Bcl-2: Prolonging Life in a Transgenic Mouse Model of Familial Amyotrophic Lateral Sclerosis

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Mutations in the gene encoding copper/zinc superoxide dismutase enzyme produce an animal model of familial amyotrophic lateral sclerosis (FALS), a fatal disorder characterized by paralysis. Overexpression of the proto-oncogene *bcl-2* delayed onset of motor neuron disease and prolonged survival in transgenic mice expressing the FALS-linked mutation in which glycine is substituted by alanine at position 93. It did not, however, alter the duration of the disease. Overexpression of *bcl-2* also attenuated the magnitude of spinal cord motor neuron degeneration in the FALS-transgenic mice.

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in humans (*1*). It is characterized by a progressive loss of motor neurons that causes skeletal muscle wasting, paralysis, and ultimately death (*1*). About 15% of FALS cases, which are clinically indistinguishable from the more common sporadic ALS (*1*), are associated with dominantly inherited missense mutations in the gene encoding the free radical–scavenging enzyme copper/zinc superoxide dismutase (Cu/Zn-SOD; E.C. 1.15.1.1) (*2*, *3*). Cu/Zn-SOD enzymatic activity is reduced in FALS patients carrying the mutations (*3*, *4*) as a result of the enzyme's instability (*5*, *6*). Several lines of transgenic mice that express a human Cu/Zn-SOD mutant develop a dominantly inherited adult-onset paralytic disorder that replicates many of the clinical and pathological hallmarks of FALS (*7*, *8*). To date, mechanical ventilation and, to a lesser extent, riluzole, a glutamate inhibitor, are the only approved therapies that prolong the lives of ALS patients (*9*).

The product of the *bcl-2* proto-oncogene (Bcl-2) is the most fully characterized member of a family of proteins that are important in regulating cell death (*10*). In cultures, Bcl-2 inhibits the death of cultured cells induced by mechanisms as diverse as growth factor withdrawal, addition of calcium ionophore, glucose withdrawal, membrane peroxidation, free radical attack, and hypoxia (*11*, *12*). Its overexpression in transgenic mice is associated with a reduction in the volume of brain infarction in an experimental model of ischemia (*13*), with protection against axotomy-induced motor neuron loss after transection of the facial nerve (*14*), and with prevention of neuronal cell body loss but not axonal loss in a mouse model of progressive motoneuronopathy (*15*). Bcl-2 mRNA expression is decreased in spinal cord motor neurons from ALS patients (*16*), whereas Bcl-2 protein content is increased (*17*).

To test whether the overexpression of human Bcl-2 can protect in an animal model of ALS, we used FALS-transgenic mice that carry about eight copies (*18*) of the transgene encoding for the human Cu/Zn-SOD mutant in which glycine is substituted by alanine at position 93 (G93A) (*19*). This mutation is associated with ALS in humans (*2*, *3*). To produce transgenic G93A mice that overexpress human Bcl-2, hemizygote transgenic G93A mice were crossed with hemizygote transgenic *bcl-2* mice that carry about 16 copies of the human *BCL-2* gene with the promoter sequence of the rat neuron–specific enolase (*13*, *14*). After birth, transgenic *bcl-2* mice express Bcl-2 throughout the nervous system, including the motor neurons of the spinal cord (*13*, *14*).

By postnatal days 2 to 6, pups could be identified as wild type (−/−), transgenic for G93A only (G93A/−), transgenic for *bcl-2* only (−/*bcl-2*), or transgenic for both G93A and *bcl-2* (G93A/*bcl-2*) (*20*); each litter in the study contained at least one pup from each category. These pups did not differ in size, weight, gross morphology, or behavior, and all thrived in a similar fashion. One-month-old mice from the four groups had a comparable number of motor neurons in the spinal cord (Table 1).

