

# Concurrent Administration of Neu2000 and Lithium Produces Marked Improvement of Motor Neuron Survival, Motor Function, and Mortality in a Mouse Model of Amyotrophic Lateral Sclerosis<sup>[S]</sup>

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## ABSTRACT

The Fas pathway and oxidative stress mediate neuronal death in stroke and may contribute to neurodegenerative disease. We tested the hypothesis that these two factors synergistically produce spinal motor neuron degeneration in amyotrophic lateral sclerosis (ALS). Levels of reactive oxygen species were increased in motor neurons from ALS mice compared with wild-type mice at age 10 weeks, before symptom onset. The proapoptotic proteins Fas, Fas-associated death domain, caspase 8, and caspase 3 were also elevated. Oral administration of 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-ben-

zylamino)-benzoic acid (Neu2000), a potent antioxidant, blocked the increase in reactive oxygen species but only slightly reduced activation of proapoptotic proteins. Administration of lithium carbonate ( $\text{Li}^+$ ), a mood stabilizer that prevents apoptosis, blocked the apoptosis machinery without preventing oxidative stress. Neu2000 or  $\text{Li}^+$  alone significantly enhanced survival time and motor function and together had an additive effect. These findings provide evidence that jointly targeting oxidative stress and Fas-mediated apoptosis can prevent neuronal loss and motor dysfunction in ALS.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by degeneration of upper and lower motor neurons, progressive paralysis, and an average mortality of 5 years after onset. Extensive evidence supports a causative role for oxidative stress in the motor neuron loss in ALS. Elevated levels of protein carbonyl groups and increased nitration have been found in the motor cortex and spinal cord in patients with ALS (Bowling et al., 1993; Beal et al., 1997). In addition, studies have shown increased protein oxidation by 4-hydroxynonenal and 8-hydroxyl-2-deoxy-

guanosine in the cortex and spinal cord in patients with ALS (Fitzmaurice et al., 1996; Pedersen et al., 1998).

In addition to oxidative stress, apoptosis probably contributes to motor neuron degeneration in ALS. Degenerating spinal cord and motor cortex neurons are characterized by dark and shrunken cytoplasm and nuclei, chromatin condensation, and apoptotic bodies (Martin, 1999). The ratio of apoptotic cell death genes Bax to Bcl-2 is increased at both the mRNA and protein level in spinal motor neurons from patients with ALS and from SOD1-G93A mice (Mu et al., 1996; Vukosavic et al., 1999). Mutant SOD1-G93A has been observed to aggregate in spinal cord mitochondria, but not liver mitochondria, and binds to Bcl-2 (Pasinelli et al., 2004). Altered expression and dysfunction of Bcl-2 may contribute to the activation of mitochondrial apoptosis machinery such as caspase-9, caspase 3, and cytochrome *c* in spinal motor neurons of ALS transgenic mice and humans with ALS (Guegan et al., 2001; Inoue et al., 2003). In support of this idea,

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**ABBREVIATIONS:** ALS, amyotrophic lateral sclerosis; PaGE, paw grip endurance; MFR, mitochondrial free radicals; DIV, days in vitro; BSO, DL-buthionine-[S,R]-sulfoximine; SOD, superoxide dismutase; LDH, lactate dehydrogenase; FADD, Fas-associated death domain.

overexpression of Bcl-2 or the caspase inhibitory protein XIAP prolongs survival and improves motor performance in ALS mice expressing the SOD1-G93A mutation (Kostic et al., 1997; Inoue et al., 2003). In addition, prevention of apoptosis using caspase inhibitors prolongs survival and delays disease progression in transgenic ALS mice (Li et al., 2000).

Evidence has accumulated showing that oxidative stress can induce neuronal death different from apoptosis. For example, neurons exposed to oxidative stress in vitro and in vivo undergo necrosis evident by cell body swelling, scattering condensation of nuclear chromatin, and early plasma membrane rupture (Bonfoco et al., 1995; Won et al., 2000). Surprisingly, insulin-like growth factor 1 prevents neuronal cell apoptosis and protects spinal motor neurons in ALS mice (Ryu et al., 1999; Kaspar et al., 2003) but markedly potentiates neuronal cell necrosis induced by hydroxyl radical or glutathione depletion (Gwag et al., 1997). A cocktail of neuroprotective drugs with different modes of action has been shown to produce greater improvement of survival and motor function than monotherapy in transgenic mouse models of ALS (Zhang et al., 2003; Petri et al., 2006). Given that oxidative stress and apoptosis play a central role in motor neuron degeneration and can contribute to neuronal death through distinctive routes in ALS, we hypothesize that a therapeutic approach targeting both oxidative stress and apoptosis would probably have additive effects on neuronal survival and motor function. For the pharmacological prevention of oxidative stress and apoptosis, Neu2000, a novel antioxidant, and  $\text{Li}^+$ , a well known antiapoptotic agent, were used. The former, a chemical derivative of aspirin and sulfasalazine, has been developed to protect neurons from oxidative stress with greater potency and safety and shown to be a potent and secure antioxidant in vitro and in animal models of hypoxic ischemia (Gwag et al., 2006).

## Materials and Methods

**Mice and Treatment Regimens.** G93A transgenic mice carrying the G93A human SOD1 mutation were obtained from the Jackson Laboratory (Bar Harbor, ME). Male G93A transgenic mice were cross-bred with B6SJL/F1/J hybrid females as described previously (Gurney et al., 1994). Mice were fed with a diet supplemented beginning at 8 weeks of age with Neu2000 (30 mg/kg/day) or 0.2% lithium carbonate. Nontransgenic littermates were used as control animals for behavior tests and histological experiments.

In experiments investigating oxidative stress and activation of the Fas pathway, mice received Neu2000 (30 mg/kg/day), 0.2% lithium carbonate (200 mg/kg/day), or 0.9% saline orally, using a feeding needle, from 8 weeks of age.

**Evaluation of Motor Function and Neuronal Death.** Motor strength and coordination were evaluated with a Rota Rod (Columbus Instruments, Columbus, OH), beginning at 8 weeks of age. Mice were evaluated at 16 rpm. The time mice remained on the Rota Rod was recorded. Maximum time was adjusted to 5 min. Mice were tested twice a week until they could no longer perform the task.

To measure paw grip endurance (PaGE), the wire lid was gently shaken to prompt the mouse to grip the grid, and the lid was swiftly turned upside down. The time the mouse held on to the inverted lid with both hind limbs was recorded. Each mouse was given up to three attempts to hold on to the inverted lid for an arbitrary maximum of 90 s, and the longest latency was recorded. Extension reflex was analyzed by scoring retraction of the hind limb: a score of 2 for normal extension of hind limbs, 1 for retraction of one hind limb, and 0 for the absence of any hind limb extension.

Neuronal death was analyzed by staining with 0.5% cresyl violet and counting viable neurons larger than 20  $\mu\text{m}$ . Number of motor neurons was counted in five sections randomly chosen from L2 to L4 for each animal.

**Evaluation of Disease Onset and Survival.** Disease onset was defined as the first day that a mouse showed a motor function deficit on the Rota Rod test. The time of death was defined as the date on which G93A mice showed complete paralysis of body and could not roll over within 20 s of being placed on their side. Death follows within a few hours after such an extreme morbidity.

**Determination of Mitochondrial Free Radical Generation.** Mitochondrial free radicals (MFR) generation was determined as described previously (Kim et al., 2002). In brief, anesthetized animals received 0.8- $\mu\text{l}$  injections of 0.2 nmol of Mitotracker Red CM-H<sub>2</sub>XRos (Invitrogen, Carlsbad, CA) dissolved in dimethyl sulfoxide and saline [1:1 (v/v)] via a Hamilton syringe in the lateral ventricle. Animals were euthanized 24 h later. Spinal cords were sectioned at a thickness of 30  $\mu\text{m}$ . MFRs were determined by detection of the oxidized fluorescence product (excitation at 554 nm, emission at 576 nm) of Mitotracker Red CM-H<sub>2</sub>XRos under a fluorescence microscope equipped with a cooled charge-coupled device system (Zeiss, Göttingen, Germany). MFR intensity was analyzed by Image Gauge 3.12 (Fuji Photo Film Co., Tokyo, Japan). To determine MFRs in spinal motor neurons, sections were immunolabeled with mouse monoclonal antibody for NeuN, a neuronal marker protein.

