93A-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 SOD1G93A-Daxx-DN mice, only 56.4 ± 3.9% of L4 motoneurons were FasL-positive, as compared with 70 ± 1.9% in SOD1G93A and 32.6 ± 7.9% in Daxx-DN mice (mean ± SD, n = 3 each, P < 0.001; Fig. 5E). Thus, expression of Daxx-DN leads to a reduction of 36% in the mutant SOD1G93A-induced increase in Fas-positive motoneurons. These 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
Daxx accumulates in the nuclei of motoneurons and forms nuclear bodies. The number of Daxx nuclear bodies (Daxx-NBs) in L4 motoneurons was higher in SOD1G93A and SOD1G85R mice than in C57BL/H11021 littermates and SOD1WT mice were analyzed at age 75 days; SOD1G85R and C57BL/H11021 litter mice (C.R., data not shown) were analyzed at age 120 days. (Scale bar: 20 μm.) (B) Daxx nuclear bodies (arrows) are associated with a subpopulation of nuclear speckles, as detected by confocal analysis of Daxx- and sc35-immunostained SOD1G93A lumbar spinal cords at age 75 days. (C) The number of Daxx nuclear bodies (Daxx-NBs) in L4 motoneurons was higher in SOD1G93A and SOD1G85R mice than in C57BL/6 or SOD1WT mice of the same age. Values represent means ± SD from three mice per genotype; ***, \( P < 0.001 \), Student’s t test. (D and E) Analysis of double transgenic SOD1G93A/Daxx-DN mice. (D) Western blots of protein extracts from lumbar spinal cords show that the dominant negative form of Daxx (Daxx-DN) and the human SOD1G93A are expressed at similar levels in double transgenic mice and in the parental mice at age 75 days. Daxx-DN was revealed with an anti-FLAG antibody that also detects a nonspecific (ns) upper band. Daxx-DN expression did not modify expression of endogenous Daxx because the three known Daxx isoforms of 70, 97, and 110 kDa (43) were detected at similar levels in all genotypes. (E) Transgenic Daxx-DN expression significantly reduced the percentage of FasL-positive motor neurons in double transgenic mutant SOD1G93A/Daxx-DN mice as compared with values in age-matched 75-day-old SOD1G93A mice. Values are means ± SD, \( n = 3 \) per genotype; ***, \( P < 0.001 \), one-way ANOVA followed by Newman–Keuls post hoc analysis.

Fig. 5. Daxx activation in lumbar motoneurons of mutant SOD1 mice and its functional relevance in the Fas/NO loop. (A) Immunolabeling reveals that Daxx accumulates in the nuclei of motoneurons and forms nuclear bodies. SOD1G93A C57BL/6 littermates and SOD1WT mice were analyzed at age 75 days; SOD1G85R and C57BL/6 litter mice (C.R., data not shown) were analyzed at age 120 days. Values are means ± SD from three mice per genotype; ***, \( P < 0.001 \), Student’s t test. (B and C) Immunofluorescence staining and immunodetection of Daxx in lumbar spinal cords from double transgenic SOD1G93A/Daxx-DN mice. (B) Daxx nuclear bodies (arrows) are associated with a subpopulation of nuclear speckles, as detected by confocal analysis of Daxx- and sc35-immunostained SOD1G93A lumbar spinal cords at age 75 days. (C) The number of Daxx nuclear bodies (Daxx-NBs) in L4 motoneurons was higher in SOD1G93A and SOD1G85R mice than in C57BL/6 or SOD1WT mice of the same age. Values represent means ± SD from three mice per genotype; ***, \( P < 0.001 \), Student’s t test. (D and E) Analysis of double transgenic SOD1G93A/Daxx-DN mice. (D) Western blots of protein extracts from lumbar spinal cords show that the dominant negative form of Daxx (Daxx-DN) and the human SOD1G93A are expressed at similar levels in double transgenic mice and in the parental mice at age 75 days. Daxx-DN was revealed with an anti-FLAG antibody that also detects a nonspecific (ns) upper band. Daxx-DN expression did not modify expression of endogenous Daxx because the three known Daxx isoforms of 70, 97, and 110 kDa (43) were detected at similar levels in all genotypes. (E) Transgenic Daxx-DN expression significantly reduced the percentage of FasL-positive motor neurons in double transgenic mutant SOD1G93A/Daxx-DN mice as compared with values in age-matched 75-day-old SOD1G93A mice. Values are means ± SD, \( n = 3 \) per genotype; ***, \( P < 0.001 \), one-way ANOVA followed by Newman–Keuls post hoc analysis.
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 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. SOD1G93A mice, line 148 (3), were maintained as homozygotes, SOD1G93A mice (4) and SOD1WT mice, line 6012/H20841.

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