Virtually all motor neurons of the spinal cord in the 1-month-old transgenic G93A/*bcl-2* mice showed strong immunoreactivity for human Bcl-2 (Fig. 1B) comparable to that in the transgenic −/*bcl-2* mice (Fig. 1A); no immunoreactivity for human Bcl-2 was observed in the transgenic G93A/− (Fig. 1C) or wild-type mice. Overexpression of human Bcl-2 prevented the loss of motor neurons of the facial nucleus after the transection of the nerve (*21*) to a comparable extent in the transgenic G93A/*bcl-2* (93%) and −/*bcl-2* mice (94%), thus confirming that the human Bcl-2 protein in the G93A/*bcl-2* mice is functional. Furthermore, the level of expression of the Cu/Zn-SOD mutant protein, as evidenced by the measurement of Cu/Zn-SOD enzymatic activity (*22*), was not altered by the overexpression of human Bcl-2, as the transgenic G93A/*bcl-2* (660 ± 9 U per milligram of protein) and G93A/− (634 ± 6 U per milligram of protein) mice had spinal cord Cu/Zn-SOD activity about five times higher than that in the transgenic −/*bcl-2* (129 ± 9 U per milligram of protein) and wild-type mice

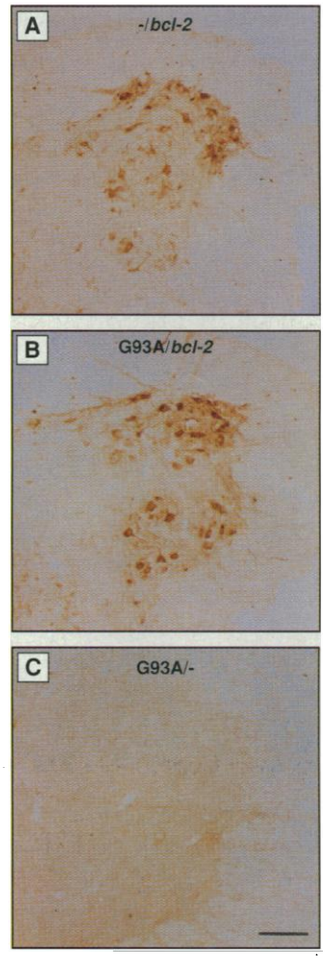

Fig. 1. Expression of human Bcl-2 in motor neurons of the spinal cord is confirmed by immunohistochemistry (monoclonal antibody to human Bcl-2; DAKO, Carpinteria, California) as in (*23*). Both the transgenic −/*bcl-2* (**A**) and G93A/*bcl-2* mice (**B**) show strong immunoreactivity for human Bcl-2 in spinal cord motor neurons. In contrast, no specific immunostaining is observed in spinal cords of the transgenic G93A/− mice (**C**). Scale bar, 132 μm.

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Table 1. Motor neuron numbers in 1-month-old mice and effects of Bcl-2 overexpression on Cu/Zn-SOD mutant–induced neurodegeneration in older mice.

	Mouse category (n = four to seven mice per group)				
	−/−	−/*bcl-2*	G93A/−	G93A/*bcl-2*	
Age (days)	30	30	30	30	
Spinal cord motor neurons					
Nissl-stained cells (number per section)	78.0 ± 1.2	75.2 ± 1.8	77.6 ± 1.6	76.8 ± 1.4	
ChAT-stained cells (number per section)	74.8 ± 1.0	76.2 ± 1.4	73.4 ± 1.2	76.8 ± 1.0	
Age (days)	239 ± 5*	239 ± 5*	239 ± 5 (end stage)	239 ± 5*	282 ± 4 (end stage)
Spinal cord motor neurons					
Nissl-stained cells (number per section)	76.8 ± 0.9	75.5 ± 1.2	38.3 ± 0.7†	56.7 ± 1.3†‡	38.7 ± 0.6†
ChAT/Nissl ratio (%)	102 ± 2	99 ± 1	98 ± 1	96 ± 2	99 ± 2
Cross-sectional area (μm^2)	239.5 ± 8.9	243.1 ± 8.8	225.7 ± 6.2	230.6 ± 6.1	235.1 ± 5.9
Myelinated peripheral axons					
Phrenic nerve (number per section)	213 ± 10	213 ± 16	99 ± 8†	146 ± 8†‡	84 ± 7
End plate in gluteus muscle					
Innervated (%)	99.3 ± 0.5	99.5 ± 0.6	21.6 ± 1.3†	47.6 ± 2.9†‡	22.7 ± 1.7†
Denervated (%)	0.2 ± 0.1	0.1 ± 0.1	49.6 ± 5.5†	27.9 ± 2.5†‡	49.0 ± 2.1†
Reinnervated (%)	0.5 ± 0.3	0.4 ± 0.2	28.8 ± 1.0†	24.5 ± 1.8†	28.4 ± 2.8†