**Immunohistochemistry.** Spinal cord sections were fixed in 3% paraformaldehyde, washed in phosphate-buffered saline, incubated in 0.3% H<sub>2</sub>O<sub>2</sub> and 0.25% Triton X-100 for 10 min at room temperature, and reacted with 10% horse serum for 1 h. Sections were then reacted overnight at 4°C with the primary antibodies: mouse anti-Fas (BD Biosciences, San Jose, CA), anti-nitrotyrosine (4  $\mu\text{g}/\text{ml}$ ; Upstate Biotechnology, Lake Placid, NY), anti-cleaved-caspase 3 (Cell Signaling Technology, Danvers, MA), and anti-NeuN. Next, the sections were reacted with anti-mouse or anti-rabbit immunoglobulin (IgG) fluorescent- or biotin-conjugated (Vector Laboratories, Burlingame, CA) antibody for 2 h. The biotin-labeled sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h and then visualized using 3,3'-diaminobenzidine tetrahydrochloride dihydrate. Nitrotyrosine intensity was determined using Image Gauge 3.12 (Fuji Photo Film Co.).

**Western Blotting.** Lumbar cords were lysed in radioimmunoprecipitation assay buffer with protease inhibitors, including 0.5 mM EDTA, 500  $\mu\text{M}$  4-(2-aminoethyl)benzenesulfonyl fluoride, 150 nM aprotinin, 1  $\mu\text{M}$  E-64, and 1  $\mu\text{M}$  leupeptin. Protein samples were electrophoresed on a 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was preincubated with 5% nonfat dry milk, reacted with primary antibodies, and incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Cell Signaling Technology). Target proteins were detected with enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK) on X-ray film or with an LAS 1000 image analyzer (Fuji Photo Film Co.). The intensity of the bands was quantified using Image Gauge 3.12 (Fuji Photo Film Co.). The following primary antibodies were used: Fas, FADD (BD Bioscience, Franklin Lakes, NJ), cleaved caspase 3, and cleaved caspase 8 (1  $\mu\text{g}/\text{ml}$ ; Cell Signaling Technology).

**Immunoprecipitation.** Protein samples were incubated with 1  $\mu\text{g}$  of anti-Fas antibody overnight at 4°C. Complexes formed were immunoprecipitated using protein A-Sepharose. The Sepharose beads were boiled in SDS-PAGE sample buffer, and the protein samples were subjected to Western blot using anti-Fas and anti-FADD antibody.

**Neurotoxicity in Mouse Cortical Cell Cultures.** Mixed cortical cell cultures containing neurons and glia were prepared as described previously (Ryu et al., 1999). For neuron-rich cortical cell cultures, 2.5  $\mu\text{M}$  cytosine arabinoside was added to cultures at 3 days in vitro (DIV 3) to halt the growth of non-neuronal cells. Oxidative stress was induced by addition of 30  $\mu\text{M}$  FeCl<sub>2</sub> or 10 mM BSO to

mixed cortical cell cultures (DIV 12–14). Neuronal death was determined 24 h later by measuring LDH release into the bathing media, scaled to a mean LDH value after 24-h exposure to 500  $\mu$ M *N*-methyl-D-aspartate (100%) or sham control (0%). Neuronal apoptosis was induced by serum deprivation in neuron-rich cortical cell cultures and analyzed 24 h later by counting viable neurons excluding trypan blue.

**Preparation of Neu2000.** Neu2000 was synthesized at Neurotech Pharmaceuticals Co. (Suwon, South Korea). In brief, to a solution of 5-aminosalicylic acid (1.02 g) and triethylamine (1 ml) was added 2,3,5,6-tetrafluoro-4-trifluoromethylbenzyl bromide (1.23 g) at room temperature under a nitrogen atmosphere. The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was extracted with ethyl acetate. The organic layer was washed with water and then dried over anhydrous  $\text{MgSO}_4$ . After evaporation of the solvent, the residue was recrystallized from ether/hexane (1:10) to give 1.60 g (64% yield) of 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethylbenzylamino)benzoic acid as a white solid.

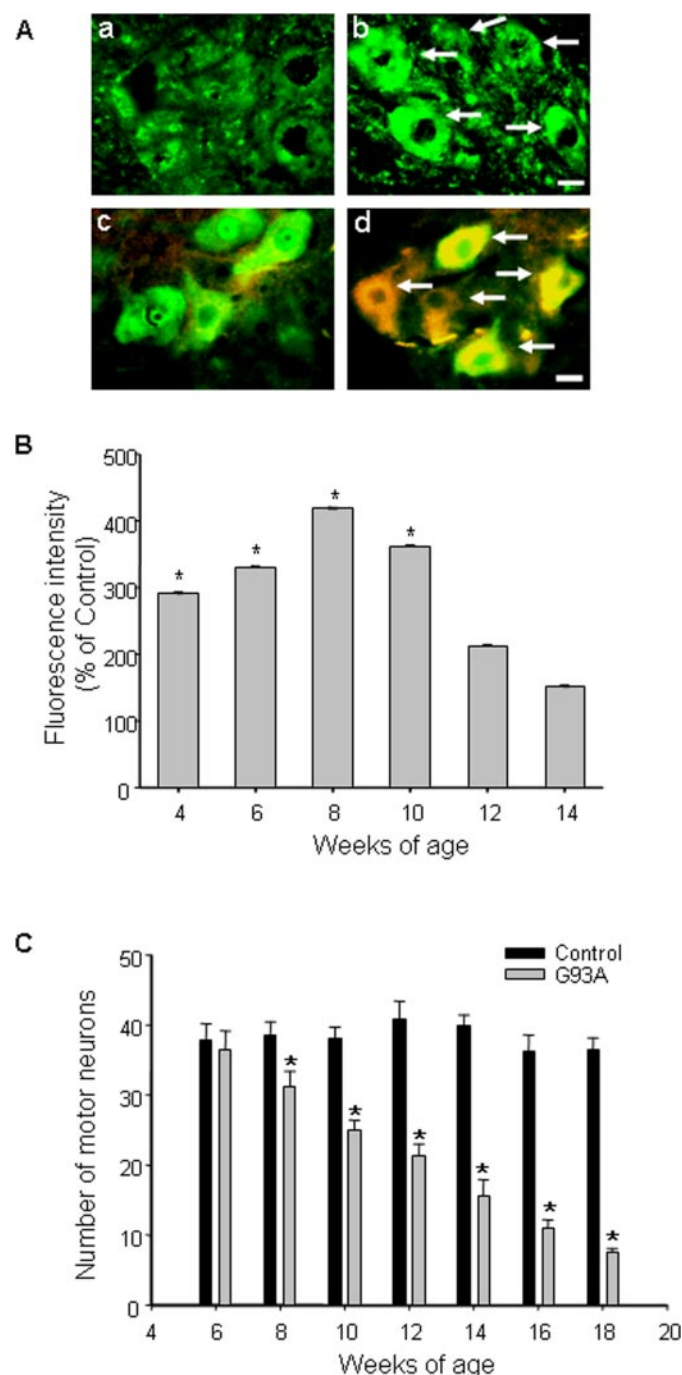
**Statistical Analysis.** Results of experiments performed on cell cultures and animals are expressed as the mean  $\pm$  S.E.M. An independent-samples *t* test was used to compare two samples. Analysis of variance and the Student-Newman-Keuls test were used for multiple comparisons. Statistical significance was set at  $P < 0.05$ .