*Killed simultaneously with G93A/− end stage littermates. All tissue processing, staining, and counting methods are given in (*43*). Values are means ± SEM. †Significantly different (Newman-Keuls post hoc test, $P < 0.01$) from −/− and −/*bcl-2* mice. ‡Significantly different (Newman-Keuls post hoc test, $P < 0.01$) from G93A/− end stage and G93A/*bcl-2* end stage mice.

(123 ± 7 U per milligram of protein).

In both the transgenic G93A/− and G93A/*bcl-2* mice, the first abnormal behaviors were a fine tremor in and posturing of at least one limb when the animal was held in the air by the tail (*7*, *23*). In the transgenic G93A/− mice, the onset of these signs occurred by the mean age of 170 ± 3 days (±SEM), whereas in the transgenic G93A/*bcl-2* mice, the onset occurred significantly later (log rank = 20.7, $P < 0.0001$), by the mean age of 203 ± 6 days (Fig. 2A). After onset, weakness and atrophy of the skeletal muscles that predominated in the hind limbs developed in both the transgenic G93A/− and G93A/*bcl-2* mice. At the end stage, paralysis was so severe and generalized that the transgenic G93A/− mice could not eat, drink, or move freely and were therefore killed (at 240 ± 2 days). Although identical end stage criteria were applied to the transgenic G93A/*bcl-2* mice, they reached the end stage significantly later (275 ± 4 days; log rank = 41.1, $P < 0.0001$) (Fig. 2B). The duration of the disease, as evaluated by the number of days that elapsed from onset to end stage, did not, however, differ between the transgenic G93A/− (70 ± 5 days) and G93A/*bcl-2* (72 ± 8 days; t test, $P = 0.57$) mice. None of the wild-type or transgenic −/*bcl-2* mice (n = 10 mice per group) developed any behavioral abnormality or died by 370 days of age. These findings indicate that the overexpression of human Bcl-2 delayed the onset of the disease by about 19% and prolonged the survival of the transgenic G93A mice by about 15%.

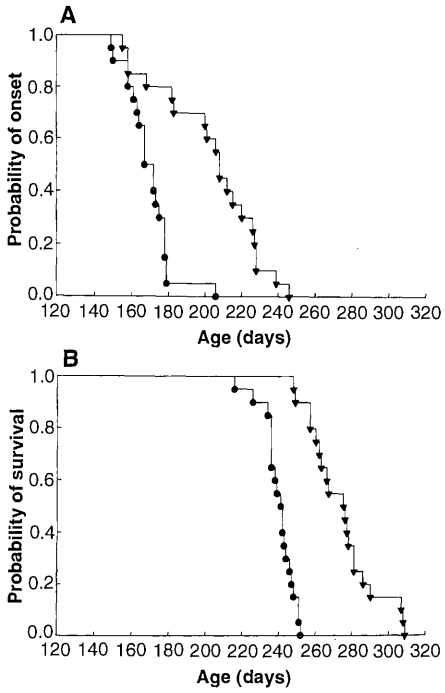


Fig. 2. Comparison of age at the onset of clinical signs (**A**) and at the end stage of the disease (**B**) in the transgenic G93A/− (circles) and G93A/*bcl-2* (triangles) mice (n = 20 mice per group). The onset and end stage are defined as in (*23*); this experimental protocol is approved by the Animal Care and Use Committee of Columbia University and is in accordance with NIH guidelines for the use of live animals. The data were analyzed by the Kaplan-Meier life test and by the log-rank test for equality over strata (G93A/− versus G93A/*bcl-2* genotype).

To determine whether Bcl-2 protects against the effect of a greater quantity of the mutant enzyme, we crossed another line of transgenic G93A mice (G1H) that carry about three times the number of copies of the human Cu/Zn-SOD G93A mutant transgene (*7*) with the same transgenic *bcl-2* mice. Consistent with previous studies (*7*, *24*), the transgenic G1H/− mice (n = six mice per group) became symptomatic by 97 ± 2 days and reached the end stage by 146 ± 4 days, which is substantially earlier than did the transgenic G93A/− mice. Nevertheless, overexpression of human Bcl-2 was still effective in protecting against the clinical expression of motor neuron disease induced by the Cu/Zn-SOD mutant, as the transgenic G1H/*bcl-2* mice (n = six mice per group) reached onset (113 ± 6 days) and end stage (164 ± 5 days) significantly later (t test, $P < 0.03$) than did the transgenic G1H/− mice. The overexpression of human Bcl-2 seems to protect the transgenic G93A mice better than the transgenic G1H mice. However, relative to the respective life-span of each transgenic line, the effect of Bcl-2 is equally potent.

The end stage transgenic G93A/− mice showed about 50% fewer motor neurons in the anterior horn of the spinal cord than did the age-matched wild-type or transgenic −/*bcl-2* mice (Table 1 and Fig. 3, A and B). Residual motor neurons had normal cross-sectional areas and retained their cholinergic phenotypic expression (Fig. 3B). Counts of myelinated axons in the phrenic nerve were 46% of that of the age-matched wild-type or transgenic −/*bcl-2* mice (Table 1 and Fig. 3, D and E). The end stage transgenic G93A/− mice had severe muscle atrophy, and microscopic examination of gluteus muscle stained for silver and cholinesterase (*24*) showed severe loss of intramuscular myelinated axons and predominantly denervated and reinnervated end plates (Table 1). In striking contrast, the

age-matched transgenic G93A/*bcl-2* mice showed significantly more spinal cord motor neurons than did the end stage transgenic G93A/− mice (Table 1 and Fig. 3C). They also showed significantly more myelinated axons in the phrenic nerve (Table 1 and Fig. 3F) and less denervation in the gluteus muscle preparations (Table 1). The proportions of reinnervated end plates did not differ between the age-matched transgenic G93A/*bcl-2* mice and end stage transgenic G93A/− mice, which indicates that overexpression of human Bcl-2 does not delay paralysis by stimulating excessive compensatory reinnervation. Our interpretation is consistent with the fact that no evidence of aberrant sprouting from end plates or nodes of Ranvier was seen in gluteus muscle preparations from the transgenic −/*bcl-2* mice (Table 1). This situation differs from that reported in retinal ganglion cells where overexpression of Bcl-2 prevents death and stimulates axonal regeneration after optic tract transection in mouse neonates (*25*). Therefore, Bcl-2 appears to promote axonal regeneration in immature retinal ganglion cells subjected to an acute injury but appears to fail in this respect in adult spinal cord motor neurons subjected to a chronic insult.

Despite the overexpression of human Bcl-2, the transgenic G93A/*bcl-2* mice eventually reached end stage. At this time, morphological assessment demonstrated a loss of motor neurons in the spinal cord and of myelinated axons in the phrenic nerve, as well as a proportion of end plate denervation in the gluteus muscle similar to that observed in the end stage transgenic G93A/− mice (Table 1). Again, the remaining motor neurons had normal cross-sectional areas and choline acetyltransferase (ChAT) expression (Table 1) and were still heavily immunostained for human Bcl-2 (Fig. 3G). This latter finding makes it unlikely that the transgenic G93A/*bcl-2* mice failed to delay neurodegeneration longer because they stopped expressing human Bcl-2.