## Results

**Oxidative Stress Precedes Motor Neuron Degeneration in G93A Transgenic Mice.** We examined levels of oxidative stress in the spinal cords of littermate control mice and G93A transgenic mice before the appearance of behavioral deficits and motor neuron degeneration. Oxidative stress was markedly increased in large motor neurons in the lumbar ventral horn at 8 weeks of age in G93A transgenic mice compared with control mice, as demonstrated by increased immunoreactivity to the nitrotyrosine antibody (Fig. 1A). Fluorescence intensity of oxidized MitoTracker CM-H<sub>2</sub>XRos, a mitochondrion-selective free radical probe, was also increased in spinal motor neurons of transgenic mice compared with control mice, suggesting free radical generation and protein nitration (Fig. 1A). Control and G93A transgenic mice showed similar levels of nitrotyrosine and MFRs in dorsal horn neurons and white matter (data not shown). Analysis of nitrotyrosine showed that at 4 weeks of age in motor neurons from transgenic mice oxidative stress was increased by up to three times above that of control mice, a significant difference (Fig. 1B). The increase in nitrotyrosine levels peaked to four times at 8 weeks of age and then declined over the subsequent 6 weeks. Neuronal death was slightly elevated in the ventral horn of transgenic mice at 8 weeks of age, the time of peak oxidative stress (Fig. 1C). Neuronal death then gradually increased until animal death. This suggests that G93A transgenic mice undergo oxidative stress selectively in spinal cord motor neurons at the early stage of disease, which may in turn cause motor neuron degeneration in the lumbar ventral horn.

**The Fas-Mediated Apoptosis Signaling Pathway Is Activated in G93A Transgenic Mice.** Fas- and Fas ligand-mediated apoptosis plays a role in neuronal loss in animal models of stroke (Martin et al., 2001). Fas and Fas ligand are induced in the brain of patients with Alzheimer's disease and are reported to mediate  $\beta$ -amyloid neurotoxicity in cultured cortical neurons (Su et al., 2003). We examined whether the Fas pathway would mediate apoptosis in ALS mice. Expression of Fas and its cytoplasmic adaptor protein FADD and

Fas-FADD interaction were also increased in the lumbar spinal cord of G93A transgenic mice at 12 weeks of age compared with control mice (Fig. 2A). Immunohistochemis-



**Fig. 1.** Neuronal oxidative stress and neuronal death in the ventral spinal cord of G93A transgenic mice. **A**, fluorescent photomicrographs of lumbar ventral sections from the control (**a** and **c**) and G93A ALS transgenic mice (**b** and **d**) at 8 weeks of age. Sections were immunolabeled with nitrotyrosine antibody (top, green) or double-labeled with MitoTracker CM-H<sub>2</sub>XRos (red) and NeuN antibody (green) (bottom). Note increased oxidative stress in the motor neurons (arrows) from G93A mice. Scale bar, 20  $\mu$ m. **B**, the fluorescence intensity of nitrotyrosine was analyzed in ventral motor neurons at 4 to 14 weeks of age; means  $\pm$  S.E.M. are shown ( $n = 25$  sections, five mice per group). \*, significant difference between control and G93A mice,  $p < 0.05$ . **C**, the number of viable motor neurons in the ventral horn from L2 to L4 was determined after staining with cresyl violet, means  $\pm$  S.E.M. are shown ( $n = 25$  sections, five mice per group). \*, significant difference between control and G93A mice,  $p < 0.05$ .



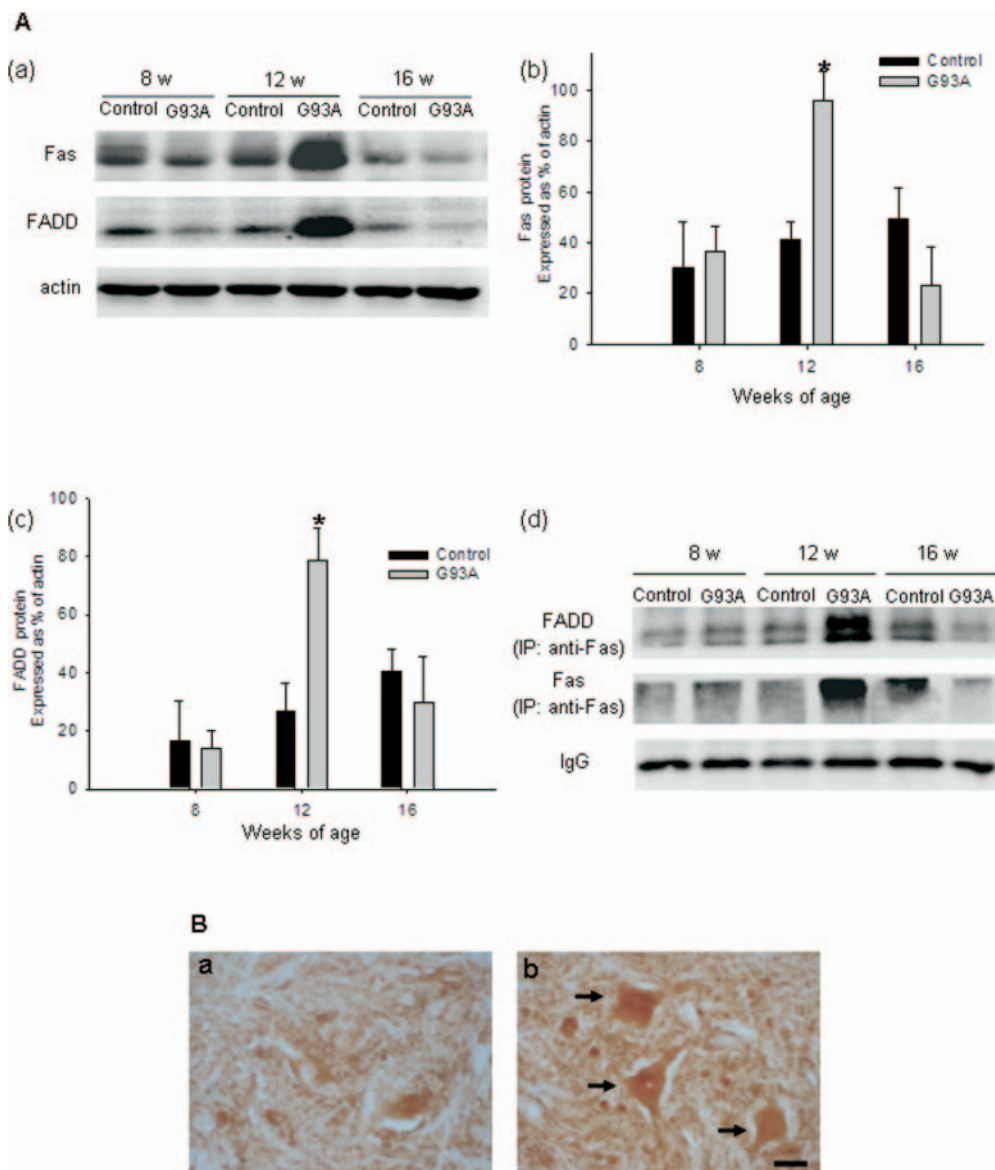
try revealed that Fas expression was increased selectively in large spinal motor neurons of G93A mice (Fig. 2B). Increased expression of the apoptosis-inducing signaling complex was followed by activation of caspase 8 and caspase 3 in the lumbar spinal cord (Fig. 2C). The active form of caspase 3 was observed in spinal motor neurons from G93A mice (Fig. 2D). These findings suggest that Fas, FADD, caspase 8, and caspase 3 are activated in spinal motor neurons and mediate subsequent neuronal apoptosis in ALS mice. No activation of the Fas-signaling molecules in G93A mice was detectable at 16 weeks of age when most motor neurons died.

**Neu2000 and Lithium Carbonate Prevent Oxidative Stress and Apoptosis in Cortical Cell Cultures and G93A Transgenic Mice.** We performed additional experiments to examine whether concurrently targeting oxidative stress and Fas-mediated apoptosis would provide additive neuroprotection in G93A transgenic mice. The selectivity of Neu2000 and lithium carbonate ( $\text{Li}^+$ ) in preventing oxidative stress and apoptosis has been verified in cortical cell cultures. Administration of  $\text{Fe}^{2+}$ , a hydroxyl radical-producing transition metal ion, or DL-buthionine-[S,R]-sulfoximine

(BSO), a glutathione-depleting agent, caused widespread neuronal cell necrosis within 24 h in cortical cell cultures containing neurons and glia (Fig. 3A).  $\text{Fe}^{2+}$ - and BSO-induced neuronal death was completely prevented by concurrent administration of Neu2000, a novel neuroprotectant derived from aspirin and sulfasalazine that was developed to block oxidative stress at submicromolar concentrations. Neu2000 was approximately 300 times more potent than vitamin E in protecting cortical neurons from  $\text{Fe}^{2+}$ -induced oxidative stress. Oxidative neuronal death was not attenuated by addition of  $\text{Li}^+$ , a mood-stabilizing agent that was reported to selectively prevent neuronal cell apoptosis without protective effects against excitotoxic neuronal cell necrosis (Kang et al., 2003; Chuang, 2005).

Neuronal cell apoptosis was induced by serum deprivation in neuron-rich cortical cell cultures; apoptosis was prevented by addition of 5 mM  $\text{Li}^+$  or 100  $\mu\text{M}$  *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, a broad-spectrum caspase inhibitor, as previously reported (Fig. 3B). However, Neu2000 had no effect.

We also investigated whether serum deprivation would



**Fig. 2.** Activation of Fas-mediated apoptosis pathways in G93A transgenic mice. **A**, Western blot analysis showing expression of Fas, FADD, and actin in lumbar segments from control or G93A transgenic mice at ages indicated (a). Levels of Fas (b) and FADD (c) were measured and scaled to actin, mean  $\pm$  S.E.M. ( $n = 4$  per group). Western blot analysis of FADD and Fas after immunoprecipitation with Fas antibody in the same samples shown above (d). **B**, bright-field photomicrographs of lumbar ventral sections from control (a) and G93A mice (b) at 12 weeks of age after immunolabeling with Fas antibody. Note increased levels of Fas in the motor neurons (arrows) from G93A mice. Scale bar, 20  $\mu\text{m}$ . **C**, Western blot analysis showing expression of cleaved caspase-8, cleaved caspase-3, and actin in lumbar segments from control or G93A mice at ages indicated (a). Levels of cleaved caspase 8 (b) and caspase 3 (c) were measured and scaled to actin, mean  $\pm$  S.E.M. ( $n = 4$  per group). **D**, fluorescence photomicrographs of lumbar ventral sections from control (a) and G93A mice (b) at 12 weeks of age immunolabeled with an antibody for cleaved caspase-3. Note activation of caspase-3 in the motor neurons (arrows) from G93A mice. Scale bar, 20  $\mu\text{m}$ . \*, significant difference between control and G93A mice,  $p < 0.05$ .

activate the Fas apoptosis pathway and whether this activation was sensitive to  $\text{Li}^+$ . Interaction of FADD with Fas, cleaved caspase 8, and cleaved caspase 3 were all increased in neuron-rich cortical cell cultures deprived of serum for 8 h, and these changes were blocked by the addition of  $\text{Li}^+$ , but not Neu2000 (Fig. 3C). Thus, it seems that Neu2000 and  $\text{Li}^+$  block oxidative neuronal cell necrosis and Fas-mediated apoptosis, respectively.

In G93A transgenic mice that had received a diet supplemented with Neu2000 (30 mg/kg/days) from 8 weeks of age the increase in nitrotyrosine and MFR in lumbar spinal motor neurons at 10 weeks of age was significantly blocked compared with control mice (Fig. 3, D and E). Administration of  $\text{Li}^+$  did not attenuate levels of nitrotyrosine increased in the motor neurons from G93A mice. Daily administration of Neu2000 in the diet slightly but statistically insignificantly attenuated the increase in Fas, FADD, and cleaved caspase 8 and caspase 3 in the lumbar spinal cords of G93A transgenic mice at 12 weeks of age (Fig. 3F). It is noteworthy that daily administration of  $\text{Li}^+$  completely blocked activation of Fas and its downstream mediators in G93A mice. This implies that concurrent administration of  $\text{Li}^+$  and Neu2000 can block both oxidative stress and activation of the Fas apoptosis pathway induced in the spinal cords of G93A mice.

**Neu2000 and  $\text{Li}^+$  Additively Delay Progression of Motor Function Deficit in G93A Transgenic Mice.** We examined motor strength, coordination, and the extension reflex in mice twice a week after drug administration, beginning at 8 weeks of age. Results of the Rota Rod test showed

that in vehicle-treated G93A mice coordination and strength was increasingly impaired beginning at 12 weeks of age. The motor function deficit was significantly alleviated in G93A mice treated with either Neu2000 or  $\text{Li}^+$ , and motor function was further improved with concurrent administration of these agents (Fig. 4A). The beneficial effects of Neu2000 and  $\text{Li}^+$  on motor strength were also demonstrated by a PaGE test showing that the average grip time was additively and significantly increased with concurrent administration of Neu2000 and  $\text{Li}^+$  (Fig. 4B). In addition, G93A mice treated with Neu2000 or  $\text{Li}^+$  demonstrated significant improvement in the extension reflex compared with vehicle-treated mice, and concurrent administration of these agents produced an additive effect (Fig. 4C). These findings suggest that dual blockade of oxidative stress and Fas-mediated apoptosis additively improves motor performance in ALS mice.

**Neu2000 and Lithium Have Synergistic Effects on Mortality and Motor Neuron Survival in G93A Transgenic Mice.** Average onset of impaired Rota Rod performance was 98.7 days in the vehicle-treated G93A mice. The onset was 112.3 and 114.7 days in G93A mice treated with Neu2000 and  $\text{Li}^+$ , respectively; the onset was further significantly delayed to 121.5 days after coadministration of Neu2000 and  $\text{Li}^+$  (Fig. 5A and Table 1). In the PaGE test, the average onset of behavioral deficiency was 104 days in vehicle-treated G93A mice, significantly delayed to 114.1 and 113.3 days in G93A mice treated with Neu2000 and  $\text{Li}^+$ , respectively (Table 1). The onset was delayed yet further to 127.6 days in G93A mice treated with both Neu2000 and  $\text{Li}^+$ .