Next, we examined whether the overexpression of Bcl-2 protects by hampering Cu/Zn-SOD mutant–induced cytotoxicity or by enabling injured motor neurons to last longer. We assessed the expression of c-Jun and ubiquitin, two markers of cell injury whose expression is increased in motor neurons of the spinal cord in ALS (*26*). In the age-matched wild-type and transgenic −/*bcl-2* mice, a small number of c-Jun–positive and even fewer ubiquitin-positive motor neurons were seen (Fig. 4E). In all other groups of mice, immunoreactivity for both proteins was markedly increased (Fig. 4E), appearing as intense immunostaining that predominated in the nucleus for c-Jun (Fig.

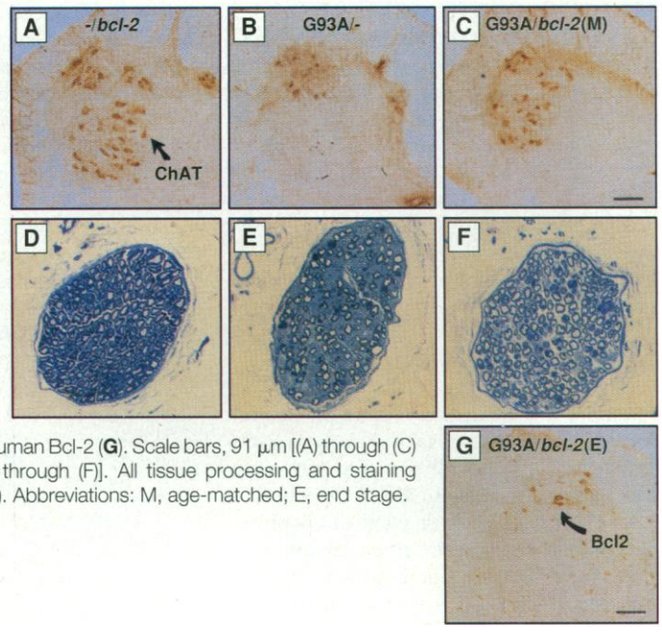


Fig. 3. Relative to the age-matched transgenic −/*bcl-2* control mice (**A**), the end-stage transgenic G93A/− mice (**B**) have markedly fewer ChAT-positive spinal cord motor neurons than do the age-matched G93A/*bcl-2* mice (**C**). Similar differences among groups of mice are seen in the magnitude of degeneration of myelinated axons in the phrenic nerve (**D** through **F**). Also, in the end-stage G93A/*bcl-2* mice, spared motor neurons continue to express human Bcl-2 (**G**). Scale bars, 91 μm [(A) through (C) and (G)] and 21 μm [(D) through (F)]. All tissue processing and staining procedures were as in (*43*). Abbreviations: M, age-matched; E, end stage.

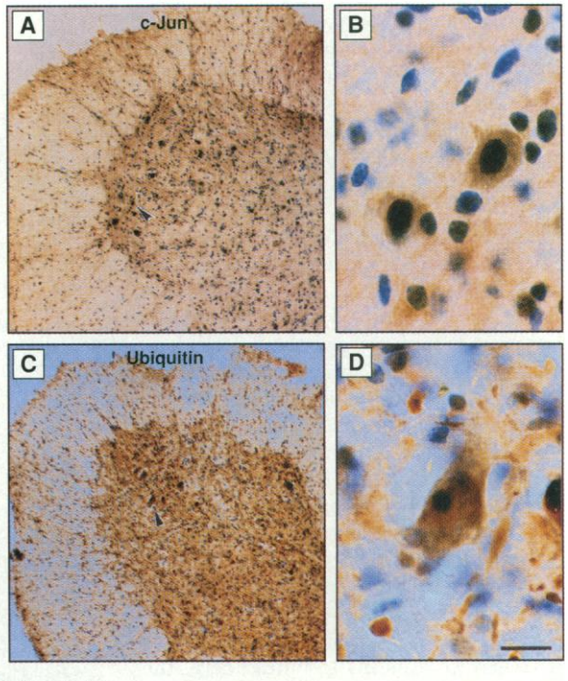
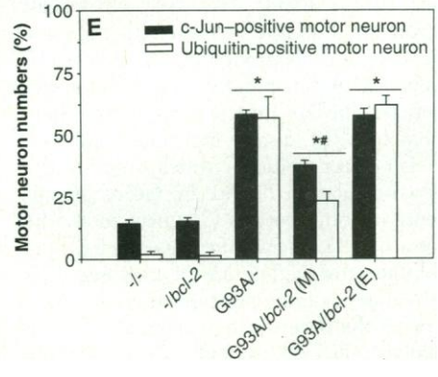