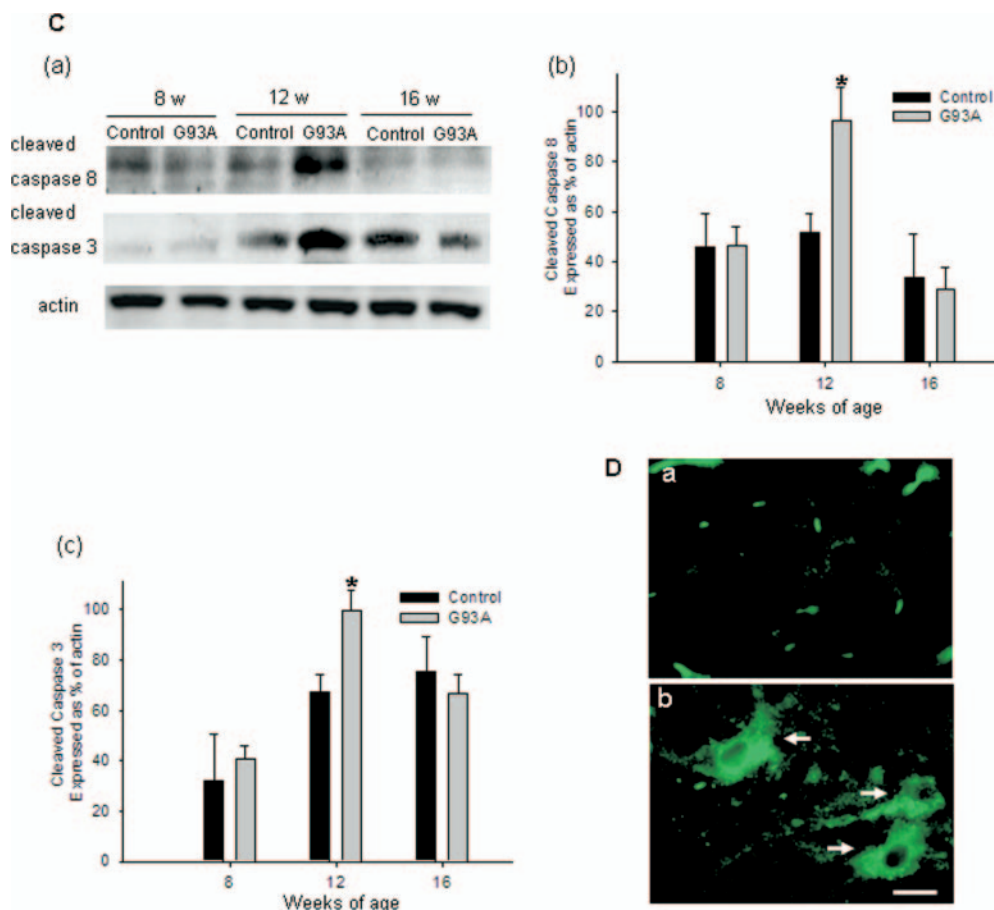
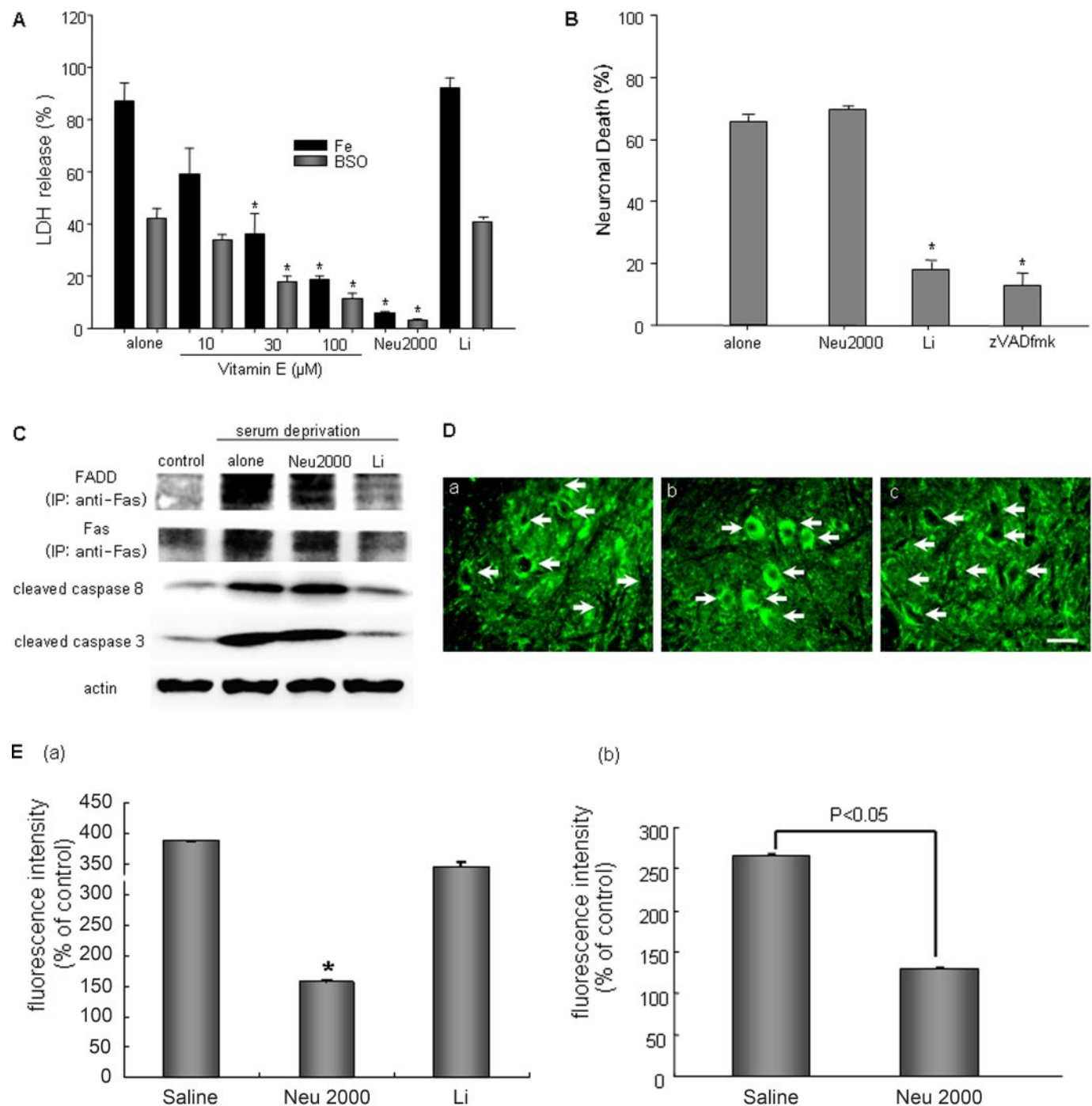


Fig. 2. Continued.



**Fig. 3.** Neu2000 and  $\text{Li}^+$  prevent oxidative stress and apoptosis, respectively, in vitro and in vivo. **A**, cortical cell cultures containing neurons and glia (DIV 12–14) were exposed to  $30 \mu\text{M}$   $\text{Fe}^{2+}$  (Fe) or  $10 \text{ mM}$  BSO, alone or with indicated doses of vitamin E,  $1 \mu\text{M}$  Neu2000, or  $5 \text{ mM}$   $\text{Li}^+$ . Neuronal death was determined 24 h later by measuring LDH efflux in the bathing media (shown as mean  $\pm$  S.E.M.,  $n = 12$ ). \*, significant difference compared with relevant control ( $\text{Fe}^{2+}$  or BSO alone),  $p < 0.05$ . **B**, neuron-rich cortical cell cultures (DIV 7) were deprived of serum, alone or with addition of  $1 \mu\text{M}$  Neu2000,  $5 \text{ mM}$   $\text{Li}^+$ , or  $100 \mu\text{M}$  *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVADfmk). Neuronal death was analyzed 24 h later by counting viable neurons excluding trypan blue (shown as mean  $\pm$  S.E.M.,  $n = 4$  culture wells per condition). \*, significant difference compared with control (alone),  $p < 0.05$ . **C**, Western blot analysis of FADD after immunoprecipitation (IP) with Fas antibody, cleaved caspase 8, cleaved caspase 3, and actin in neuron-rich cortical cell cultures deprived of serum for 12 h, alone or in the presence of  $1 \mu\text{M}$  Neu2000 or  $5 \text{ mM}$   $\text{Li}^+$ . **D**, fluorescent photomicrographs of lumbar ventral sections immunolabeled with nitrotyrosine antibody from control (a) and G93A transgenic mice treated with saline (b) or Neu2000 (c) ( $30 \text{ mg/kg/day}$ ) for 2 weeks starting from 8 weeks of age. Arrows indicate motor neurons. Scale bar,  $50 \mu\text{m}$ . Bottom, fluorescence intensity of nitrotyrosine in the motor neurons, shown as the mean  $\pm$  S.E.M. ( $n = 15$  sections, three mice per condition). **E**, fluorescence intensity of nitrotyrosine (a) or oxidized MitoTracker red CM-H2XRos (b) in the motor neurons from control and G93A mice treated with saline or Neu2000 shown as the mean  $\pm$  S.E.M. ( $n = 15$  sections, three mice per condition). **F**, Western blot analysis of Fas, FADD, cleaved caspase 8, cleaved caspase 3, and actin in lumbar segments from control and G93A transgenic mice treated with saline, Neu2000 ( $30 \text{ mg/kg/day}$ ), or  $\text{Li}^+$  ( $200 \text{ mg/kg/day}$ ) for 4 weeks starting from 8 weeks of age (a). Level of each protein was analyzed and scaled to actin, mean  $\pm$  S.E.M. ( $n = 4$  per group).