Fig. 4. A marker of cell injury is the gene product of *c-jun*, whose expression is increased in motor neurons of the end stage transgenic G93A/− mice (**A**); c-Jun immunostaining appears as intense labeling (**B**). The section is counterstained with thionin (blue). Another pathological marker is ubiquitin (**C**), which appears as a strong and diffuse cytoplasmic labeling (**D**); the section is counterstained with hematoxylin (blue). Spinal cord tissue samples for both c-Jun and ubiquitin immunostaining were prepared and processed as for ChAT immunohistochemistry with substitution for antibody to ChAT either with a polyclonal antibody to c-Jun (1:5000 dilution; Santa Cruz; Santa Cruz, California) or a monoclonal antibody to ubiquitin (1:2000 dilution; DAKO). Scale bar, 132 μm (A and C) and 21 μm (B and D). The proportion of motor neurons that express c-Jun or ubiquitin is significantly greater in the end stage transgenic G93A/− mice than in the age-matched G93A/*bcl-2* mice (**E**). Values are means ± SEM (*n* = three to four mice per group). Statistical analyses were performed by one-way ANOVA followed by a Newman-Keuls post hoc test. *$P < 0.01$ compared to the wild-type (−/−) and transgenic −/*bcl-2* mice; #$P < 0.01$ compared to the end stage transgenic G93A/− and end stage transgenic G93A/*bcl-2* mice. Abbreviations: M, age-matched; E, end stage.



4, A and B) and in the entire neuronal cell body and processes for ubiquitin (Fig. 4, C and D). In the end stage transgenic G93A/− and G93A/*bcl-2* mice, more than half of the remaining motor neurons were positive for c-Jun or ubiquitin (Fig. 4E). In contrast, in the age-matched transgenic G93A/*bcl-2* mice, significantly fewer ($P < 0.01$) remaining motor neurons were positive for c-Jun or ubiquitin (Fig. 4E). This finding suggests that by the time the transgenic G93A/− mice reach end stage, the age-matched transgenic G93A/*bcl-2* mice have more and healthier motor neurons.

There is little doubt that Cu/Zn-SOD mutations are the initiators of motor neuron disease, whereas other factors such as excitotoxicity (*27*) and nitrosylation (*28*) may underlie its progression. Bcl-2, which is a potent inhibitor of most types of apoptotic cell death (*10*), may prevent degeneration of motor neurons by impeding the activation of apoptosis that results from the Cu/Zn-SOD mutation (*29*). Supporting the role of apoptosis in ALS is the observation that in postmortem spinal cord samples from ALS patients, but not from controls, DNA fragmentation (a biochemical feature of apoptosis) is evidenced by in situ end labeling (*17, 30*). Moreover, Cu/Zn-SOD mutants enhance apoptosis in immortalized rat nigral neurons (*31*) and in postnatal midbrain neurons (*32*). Bcl-2 also has potent antioxidant properties (*11, 33*). It is thus plausible that overexpression of human Bcl-2 prevents degeneration of motor neurons by inhibiting free radical–mediated damage. The view that Cu/Zn-SOD mutants subject cells to oxidative stress is further supported by the demonstration that the antioxidant vitamin E delays the onset of clinical signs and slows the progression of the disease in FALS-transgenic mice (*34*) and that Cu/Zn-SOD mutants kill not only spinal cord motor neurons but also midbrain dopaminergic neurons (*23*), a cell type that is particularly sensitive to free radical attack.