Administration of Neu2000 and  $\text{Li}^+$  significantly extended the average survival time in G93A mice from 125.6 days to 143.8 and 137.2 days, respectively (Figure 5B and Table 1).

Survival was further extended to 152.1 days in G93A mice treated with both Neu2000 and  $\text{Li}^+$ , a significant increase relative to either monotherapy.

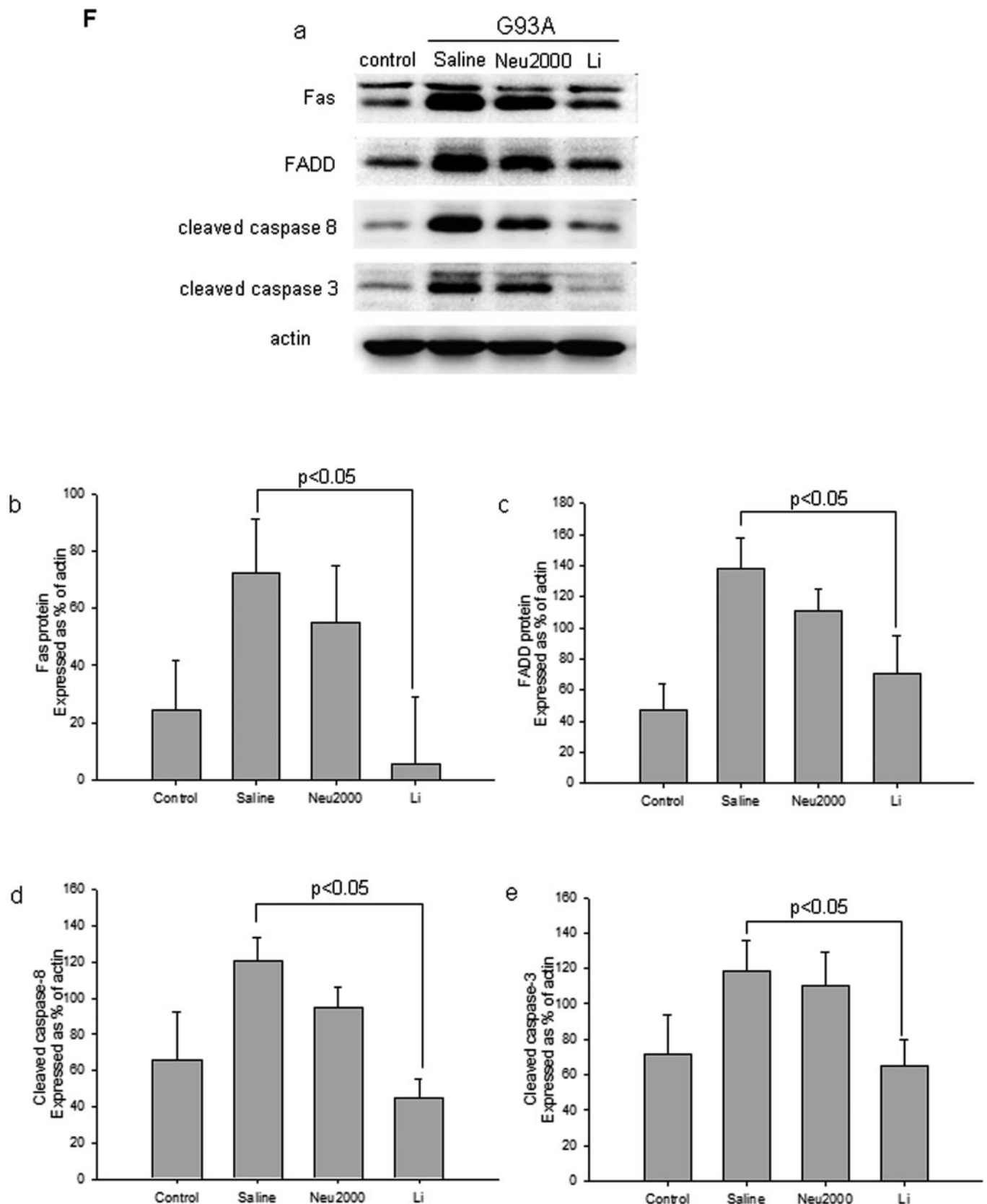
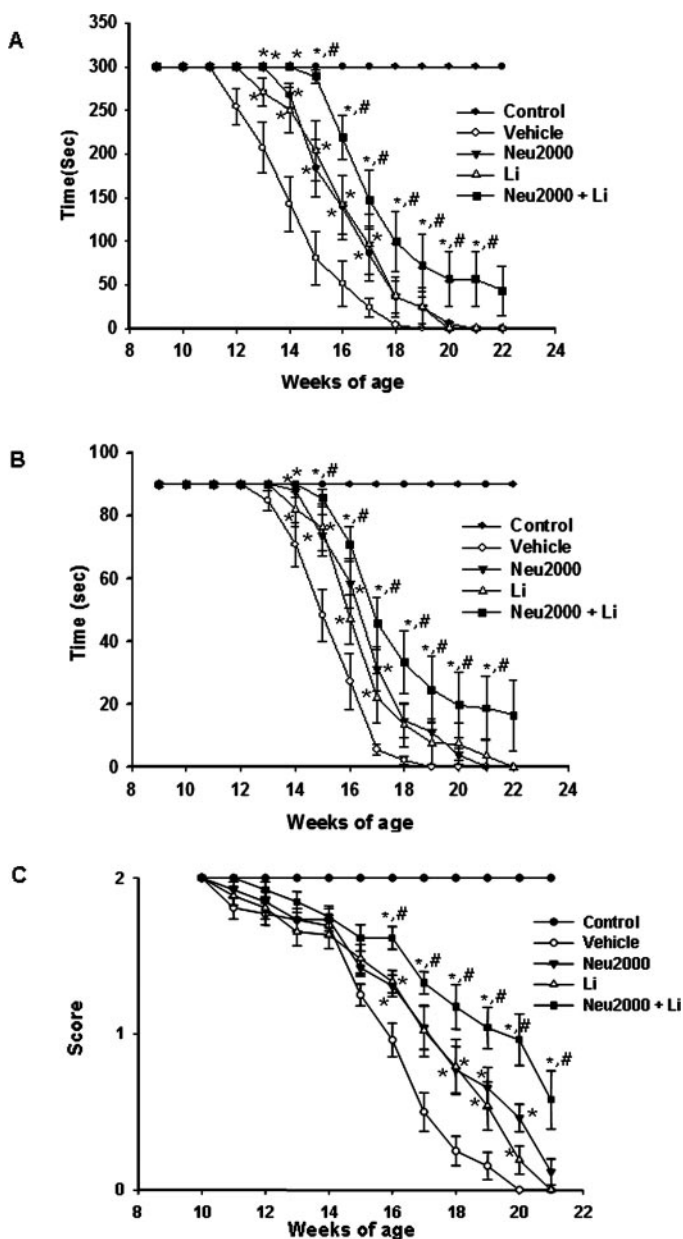


Fig. 3. Continued.





**Fig. 4.** Neu2000 and  $\text{Li}^+$  additively improve motor function in G93A transgenic mice. Motor function is shown for wild-type mice (control), G93A mice treated with vehicle, Neu2000 (30 mg/kg/days), 0.2% lithium carbonate (Li), or a combination of both (Neu2000 + Li) in the diet from 8 weeks of age. Rota Rod test (A), PaGE test (B), and extension reflex (C) were analyzed at the ages indicated. Results are shown as the mean  $\pm$  S.E.M. ( $n = 13$  per group). \*, significant difference compared with vehicle,  $p < 0.05$ ; #, significant difference compared with Neu2000 or Li alone,  $p < 0.05$ .

**TABLE 1**

Onset of Rota Rod deficit, PaGE deficit, and mortality in G93A transgenic mice

G93A transgenic mice received Neu2000 (30 mg/kg/d), 0.2% lithium carbonate (Li), or a combination of both (Neu2000 + Li) in the diet from 8 weeks of age. Onset of motor deficit and mortality were analyzed, means  $\pm$  S.E.M. ( $n = 13$  per group).

	Vehicle	Neu 2000	Li	Neu 2000 + Li
	<i>days</i>			
Onset from Rota Rod	98.7 $\pm$ 3.30	112.3 $\pm$ 2.89 <sup>a</sup>	114.7 $\pm$ 2.23 <sup>a</sup>	121.5 $\pm$ 4.67 <sup>ab</sup>
Onset from PaGE	104 $\pm$ 2.70	114.1 $\pm$ 2.02 <sup>a</sup>	113.3 $\pm$ 2.28 <sup>a</sup>	127.6 $\pm$ 7.39 <sup>ab</sup>
Onset from Extension reflex	113.69 $\pm$ 2.60	121.30 $\pm$ 2.63	120.61 $\pm$ 3.85	133.62 $\pm$ 3.76 <sup>ab</sup>
Mortality	125.3 $\pm$ 2.10	143.8 $\pm$ 2.83 <sup>a</sup>	137.2 $\pm$ 2.20 <sup>a</sup>	152.1 $\pm$ 5.87 <sup>ab</sup>

<sup>a</sup> Significant difference compared to vehicle,  $P < 0.01$ .

<sup>b</sup> Significant difference between Neu2000 (or Li) alone and combination of Neu2000 and Li,  $P < 0.05$ .

Finally, the neuroprotective effects of Neu2000 and  $\text{Li}^+$  were examined in lumbar spinal ventral motor neurons at 16 weeks of age. In control G93A mice, motor neurons underwent widespread degeneration of up to 74% (Fig. 5, C and D). Degeneration of motor neurons was significantly reduced to 57% and 58% in G93A mice treated with Neu2000 and  $\text{Li}^+$ , respectively. Neuronal loss was reduced significantly further, to 17%, in G93A mice treated with a combination of these agents.

## Discussion

Studies in both human patients and the transgenic mouse model of ALS have delineated multiple pathological mechanisms of neuronal death in ALS. These mechanisms include mitochondrial dysfunction, SOD1 mutations, and activation of  $\text{Ca}^{2+}$ -permeable ionotropic glutamate receptors, which result in toxic generation of superoxide, hydrogen peroxide, and nitric oxide (Williams et al., 1997; Kong and Xu, 1998). Riluzole, the only therapeutic drug approved for ALS, extends survival by approximately 3 months and is thought to reduce glutamate neurotoxicity and oxidative stress without preventing apoptosis (Koh et al., 1995; Gurney et al., 1996). Apoptosis propagates through mechanisms and morphology distinct from glutamate and free radical neurotoxicity and probably constitutes an additional route toward neuronal death in ALS. In this study, we provide the first evidence that combination therapy targeting oxidative stress and apoptosis together additively delay onset and progression of motor function deficit and extend survival in ALS transgenic mice.

Oxidative stress in ALS seems to be attributable to multiple factors, including mitochondrial dysfunction, reduced glutathione peroxidase activity, and point mutations in the  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  superoxide dismutase (SOD1) gene, the last of which are present in approximately 20% of familial ALS cases (Rosen et al., 1993). Two findings in particular suggest a strong link between the SOD1 gene mutation and oxidative stress. First, expression of the SOD1-G93A mutation has been found to elevate free radical generation in vitro and in transgenic mice (Liu et al., 1998). Second, in transgenic mice expressing the SOD1-G93A mutation (transgenic ALS mice), administration of antioxidants such as coenzyme Q10, a component of the mitochondrial respiratory chain, and creatine, an inhibitor of the mitochondrial transition pore, reduces free radical formation and increases life span and motor performance (Matthews et al., 1998; Klivenyi et al., 1999).

The temporal pattern of oxidative stress in G93A transgenic mice supports the hypothesis that such stress causes

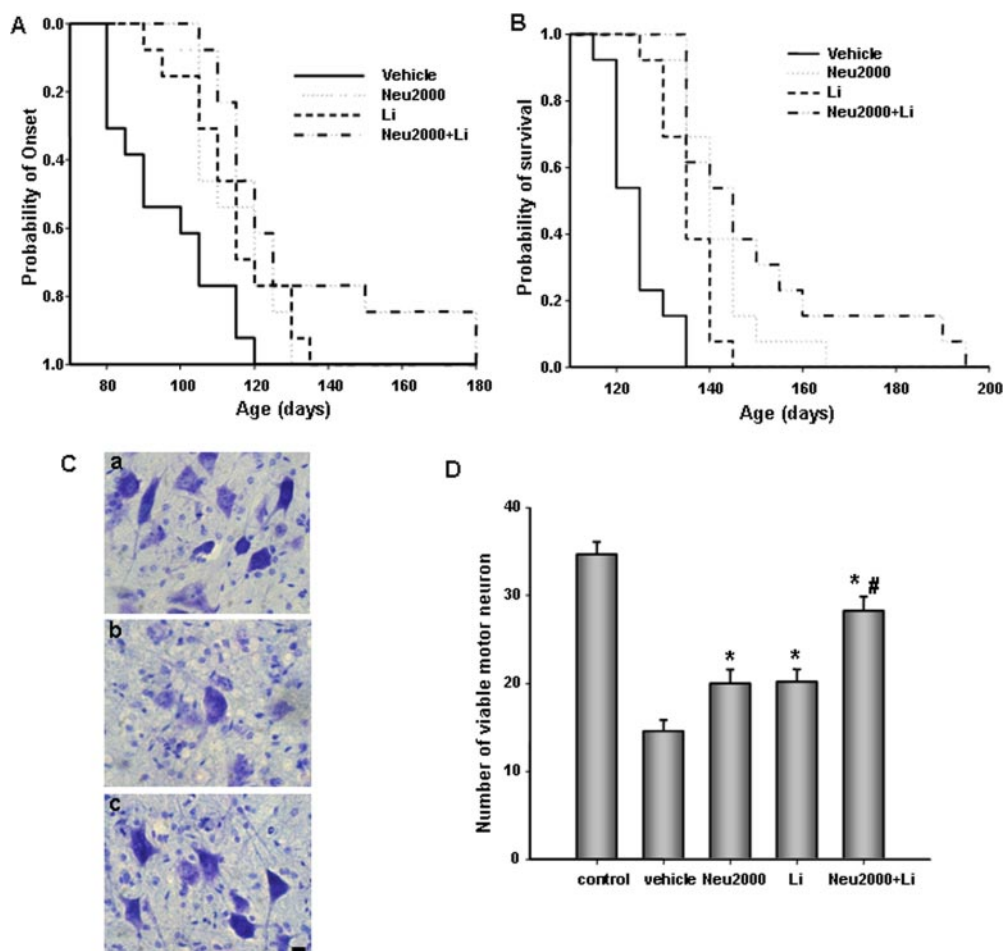


degeneration of spinal motor neurons in ALS. Levels of nitrotyrosine and MFRs were increased before neuronal death in the lumbar spinal cord of G93A mice. In control mice, oxidative stress in spinal motor neurons increased gradually over the period of 6 to 14 weeks of age (Supplemental Material 1). Oxidative stress reached a near maximal level in the motor neurons of G93A mice at 10 weeks of age, a time when mild neuronal death was observed. It is noteworthy that oxidative stress in the spinal cord of control mice at 14 weeks of age was comparable with that in G93A mice at 8 to 10 weeks of age but was not followed by neuronal loss. This raises the possibility that the SOD1 mutation not only enhances oxidative stress in lumbar motor neurons but also may render motor neurons more vulnerable to oxidative stress. The latter effect may be attributable to interaction of mutant SOD1 and Bcl-2, causing mitochondrial dysfunction and subsequently increased sensitivity to oxidative stress (Pasinelli et al., 2004). We found that administration of Neu2000 completely blocks oxidative stress but partially reduces neuronal death in the lumbar spinal cords of G93A mice. Therefore, blockade of oxidative stress with Neu2000 delays onset of motor deficits and mortality to some extent. This suggests that there are additional pathways to cell death, insensitive to Neu2000, contributing to degeneration of spinal motor neurons in ALS mice.