The amount of Cu/Zn-SOD mutant protein affects the duration of Bcl-2 protection, so an increase in the quantity of endogenous Bcl-2 may be a means to further delay the onset of the disease. This study also suggests a role for gene intervention with the use of Bcl-2 or anti-apoptotic Bcl-2 homologs (*10*) as potential therapies for ALS. Mutant mice deficient in Bax (*35*), a pro-apoptotic Bcl-2 homolog (*10*), display increased numbers of facial motor neurons, which suggests that down-regulation of Bax by means of antisense oligonucleotides (*36*) may be another form of gene therapy for this disease. Bcl-2 inhibits the activation of caspases (*37*), which are cysteine proteases that are important in the occurrence of apoptosis in mammalian cells (*38*). Should caspase inhibition mimic Bcl-2 protection on motor neuron disease in transgenic G93A mice, the use of synthetic caspase antagonists (*38*) would provide a more feasible therapeutic approach (*39*). Because Bcl-2 overexpression delays the onset of motor neuron disease in transgenic G93A mice but does not affect the progression of the disease, anti-apoptotic therapy may only be beneficial to ALS patients in whom treatment can be started before the onset of symptoms.

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19. We confirmed that transgenic G93A mice from Jackson Laboratory (Bar Harbor, ME) harbor the point mutation GGT → GCT in codon 93 of the human Cu/Zn-SOD gene using specific primers for exon 4 (*2*), followed by a standard sequencing method.
20. Each mouse pup was genotyped at postnatal days 2 to 6 by activity gel to detect human Cu/Zn-SOD activity, by single-strain conformational polymorphism (SSCP) to detect the human Cu/Zn-SOD G93A mutant transgene, and by polymerase chain reaction (PCR) to detect the human *BCL-2* transgene. Activity gel was performed on hemolysates (*40*), and SSCP (*2*) and PCR (*14*) were performed on purified genomic DNA (QIAamp; Qiagen, Chatsworth, CA) prepared from 200 μl of blood and 1 cm of tail. All genotyping was performed at least twice for each pup. After completion of the genotyping, each pup was given an arbitrary code and, from then on, was assessed for clinical and morphological characteristics by investigators blinded to the genotype; the blind was broken only at the end of the study.
21. Transection of the facial nerve was performed in 2-day-old pups, and the extent of the lesion was assessed 1 week later in Nissl-stained tissue sections from the facial nucleus (*14*). Values are means + SEM (n = three mice per group). Statistical analyses were performed by one-way analysis of variance (ANOVA).
22. SOD activity was measured in whole spinal cord tissue homogenates prepared as in (*23*) and assayed as in (*41*). Values are means ± SEM (n = three mice per group). Statistical analyses were performed by one-way ANOVA.
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43. For spinal cord morphology, mice (three to seven per group) were prepared (*22*), and stainings and cell counts were done as in (*23*) at the cervical (C7) and the lumbar (L3) levels; the lumbar counts (not shown) revealed changes similar to those in cervical counts. We determined cross-sectional areas using a computerized image-analysis system as in (*42*) by digitizing 50 to 200 random motor neurons per mouse (n = three mice per group). For myelinated peripheral axon counts, about 2 cm of phrenic nerve was dissected out and immersed in 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (overnight). Then, nerve samples were processed as in (*15*). Two-micrometer-thick sections were collected at 0.5 cm from the entry of the nerve into the diaphragm and were stained with toluidine blue before light microscopy examination (×800 to 1000). For end plate counts, gluteus muscle was dissected out (*24*) and stained for silver and cholinesterase (*24*). The muscles, mounted between two cover slips for viewing, were coded and scored without knowledge of age or genotype of the following as in A. Y. Chiu *et al.* [*Mol. Cell. Neurosci.* **6**, 349 (1995)]: (i) normal end plates innervated by a single myelinated axon, (ii) denervated end plates identified by cholinesterase staining that were not contacted by an axon, and (iii) end plates that were in the process of reinnervation. End plates undergoing reinnervation were contacted by one or more unmyelinated nodal sprouts that could be traced back to the intramuscular nerve or by two or more myelinated axons. At least 100 end plates per muscle were scored.

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