Several lines of evidence support a potential role of apoptosis in ALS. Cultured spinal motor neurons from G93A

transgenic mice have been shown to be highly susceptible to Fas-induced apoptosis but not to excitotoxic insults that would cause neuronal cell necrosis or to trophic factor deprivation-induced apoptosis (Gwag et al., 1997; Raoul et al., 2002). We found that expression of Fas and FADD were increased selectively in the ventral motor neurons of G93A transgenic mice and that this led to activation of caspase 8 and caspase 3. In motor neurons of ALS mice, the Fas-signaling pathway remained activated after complete blockade of oxidative stress by Neu2000. This suggests that the Fas-mediated apoptosis pathway can be activated independently of oxidative stress and represents a separate path to neuronal death in the ventral horn of G93A mice. In support of this,  $\text{Li}^+$  blocked activation of the Fas pathway during serum deprivation-induced apoptosis and attenuated motor neuron degeneration as well as activation of Fas, caspase 8, and caspase 3 in the spinal cords of ALS mice. However, treatment with  $\text{Li}^+$  did not attenuate oxidative stress induced in cultured neurons and the spinal motor neurons from G93A mice.

Neu2000 is a rational therapeutic drug derived from sulfasalazine, a conjugate of 5-aminosalicylic acid and sulfapyridine designed to protect neurons from oxidative stress in the central nervous system. Neu2000 blocks free radical neurotoxicity in cortical cell cultures at a dose as low as  $0.3 \mu\text{M}$  and completely blocks free radical production after focal cerebral (Gwag et al., 2006) and global forebrain ischemia (data not shown). Its antioxidant po-



**Fig. 5.** Neu2000 and  $\text{Li}^+$  additively extend symptom onset, life expectancy, and motor neuron survival in G93A transgenic mice. Animals received Neu2000 (30 mg/kg/days), 0.2% lithium carbonate (Li), or a combination of both (Neu2000 + Li) in the diet from 8 weeks of age. The cumulative probability of onset of motor deficits from Rota Rod (A) and mortality (B) in G93A transgenic mice are shown. C, bright-field photomicrographs of cresyl violet-stained lumbar motor neurons at 16 weeks of age from control (a) or G93A transgenic mice treated with vehicle (b) or Neu2000 +  $\text{Li}^+$  (c). D, the number of viable motor neurons in the lumbar ventral horn for control and G93A mice at 16 weeks of age, means  $\pm$  S.E.M. are shown ( $n = 20$  sections, four mice per group). \*, significant difference compared with vehicle,  $p < 0.01$ ; #, significant difference between Neu2000 (or Li) alone and combination of Neu2000 and Li,  $p < 0.01$ .

tency against  $\text{Fe}^{2+}$ -induced oxidative stress is remarkably higher than that of vitamin E, a scavenger of peroxy radicals that has been widely investigated for amelioration of neurodegenerative diseases but failed to show beneficial effects in clinical trials of Parkinson's disease and Alzheimer's disease, possibly because of poor bioavailability in the brain (Morens et al., 1996; Luchsinger et al., 2003). We showed here that long-term dietary administration of Neu2000 completely blocked oxidative stress in spinal motor neurons from G93A transgenic mice, modestly increasing motor neuron survival, motor function, and life expectancy. The neuroprotective effects of Neu2000 against oxidative stress were much more potent and efficacious than those of experimental ALS drugs such as minocycline and creatine (Zhu et al., 2002) (Supplemental Material 2). This suggests that Neu2000 can be applied to effectively prevent oxidative stress in ALS and other neurological disorders. In addition to its clinical use in treating mood disorder,  $\text{Li}^+$  has been shown to prevent apoptosis through mechanisms involving up-regulation of Bcl-2, inhibition of glycogen synthase kinase-3 $\beta$ , and activation of phosphatidylinositol 3-kinase that result in activation of the serine/threonine kinase Akt-1 and phospholipase C $\gamma$  (Chalecka-Franaszek and Chuang, 1999; Kang et al., 2003). In addition, the Fas apoptosis signaling complex seems to be a molecular target of  $\text{Li}^+$  against apoptosis, as shown here by its blockade of Fas expression and activation. The neuroprotective effects of  $\text{Li}^+$  have been demonstrated in various animal models of neurological disease. Chronic treatment with  $\text{Li}^+$  reduces apoptotic cell death after focal cerebral ischemia (Xu et al., 2003). Administration of  $\text{Li}^+$  prevented neuronal death induced by injections of the dopaminergic neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or the excitotoxin quinolinic acid (Senatorov et al., 2004; Youdim and Arraf, 2004). In the present study, long-term administration of  $\text{Li}^+$  attenuated degeneration of spinal motor neurons from G93A transgenic mice. The neuroprotective effects of  $\text{Li}^+$  in vivo were observed at doses ( $\sim 0.425 \pm 0.05$  mEq/l in blood) below the therapeutic range (0.6–1.5 mEq/l in blood) for treatment of manic episodes and depression in humans (Ross and Frank, 2001). This suggests that  $\text{Li}^+$  can be safely used to prevent neuronal cell apoptosis in acute and chronic neurological diseases.

Long-term treatment with  $\text{Li}^+$  increases expression of brain-derived neurotrophic factor in the hippocampus and neocortex, which mediates the antiapoptotic action of  $\text{Li}^+$  (Fukumoto et al., 2001). The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3 (NT-3), and NT-4/5 promote neuronal survival by preventing programmed cell death or apoptosis, but they markedly enhance necrotic degeneration of neurons exposed to oxidative stress (Koh et al., 1995; Won et al., 2000). Neurotrophins can induce oxidative stress through up-regulation of NADPH oxidase, leading to neuronal cell necrosis (Kim et al., 2002). Like neurotrophins,  $\text{Li}^+$  was shown to potentiate free radical neurotoxicity in cortical cell cultures (Kang et al., 2003). This suggests that the neuroprotective effects of  $\text{Li}^+$  as well as neurotrophins can be enhanced with blockade of oxidative stress.

In conclusion, the present study suggests that oxidative stress and the Fas death pathway constitute two separate

routes of the motor neuron degeneration in G93A mice. The former is rapidly induced and probably mediates necrotic degeneration of the motor neurons. The Fas pathway is slowly activated even in the blockade of oxidative stress and seems to cause apoptotic degeneration of the motor neurons in the lumbar spinal cord. Concurrent administration of Neu2000 and  $\text{Li}^+$ , which block free radical-mediated necrosis and Fas-mediated apoptosis, respectively, markedly delayed onset and progression of motor neuron degeneration and motor function deficits. Thus, targeting both oxidative stress and the Fas apoptosis pathway with concurrent treatment with Neu2000 and  $\text{Li}^+$  may additively improve neurological function and neuronal survival in ALS and possibly other neurological diseases, including stroke, Alzheimer's disease, and Parkinson's disease.

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